

Regulation of flagellar motility during biofilm formation

Sarah B. Guttenplan & Daniel B. Kearns

Department of Biology, Indiana University, Bloomington, IN, USA

Correspondence: Daniel B. Kearns,
Department of Biology, Indiana University,
Bloomington, IN 47405, USA.
Tel.: 812 856 2523; fax: 1-812-855-6705;
e-mail: dbkearns@indiana.edu

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Abstract

Many bacteria swim in liquid or swarm over solid surfaces by synthesizing rotary flagella. The same bacteria that are motile also commonly form nonmotile multicellular aggregates called biofilms. Biofilms are an important part of the lifestyle of pathogenic bacteria, and it is assumed that there is a motility-to-biofilm transition wherein the inhibition of motility promotes biofilm formation. The transition is largely inferred from regulatory mutants that reveal the opposite regulation of the two phenotypes. Here, we review the regulation of motility during biofilm formation in *Bacillus*, *Pseudomonas*, *Vibrio*, and *Escherichia*, and we conclude that the motility-to-biofilm transition, if necessary, likely involves two steps. In the short term, flagella are functionally regulated to either inhibit rotation or modulate the basal flagellar reversal frequency. Over the long term, flagellar gene transcription is inhibited and in the absence of *de novo* synthesis, flagella are diluted to extinction through growth. Both short-term and long-term motility inhibition is likely important to stabilize cell aggregates and optimize resource investment. We emphasize the newly discovered flagellar functional regulators and speculate that others await discovery in the context of biofilm formation.

Introduction

Bacteria move by a variety of mechanisms, but the most studied form of bacterial motility involves the assembly and rotation of propeller-like flagella (Jarrell & McBride, 2008). Each flagellum is assembled from the inside-out starting with a “basal body” inserted into the cytoplasmic membrane (Fig. 1) (Macnab, 2003). Within the basal body, a type III secretion apparatus secretes subunits of the axle-like rod that extends through the peptidoglycan and outer membrane (if present). Next, a short curved hook is polymerized from approximately 200 subunits of the hook structural protein. Finally, the long helical filament is polymerized from approximately 20 000 units of a structural protein called flagellin. In total, flagella are spontaneously assembled from over 30 proteins consuming approximately 2% of a cell’s metabolic resources, and the high cost of synthesis may have selected for the most economical amino acids being favored in the most abundant structural components during evolution (Macnab, 1996; Smith & Chapman, 2010).

In addition to synthesis cost, motile bacteria must make further investment by consumption of ion motive

forces to power flagellar rotation (Macnab, 1996). Connected to each basal body is a motor complex composed of a rotor and a stator that interact to turn the flagellum (Berg & Anderson, 1973; Silverman & Simon, 1974; Macnab, 1977). The rotor is a cytoplasmic circular gear connected to the basal body and polymerized from monomers of a protein called FliG (Lowder *et al.*, 2005; Sowa *et al.*, 2005; Thomas *et al.*, 2006) (Fig. 1). Anchored around the basal body are stator complexes made of MotB, a membrane protein that is anchored to the peptidoglycan, and MotA, a membrane protein that interacts with FliG (Blair & Berg, 1990; Zhou *et al.*, 1998). MotA and MotB combine to create an ion channel where ion flow through the channel induces a conformational change in MotA that interacts with FliG to generate torque (Garza *et al.*, 1995; Kojima & Blair, 2001; Berg, 2003; Gabel & Berg, 2003). When rotating, the bacterial flagellum is one of the most powerful motors on earth, revolving at 100–1500 Hz and generating 400–3800 pN·nm of torque depending on the species (Magariyama *et al.*, 1994; Sowa *et al.*, 2003; Li & Tang, 2006; Reid *et al.*, 2006; Darnton *et al.*, 2007). The torque generated by the filament powers swimming through liquid and swarming

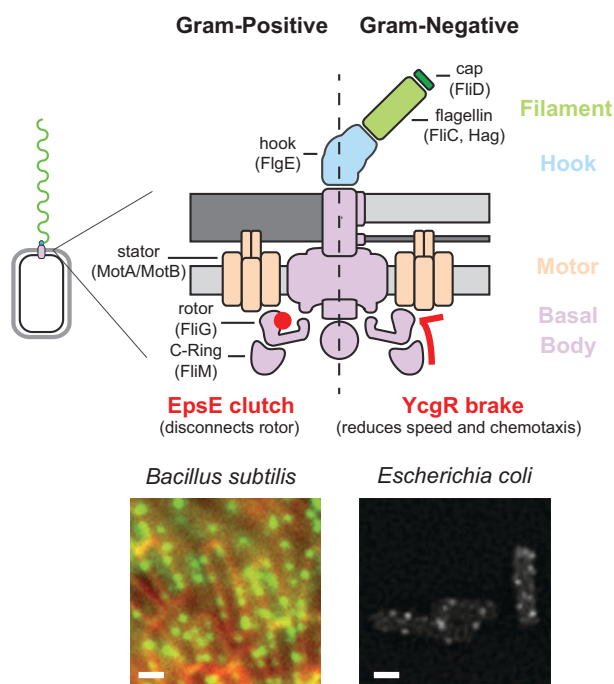


Fig. 1. Flagellar structure and functional regulation of the flagellum. Schematic of the Gram-positive and Gram-negative flagellar structures with the filament (green), hook (light blue), basal body (pink), and motor (tan) indicated. The site of clutch or brake motility inhibition is indicated in red. Micrographs of EpsE-GFP and YcgR-GFP localizing as puncta at the flagellum are reprinted from Blair *et al.* (2008) and Boehm *et al.* (2010), respectively. Scale bars are 2 μ m.

over solid surfaces (Kearns, 2010). Although advantageous under certain conditions, both the high-energy investment of synthesis and output power of flagella may account for the observation that motility is extensively regulated at multiple levels.

One way in which flagella are regulated is at the level of gene expression. Flagella genes are often organized into regulatory hierarchies called classes I, II, and III (and in some cases class IV) depending on the organism in question (Kutsukake *et al.*, 1990; Macnab, 1992). Class I genes encode master transcriptional regulators that activate a regulon of genes including the Class II genes. Class II genes typically encode flagellar structural components that form the basal body, the secretion machinery, and the hook. Class III genes typically encode flagellar structural components that form the filament and the motor proteins. Regulation typically occurs at the junctions between the different classes. For example, class I master regulators can be controlled as to whether or not they activate class II gene expression, and regulatory modules that sense class II flagellar assembly states either permit or deny class III gene expression accordingly (Hughes *et al.*, 1993; Kutsukake, 1994; Mukherjee *et al.*, 2011;

Patrick & Kearns, 2012). Also commonly encoded among the class III genes are proteins that form the chemoreceptors and the signal transducers for bacterial chemotaxis.

A second way in which flagella are regulated is at the functional level where motile behavior is controlled by chemotaxis (for review see Wadhams & Armitage, 2004; Sourjik & Wingreen, 2011). Chemotaxis is the directed movement up a chemical gradient, and in bacteria, it is mediated by a biased random walk controlled by the frequency with which the flagellum changes the direction of rotation (Berg & Brown, 1972). Flagellar motors typically rotate both clockwise and counterclockwise, where rotation in one direction generates straight swimming of the bacterium and the other direction causes cells to acquire a new orientation (Larsen *et al.*, 1974). Gradients of chemoattractants are sensed by binding to chemoreceptors and are transduced by a cytoplasmic two-component regulatory system to bias the motor toward either the clockwise or the counterclockwise state. In this way, cells reinforce smooth swimming when attractants are increasing and encourage new direction sampling when attractants are decreasing. Although metabolism of effectors is unnecessary for chemotaxis, attractants tend to resemble food and energy sources, and repellents tend to resemble toxins (Adler, 1966, 1969).

Recently, a third form of flagellar regulation has been discovered that operates at the functional level of the motor to either stop or slow rotation. Traditionally, mutants that disrupt motility have been organized into three phenotypic classes called *fla*, *che*, and *mot* (Macnab, 1992). Mutants with a *fla* phenotype were aflagellate and disrupted for either the expression or synthesis of flagellar structural components. Mutants with a *che* phenotype had functional flagella that were defective in chemotaxis and disrupted either the cytoplasmic signal transduction system or the chemoreceptors. Mutants with a *mot* phenotype were rare mutants and disrupted either MotA or MotB of the stator complex or were point mutants in the rotor FliG. Recently, there has been an increase in the discovery of mutants that have a *mot* phenotype outside of the structural components of the motor (Ko & Park, 2000; Blair *et al.*, 2008; Pilizota *et al.*, 2009; Boehm *et al.*, 2010). Some of the new *mot* phenotypes are related to biofilms suggesting that impedance of flagellar rotation is important for biofilm formation.

Biofilms are multicellular aggregates of bacteria bound by a matrix of extracellular polymers (O'Toole *et al.*, 2000; Kolter & Greenberg, 2006; Monds & O'Toole, 2009). The matrix often consists of a complex mixture of extracellular polysaccharide (EPS), protein, and DNA and not only allows the bacteria to cohere to one another but also adhere to solid surfaces (Flemming & Wingender, 2010). The multicellular group of aggregated bacteria may enjoy advantages that their planktonic (dispersed,

free-floating) counterparts do not. Biofilms have been reported to experience increased tolerance to a variety of environmental parameters including antibiotic challenge and grazing by unicellular eukaryotes (Hall-Stoodley *et al.*, 2004; Matz & Kjelleberg, 2005). As we learn more about biofilms, it is becoming clear that biofilms are predominantly beneficial in the environment but can be particularly problematic when associated with biofouling and pathogenesis (Hall-Stoodley *et al.*, 2004; Shi & Zhu, 2009; Simões *et al.*, 2010). Importantly, bacteria in biofilms are nonmotile and thus, it is implicitly assumed that motile bacteria must become nonmotile when transitioning to the biofilm state. In support of the notion that the motility-to-biofilm transition is important, mutations in many regulatory genes have diametrically opposed effects on biofilm formation and motility (Yildiz *et al.*, 2001, 2004; Sauer *et al.*, 2002; Blair *et al.*, 2008; Verstraeten *et al.*, 2008).

A key regulator of the motility-to-biofilm transition is the small cytoplasmic signaling molecule cyclic-di-GMP (c-di-GMP) (Römling *et al.*, 2005; Römling & Amikam, 2006; Wolfe & Visick, 2008; Hengge, 2009). Elevated levels of c-di-GMP have been associated with inhibition of motility and activation of biofilm formation. C-di-GMP is the product of the condensation and cyclization of two molecules of GTP catalyzed by diguanylate synthase enzymes containing a GGDEF amino acid motif (Paul *et al.*, 2004; Simm *et al.*, 2004; Ryjenkov *et al.*, 2005). C-di-GMP is hydrolyzed to two molecules of GMP by the action of phosphodiesterase enzymes containing an EAL or HD-GYP amino acid motif (Simm *et al.*, 2004; Christen *et al.*, 2005; Ryan *et al.*, 2006). Organisms can encode dozens of different GGDEF and EAL domain-containing proteins and the environmental inputs that govern c-di-GMP levels are poorly understood. C-di-GMP output can be exerted by binding to RNA riboswitches or by binding allosterically to proteins containing PilZ, HD-GYP or degenerate GGDEF/EAL domains to regulate physiological targets by protein–protein interaction (Amikam & Galperin, 2006; Benach *et al.*, 2007; Christen *et al.*, 2007; Hickman & Harwood, 2008; Sudarsan *et al.*, 2008). How one pool of c-di-GMP controls many different phenotypes, and why different mutants that disrupt c-di-GMP do not always conform to the general phenotypic consequences described above remain poorly understood.

Here, we draw upon four model systems, *Bacillus*, *Vibrio*, *Pseudomonas*, and *Escherichia* and review what is known about the regulation of flagellar-mediated motility during biofilm formation. We arrive at the conclusion that the inhibition of motility is likely advantageous in the environment but may be either dispensable or easily bypassed in the laboratory. Nonetheless, motility is regu-

lated during the transition to the biofilm and appears to happen in two stages: a fast inhibition at the level of flagellar function and a slow inhibition at the level of inactivation of flagellar gene expression (Fig. 2).

Bacillus subtilis

Bacillus subtilis is a Gram-positive bacterium that is motile by assembling many peritrichous flagella arranged along the body of the cell. While the structure of Gram-positive flagella is far less understood than that of

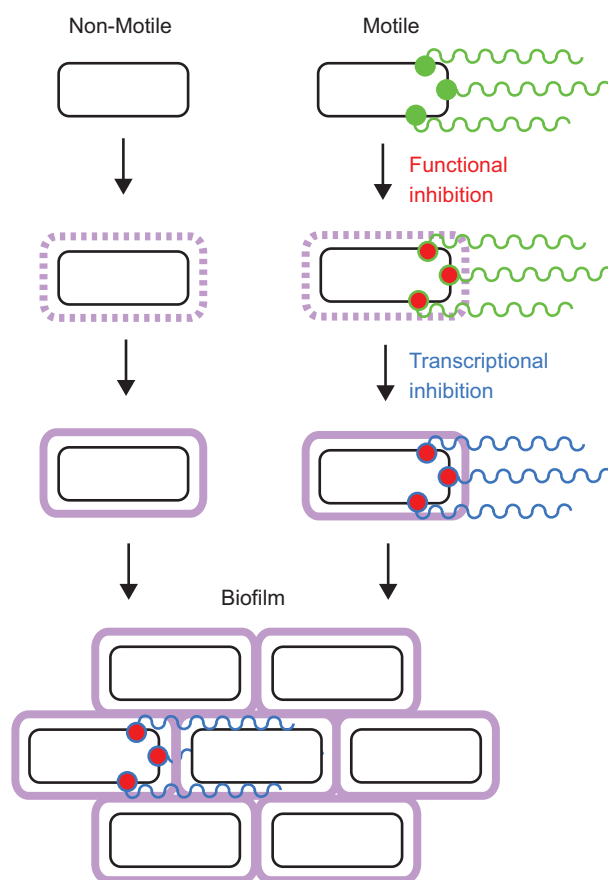


Fig. 2. General steps of the motility-to-biofilm transition. Biofilms may be assembled from motile or non-motile cells. We propose that non-motile cells may proceed to biofilm assembly directly whereas motile cells must first inhibit motility. Flagella that are rotating are labeled in green, functional inhibition of the flagellum is labeled in red, decreased transcription of flagella genes is denoted by labeling the flagellum blue, and the extracellular matrix is shown in purple. Nonmotile cells increase transcription of and synthesize the extracellular matrix components (purple), while motile cells first inhibit motility functionally at the flagella basal body (red), then decrease transcription of flagella genes (blue) while increasing expression of the extracellular matrix components (purple). Finally, both types of cells form a biofilm together.

Gram-negative flagella, genes homologous to Gram-negative flagellar genes are encoded in the genome, with many concentrated in the long 31 gene *fla/che* operon (Márquez-Magaña & Chamberlin, 1994). Only a subpopulation of cells synthesize flagella due to a failure to express and activate the Class II encoded sigma factor, σ^D , that directs the expression of Class III genes encoding, among other things, flagellin (Kearns & Losick, 2005; Cozy & Kearns, 2010). The relative subpopulation that is motile is genetically determined and varies from strain to strain (Kearns & Losick, 2005). *Bacillus subtilis* laboratory strains have the capacity to swim in liquid media, but the ancestral strain also has the ability to swarm over solid surfaces (Kearns & Losick, 2003; Patrick & Kearns, 2009). Ancestral strains swarm because they encode functional alleles of two proteins that domesticated strains lack: Sfp that promotes synthesis of a surfactant and SwrA that activates the *fla/che* operon (Kearns *et al.*, 2004; Kearns & Losick, 2005; Patrick & Kearns, 2009). The ancestral strain also has the ability to form nonmotile biofilms (Branda *et al.*, 2001; McLoon *et al.*, 2011).

Bacillus subtilis forms biofilms either as floating pellicles when grown in liquid, or as colonies with complex architecture when grown on a solid surface (Branda *et al.*, 2001). The biofilm extracellular matrix is complex and composed of many proteins and polysaccharides (Marvasi *et al.*, 2010). One secreted protein found in the matrix is BslA (formerly YuaB) that confers hydrophobicity to the biofilm aggregate (Verhamme *et al.*, 2009; Ostrowski *et al.*, 2011; Kobayashi & Iwano, 2012). The *tapA-sipW-tasA* operon (*tasA* operon) encodes other protein components of the matrix including the protein TasA that forms amyloid fibers anchored to the cell surface by the protein TapA (Branda *et al.*, 2006; Chu *et al.*, 2006; Romero *et al.*, 2010, 2011). Together TapA and TasA tether and/or organize another major component of the matrix, EPS. The EPS is of unknown chemical structure and is synthesized by the proteins encoded within the long 15 gene *eps* operon (Branda *et al.*, 2001; Kearns *et al.*, 2005). The *eps* operon contains a pseudoknot RNA-structure called EAR (eps-associated RNA) that acts to promote processive antitermination of RNA polymerase and ensure complete operon transcription (Irnov & Winkler, 2010). Also encoded within the *eps* operon is the bifunctional protein EpsE that inhibits flagellar motility (Fig. 3; Table 1).

EpsE inhibits motility by acting as a clutch on the flagellar rotor (Blair *et al.*, 2008; Guttenplan *et al.*, 2010). When EpsE is expressed, either alone from an artificial promoter or expressed together with the rest of the *eps* operon during biofilm formation, flagellar rotation comes to a halt. EpsE is thought to inhibit the flagella rotor FliG by direct protein-protein interaction because an

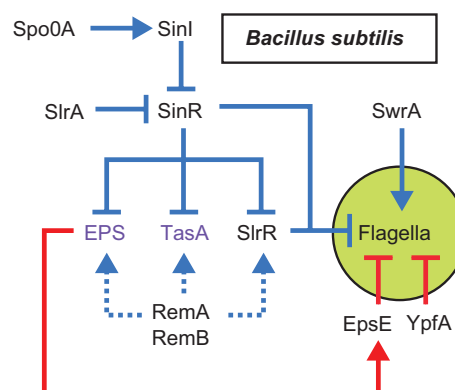


Fig. 3. Network of *Bacillus subtilis* biofilm-related motility regulation. Transcriptional regulation is labeled in blue, functional regulation labeled in red, and the biofilm matrix products in purple. Green circle highlights the flagellum. Solid lines indicate a confirmed direct interaction, while dashed lines indicate that the interaction is poorly understood and could be indirect or direct.

EpsE-GFP fusion localizes as puncta along the cell membrane, reminiscent of peritrichously arranged flagellar basal bodies (Fig. 1). Furthermore, EpsE puncta colocalize with flagellar basal bodies and are dependent upon critical amino acids on an exposed surface of FliG (Blair *et al.*, 2008; Guttenplan *et al.*, 2010, 2013). EpsE could have inhibited flagellar rotation either like a brake that caused rotation to lock up, or like a clutch that resulted in unpowered flagella that could be externally rotated. A clutch-like mechanism was supported because when cells were tethered by one flagellum and EpsE was expressed, the cell bodies rotated from the external torque of Brownian motion as freely as one would expect by diffusion. Thus, EpsE acts like a clutch by separating the flagellar drive train (the rotor FliG) from the power source (the proton channel MotA/MotB) thereby disabling rotation. EpsE is the first flagellar clutch protein identified, and it has been speculated that the clutch might be required for a rapid loss of motility during early biofilm formation.

Mutation of EpsE results in a severe defect in biofilm formation but attributing the defect to the clutch activity was complicated by the fact that EpsE is homologous to members of the group II glycosyltransferases. Glycosyltransferase enzymes are commonly encoded within operons that synthesize EPS, and EpsE contains the highly conserved glycosyltransferase active site residues (Guttenplan *et al.*, 2010). Mutation of the active site residues abolish synthesis of the biofilm EPS and biofilm formation but do not abolish flagellar clutch function (Blair *et al.*, 2008; Guttenplan *et al.*, 2010). Clutch activity, rather, requires a series of residues predicted to occupy a common surface of an exposed alpha helix that may represent the contact site with FliG, proving that EpsE is

Table 1. Biofilm-related proteins that regulate motility

Protein	Function	Annotation	References
<i>Bacillus subtilis</i> functional regulators			
EpsE	Flagellar clutch, inhibits FlhG	Glycosyltransferase	Blair <i>et al.</i> (2008) and Guttenplan <i>et al.</i> (2010)
RemA	Activator of EpsE	No homology/domains	Winkelman <i>et al.</i> (2009)
RemB	Activator of EpsE	No homology/domains	Winkelman <i>et al.</i> (2009)
YpfA	Inhibits MotA	YcgR homolog/PilZ domain	Chen <i>et al.</i> (2012a, b)
<i>Bacillus subtilis</i> transcriptional regulators			
SinI	Antagonist of SinR	Small protein	Bai <i>et al.</i> (1993) and Scott <i>et al.</i> (1999)
SinR	Repressor of EpsE and co-inhibitor of <i>fla/che</i> with SlrR	DNA-binding protein	Kearns <i>et al.</i> (2005), Chai <i>et al.</i> (2010) and Cozy <i>et al.</i> (2012)
SlrA	Antagonist of SinR	Small protein	Kobayashi (2008), Chai <i>et al.</i> (2009) and Cozy <i>et al.</i> (2012)
SlrR	Co-inhibitor of <i>fla/che</i> with SinR	DNA-binding protein	Chu <i>et al.</i> (2008), Chai <i>et al.</i> (2010) and Cozy <i>et al.</i> (2012)
SwrA	Activator of <i>fla/che</i> operon	No homology/domains	Kearns <i>et al.</i> (2004), Kearns & Losick (2005) and Patrick & Kearns (2009)
<i>Pseudomonas aeruginosa</i> functional regulators			
BifA	Increase flagella reversals	c-di-GMP hydrolase	Kuchma <i>et al.</i> (2007)
FlhD	Binds mucin, tethers flagella	Cap of flagellar filament	Arora <i>et al.</i> (1998) and Landry <i>et al.</i> (2006)
SadC	Decrease flagella reversals	c-di-GMP synthase	Caiazza & O'Toole (2004) and Merritt <i>et al.</i> (2007)
<i>Pseudomonas aeruginosa</i> transcriptional regulators			
ArmZ	Repress <i>fleQ</i> transcription	DNA-binding protein	Garrett <i>et al.</i> (1999), Tart <i>et al.</i> (2005, 2006)
FleQ	Activator of flagellar genes/regulator of <i>pel</i> transcription	DNA-binding protein	Arora <i>et al.</i> (1997), Hickman & Harwood (2008) and Baraquet <i>et al.</i> (2012)
<i>Vibrio</i> sp. transcriptional regulators			
FlrA	Activates flagellar genes	NtrC homolog	Prouty <i>et al.</i> (2001) and Correa & Klose (2005)
HapR	Activates motility	LuxR homolog	Yildiz <i>et al.</i> (2004) and Waters <i>et al.</i> (2008)
OpaR	Activates motility	HapR homolog	McCarter (1998)
ScrC	Activates motility	c-di-GMP synthase/hydrolase	Boles & McCarter (2002), Ferreira <i>et al.</i> (2008) and Trimble & McCarter (2011)
ScrG	Activates motility	c-di-GMP hydrolase	Kim & McCarter (2007)
VpsT	Repressor of flagellar gene expression.	DNA-binding protein	Casper-Lindley & Yildiz (2004) and Krasteva <i>et al.</i> (2010)
<i>Escherichia coli</i> functional regulator			
YcgR	Slows rotation and changes reversal frequency at MotA/FlhG/FlhM	c-di-GMP-binding protein	Boehm <i>et al.</i> (2010), Fang & Gomelsky (2010) and Paul <i>et al.</i> (2010)
<i>Escherichia coli</i> transcriptional regulators			
CsrA	Stabilizes <i>flhDC</i> mRNA	RNA-binding protein	Wei <i>et al.</i> (2001)
OmpR	Represses <i>flhDC</i> expression	DNA-binding protein	Shin & Park (1995) and Prigent-Combaret <i>et al.</i> (1999)
RcsBA	Represses <i>flhDC</i> expression	DNA-binding complex	Gottesman <i>et al.</i> (1985), Francez-Charlot <i>et al.</i> (2003) and Fredericks <i>et al.</i> (2006)

bifunctional (Guttenplan *et al.*, 2010). Interestingly, the residues in EpsE necessary for clutch function are more highly conserved among related glycosyltransferases (even those from nonmotile organisms) than the residues in FlhG found to be necessary for clutch contact. Thus, under selection for motility inhibition, FlhG may have evolved to interact with EpsE, an enzyme that happened to be present during biofilm formation. Finally, both the EPS biosynthetic and clutch functions of EpsE contribute to biofilm formation as mutation of both activities synergize to create a greater defect than either alone (Guttenplan *et al.*, 2010).

What is the function of the clutch during biofilm formation? First, functional inhibition of the flagellum is a rapid mechanism to inhibit motility and may either act early in biofilm formation and/or at intermediate stages

to stabilize cell aggregates under stress. Second, if the metaphor of a clutch in an automobile is accurate, EpsE may be reversible if biofilm formation is prematurely aborted. Third, EpsE is the last protein encoded in the *eps* operon upstream of a series of internal terminators that are counteracted by the EAR antitermination *cis*-element (Irnov & Winkler, 2010). If EAR-dependent antitermination is regulated, there may be conditions under which EpsE inhibits motility without cells progressing immediately to biofilm formation due to termination prior to transcription of the rest of the operon. Finally, the function of the clutch may have a long-term advantage in preserving flagellar function by relieving selective pressure against the enrichment of motility mutants. For example, a strain that is clutch-deficient forms biofilms, but these biofilms accumulate mutations in genes

required for flagellar motility (Guttenplan *et al.*, 2010). Thus, in the absence of the clutch, the selection for biofilm formation comes at the genetic cost of heritable flagellar defects such that the motility of the biofilm descendants is compromised. The very fact that selective pressure acts against motility in the biofilm provides direct evidence that motility inhibition is indeed an important step (Guttenplan *et al.*, 2010).

A second functional regulator of flagella has recently been discovered in *B. subtilis*. YpfA is a protein that contains a PilZ domain attributed to c-di-GMP binding (Chen *et al.*, 2012a) (Table 1). Although c-di-GMP has only been directly observed in Gram-positives once, in *Clostridium difficile*, indirect reporters suggest it may also be present in *B. subtilis* (Sudarsan *et al.*, 2008; Bordeleau *et al.*, 2011; Chen *et al.*, 2012a; Purcell *et al.*, 2012). Mutation of a phosphodiesterase homolog called YuxH abolished motility, and motility was restored to the YuxH mutant by mutation of YpfA (Chen *et al.*, 2012a). Interestingly, YpfA interacted with MotA in bacterial two-hybrid analysis and both MotA interaction and motility inhibition was abolished by mutations in the c-di-GMP-binding PilZ domain (Fig. 3). Whether c-di-GMP is actually present in *B. subtilis* has yet to be demonstrated and whether either YuxH or YpfA are related to biofilms is unclear. Mutation of either YuxH or YpfA had little to no detectable effect on biofilm formation, but we note that biofilm inhibition could have been masked by the rapid selection of nonmotile suppressor mutations, as was found with EpsE clutch activity. Whether YpfA acts as a clutch, brake, or other mechanism remains to be elucidated.

Motility in *B. subtilis* is also inhibited at the level of transcription during biofilm formation but when and where transcriptional regulation occurs is a complex issue. Expression of flagellar basal body genes decreases as biofilm formation progresses and flagellar filament gene expression is repressed in the mature biofilm (Kobayashi, 2007a, b; Vlamakis *et al.*, 2008). Furthermore, the control of flagellar gene expression within the biofilm is spatially partitioned. By fluorescently labeling subpopulations with different fluorophores, Vlamakis *et al.* (2008) showed that in a cross-section of a biofilm, cells that express the flagella filament are localized to the periphery and the region directly adjacent to the substrate. As cells increased their distance from the substrate in three dimensions, flagellar filament expression decreased and gave rise to the expression of the biofilm matrix genes. There appears to be a tight and inverse coupling of the transcriptional regulation of motility and biofilm matrix genes that occurs in both space and time.

Multiple regulators converge to spatially regulate motility and biofilm gene expression. The master regulator of

biofilm formation, SinR, represses expression of both the *eps* and *tasA* operons (Kearns *et al.*, 2005; Branda *et al.*, 2006; Chu *et al.*, 2006). SinR is a phage-like repressor protein that dimerizes and binds to a consensus sequence (GTTCTYT) to control a relatively small set of genes mostly dedicated to biofilm formation (Gaur *et al.*, 1991; Lewis *et al.*, 1998; Kearns *et al.*, 2005) (Table 1). Encoded immediately upstream of *sinR* is SinI, a small protein that binds to SinR and inhibits SinR dimerization (Bai *et al.*, 1993; Lewis *et al.*, 1998; Scott *et al.*, 1999) (Table 1). In response to biofilm-initiating signals, the response regulator Spo0A activates the expression of SinI, SinR is antagonized, and the biofilm genes are derepressed (Gaur *et al.*, 1988; Branda *et al.*, 2001; Hamon & Lazazzera, 2001; Chai *et al.*, 2011) (Fig. 3). Furthermore, Spo0A activation is heterogeneous in the population and heterogeneous activation likely contributes to the three-dimensional spatial patterning of biofilm gene expression (Chai *et al.*, 2008; Vlamakis *et al.*, 2008).

The genes expressed by SinI antagonism of SinR not only include the matrix synthesizing operons but also include SlrR, a paralog of SinR. Indeed, SinR and SlrR are sufficiently similar that they cross-react with SinR-specific polyclonal antibodies (Chu *et al.*, 2008). SlrR activates expression of the *tasA* operon and redirects SinR function by forming a SinR/SlrR heterodimer that corepresses expression of the autolysin genes and the *fla/che* operon that codes for the flagellar basal body proteins (Chu *et al.*, 2008; Chai *et al.*, 2010; Cozy *et al.*, 2012) (Fig. 3; Table 1). A third protein, YmdB, is also involved in the regulation of flagellar gene expression through its activation of SlrR (Diethmaier *et al.*, 2011). Therefore, the repression of motility gene expression is concomitant with activation of extracellular matrix genes and in the absence of new transcription; the flagella are presumably diluted to extinction through cell growth within the biofilm.

There may be additional mechanisms and pathways to inhibit motility in the biofilm. Other regulators are involved in joint biofilm and motility regulation, but the mechanism of activation or repression is less understood (Fig. 3). SlrA is a paralog of the SinI protein and can antagonize SinR to derepress biofilm genes and inhibit *fla/che* operon transcript levels (Kobayashi, 2008; Chai *et al.*, 2009; Cozy *et al.*, 2012) (Table 1). RemA and RemB are two other transcriptional activators of biofilm formation that act in parallel to SinR, but the stimulus input and mechanisms of regulatory output of either protein is unknown (Winkelman *et al.*, 2009) (Table 1). DegU is a response regulator that regulates both motility and biofilm formation in a complex manner (Kobayashi, 2007b; Verhamme *et al.*, 2007). When the concentration of phosphorylated DegU is low in the cell, swarming

motility and flagellar basal body gene expression are activated (Kobayashi, 2007b; Verhamme *et al.*, 2007; Tsukahara & Ogura, 2008). However, as the amount of phosphorylated DegU increases, DegU-P represses expression of flagellar basal body genes and activates the anti-sigma factor FlgM, which in turn antagonizes the σ^D sigma factor that expresses the flagellar filament protein (Amati *et al.*, 2004; Hsueh *et al.*, 2011). Additionally, phosphorylated DegU activates genes required for biofilm formation (Verhamme *et al.*, 2009; Ostrowski *et al.*, 2011). DegU is phosphorylated by a cytoplasmic histidine kinase DegS, but the stimulus that controls the amount of phosphorylated DegU remains poorly understood (Mukai *et al.*, 1990).

Motility inhibition during *B. subtilis* biofilm formation is presumed to be initiated by extracellular signals that are transduced to activate SinI (Kearns *et al.*, 2005; Lopez *et al.*, 2009, 2010; Shemesh *et al.*, 2010; Chen *et al.*, 2012b) (Fig. 3). SinI antagonizes SinR to derepress the *eps* and *tasA* operons that encode proteins that begin assembling the extracellular matrix and the gene that encodes SlrR. As SlrR accumulates, it redirects SinR to repress the *fla/che* operon transcript abundance, thereby inhibiting flagellar gene expression. Flagella are numerous and stable, and thus, inhibiting transcript is insufficient to inhibit motility. EpsE is also made and inhibits flagellar rotation in the short term, while *de novo* flagellar synthesis is inhibited at the transcriptional level over the long term. Finally, because flagellar gene expression in *B. subtilis* is heterogeneous, there is a pre-existent nonmotile subpopulation that need not inhibit motility and may proceed to biofilm formation directly (Kearns & Losick, 2005; Chai *et al.*, 2008, 2010; Lopez *et al.*, 2009).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes dangerous infections of the lungs in patients with cystic fibrosis (CF). *Pseudomonas aeruginosa* synthesizes a single polar flagellum to swim in liquid media and also swarms over solid surfaces. During swarming, either the number of flagella at the pole increases and/or the single polar flagellum is augmented by an alternative swarming-specific stator complex (Köhler *et al.*, 2000; Rashid & Kornberg, 2000; Doyle *et al.*, 2004; Toutain *et al.*, 2005). Flagellar biosynthesis is organized in a Class I-IV hierarchy where Class I is represented by the master regulator protein FleQ, classes II and III encode the hook-basal body genes, and Class IV encodes flagellin and the chemotaxis proteins (Arora *et al.*, 1997; Jyot *et al.*, 2002; Dasgupta *et al.*, 2003). *Pseudomonas aeruginosa* also uses type IV pilus-based twitching motility to move over solid surfaces but this form of

motility will not be discussed further here (for review see Burrows, 2012).

Pseudomonas aeruginosa forms pellicle biofilms at air–liquid interfaces as well as liquid–solid submerged biofilms attached to glass or other substrates. The primary structural component of the *P. aeruginosa* biofilm is extracellular polysaccharides (EPS) that depend not only on the strain but also on the conditions under which the biofilm forms. EPS biosynthesis proteins are encoded by genes within either the *pel* and/or the *psl* operons depending on the strain considered (Friedman & Kolter, 2004a, b; Jackson *et al.*, 2004; Matsukawa & Greenberg, 2004). The chemical structure of the PSL polysaccharide has recently been elucidated as a repeating pentasaccharide of D-mannose, D-glucose, and L-rhamnose, but the chemical structure of PEL is unknown (Ma *et al.*, 2007; Byrd *et al.*, 2009). Finally, some clinical isolates of *P. aeruginosa* isolated directly from the lung of a CF patient synthesize the abundant EPS alginate using enzymes encoded within the *alg* operons (Govan & Deretic, 1996; Wozniak *et al.*, 2003b; Ramsey & Wozniak, 2005). The chemical structure of alginate is an unbranched heteropolymer of O-acetylated β -1,4-linked D-mannuronic and L-guluronic acids (Linker & Jones, 1966; Ramsey & Wozniak, 2005). Other components of the extracellular matrix include DNA and proteins, but the exact composition and contribution of these structures is unknown (Whitchurch *et al.*, 2002; Matsukawa & Greenberg, 2004; Borlee *et al.*, 2010).

Pseudomonas aeruginosa requires cell motility to form a biofilm, but unregulated motility during biofilm formation destabilizes the biofilm structure (O'Toole & Kolter, 1998; Vallet *et al.*, 2001; Whitely *et al.*, 2001; Sauer *et al.*, 2002; Klausen *et al.*, 2003). Cells that are enhanced for swarming motility form a flat and featureless biofilm, while cells that are deficient for swarming motility form a more structured biofilm with large tower-like aggregates (Shrout *et al.*, 2006). Thus, a decrease in motility during biofilm formation may promote and stabilize complex three-dimensional architectural development. Consistent with motility antagonizing biofilms, *P. aeruginosa* colony morphology variants that inherently form more robust biofilms have a decrease in motility compared with their parental strains (Kirisits *et al.*, 2005). Thus, the proper timing of motility control is required for forming wild-type biofilm architecture, but motility *per se* can be bypassed entirely when cells are genetically locked in a biofilm-proficient state.

In *P. aeruginosa*, flagella are functionally regulated at the level of flagellar reversal frequency (Fig. 4). Strains mutated for the protein SadC were 'surface attachment defective', pellicle-deficient and had increased motility in swarming assays (Merritt *et al.*, 2007). Increased swarming

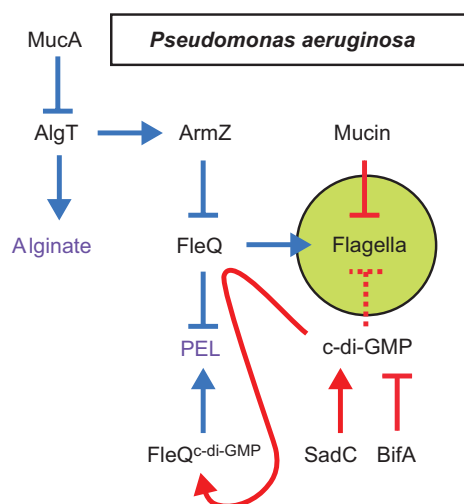


Fig. 4. Network of *Pseudomonas aeruginosa* biofilm-related motility regulation. Transcriptional regulation is labeled in blue, functional regulation labeled in red, and the biofilm matrix products in purple. Green circle highlights the flagellum. C-di-GMP in superscript indicates that the protein needs to be bound to c-di-GMP to carry out the indicated activity. Solid lines indicate a confirmed direct interaction, while dashed lines indicate that the interaction is poorly understood and could be indirect or direct.

migration in the *sadC* mutant was correlated with an increase in basal flagella reversal frequency in a high-viscosity medium (Caiazza *et al.*, 2007; Merritt *et al.*, 2007). SadC is a diguanylate cyclase that synthesizes c-di-GMP and *sadC* mutants showed a reduction in the cytoplasmic c-di-GMP levels (Merritt *et al.*, 2007) (Table 1). Conversely, mutants defective in the phosphodiesterase BifA exhibited decreased swarming motility that was correlated with a decrease in basal flagella reversal frequency and an increase in c-di-GMP levels (Kuchma *et al.*, 2007) (Table 1). The output of elevated c-di-GMP levels that controls the flagellar reversal frequency is unknown, but the inputs that elevate c-di-GMP seem to be related to the type IV pili that mediate twitching motility and the EPS matrix itself (Kuchma *et al.*, 2010, 2012; Irie *et al.*, 2012). The role of controlling flagellar reversal frequency early in biofilm formation is also unclear but maintenance of a critical frequency of basal reversals has been correlated with a cell's ability to navigate pores in soft agar (Wolfe & Berg, 1989). Thus, reversal frequency control may be important to facilitate spread through a matrix such as the mucus of the CF lung even in the absence of chemotactic signals.

In isolates from CF lungs, motility of *P. aeruginosa* is mechanically inhibited by the interaction with host mucin, the protein component of mucus found abundantly in the airway of CF patients. When *P. aeruginosa* is inoculated onto a glass surface coated with mucin, sur-

face motility is inhibited and biofilm formation is enhanced (Landry *et al.*, 2006). One reason for motility inhibition is that the FliD flagellar cap protein found at the tip of the filament binds to mucin to tether the cells and constrain rotation (Arora *et al.*, 1998; Landry *et al.*, 2006) (Table 1). The FliD–mucin interaction may be a mechanism for the cell to abolish motility and promote adherence in a single step (Fig. 4). That FliD also acts as a mucin receptor may contribute to the persistence of biofilms in CF patients' lungs due to a reduced ability of the cells to disperse.

Once biofilm formation has been initiated, flagellar gene expression decreases as a biofilm matures (O'Toole & Kolter, 1998; Vallet *et al.*, 2001; Whitely *et al.*, 2001; Sauer *et al.*, 2002; Klausen *et al.*, 2003; Wolfgang *et al.*, 2004). One way in which cells coordinate flagellar and biofilm gene expression is by controlling the transcriptional regulator FleQ. FleQ activates flagellar genes and represses *pel* operon expression (Arora *et al.*, 1997; Hickman & Harwood, 2008) (Fig. 4). However, when c-di-GMP levels rise in the cell, FleQ binds to c-di-GMP and activates rather than represses *pel* transcription (Hickman & Harwood, 2008; Baraquet *et al.*, 2012). The mechanism of the FleQ functional switch is unclear, but it is likely due to conformational changes caused by the interactions with both c-di-GMP and FleN, the antagonist of FleQ (Baraquet *et al.*, 2012). Binding of FleQ to c-di-GMP does not inhibit flagellar gene expression significantly and presumably, other mechanisms are involved in the decrease in flagellar gene expression during biofilm formation.

Most patients are infected with motile strains of *P. aeruginosa*, but bacteria re-isolated from infected CF lungs are typically nonmotile (Luzar *et al.*, 1985; Burke *et al.*, 1991; Mahenthalingam *et al.*, 1994). Loss of motility is genetic, as selective pressure enriches for a frameshift mutation in a gene *mucA* that reduces flagellar gene expression and enhances biofilm formation (Martin *et al.*, 1993). The *mucA* gene encodes the protein MucA, the antisigma factor of an alternative sigma factor called AlgT (Govan & Deretic, 1996; Tart *et al.*, 2005). Unchecked, AlgT directs RNA polymerase to express the genes for alginate biosynthesis and the gene *armZ* (formerly *algZ*), encoding the transcription factor ArmZ (Wozniak *et al.*, 2003a; Tart *et al.*, 2006). ArmZ in turn represses expression of FleQ, thereby inactivating expression of flagellar biosynthesis genes and inhibiting motility (Garrett *et al.*, 1999; Tart *et al.*, 2005, 2006) (Fig. 4; Table 1). The mutation in *mucA* occurs at high frequency in a homopolymeric tract and therefore can be reselected for gain-of-motility reversion by restoring the wild-type frame (Martin *et al.*, 1993; Henderson *et al.*, 1999). High frequency, reversible frameshifting may reduce selective

pressure on the mutation of the flagellar genes themselves and ensures that if the cells are reselected to resume flagellum biosynthesis; all of the required genes are intact.

Late in biofilm maturation, motility is re-activated to permit cell dispersal (Sauer *et al.*, 2002; Davies & Marques, 2009). Biofilm dispersion is triggered by the cellular release of a small chemical signal, *cis*-2-decenoic acid (Davies & Marques, 2009). The precise cellular target of *cis*-2-decenoic acid remains unknown, but one protein that has been identified as being involved in dispersion is BdlA (Morgan *et al.*, 2006). In *bdlA* mutants, biofilms do not disperse upon nutrient starvation conditions, and BdlA mutants have a higher intracellular concentration of c-di-GMP compared with wild type (Morgan *et al.*, 2006). The mechanism of BdlA involvement in dispersion is presumably indirect since the protein does not contain a GGDEF, EAL, or known c-di-GMP-binding domain. Other compounds have been discovered to disperse biofilms but if and how these compounds activate motility is unknown (Chen & Stewart, 2000; Banin *et al.*, 2006; Barraud *et al.*, 2006; Kolodkin-Gal *et al.*, 2010; Hibbing & Fuqua, 2012).

The motility regulation in *P. aeruginosa* biofilms is controlled at multiple levels (Fig. 4). Perhaps, early in an infection, extracellular stimuli signal for an increase in the cellular levels of c-di-GMP, which decreases the flagellar reversal rate, impairs dispersal and promotes aggregation. Binding of the flagellar cap to mucin may constrain flagellar rotation and tether the bacteria as c-di-GMP levels continue to rise in the cytoplasm. High levels of c-di-GMP inhibit flagellar gene expression while activating EPS biosynthesis to construct the biofilm matrix. At some point in the process, selective pressure may select for MucA mutants to genetically trap a subpopulation of bacteria in a nonmotile, biofilm-producing state. Late in biofilm formation, the synthesis of *cis*-2-decenoic acid may reactivate motility and stimulate dispersal of the biofilm.

***Vibrio* species**

Vibrio cholerae is a Gram-negative bacterium that is the causative agent of the severe gastrointestinal infection cholera that swims in liquid by synthesizing a single polar flagellum powered by the sodium motive force (Kojima *et al.*, 1999). Flagellar biosynthesis genes are organized in a four-level hierarchy (Class I–IV) (Prouty *et al.*, 2001). The master regulator of flagellar biosynthesis is the NtrC-like FlrA protein that activates σ^{54} RNA polymerase to express class II basal body genes. FlrA also activates both a second NtrC-like regulator FlrC that redirects RNA polymerase to express Class III rod and hook genes, and an alternative sigma factor σ^{28} that directs RNA polymerase to express the Class IV filament

genes (Prouty *et al.*, 2001; Correa & Klose, 2005). Unlike many other bacteria, the flagellum is sheathed by an extension of the outer membrane that surrounds the filament, but the function of the sheath is unknown (Sjölblad *et al.*, 1983). Virulence factor adhesins are coactivated with the flagellar biosynthesis genes and motility *per se* is required for *V. cholerae* virulence (Lee *et al.*, 2001; Butler & Camilli, 2005; Morris *et al.*, 2008; Syed *et al.*, 2009).

Vibrio cholerae forms biofilms that manifest as pellicles at air–liquid interfaces, as surface-associated submerged biofilms that attach to glass or other substrates and finally as rugose colonies of complex architecture (Yildiz & Visick, 2009). The biofilm extracellular matrix is complex and is composed of *Vibrio* polysaccharide (VPS) and proteins. Two of the proteins, RbmC and Bap1, are seemingly redundant paralogs that coat substrate surfaces and promotes cell adhesion, while another protein RbmA coats cell surfaces and promotes cell–cell cohesion (Fong *et al.*, 2006; Fong & Yildiz, 2007; Absalon *et al.*, 2011; Berk *et al.*, 2012). VPS is synthesized by proteins encoded in 18 genes divided between two operons called *vps-I* and *vps-II* (Yildiz & Schoolnik, 1999). While the precise structure of the VPS is unknown, sugar composition analysis suggests that it is composed primarily of glucose and galactose with some xylose, mannose, and *N*-acetyl glucosamine (Yildiz & Schoolnik, 1999). Mutations that disrupt either VPS or matrix protein synthesis disrupt biofilm formation. Extracellular DNA is also a component of the biofilm matrix, but how it interacts with the VPS and matrix proteins is unknown (Seper *et al.*, 2011). Biofilm formation is thought to not be essential for virulence *per se* but is rather thought to be essential for survival of cholera-causing bacteria in the environmental water reservoir (Cowell, 1996; Faruque *et al.*, 2006).

Like other bacteria, production of c-di-GMP is correlated with the motility-to-biofilm transition. *Vibrio cholerae* encodes 53 putative diguanylate cyclase (GGDEF domain containing) and phosphodiesterase (EAL domain containing) homologs, and at least five proteins with PilZ domains that potentially serve as c-di-GMP effector proteins (Lim *et al.*, 2006; Pratt *et al.*, 2007). Mutants of the putative phosphodiesterases CdgC, CdgJ, RocS, and MbaA are less motile compared with wild type, consistent with high levels of c-di-GMP inhibiting motility (Lim *et al.*, 2006; Liu *et al.*, 2010). Conversely, mutants of putative diguanylate cyclases CdgD, CdgH, and Vpvc are more motile compared with wild type, consistent with low levels of c-di-GMP promoting motility (Lim *et al.*, 2006; Beyhan & Yildiz, 2007; Beyhan *et al.*, 2008). Finally, mutations in the PilZ domain-containing proteins PlzB and PlzC resulted in a decrease in motility and biofilm formation compared with wild type (Pratt *et al.*, 2007). Taken together, c-di-GMP regulates both motility and

VPS production, but how the small molecule information is integrated over time remains mysterious.

The study of the transition from motility-to-biofilm formation has been aided by the isolation of spontaneous mutants that produce smooth or rugose colony variants (White, 1938). The rugose variant is enhanced for biofilm formation, exhibits increased VPS synthesis and has reduced motility (Yildiz *et al.*, 2004). By contrast, the smooth colony variant is motile but biofilm-deficient (Yildiz *et al.*, 2004). One mutation that mediates the smooth-to-rugose genetic transition is a gain-of-function allele of the VpvC diguanylate cyclase that increases c-di-GMP levels in the cell (Beyhan & Yildiz, 2007) (Fig. 5). Thus, the rugose variants are locked in a non-motile biofilm state because c-di-GMP is constitutively high. Increased c-di-GMP levels decrease motility and increase colony rugosity in part by increasing the levels of two cytoplasmic regulatory proteins VpsR and VpsT (Yildiz *et al.*, 2004; Lim *et al.*, 2006).

The two-component response regulator proteins VpsR and VpsT are required to activate the expression of VPS biosynthesis genes, and mutation of either gene abolishes biofilm formation (Yildiz *et al.*, 2001; Casper-Lindley & Yildiz, 2004) (Fig. 5). VpsR binds to the *vps* promoter region when phosphorylated on a conserved aspartate residue, but the histidine kinase responsible for phosphorylation is unknown (Lauriano *et al.*, 2004). VpsT on the other hand, only binds to the *vps* promoter region when c-di-GMP is bound (Krasteva *et al.*, 2010) (Fig. 5; Table 1). Although VpsR and VpsT have very similar regulons, VpsR exerts larger effects on gene expression perhaps because VpsR also activates VpsT expression (Beyhan *et al.*, 2007; Srivastava *et al.*, 2011). Mutation of genes required for VPS synthesis in a rugose variant abolishes rugose colony morphology and biofilm formation (Yildiz *et al.*, 2001, 2004).

The regulation of motility during biofilm formation in *V. cholerae* is complex and relatively poorly understood. Flagella are required for early stages of biofilm development and mutants lacking the flagellum have a decrease in biofilm formation (Watnick & Kolter, 1999). Flagella function itself may be directly required for *vps* gene expression, as paralysis of flagellar rotation abolished *vps* gene expression in a manner that could be bypassed by constitutive expression of VpsR (Lauriano *et al.*, 2004) (Fig. 5). As the biofilm matures, however, motility appears to be lost or at least diminished. One possible explanation for reduced motility is due to flagella tethering by VPS overproduction and indeed, four flagellins comprising the flagellar filament copurified with the biofilm matrix (Absalon *et al.*, 2011). Simply constraining flagella, however, is likely not sufficient as mutation of genes required for VPS synthesis in a rugose variant

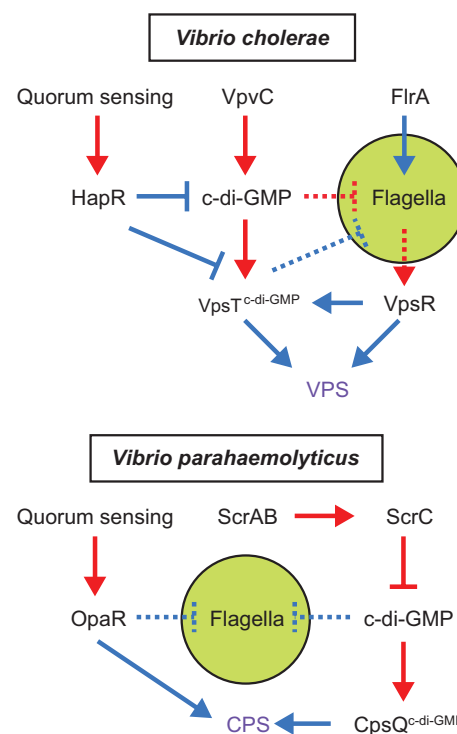


Fig. 5. Network of *Vibrio cholerae* and *V. parahaemolyticus* biofilm-related motility regulation. Transcriptional regulation is labeled in blue, functional regulation labeled in red, and the biofilm matrix products in purple. Green circle highlights the flagellum. C-di-GMP in superscript indicates that the protein needs to be bound to c-di-GMP to carry out the indicated activity. Solid lines indicate a confirmed direct interaction, while dashed lines indicate that the interaction is poorly understood and could be indirect or direct.

abolishes biofilm formation but did not restore motility (Yildiz *et al.*, 2001, 2004). Furthermore, the rugose variant retained polar flagella, despite being nonmotile suggesting a level of as-yet-unidentified functional regulation on the flagellar motor (Yildiz *et al.*, 2004). Finally, cells in a mature biofilm decrease expression of the flagella filament, *flaA* (Watnick *et al.*, 2001; Moorthy & Watnick, 2004). VpsT in its c-di-GMP bound state inhibits flagellar gene expression by an unknown mechanism that could be mediated by direct binding to flagellar promoters or by controlling a secondary regulator (Fig. 5; Lim *et al.*, 2006; Beyhan *et al.*, 2006; Krasteva *et al.*, 2010).

In addition to decreasing motility to promote biofilm formation, *V. cholerae* may also increase motility late in the cycle to induce dispersion from a mature biofilm. *Vibrio cholerae* is found in the aquatic environment attached to surfaces as well as in the planktonic form between human outbreaks (Islam *et al.*, 1994). Quorum sensing may be involved in the transition back to the planktonic state since a LuxR homolog, HapR, promotes motility and directly represses expression of VpsT at high

cell densities (Yildiz *et al.*, 2004; Waters *et al.*, 2008) (Table 1). Mutation of HapR increases biofilm formation, VPS production, and decreases motility (Zhu *et al.*, 2002; Hammer & Bassler, 2003; Zhu & Mekalanos, 2003). HapR also decreases the amount of c-di-GMP in the cell by regulating expression of putative diguanylate cyclases and phosphodiesterases (Waters *et al.*, 2008; Hammer & Bassler, 2009). Thus, as cell density increases quorum signals activate HapR to mediate the transition from the biofilm state back toward motility.

Flagella and motility are required early in biofilm formation perhaps for locating and interacting with an appropriate surface. Motility appears to be promoted by low levels of c-di-GMP potentiated by PilZ-containing proteins such as PlzB and PlzC. The environmental stimuli to initiate biofilm formation are diverse and include low salt, indole, and polyamines (Karatan *et al.*, 2005; Mueller *et al.*, 2009; Shikuma & Yildiz, 2009; Shikuma *et al.*, 2012). Once biofilm formation is initiated, c-di-GMP levels rise, VpsR and VpsT activate matrix biosynthesis genes, and cells begin to aggregate in the biofilm. Flagella become inhibited at the functional level; perhaps either paralyzed, like *B. subtilis*, or altered for behavior, like *P. aeruginosa*. Furthermore, flagella gene expression is inhibited by some mechanism involving, at least in part, c-di-GMP and VpsT. Finally, very late in biofilm formation quorum sensing signals reprogram the regulatory state of the cells, reactivate motility and promote biofilm dispersal. Biofilms and motility are clearly intertwined, and the pathogenesis of *V. cholerae* may hang on the precise alternation between the biofilms that promote survival in the environment and the planktonic cells that are virulent (Rice *et al.*, 1992; Wai *et al.*, 1998; Yildiz & Schoolnik, 1999; Tamayo *et al.*, 2010).

Another *Vibrio* species, which was among the earliest studies to investigate the regulation of the motility during biofilm formation, is *Vibrio parahaemolyticus*. *Vibrio parahaemolyticus* is motile and synthesizes two entirely separate flagellar systems: a sheathed polar flagellum driven by the sodium motive force and unsheathed lateral flagella driven by the proton motive force (Atsumi *et al.*, 1992; Kim & McCarter, 2000; Stewart & McCarter, 2003). The two flagellar systems have different functions where the polar flagellum powers swimming in liquid media and the lateral flagella powers swarming over solid surfaces (for review see McCarter, 2004). *Vibrio parahaemolyticus* forms nonmotile biofilms that manifest as a pellicle on a liquid interface as well as colonies with complex architecture that grow on solid surfaces (Güvençer & McCarter, 2003). The biofilm matrix is composed of EPS that is referred to as capsule polysaccharide (CPS) due to the tight association with the cell surface (Enos-Berlage & McCarter, 2000). CPS is synthesized

from the 11-gene *cps* operon and is composed of fucose, galactose, glucose, and *N*-acetylglucosamine (Enos-Berlage & McCarter, 2000; Güvener & McCarter, 2003).

Like *V. cholerae*, *V. parahaemolyticus* produces high-frequency genetic subpopulations that grow to either translucent or opaque colonies. Opaque colony variants contain mutations in a HapR homolog called OpaR, and *opaR* mutants confer an opaque phenotype that is enhanced for biofilm formation and reduced for motility (McCarter, 1998) (Table 1). Similarly, mutation of two proteins ScrC and ScrG that contain both GGDEF and EAL domains also result in enhanced biofilm formation and decreased motility (Boles & McCarter, 2002; Kim & McCarter, 2007; Ferreira *et al.*, 2008) (Fig. 6; Table 1). Simultaneous mutation of ScrC and ScrG resulted in a synergistically enhanced biofilm formation (Kim & McCarter, 2007). Thus, OpaR, ScrG, and ScrC normally function to promote motility in *V. parahaemolyticus*.

ScrC regulates motility by controlling c-di-GMP in response to quorum-sensing signals that accumulate in the environment. ScrC is encoded within a three gene operon *scrABC*, which also encodes ScrA, a periplasmic putative aminotransferase and ScrB a periplasmic solute binding protein (Boles & McCarter, 2002). ScrC is a transmembrane protein with cytoplasmic GGDEF and EAL domains, and ScrC has the capacity to function as both a diguanylate cyclase and a phosphodiesterase (Ferreira *et al.*, 2008). Regulation of ScrC, however, is

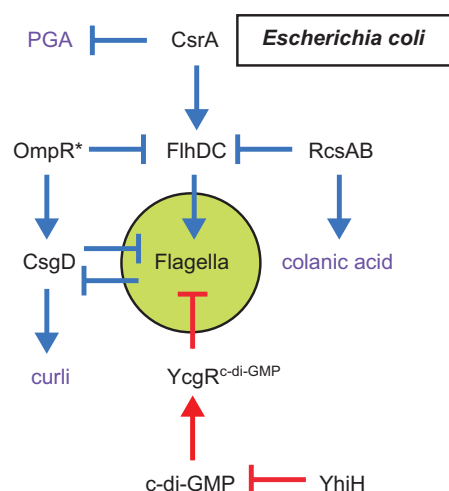


Fig. 6. Network of *Escherichia coli* biofilm-related motility regulation. Transcriptional regulation is labeled in blue, functional regulation labeled in red, and the biofilm matrix products in purple. Green circle highlights the flagellum. C-di-GMP in superscript indicates that the protein needs to be bound to C-di-GMP to carry out the indicated activity. Solid lines indicate a confirmed direct interaction, while dashed lines indicate that the interaction is poorly understood and could be indirect or direct.

complex and is modulated by spent media that accumulates an as-yet-unidentified signal (S signal), the synthesis of which depends of ScrA (Trimble & McCarter, 2011) (Fig. 5). Genetic evidence suggests that ScrB is a receptor for the S signal that specifically enhances ScrC phosphodiesterase activity (Trimble & McCarter, 2011). Therefore, at high cell density, a pheromone modulates the activity of the bifunctional ScrC to degrade c-di-GMP and enhance motility by an unknown effector. Conversely, at low cell density, c-di-GMP is high and promotes biofilm formation through a recently discovered VpsT homolog, CpsQ that activates CPS gene expression (Ferreira *et al.*, 2011) (Fig. 5). *Vibrio cholerae* does not seem to encode the Scr operon (Ferreira *et al.*, 2008).

Vibrio cholerae and *V. parahaemolyticus* are two closely related species that both inversely regulate motility and biofilm formation. Both species even seem to have quorum-sensing systems that coordinate the lifestyle transition, and yet despite these similarities, the regulatory mechanisms differ. Each model organism contributes to our understanding of the motility-to-biofilm transition. Biofilm formation is better understood in *V. cholerae* and it will be interesting to learn how motility is inhibited in the biofilm. Motility is better understood in *V. parahaemolyticus*, and it will be interesting to learn how biofilm formation is coordinated between the two different flagellar systems that govern swimming and swarming.

Escherichia coli

Escherichia coli and its close relative *Salmonella enterica* are the paradigms of bacterial flagellar motility; in these organisms, flagellar synthesis, structure, regulation, and chemotaxis have been extensively studied. *Salmonella enterica* synthesizes between 3 and 8 flagella per cell organized in a peritrichous arrangement (Iino, 1969; Erhardt & Hughes, 2010). Flagellar number is controlled by the master transcriptional regulator FlhDC which binds to and activates promoters that drive basal body gene expression (Fig. 6; Claret & Hughes, 2000, 2002). Both *E. coli* and *S. enterica* swim in liquid media and both are capable of swarming motility under specific permissive conditions (Harshey & Matsuyama, 1994; Kim & Surette, 2005; Girgis *et al.*, 2007).

Escherichia coli biofilms manifest as aggregates at the meniscus liquid–air surface found on partially submerged coverslips or on the sides of microtiter plate wells. The biofilm extracellular matrix is composed of EPS and proteins, but the identity and role of each component differs depending on the strain background. In strain K-12, curli are required for initial biofilm attachment. Curli are proteinaceous amyloid fibers that are nucleated by the outer membrane protein CsgB and assembled by polymer-

ization of CsgA monomers (Hammar *et al.*, 1996; Bian & Normark, 1997). Following curli formation, an EPS called colanic acid is synthesized by proteins expressed from the 19 gene *wca* cluster and is required for biofilm maturation (Stevenson *et al.*, 1996; Danese *et al.*, 2000). By contrast, in strain MG1665, the products of the *pgaABCD* operon synthesize β -1,6-poly-*N*-acetylglucosamine (PGA) that is thought to act as an adhesin during early attachment and subsequent biofilm maturation (Wang *et al.*, 2004). The cells within an *E. coli* biofilm are non-motile.

Some *E. coli* strains require motility early in biofilm formation. By comparing multiple strains of *E. coli* in flow cells, motility and biofilm formation were found to be correlated such that strains with the most robust biofilm formation also displayed the most vigorous motility as planktonic cells (Wood *et al.*, 2006). At the molecular level, mutations in *flhD*, which encodes the FlhD subunit of the master regulator for flagella gene transcription, and mutations in *fliC*, which encodes the flagella filament protein flagellin, result in severe biofilm defects (Pratt & Kolter, 1998). Likewise, mutations that paralyze the flagellar motor but leave the structure intact are also crippled for biofilm formation (Pratt & Kolter, 1998; Vallet *et al.*, 2001). Thus, it appears that motility is important, at least at the early stages, of initiating biofilm formation.

At some point in biofilm formation, it is inferred that flagella become functionally inhibited by the protein YcgR. YcgR was first shown to inhibit motility in cells mutated for the histone-like DNA-binding protein H-NS and later in cells mutated for YhjH, an EAL domain-containing c-di-GMP phosphodiesterase (Ko & Park, 2000; Simm *et al.*, 2004; Ryjenkov *et al.*, 2006). In the absence of YhjH, c-di-GMP levels rise, YcgR binds to c-di-GMP via a PilZ-domain and inhibits motility by interacting with the flagellar motor (Amikam & Galperin, 2006; Ryjenkov *et al.*, 2006) (Fig. 6; Table 1). YcgR has been reported to make direct contact with components of the flagellar motor including the FliG rotor, the MotA stator protein, and the FliM protein that couples the chemotaxis machinery to the direction of flagellar rotation (Boehm *et al.*, 2010; Fang & Gomelsky, 2010; Paul *et al.*, 2010). Supporting these interactions, translational fusions between YcgR and a fluorescent fluorophore localize as puncta along the cell membrane in a manner dependent upon FliG, FliM, and MotA respectively (Fig. 1; Boehm *et al.*, 2010; Paul *et al.*, 2010). The complex interaction between YcgR and multiple pieces of the flagellum perhaps accounts for the observation that YcgR both reduces swimming speed (invoking FliG and MotA) and impairs chemotaxis by biasing flagellar rotation in favor of the counterclockwise direction (invoking FliG and FliM) (Boehm *et al.*, 2010; Fang & Gomelsky, 2010; Paul *et al.*, 2010). In sum, YcgR is thought to functionally inhibit

motility in the presence of c-di-GMP at a time when cells are presumably preparing to form a biofilm. The role of YcgR-dependent inhibition of motility on biofilm formation has not been directly investigated.

As shown in other systems, transcriptional regulation of motility also occurs in the transition from motile to sessile cells in a biofilm. Prigent-Combaret *et al.* (1999) found that cells in a biofilm decrease expression of the flagella filament, *flhC*. One potential candidate for the inhibition of motility during biofilm forming conditions is the Rcs phosphorelay system. The Rcs system consists of a transmembrane kinase RscC, a membrane bound phosphotransfer protein RcsD, and a response regulator transcription factor RcsB (Majdalani & Gottesman, 2006). RcsB along with the accessory protein RcsA activates expression of colanic acid CPS synthesis genes and inhibits motility by directly repressing expression of the *flhDC* promoter (Gottesman *et al.*, 1985; Francez-Charlot *et al.*, 2003; Fredericks *et al.*, 2006) (Fig. 6; Table 1). Furthermore, mutation of Rcs components impairs biofilm formation and enhances motility (Ferrières & Clarke, 2003; Girgis *et al.*, 2007). Thus, the Rcs signal transduction system appears to coordinate both phenotypes in the motility-to-biofilm transition at the level of gene transcription.

Inverse regulation of the flagella and EPS can also occur at the post-transcriptional level. CsrA is a small RNA-binding protein that acts as a global regulator in many bacteria by post-transcriptional regulation (Romeo, 1998) (Fig. 6; Table 1). In *E. coli*, CsrA increases transcription of *flhDC* by directly binding the mRNA to stabilize the transcript (Wei *et al.*, 2001). Conversely, CsrA decreases expression of the *pgaABCD* PGA polysaccharide synthesis operon by binding to the mRNA and occluding the ribosome-binding site (Wang *et al.*, 2005). Consistent with the reciprocal regulation, mutants of CsrA are enhanced for biofilm formation and impaired for motility (Wei *et al.*, 2001; Jackson *et al.*, 2002; Jonas *et al.*, 2008). Biologically, CsrA may be important in biofilm dispersion since induction of CsrA in a mature biofilm causes disaggregation within hours (Jackson *et al.*, 2002). In support of the artificial induction results, CsrA levels gradually increase during the course of biofilm development (Jackson *et al.*, 2002). The signal for the increase in CsrA levels is unknown.

Many commonly studied laboratory strains of *E. coli* do not form biofilms until they acquire a gain-of-function point mutation in the gene *ompR* that codes for an enhanced allele of the OmpR transcription factor (Vidal *et al.*, 1998). Enhanced OmpR has increased binding to the promoter of *csgD*, encoding the CsgD master transcriptional activator of CsgA and curli synthesis (Hammar *et al.*, 1995; Prigent-Combaret *et al.*, 2001). In addition, enhanced OmpR inhibits expression of the

flagellar filament over the course of biofilm formation as it binds to and directly represses expression of the promoter for *flhDC* (Shin & Park, 1995; Prigent-Combaret *et al.*, 1999) (Fig. 6; Table 1). Consequently, the enhanced OmpR strains over-produce curli and are defective in flagellar motility, yet form biofilms that are motility-independent (Prigent-Combaret *et al.*, 2000). It appears that *E. coli* strains that over-produce curli during biofilm formation either undergo a different developmental pathway or the excess curli override the importance of motility in biofilm formation.

Curli and flagella inhibit each other in a reciprocal fashion. Motile cells co-express the protein FlhZ with other flagellar genes. FlhZ is a DNA-binding protein that inhibits curli production by decreasing expression of CsgD and CsgD-activators (Pesavento *et al.*, 2008; Pesavento & Hengge, 2012). FlhZ also serves as a negative feedback mechanism that inhibits FlhDC at multiple levels (Wada *et al.*, 2011; Pesavento & Hengge, 2012). As cells transition into the biofilm forming state, c-di-GMP levels rise, activating CsgD, which in turn activates curli gene expression (Pesavento *et al.*, 2008). How c-di-GMP activates CsgD expression is unknown, but once activated, CsgD also directly represses flagellar basal body gene operons to decrease flagellar synthesis (Ogasawara *et al.*, 2011). Finally, a small RNA called McaS inhibits curli by repression of CsgD and activates motility by activating *flhDC* expression (Thomason *et al.*, 2012). The inverse regulation of flagella genes and curli is similar to the inverse regulation of flagella and EPS in other bacteria and perhaps ensures that the cell does not waste energy on two opposing behaviors.

While we must draw on information from multiple *E. coli* strains and even extrapolate from *S. enterica*, the inhibition of motility during biofilm formation may proceed as follows. Motility is required early perhaps to locate a suitable environment or surface on which to form a biofilm. Once biofilm formation is initiated, c-di-GMP levels rise, perhaps by the inhibition of phosphodiesterases like YhjH. Elevated c-di-GMP levels activate YcgR that interacts with the flagellar rotor to decrease rotation speed and increase the frequency of direction reversals. In parallel, c-di-GMP activates CsgD, which in turn activates curli biosynthesis and inhibits flagellar gene expression. As the biofilm matures, post-transcriptional regulators like CsrA may accumulate to reactivate motility and antagonize matrix expression and thereby promote cellular dispersal for a new round of biofilm formation.

Concluding remarks

It is important to acknowledge that the notion of the motility-to-biofilm transition is largely theoretical. Biofilm

formation takes place over prolonged periods of time during which the event of a cell or cells transiting between physiological states is difficult to capture. In addition, many bacteria are nonmotile, and some motile bacteria generate nonmotile subpopulations that may proceed directly to biofilm formation without further motility regulation (Kearns & Losick, 2005; Guttenplan *et al.*, 2010). Despite these complications, the fact that some bacterial species can exist in two diametrically distinct modes of growth, either as motile individual cells or as nonmotile aggregates of a biofilm, prompts the inference that the bacteria must switch physiologies at some point. The inference of a transition is supported by mutations that enhance biofilm formation and reduce motility thereby revealing the reciprocal regulation of the two phenotypes (other examples Wei & Bauer, 1999; Cano *et al.*, 2002; Yao *et al.*, 2004; Bahlawane *et al.*, 2008). Thus, it has become clear that motility and biofilms are oppositely controlled but precisely when, where, and how the transition takes place during biofilm formation is unclear.

The inhibition of motility is often thought to be an essential event in biofilm formation perhaps because motility is sometimes required early but inhibited late in mature biofilms. Furthermore, some mutants with enhanced motility are associated with decreased biofilm formation suggesting that the maintenance of motility may destabilize nascent multicellular aggregates. Empirically proving that motility inhibition is required for biofilm formation, however, is challenged by the fact that in many cases the mechanisms of motility inhibition are unknown, flagellar regulation is complex, and matrix biosynthesis gene expression is often coregulated with motility regulons. Even in instances where motility inhibition can be genetically separated from matrix synthesis, biofilm inhibition may appear transient due to rapidly selected suppressors that abolish motility through independent pathways (Guttenplan *et al.*, 2010). In fact, the arrival and dominance of nonmotile suppressors may be the best evidence that motility inhibition is important because they suggest that, under biofilm forming conditions, the selective pressure to inhibit motility is strong (Tart *et al.*, 2005, 2006; Guttenplan *et al.*, 2010).

The concept of the motility-to-biofilm transition, regardless of biological importance, has proven powerful for the study of each individual phenotype. Understanding that the regulation of motility and biofilms is diametrically opposed has been critical in revealing the widespread importance of the secondary-messenger molecule c-di-GMP. Bacteria encode dozens of enzymes homologous to c-di-GMP synthases and hydrolases that control the cytoplasmic accumulation of c-di-GMP, which in turn promotes a biofilm-like state where motility is inhibited at multiple levels. The inputs that control

c-di-GMP pools and the reason that mutation of various enzymes acting on a common pool gives rise to a spectrum of diverse phenotypes are unclear. Many output effectors of c-di-GMP remain to be discovered (Roelofs *et al.*, 2011).

The relationship of motility to biofilms is complicated and likely invokes other regulatory systems like quorum sensing. Quorum sensing is a general term describing the process by which genes are regulated at high cell density in response to a secreted pheromone and is relevant as biofilms maintain cells at high density. In some systems, quorum sensing is necessary for EPS synthesis and biofilm formation whereas in other systems quorum sensing induces motility and biofilm dispersal (Quiñones *et al.*, 2005; Hoang *et al.*, 2008; Waters *et al.*, 2008). The differences in the way that quorum sensing regulates biofilms may be due to ecology. Inducing dispersion in *V. cholerae* may be advantageous in the water reservoir for colonizing new regions and in scavenging more nutrients (Yildiz *et al.*, 2004; Waters *et al.*, 2008). In *P. syringae* and *S. meliloti*, biofilms are formed in the nutrient-rich environments of plants, and thus colonizing new areas to scavenge nutrients may be unnecessary (Quiñones *et al.*, 2005; Hoang *et al.*, 2008). In any case, the relationship of motility and biofilms may be further complicated by the density of cells being considered.

Most regulation of the flagellum has historically focused on mechanisms that govern the expression of flagellar genes and ensure the ordered assembly of the machine. Thus, regulation has essentially focused on the nonmotile-to-motile transition, and biofilms introduce a new regulatory problem: the inhibition of motility. Motility inhibition by transcriptional repression is slow but may be important for the long-term stability of the biofilm. Flagella, however, are complex, stable structures integrated into the fabric of the cell envelope and abolishing *de novo* synthesis does not necessarily inhibit motile behavior in the short term. Thus, the pre-existent flagella must either be segregated to extinction by growth or there must be a parallel level of control that alters flagellar function in the short term. The context of biofilm formation has enabled the discovery and interpretation of regulators of flagellar function.

Functional regulators of the flagellum are still relatively rare in the literature and are poorly understood (Table 1). EpsE of *B. subtilis* acts like a clutch likely by binding to the inside surface of FliG, bending the rotor, and disengaging it from the stator (Fig. 1; Blair *et al.*, 2008; Guttenplan *et al.*, 2010). YcgR of *E. coli* reduces rotation speed and changes basal reversal frequency likely by binding to the outside surface of FliG and deforming the rotor (Fig. 1; Boehm *et al.*, 2010; Fang & Gomelsky, 2010; Paul *et al.*, 2010). The YcgR homolog YpfA in *B. subtilis* inhibits motility by

interacting with the MotA stator protein (Chen *et al.*, 2012a). In *Caulobacter crescentus*, DgrA is a c-di-GMP-binding protein that inhibits flagellar rotation perhaps through another regulator of flagella function, FliL (Jenal *et al.*, 1994; Christen *et al.*, 2007). Fumarate reductase and the F₁F₀ ATPase of *E. coli* interacts with FliG to increase the basal tumbling frequency of motile behavior, and CheY locks the flagellar rotor like a brake by interacting with FliM in *Rhodobacter sphaeroides* (Cohen-Ben-Lulu *et al.*, 2008; Pilizota *et al.*, 2009; Zarbiv *et al.*, 2012). Finally, the mutant phenotypes of cells lacking SadC in *P. aeruginosa* and VpsR/VpsT in rugose *V. cholerae* suggest as-yet-undiscovered regulators that act during biofilm formation to inhibit motility (Yildiz *et al.*, 2001, 2004; Caiazza & O'Toole, 2004; Merritt *et al.*, 2007). Functional regulation of the flagellum is most likely present in many more bacteria and the relationship of this regulatory strategy to biofilm formation remains to be clarified (Wolfe & Visick, 2008).

How motility is regulated during biofilm formation is under studied for a variety of reasons. Research that explores the motility-to-biofilm transition generally favors regulatory mechanisms governing biofilm matrix synthesis. Studying two complex processes simultaneously is difficult, and it is typically easier to document the induction of a phenotype than its disappearance. Furthermore, flagellar biosynthesis and regulation is so extensively understood in a relatively narrow group of organisms, *E. coli* and *S. enterica* specifically, that the flagella and motile behavior of other model organisms may be taken for granted and understudied in their own right. While it is true that flagellar biosynthesis genes are highly conserved, individual bacteria encode novel elements particularly with respect to the regulators of flagellar gene expression and function. Finally, besides analogies to automobile mechanics, there are few conceptual models for how the functional inhibition of motility is mediated. Inhibition is a new frontier of motility regulation and biofilms have already proven to be a powerful context for the discovery of new regulatory mechanisms. Flagella are classic models of bacterial machines, and by understanding their regulation, we might discover new paradigms for the regulation of other machines like secretion apparatus and polymerases.

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