MINIREVIEW

Get the Message Out: Cyclic-Di-GMP Regulates Multiple Levels of Flagellum-Based Motility[▽]

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Flagella are complex organelles whose synthesis depends upon approximately 50 gene products. Not surprisingly, multiple mechanisms control and direct flagellar biogenesis (reviewed in references 1, 15, and 55). Perhaps best understood is the control exerted at the level of transcription: hierarchies exist, such that the flagellar structural proteins are synthesized only under proper conditions and at the time each is needed for assembly. Many bacteria also possess posttranscriptional mechanisms that control translation or protein stability. Assembly itself also occurs in a highly ordered fashion, with the insertion of one component building on the insertion of a previous subunit. In the best-characterized bacteria, flagellar biogenesis begins with the insertion of an inner membrane protein called FliF (Fig. 1). Building on the assembled FliF subunits are proteins that comprise the flagellar type III secretory (TTS) apparatus. This device exports the protein subunits required to build the basal body, which includes both a cytoplasmic switching device and a complex of rod and ring proteins that spans the two membranes of the gram-negative bacterium. The completed basal body provides a narrow channel through which the more external components exit the cell for assembly. These external components include the hook (a flexible linker comprised of over 100 identical subunits) and the filament (a semirigid propeller built from thousands of flagellin subunits). Once assembled, the flagellum rotates, a process powered by the proton (or sodium) motive force via motor proteins localized in the cytoplasmic membrane in close proximity to the switching device. The direction of rotation is dictated by the switching device, which interfaces with a signaling pathway that delivers information about the cell's physicochemical environment. Chemotaxis, the resulting behavior, permits cells to migrate toward favorable environments. Finally, some bacteria eject their fully functional flagellum in coordination with the cell cycle.

In recent years, researchers have discovered a new layer of regulation on top of the already sophisticated control of flagellar biogenesis and function. This layer depends on bis-(3′,5′)-cyclic diguanylic acid (also known as cyclic diguanylate, or c-di-GMP), a novel second messenger that apparently is unique to bacteria. This newly appreciated second messenger modulates diverse cellular processes, including virulence (40, 82). First identified as a positive effector of cellulose synthase in *Gluconacetobacter xylinus* (reviewed in reference 84), c-di-GMP regulates the transition from the motile, planktonic state to sessile, community-based behaviors, such as biofilm development. It tends to enhance biosynthesis of capsular and fimbrial components required by developing biofilms, while inhibiting flagella and pili that permit movement (33, 38, 81, 83, 92, 107).

Diguanylate cyclases (DGCs) synthesize c-di-GMP, while phosphodiesterases (PDEs) degrade it. Together, these activities maintain the steady-state concentration of c-di-GMP (84) (Fig. 2). DGC activity depends upon the highly conserved GGDEF (formerly DUF 1) domain (73, 74, 89, 92, 96), while PDE activity requires either the EAL (formerly DUF 2) domain (9, 19, 98) or the HD-GYP domain (26, 85). Such domains have been found associated with many diverse input and output domains (reviewed in reference 83), suggesting that they receive many different types of stimuli and respond through a variety of mechanisms. Intriguingly, hybrid proteins that possess both GGDEF and EAL domains exist. Those that have been tested, however, exhibit either DGC or PDE activity but not both (19, 45). This appears to be the case especially when one of the domains is poorly conserved. It has been proposed that these noncatalytic domains function in a regulatory capacity (19). On the basis of sheer abundance in diverse bacterial genomes, GGDEF, EAL, and HD-GYP domains represent a major family of signaling pathways that use c-di-GMP as their second messenger (28). Many bacteria possess multiple proteins with these domains (29). For example, *Shewanella oneidensis* possesses 98 of these proteins (101), *Pseudomonas aeruginosa* has 38 (50), *Vibrio cholerae* has 53 (54), *V. fischeri* has 48 (K. L. Visick, unpublished), and *Escherichia coli* has 29 (107). This abundance suggests that these bacteria possess a network of pathways that feeds c-di-GMP into a global pool or, alternatively, an array of unlinked pathways that produce small, localized, and thus highly specific concentrations of c-di-GMP (82, 83, 107).

Although it remains unclear how c-di-GMP influences behavior, some clues exist. Recently, Amikam and Galperin (5) used bioinformatics to identify a c-di-GMP-binding domain, termed PilZ after a protein that regulates type IV pilus assembly in *P. aeruginosa* (60). Subsequently, Ryjenkov and coworkers (88) showed that c-di-GMP binds the PilZ domain with a 1:1 stoichiometry. Typically, PilZ is only one domain of a multidomain protein. For example, the BcsA subunit of the

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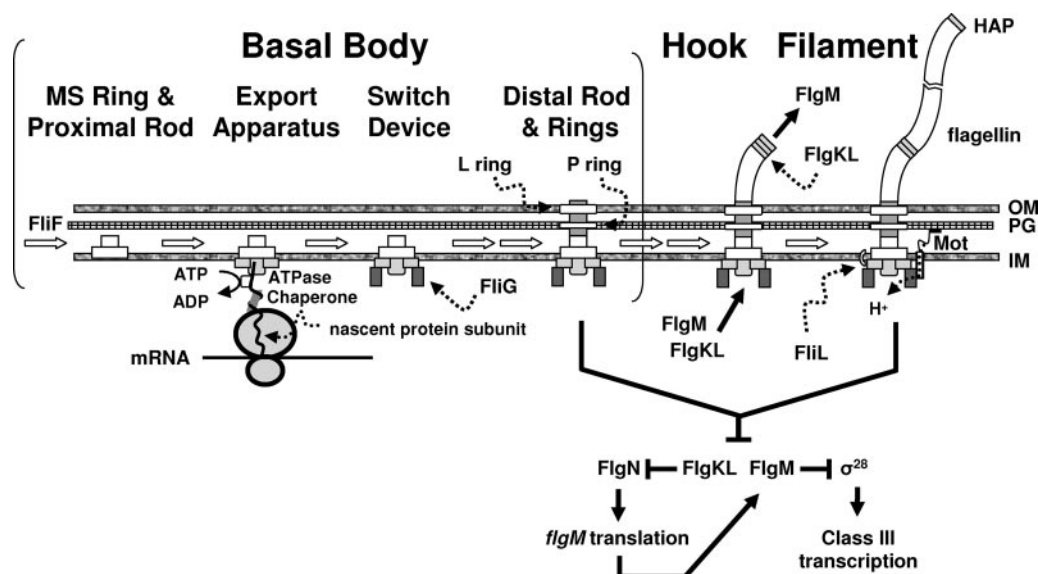
FIG. 1. Flagellar assembly. Best studied in the enterics (*Escherichia coli* and *Salmonella enterica* serovar Typhimurium), the flagellum is comprised of three major substructures: the basal body, the hook, and the filament. These substructures are assembled in order (reviewed in reference 55). The first step in assembly appears to be the insertion into the inner membrane (IM) of the MS ring and proximal rod (composed entirely of FliF subunits) because this insertion can occur independently of all other flagellar components. The second and third steps, assembly of a TSS export apparatus and the switching device, including FliG, occur within the IM and at the cytoplasmic face of the MS ring, respectively. The fourth step, assembly first of the rod and then of the P- and L-ring components, depends upon the presence of both the assembled export apparatus and the switch. Assembly of the distal rod (as well as the hook and filament) depends upon the TSS export apparatus. Secretion of nascent subunits is assisted by a subunit-specific chaperone and depends upon a flagellum-specific ATPase. The first subunits secreted through this TTS apparatus complete the rod, which now bridges the IM, peptidoglycan (PG), and outer membrane (OM). In contrast, assembly of the P ring into the PG and the L ring into the OM depends upon the general secretory apparatus. Following this assembly process, the completed basal body can now serve as a channel through which subsequent proteins travel across the envelope to their assembly site located distal to the OM. First secreted through this envelope-spanning channel are dozens of hook subunits, which are polymerized at the distal end of the basal body. Next secreted and assembled at the distal end of the hook are three HAPs, which serve as adaptors between the larger hook and smaller filament diameters. Two of these HAPs, FlgK and FlgL, bind to their chaperone FlgN and inhibit its ability to activate *flgM* translation; their secretion relieves this inhibition. This permits increased synthesis of FlgM, the anti-sigma factor for the flagellum-specific sigma factor σ^{28}. Secretion of FlgM relieves inhibition of σ^{28}, which now becomes free to transcribe class III genes, including *fliC*, which encodes flagellin. Thousands of these flagellin subunits are secreted and assembled between the HAPs to produce the filament. Once the filament is assembled, the HAPs and FlgM are no longer secreted and they resume their inhibitory roles. The result is a semirigid helix strong enough to enable propulsion through a viscous environment. Propulsion occurs because the rod, hook, and filament rotate. Rotation is driven by energy generated by proton (H^+) flow across the IM via a PG-anchored proton channel (Mot) that interacts with the switching device. Although the role of FliL is not understood, this protein is membrane bound and associated with the basal body complex (3, 55).
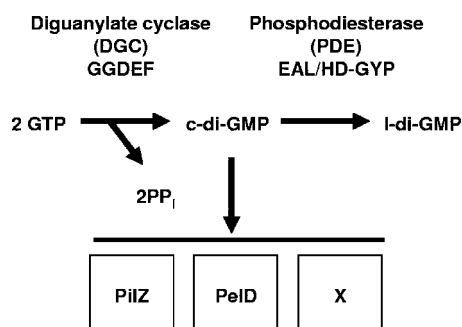

FIG. 2. Fundamental c-di-GMP pathway. A DGC containing conserved GGDEF domain catalyzes the synthesis of c-di-GMP from two GTP molecules with the release of pyrophosphate (PP$_i$). A PDE containing the conserved EAL or HD-GYP domain catalyzes the degradation of c-di-GMP to linear diguanylate (l-di-GMP). Together, these two catalytic activities set the steady-state level of c-di-GMP, which can bind to a protein with the conserved PilZ or PelD domain or another as-yet-unidentified domain (X) (21).

cellulose synthesis complex contains a PilZ domain linked to a glycosyltransferase. The docking of c-di-GMP to this PilZ domain likely activates cellulose synthesis (5, 88). Multiple additional studies support the hypothesis that PilZ domain proteins bind c-di-GMP (18, 64, 76, 80). However, PilZ domains may not be the only c-di-GMP-binding motif, as some organisms that contain DGCs and PDEs do not appear to carry PilZ domain genes (5). Indeed, Lee and coworkers have recently identified PelD as a non-PilZ domain c-di-GMP-binding protein (51).

Many studies have suggested a role for c-di-GMP in controlling flagellum-based motility. However, most have utilized overexpression constructs that may lead to artifactual results due to unnaturally high (or low) levels of c-di-GMP. Furthermore, relatively few studies have investigated the level at which c-di-GMP exerts its control. However, those few studies have revealed a variety of ways in which c-di-GMP can impact motility. These putative or established mechanisms can be classified on the basis of the affected level: (i) transcription, (ii) posttranscription, (iii) function, or (iv) ejection. Although c-di-GMP impacts pilus-based twitching motility, biofilm forma-
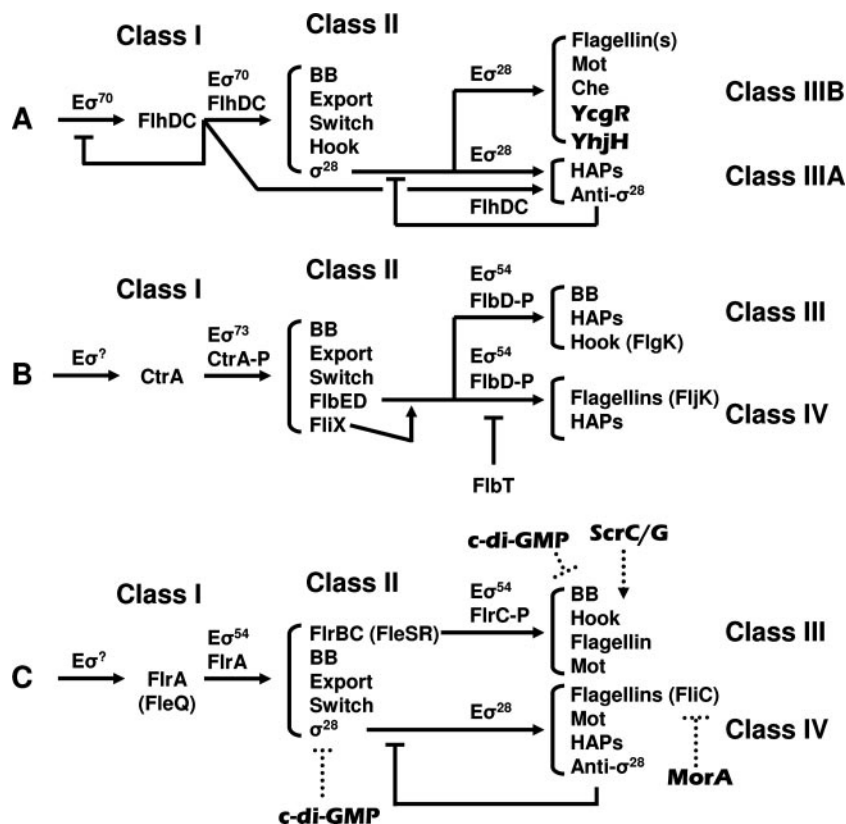
FIG. 3. Schematic of transcription hierarchies, as determined for (A) the *Enterobacteriaceae* (1, 15, 25, 78, 94), (B) *Caulobacter* (24, 68, 109), and (C) the vibrios (and pseudomonads) (22, 62, 77). Arrows indicate induction, while a straight line in place of the arrowhead indicates inhibition. Note that the hierarchy for the *Enterobacteriaceae* includes c-di-GMP-associated proteins YcgR and YhjH, which are known to be encoded by genes within the flagellar regulon in *Salmonella*. The sigma factors at the top of the hierarchies for *Caulobacter* and vibrio/pseudomonad species are as yet undefined. The levels at which c-di-GMP impacts transcription for *V. cholerae* (*cdgF* overexpression; written as c-di-GMP in the figure), *V. parahaemolyticus* (ScrC/G), and *P. putida* (MorA) are not known, but the protein that is affected is indicated as an aid for the reader. BB, basal body; Mot, motor proteins; anti-σ^{28}, FlgM; -P, phosphorylated.

tion, and related processes, a number of recent reviews have already covered these topics (12, 20, 40, 82, 83, 86, 97). Thus, they remain outside the scope of this review. Also outside this review are mechanisms that utilize homologs of the chemotaxis system to control non-flagellum-based processes, such as the WspR pathway of *Pseudomonas* (34). Instead, this minireview focuses strictly on mechanistic flagellum-based studies. At each level, we first briefly summarize the previously established mechanisms of control and then describe the predicted role of c-di-GMP.

Impact at the level of transcription. Ordered assembly is maintained by two general mechanisms (reviewed in reference 25). First, transcription of flagellar genes occurs in a hierarchical manner. This is true for all bacteria studied to date, regardless of whether they assemble a single flagellum or many and whether they possess polar or peritrichous flagella (for a review, see reference 78). This ordering permits cells to couple gene expression to the flagellar assembly process, such that each protein is synthesized only when needed. Second, the flagellar export (TTS) apparatus switches substrates from components of the rod and hook subassemblies to components of the filament, a process that is described in more detail in the next section (for reviews, see references 1, 15, 25, and 94). In this section, we first describe the hierarchical process by which

cells transcribe flagellar genes and then relate the evidence that c-di-GMP impacts this regulatory scheme.

Three major hierarchical schemes are known (reviewed in reference 78). The first (Fig. 3A), developed from studies of the enterics (primarily *E. coli* K-12 and *Salmonella enterica* serovar Typhimurium), relies on a class I master regulator (FlhDC) that acts in conjunction with the general sigma factor (σ^{70}) to direct RNA polymerase (E) to transcribe class II genes. These class II genes encode components of the switch, export apparatus, basal body, and hook. They also include the gene for the flagellum-specific sigma factor FliA (σ^{28}), which subsequently directs transcription of class III genes encoding later-assembled components, such as hook-associated proteins (HAPs), flagellin, the chemotactic signaling pathway (Che), and motor proteins (Mot). These genes are divided into two subsets on the basis of the regulators required for their transcription: class IIIA requires both σ^{28} and FlhDC, while class IIIB requires only σ^{28}. One of the class IIIA genes encodes the σ^{28}-specific anti-sigma factor FlgM, which provides feedback to σ^{28} regarding the state of flagellar assembly (reviewed in references 1, 15, and 55), while class IIIB includes two proteins (YcgR and YhjH) known to be associated with c-di-GMP (27, 47, 105).

The second and third schemes arose, respectively, from stud-

ies of *Caulobacter crescentus* (Fig. 3B) (24, 68, 109) and investigations of several vibrio and pseudomonad species (Fig. 3C) (22, 62, 77). Like the enterics, both are constructed in a hierarchical manner, each also with four major groupings of genes. The major difference lies in the regulators: the sigma factors, the master regulators, and the secondary regulators. Whereas the enterics use FlhDC, σ^{70}, and σ^{28} to coordinate hierarchical control, *C. crescentus* uses its σ^{70} homolog (σ^{73}) in conjunction with CtrA (a two-component response regulator) and σ^{54} in conjunction with FlbD (also a response regulator). In contrast, the vibrio/pseudomonad group uses σ^{54}, two σ^{54} regulators (FlrA/FleQ and the response regulator FlrC/FleR), and σ^{28} to perform similar tasks.

A number of reports have documented cases in which overexpression of a DGC or a PDE impacts motility (8, 48, 54, 63, 66, 70, 79, 92). Although in many cases the levels impacted by overexpression have not been characterized, it is not unlikely that some of these proteins influence transcription of flagellar genes. Indeed, the hypothesis that c-di-GMP production affects transcription is supported by a couple of recent microarray studies (8, 34, 63). In particular, one study performed with *V. cholerae*, which assembles a single polar flagellum, revealed changes in transcript levels of numerous flagellar genes. Overexpression of the DGC CdgF (8) in *V. cholerae* caused a 2.1- to 2.5-fold decrease in the transcript levels of many class III and IV genes, including many of the flagellin and basal body proteins (Fig. 3C). Other flagellar transcripts were reduced between 1.5- and 1.9-fold. Importantly, the transcript level for the gene encoding σ^{28} also was reduced, a result that could easily account for the observed decrease in class IV transcripts. Some of these decreases could be seen as early as 15 min following induction of CdgF, timing that is consistent with control at the level of transcription. Not surprisingly, overexpression of CdgF caused a decrease in swimming migration through soft agar (0.3%) plates, while the corresponding overexpression of a PDE increased migration. A null mutation in *cdgF*, however, did not cause defects in motility or other c-di-GMP-associated phenotypes. This suggests that the influence of *cdgF* overexpression on motility is due to elevated levels of total intracellular c-di-GMP and that the natural role for *cdgF* does not include the control of flagellar gene transcription.

ScrC/ScrG. A clearer example of transcriptional control by c-di-GMP has been documented for *V. parahaemolyticus*, an organism that serves as a model for bacterial differentiation (61). When grown in liquid media, this organism exists as a swimmer cell that assembles a single flagellum at one of its poles. This swimmer cell differentiates into a swarmer cell in response to environmental conditions that are thought to increase drag and/or decrease function of the polar flagelllum, such as increased viscosity or exposure to a surface. The swarmer cell is elongated and elaborates numerous lateral flagella arranged randomly about the cell surface, permitting the cell to migrate across wetted surfaces, a process called swarming (32, 65). A recent study (10) searched for genes that could inappropriately induce expression in liquid culture of a lateral flagellar gene, *flgE*$_L$. One cosmid that conferred high levels of transcription to the *flgE*$_L$ reporter construct contained the *scrABC* operon (*s*warmer and *c*apsular polysaccharide *r*egulation) (10). The first gene (*scrA*) encodes a putative pyridoxal phosphate-dependent enzyme, while the second (*scrB*)
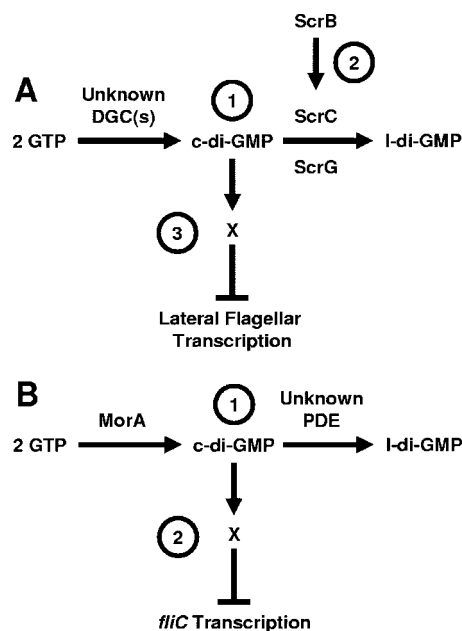

FIG. 4. Working model for how c-di-GMP influences flagellar transcription. (A) Scr pathway of *V. parahaemolyticus*. (Label 1) The PDEs ScrC and ScrG and unidentified DGCs set the steady-state levels of c-di-GMP. (Label 2) ScrB, localized to the periplasm, receives a signal that causes ScrB to modulate ScrA activity. (Label 3) c-di-GMP binds to an unknown c-di-GMP-binding protein, X, to directly or indirectly inhibit lateral flagellar gene transcription. The role of ScrA is as yet unknown and is not included here. The net effect of Scr activity is to decrease c-di-GMP levels and, thus, increase lateral gene transcription. (B) *P. putida* MorA model. (Label 1) The levels of c-di-GMP are set through the activities of the putative DGC MorA and a hypothetical PDE. (Label 2) c-di-GMP together with a c-di-GMP-binding protein may directly or indirectly inhibit *fliC* transcription. Thus, the consequence of MorA activity is to decrease *fliC* transcription. l-di-GMP, linear diguanylate.

encodes a putative periplasmic solute-binding protein. The third gene (*scrC*) encodes a membrane-associated GGDEF-EAL hybrid protein. Each of these genes appears to be involved in the observed phenotype, as chromosomal disruptions of *scrA*, *scrB*, or *scrC* substantially decreased the activity of the *flgE*$_L$ reporter fusion and significantly decreased swarming. Specifically, disruption of *scrA* by two different insertions, one predicted to be polar and a second predicted to be nonpolar, resulted in decreases of 75- and 7.5-fold, respectively, while disruption of either *scrB* or *scrC* reduced expression about 30-fold. Not surprisingly, decreases in motility and lateral gene transcription corresponded to significant decreases in lateral flagellin, the abundant subunit that comprises the external filament (Fig. 1). While complementation experiments verified the role of *scrC*, they also demonstrated that *scrA* and *scrB* are critical to operon function; thus, a full understanding of the individual roles of the last two genes awaits further characterization. However, it does appear that the activity of ScrC can be modulated by ScrB, as cooverexpression of these proteins increased *flgE*$_L$ expression while overexpression of ScrC alone appeared to decrease it. The authors proposed a model (Fig. 4A) in which ScrB, localized to the periplasm, receives a signal. In turn, ScrB communicates this information to ScrC via the latter protein's periplasmic domain. In response, ScrC controls

the production of lateral flagella. Although *V. parahaemolyticus* can express either a single polar flagellum or multiple lateral flagella, the ability of the *scrABC* operon to impact motility appears to be limited to lateral flagella: swimming motility was unaltered by *scr* mutations. Furthermore, these genes do not appear to be required for the surface sensing that is mediated by the polar flagellum, as *scr* mutations do not substantially alter the induction of swarming that occurs through the loss of polar flagellum. These data thus suggest a novel function for the *scr* genes (10).

A second cosmid also substantially increased activity of the *flgE*$_L$ reporter (46). This second cosmid encoded ScrG, a protein whose domains include not only both GGDEF and EAL (like ScrC) but also PAS, which binds cofactors such as FAD or FMN and can respond to a variety of signals, such as light, oxygen, or redox (99). The GGDEF signature, however, is poorly conserved (HDDDF), while the EAL motif is ESL. Overexpression of ScrG alone was sufficient to induce both *flgE*$_L$ expression and production of lateral flagellins. Importantly, disruption of *scrG* resulted in both decreased lateral flagellin protein (to about 60% of WT) and decreased swarming, thus suggesting that the overexpression phenotype reflects a natural function of the protein. These overexpression phenotypes depended upon the EAL domain, as the truncated version ScrGΔEAL produced stable protein but failed to induce either transcription or flagellin production. Disruption of the EAL domain by substitution of ESL to ASL similarly abrogated activation. Surprisingly, both the deletion and the substitution converted ScrG into an inhibitor of transcription, as both variants caused *flgE*$_L$ transcription to be diminished relative to the level for the vector control. In contrast, substitutions in the GGDEF signature motif had little impact on protein function, even when combined with the EAL deletion; thus, single amino acids in the poorly conserved signature motif are not critical for the inhibitory activity (46). This genetic evidence suggests that ScrG functions as a PDE. Two-dimensional thin-layer chromatography supports this assignment; overexpression of ScrG decreases the steady-state levels of c-di-GMP (46). Given the similarities in domain structure and behavior, this is probably also the case for ScrC. For example, overexpression of either ScrABC or ScrG decreased transcription of capsular polysaccharide (*cps*) genes, probably as a consequence of elevated intracellular c-di-GMP (10, 46). Furthermore, the two sets of regulators suppressed each other: overexpression of ScrG restored swarming and *flgE*$_L$ expression to the *scrABC* operon mutants in an EAL-dependent manner, while a plasmid containing *scrABC* likewise restored those phenotypes to a *scrG* mutant. The interaction between these systems was investigated through the construction of double mutants. These analyses suggested that the *scrABC* locus played the greater role in controlling swarming motility; however, loss of both *scrG* and the *scrA* locus resulted in cumulative effects on both *flgE*$_L$ transcription and swarming motility (46).

To date, the level at which these regulators impact transcription of *flgE*$_L$ is unknown. Although the impact of *scrA* disruption on *cps* expression can be attributed to a newly discovered regulator (CpsR), its impact on motility cannot (31). Perhaps it depends upon one of the newly identified regulators of swarming (37).

MorA. Another example of a GGDEF/EAL protein that impacts transcription of flagellar genes is the MorA protein of *P. putida* (Fig. 4B) (16). Disruption of *morA* led to enhanced swimming in soft agar (0.4%) motility plates, a phenotype that was largely restored by expression of *morA* from a plasmid and, in the context of numerous other studies, suggests that MorA acts as a DGC. Examination by transmission electron microscopy revealed that *morA* mutants contained substantially greater numbers of flagella. Furthermore, virtually all *morA* mutant cells contained flagella at all growth phases evaluated (early exponential, mid-exponential, and the exponential-to-stationary transition). This contrasts with wild-type cells, which restrict flagellar production largely to the exponential-to-stationary phase transition. These differences were reflected in the amounts of flagellin (FliC) protein produced by the two strains: the *morA* mutant contained substantially larger amounts of FliC protein. Similarly, the levels of *fliC* transcripts, as evaluated by Northern blot analysis, were greatly enhanced in the *morA* mutant, suggesting that MorA impacts flagellar biogenesis by controlling transcription. This effect appeared limited to *P. putida*, as a disruption of *morA* in *P. aeruginosa* failed to impact motility or levels of the *fliC* transcript. The level within the transcriptional hierarchy impacted by MorA remains to be determined.

Impact between transcription and assembly. Bacteria also exert posttranscriptional control of flagellar biogenesis (1, 15, 25, 55). The ultimate goal of this regulation is to irreversibly switch the export substrate specificity from that of the subunits that comprise the rod and hook subassemblies to that of the subunits that form the filament (25) (Fig. 1). This control is coupled to the status of flagellar assembly and can occur at the level of translation, protein stability, and/or export and assembly. For example, translation of late (class III) genes has been shown to be positively regulated by a protein with weak similarity to translation initiation factors and by TTS chaperones. The latter proteins also protect their substrates (i.e., the products of these class III genes) from degradation and/or aggregation. Furthermore, the chaperone-substrate complexes appear to dock with the ATPase complex of the TTS export apparatus (3, 100) (Fig. 1). These observations have led to the hypothesis that translation of class III gene products might be localized to the export apparatus of actively assembling flagella (reviewed in reference 1), where the ability of subunits to be assembled into the growing flagellum dictates whether the protein will be translated and, if so, whether it is stable (3, 100). In this section, we describe two examples in which c-di-GMP appears to control motility at levels beyond transcription but prior to the production of fully functional flagella. The first example shows that c-di-GMP can impact both translation and secretion of late gene products, perhaps by interacting with one of these previously characterized mechanisms. The second example implicates c-di-GMP in a very early step in the export/assembly process (Fig. 1).

TipF. The first example revolves around the TipF PDE of *C. crescentus*, an organism that has been studied extensively as a model for bacterial development and cell cycle control (14, 39, 53, 57, 95). This organism undergoes an asymmetric division that results in a motile swarmer cell and a nonmotile stalked cell. After a brief motile period, the swarmer cell ejects its
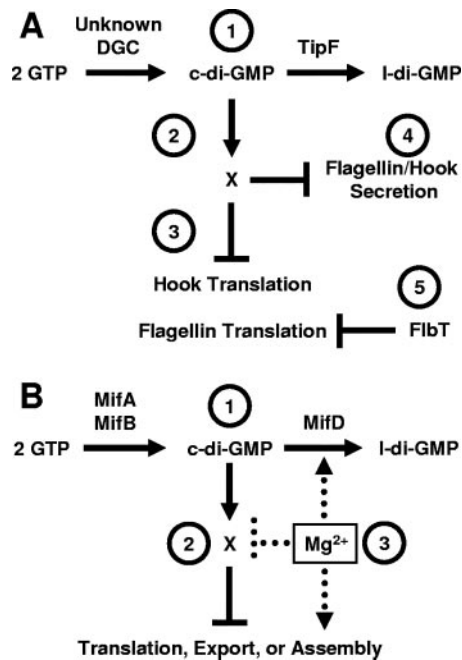
FIG. 5. Working models for how c-di-GMP influences flagellar assembly. (A) *C. crescentus* TipF. (Label 1) An unknown DGC and the PDE TipF control the level of c-di-GMP. (Label 2) c-di-GMP binds to a c-di-GMP-binding protein, X, to directly or indirectly inhibit hook translation (or stability) and flagellin translation (label 3) and the secretion of both (label 4). FlbT independently inhibits flagellin translation (label 5). The net effect of TipF activity is to enhance hook and flagellin translation and secretion. (B) Mif model in *V. fischeri*. (Label 1) The DGCs MifA and MifB and the PDE MifD set the steady-state levels of c-di-GMP, which binds to an unknown c-di-GMP-binding protein, X (label 2). This complex interferes with the translation, export, or assembly of very early flagellar components. (Label 3) Mg^{2+} inhibits this process at some step downstream of c-di-GMP synthesis, e.g., activation of MifD activity or inhibition of the c-di-GMP-binding protein. l-di-GMP, linear diguanylate.

single polar flagellum and grows a stalk at the pole previously occupied by the flagellum.

tipF was uncovered in a recent search for regulators that impact *C. crescentus* motility (36). Disruption of *tipF* resulted in nonmotile cells that lacked the hook and filament of the polar flagellum, as monitored by transmission electron microscopy. The nonmotile phenotype could be complemented by plasmid-borne wild-type TipF but not by a derivative (TipFE 211A) with a disrupted EAL domain: this behavior supports the hypothesis that this protein indeed acts as a PDE (Fig. 5A). Although the *tipF* mutant exhibited near-normal levels (58% of wild type) of *fljK* flagellin transcription, it displayed little translation (2.5% of wild type) and highly reduced steady-state levels of the FljK protein. Similarly, the *tipF* mutant possessed decreased levels of the FlgE hook protein. In *C. crescentus*, the FlbT protein serves as a negative regulator of flagellin translation (Fig. 3B). When a mutation in *flbT* was combined with a *tipF* mutation, FljK levels increased, but the protein was not properly secreted, suggesting that TipF also controls motility at an additional level.

Investigations into the localization of TipF revealed that it could be found at the division plane approximately 30 min after the appearance of TipN, a presumptive "birth scar" protein that is thought to mark the new pole (36). It is at this new pole that the flagellum is eventually assembled, a process that clearly depends on TipF. Indeed, investigations of the localization of the switch component FliG (Fig. 1) revealed that while wild-type cells contained fluorescent foci of FliG-green fluorescent protein at both poles, the *tipF* mutant contained few such foci (19% of cells), the majority of which were mislocalized. Although the function of TipN does not appear to be related to the control of c-di-GMP levels, this protein is also required for correct localization of the flagellum: disruption of *tipN* resulted in cells with mislocalized flagella. Interestingly, a *tipF tipN* double mutant appeared similar to the *tipF* mutant in that no flagella were made, indicating that TipF acts in the pathway downstream of TipN and controls flagellar assembly regardless of flagellar placement (Fig. 5A).

Mif. The second example of posttranscriptional control of motility comes from studies of *V. fischeri*. To assemble its polar tuft of flagella, this marine symbiont requires magnesium (Mg^{2+}) at concentrations similar to those found in seawater. Under Mg^{2+}-limited conditions, the vast majority of cells do not assemble flagella, apparently due to a paucity of flagellar proteins: Mg^{2+}-limited cells possess very little of the highly abundant flagellin subunits that normally comprise the filament (69). Yet, Mg^{2+} does not substantially impact transcription of a variety of flagellar genes, including those for flagellin (70). Because Mg^{2+} does not seem to affect transcription but clearly influences steady-state protein levels (69, 70), it has been proposed that Mg^{2+} exerts its influence somewhere after transcript synthesis and stability. Because transmission electron microscopy found no obvious basal body-like structures at the poles of the nonflagellated Mg^{2+}-limited cells (69), the block likely occurs just before or during an early stage of assembly, e.g., translation, protein stability, and/or export of basal body components (Fig. 5B).

Mutant screens to identify components of this novel *m*agnesium-dependent *i*nduction of *f*lagellation (Mif) pathway led to the identification of two genes that contribute to the inhibition of motility: in the absence of Mg^{2+}, mutants defective for either gene migrated in soft (0.25%) agar substantially sooner and faster than the wild-type control (70). In the presence of Mg^{2+}, however, both mutants migrated at a rate indistinguishable from that of the parent, suggesting that they were not simply hypermotile but instead no longer responded properly to the absence of Mg^{2+}. The *mifA* gene encodes a DGC: when overexpressed in *E. coli*, it promotes synthesis of c-di-GMP. The *mifB* gene likely also encodes a DGC for it shares extensive homology with MifA and other DGCs. Like Mg^{2+}, the impact of MifA and MifB appears to be posttranscriptional. The steady-state levels of flagellin subunits increased in *mifA*, *mifB*, or *mifAB* mutants and decreased when either protein was overexpressed, and yet none of these genetic manipulations exerted a substantial effect upon flagellar gene transcription (70).

Surprisingly, in the absence of Mg^{2+}, a double *mifA mifB* mutant did not exhibit the full Mg^{2+}-induced phenotype. Such behavior suggests the existence of an additional Mif component. It also supports a model in which Mg^{2+} does not feed into the system solely through MifA or MifB but leaves open the possibility that Mg^{2+} signals through another, more down-

stream component (Fig. 5B). Given the involvement of two DGCs, it seemed reasonable to hypothesize the existence of a gene that encodes a PDE that would specifically degrade the c-di-GMP synthesized by MifA and MifB. Indeed, such a gene has been identified: loss of MifD (a GGDEF-EAL hybrid with a nonconsensus GGDEF domain) results in decreased migration (even in the presence of Mg^{2+}) and restores wild-type-like behavior to a *mifB* mutant (A. J. Wolfe and K. L. Visick, unpublished data). Whether other PDEs also participate in this pathway and whether PDE activity is modulated by Mg^{2+} remain to be determined.

Impact on function. Upon completion of flagellar assembly, motor (Mot) proteins pump protons (H^+) across the cytoplasmic membrane, providing energy to drive rotation (Fig. 1), while chemotaxis components control the direction of that rotation in response to physicochemical gradients (55). For the enterics *E. coli* and *S. enterica* serovar Typhimurium (YhjH/YcgR), for *C. crescentus* (DgcA/DgrAB), and for *P. aeruginosa* (SadB/BifA), evidence that c-di-GMP inhibits motility at the level of flagellar rotation exists.

YhjH/YcgR. For *E. coli*, the evidence that c-di-GMP-related components might be involved came long before their biochemical functions became clear. An early study (47) revealed related roles in motility for two proteins: YcgR and YhjH, now known to be a PilZ domain protein (5, 18) and a PDE (90), respectively. These linked roles were identified through characterization of *hns* mutants, which exhibit motility defects (47). At the time, H-NS was known to impact motility at two levels: (i) a DNA-binding-dependent enhancement of flagellar biogenesis by promoting transcription of *flhDC*, which encode the master flagellar transcriptional regulator (Fig. 3A) (7), and (ii) a DNA-binding-independent modulation of flagellar rotation through interaction with the flagellar switch component FliG (23, 58). In an effort to separate these two functions, Ko and Park constructed *hns* mutants that transcribed *flhDC* in an H-NS-independent manner and found that they remained largely nonmotile (47). Careful phenotypic analyses of these mutants suggested that the absence of H-NS results in paralyzed flagella. Overexpression of the motor components FliG, MotB, and both MotB and MotA failed to restore motor function to the *flhDC*-constitutive *hns* mutant. In contrast, overexpression of MotA alone increased motility, albeit to a fraction of that of the wild-type parent. Since the levels of these motor components were not reduced in the *flhDC*-constitutive *hns* mutant relative to the wild type, Ko and Park argued against the lack of expression or protein instability as the cause of the H-NS-dependent motor defect. A search for transposon-based suppressors of this motor defect revealed *ycgR*, while a search for multicopy suppressors yielded *yhjH*. Although disruption of *ycgR* alone in an otherwise wild-type background did not cause a motility phenotype, loss of this gene in the *flhDC*-constitutive *hns* mutant restored flagellar rotation and, thus, promoted motility. Similar results were seen when multicopy *yhjH* was introduced into the *flhDC*-constitutive *hns* mutant. Bioinformatic analysis of the *ycgR* and *yhjH* promoter regions revealed sequences that suggested that both genes were σ^{28}-dependent (class III) members of the flagellar regulon (Fig. 3A); indeed, transcription of both genes depended on *flhDC* (47).

Recently, many of these observations were confirmed for *S. enterica* serovar Typhimurium. As with *E. coli*, both *yhjH* and *ycgR* were shown to be class III members of the flagellar regulon (27, 105). Moreover, disruptions of *yhjH* led to a decreased migration rate, while disruptions in *ycgR* did not (27, 87, 88). Importantly, however, the decreased migration rate of the *yhjH* mutant could be reversed by a second disruption, one in *ycgR*, thus providing strong genetic evidence that the PilZ domain protein YcgR functions downstream of the PDE YhjH. In support of this hypothesis, biochemical and genetic analyses showed that YcgR must be able to bind c-di-GMP to antagonize the action of YhjH. Furthermore, the inability to observe any other phenotypes associated with c-di-GMP in *Salmonella* suggests that YhjH and YcgR are dedicated to motor control and provides evidence that c-di-GMP can be very specific (88).

Taken together, these studies suggest the following model (Fig. 6A). YhjH and an unknown DGC set the levels of c-di-GMP, which binds to YcgR. In a mechanism yet to be determined, this complex interferes with the proper association of the Mot proteins with FliG and the rest of the switching device. The result is a paralyzed flagellum. If the interaction of the Mot complex with FliG is a regulated process (104), then one could easily imagine that H-NS could enhance the process while the c-di-GMP–YcgR complex could inhibit it (47).

DgcA/DgrAB. Recent work by Christen and colleagues (18) revealed that two other PilZ domain proteins (DgrA and DgrB; *di*guanylate *r*eceptors A and B) modulate flagellar function in response to high c-di-GMP levels. Overexpression of DgcA, a highly active *C. crescentus* DGC, disrupted motility (17, 18). However, overexpression of DgcA had no effect upon the levels of flagellin protein and the cells remained flagellated (18). This paralysis depended upon DgrA and DgrB: although disruption of *dgrA* or *dgrB* had no effect in wild-type cells, disruption of either alleviated the motility defect caused by DgcA overexpression. Thus, the DgcA/DgrAB story resembles that of YhjH/YcgR: in response to high levels of c-di-GMP, the PilZ domain protein appears to interfere with flagellar function rather than with the expression or assembly of the core flagellar components.

Like overexpression of *dgcA*, overexpression of either *dgrA* or *dgrB* resulted in cells with paralyzed flagella. Such cells contained normal levels of flagellar proteins chosen as representatives of each level of the flagellar hierarchy (Fig. 3). Furthermore, they elaborated seemingly complete flagella, and yet the cells appeared to be nonmotile (18). How the c-di-GMP generated by DgcA and that bound by DgrA and DgrB influence flagellar function remains to be determined; however, several intriguing clues exist. First, overexpression of DgrA, but not of DgrB, reduced the levels of a single flagellum-associated protein, FliL (18). Second, in *C. crescentus*, disruption of *fliL* was shown to result in paralyzed flagella (42). Third, in *C. crescentus*, FliL is present throughout the inner membrane rather than localized to the vicinity of the flagellum, a position that could facilitate its ability to interact with c-di-GMP wherever it exists. Fourth, FliL is the product of the first gene in a large operon that encodes motor/switch and export apparatus proteins, but it has not yet been assigned a specific function (91). Recently, however, it was shown to be involved in the transition of *Proteus mirabilis* from a vegetative swimmer cell to an elongated swarmer cell (6): polar insertions into *fliL* mutants resulted in a nonmotile, hyperelongated phenotype, while cells (including wild type) that overexpressed FliL alone
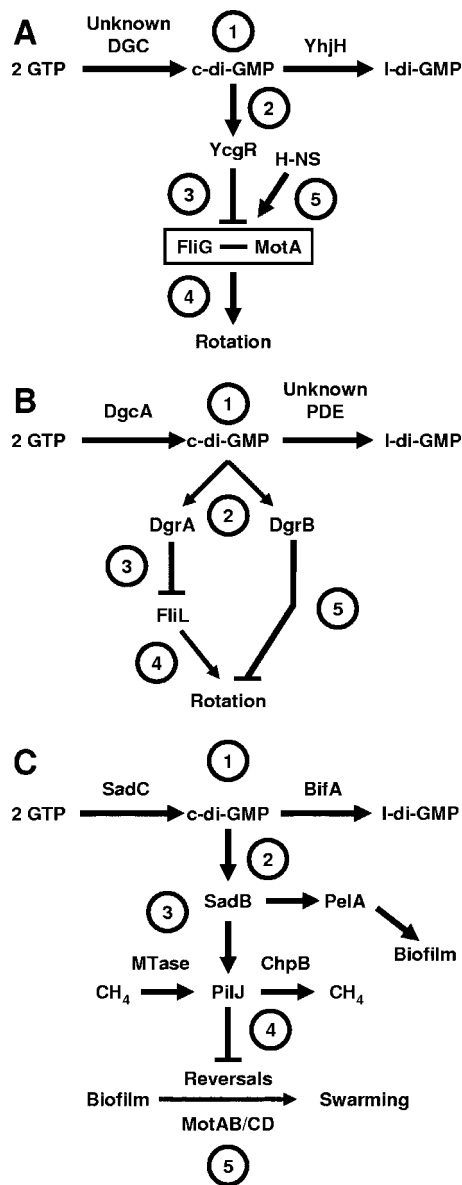
FIG. 6. Working models for how c-di-GMP influences flagellar function. (A) YhjH/YcgR model for *E. coli* and *S. enterica*. (Label 1) The PDE YhjH and an unidentified DGC set the steady-state levels of c-di-GMP, which (label 2) binds to YcgR. (Label 3) This complex interferes with the ability of cells to properly insert the MotAB energy transduction complex next to the switching device component FliG in the completed basal body, resulting in (label 4) paralyzed flagella. (Label 5) The nucleoid protein H-NS antagonizes this effect by stabilizing the FliG-MotA interaction. The net effect of YcgR activity is to impair rotation, an activity opposed by YhjH. (B) DgcA/DgrAB model for *C. crescentus*. (Label 1) The DGC DgcA and an unidentified PDE set the steady-state levels of c-di-GMP, which (label 2) binds to DgrA and DgrB. (Label 3) Overexpression of DgrA decreases the steady-state levels of FliL. (Label 4) Since *fliL* mutants assemble paralyzed flagella, FliL may be integral to DgrA-dependent inhibition of rotation. (Label 5) Like DgrA, overexpression of DgrB inhibits flagellar rotation although it has no effect on FliL. The net effect of DgcA, DgrA, and DgrB is to impair rotation. (C) SadC-BifA model for *P. aeruginosa*. (Label 1) The DGC SadC and the PDE BifA set the steady-state levels of c-di-GMP, which (label 2) modulates the activity of SadB via a presently unknown mechanism. Also not understood is how SadB influences the activity of (label 3) PelA and the rest of the EPS biosynthetic pathway or (label 4) the methylation state of the

did not elongate but remained nonmotile. Furthermore, expression of *P. mirabilis* FliL in wild-type *E. coli* resulted in reduced motility. Whether the *P. mirabilis* FliL functions in a c-di-GMP-dependent pathway and, if so, what level of regulation is impacted remain to be determined.

How might c-di-GMP influence *C. crescentus* flagellar function? The strong similarities to the YhjH/YcgR story, coupled with the observations that *fliL* mutants of *C. crescentus* assemble paralyzed flagella and that overexpression of *P. mirabilis* FliL inhibits *E. coli* motility, led Christen and coworkers to argue that FliL plays a key role in a universal mechanism that controls the transition between the motile planktonic and sessile biofilm lifestyle (18). In this model (Fig. 6B), the c-di-GMP produced by DgcA binds to DgrA and DgrB. The c-di-GMP–DgrA complex inhibits FliL, which is required for flagellar rotation. The c-di-GMP–DgrB complex also inhibits rotation, but the mechanism remains uncertain. Whether it involves the interaction between the MotAB energy transduction apparatus and FliG of the switching device, as proposed by Ko and Park (47), remains to be determined.

How does the c-di-GMP–DgrA complex regulate FliL? While the mechanism is unclear, the evidence suggests that it works posttranscriptionally. First, despite its position as the first gene in its operon, only FliL is regulated by DgrA. Furthermore, a search for extragenic suppressors of DgrA overexpression revealed *rpsA*, which encodes S1, a ribosomal protein that enhances translation initiation (18). An additional link between FliL, motility, and c-di-GMP is described later in the review.

SadC/BifA. In four recent manuscripts, O'Toole and colleagues have sketched out a c-di-GMP-dependent pathway that appears to control the initiation of biofilm development in *P. aeruginosa* in part by regulating the flagellar reversal rate of swarming cells (11, 49, 66, 102).

Biochemical assays demonstrated that SadC, a GGDEF domain protein, functions as a DGC (66), while BifA, a GGDEF-EAL domain hybrid, functions as a PDE (49). Epistasis experiments provided evidence that, although other DGCs might be involved, these two integral cytoplasmic membrane proteins primarily set the steady-state level of a c-di-GMP pool responsible for controlling biofilm formation, wrinkled colony morphology, extracellular polysaccharide (EPS) synthesis, and swarming (Fig. 6C). The cumulative data support the hypothesis that c-di-GMP accumulation enhances biofilm formation by increasing *pel*-dependent EPS synthesis and inhibiting swarming. This inhibition, however, does not involve EPS: *pelA bifA* mutants are no more motile than *bifA* mutants (49, 66).

Instead, c-di-GMP-dependent inhibition of swarming correlates with a decrease in the rate at which flagellar rotation switches between the clockwise and counterclockwise states. Flagella are intimately involved in reversible attachment, the initial step of biofilm development (11, 71, 102). Furthermore, cells that switch rapidly (e.g., *sadC* mutants) are less likely to

chemoreceptor PilJ or (label 5) how PilJ controls the reversal rate, a process that also includes the energy transduction complexes MotAB and MotCD. The net result of SadC and SadB activity is to decrease the reversals necessary for swarming. l-di-GMP, linear diguanylate.

become irreversibly attached. Thus, O'Toole and coworkers suggested that the reduced switching rate provides the cells with a longer window of opportunity to interact lengthwise with the surface and increases the likelihood of irreversible attachment, the committed step toward biofilm formation (11). They argue that this behavior is analogous to that of switching-deficient *E. coli* mutants, which tend to become trapped in blind alleys within the liquid-filled maze of channels that comprises semisolid (soft) agar (108).

How does this pool of c-di-GMP influence motor reversals and, hence, the transition between biofilm formation and swarming? The details of the mechanism remain unsolved; however, the authors have used epistasis analyses to conclude that SadB is a required downstream pathway component that regulates both EPS synthesis and motor reversals (11, 49, 66). Downstream of SadB is the CheIV chemotaxis-like cluster, which includes the chemoreceptor PilJ and the methylesterase ChpB (Fig. 6C). It appears that the methylation state of PilJ determines the reversal rate (11), a process that involves the energy transduction complexes MotAB and MotCD (102).

Flagellar ejection. In *C. crescentus*, motility is controlled at specific times in the cell cycle by flagellar assembly and subsequent ejection of a fully functional flagellum. Release of the *Caulobacter* flagellum occurs concomitantly with the proteolysis of FliF (41). FliF is the first flagellar protein assembled and comprises the MS ring that anchors the flagellum into the cytoplasmic membrane (Fig. 1) (41, 103). Given its importance in assembly, it would thus not be surprising if it also served as the target for the control of flagellar ejection (41). Indeed, upon proteolysis of FliF, the flagellar filament along with the hook and the distal portion of the rod is released into the extracellular medium (41, 43). However, recent data have failed to support this model. For example, proteolysis-resistant derivatives of FliF retained normal flagellar function and ejection (30). Similarly, a mutant that lacked ClpA, a protein that together with ClpP forms the ClpAP protease (75), failed to degrade FliF yet ejected flagella normally (30). Together, these data argue that ejection does not require cleavage of FliF but remain consistent with a model that involves the loss of one protein or a small complex of proteins located near FliF at the cytoplasmic-proximal portion of the rod (43). Other FliF-proximal flagellar proteins, the switching proteins FliG and FliM, also appear to undergo cell cycle-dependent degradation (41, 42) and thus could also participate in flagellar ejection.

Although the cause of flagellar ejection cannot be ascribed to proteolysis of FliF, this developmental event remains proximal to ejection and thus important as a marker for development. One regulator known to control FliF stability and flagellar ejection is PleD, the best-characterized regulator of c-di-GMP levels and motility in *C. crescentus* (2, 4, 33, 93). Examination of PleD provided biochemical proof that the GGDEF domain was responsible for DGC activity (73). Furthermore, PleD is the first DGC for which a crystal structure has been obtained. Most importantly, this structural work revealed the active site for c-di-GMP synthesis and identified a site for allosteric inhibition (13, 17).

Like many other regulators of c-di-GMP, PleD controls the motile-sessile transition: the evidence for its role in ejection is substantial, as are the data that this protein participates in the subsequent development of the stalk (2, 33, 52, 93). In a *pleD*
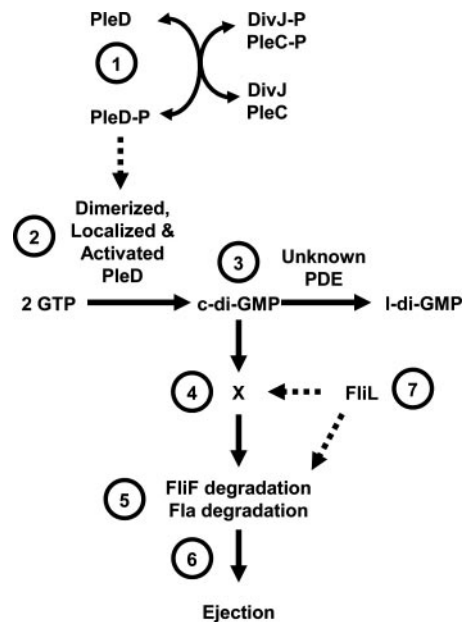

FIG. 7. Working model for how c-di-GMP influences flagellar ejection. The DGC PleD controls flagellar ejection. (Label 1) Activation of PleD depends upon phosphorylation, which is controlled by two sensor kinases, DivJ and PleC. (Label 2) Phosphorylated PleD (PleD-P) dimerizes, and these dimers can now be polarly localized. (Label 3) The dimer form of PleD is now active as a DGC, and it and an unidentified PDE set the steady-state levels of c-di-GMP. (Label 4) c-di-GMP binds to an unknown c-di-GMP-binding protein, which (label 5) helps to destabilize FliF and possibly another flagellar protein(s) in the proximity of FliF. (Label 6) Destabilization of this protein(s) leads to ejection. (Label 7) FliL appears to be a component of the PleD pathway, but its position in that pathway remains uncertain. l-di-GMP, linear diguanylate.

mutant, the flagellum is not ejected. This effect is independent of *fliF* transcription; instead, the FliF protein is not efficiently processed, i.e., the stability of the FliF protein increases from 67 min to 94 min, a change probably sufficient to account for the retention of FliF by the *pleD* mutant (2). The C-terminal GGDEF domain of PleD is required for appropriate degradation of FliF, as mutations that deleted GGDEF or changed the GG to DE failed to induce normal proteolysis (2). These data suggest that the DGC activity of PleD is important for flagellar ejection (Fig. 7).

PleD does not impact generalized cell cycle-dependent degradation, as indicated by the normal degradation in a *pleD* mutant of the cell cycle-dependent McpA chemoreceptor (2). Thus, ejection of the flagellum and degradation of the chemotaxis machinery appear to be independent events. Indeed, proteolysis of FliF is independent of a number of other flagellar proteins, including rod, hook, and switch proteins (2). Intriguingly, however, proteolysis is decreased in cells lacking FliL, a protein that plays a role in flagellar rotation (2, 42) and whose levels are decreased in cells that overexpress the PilZ domain protein DgrA (18). Thus, both PleD and FliL appear to destabilize FliF. Furthermore, the stability of FliF in a *pleD fliL* double mutant was similar to that of the *pleD* single mutant, a result that suggests they may function in the same pathway (2). Whether DgrA is also part of this pathway remains to be determined. A dissection of FliL revealed that its ability to

induce normal FliF proteolysis depends upon a highly charged internal domain (Δ*fliL1*), a region not required for motility (42). These data support the hypothesis that FliL plays distinct roles in both motility and FliF degradation.

The connection between PleD and FliL indicates that PleD may contribute to control of flagellar rotation. Indeed, this possibility was first suggested by the isolation of *pleD* as a motile suppressor of the paralyzed *pleC* mutant (93). Furthermore, flagellar biogenesis but not rotation could be restored to the *pleD* mutant by a constitutively active allele of the *P. fluorescens* WspR DGC (4), further establishing this link. However, the specific role of PleD, if any, in promoting flagellar rotation remains to be determined.

How is the activity of PleD controlled? PleD is composed of three domains: the GGDEF domain and two copies of the two-component response regulator domain. This architecture is unusual on two counts: it appears to have two input domains, and those input domains are attached to a GGDEF domain instead of a DNA-binding domain (2, 33). This structure suggests that phosphorylation modulates DGC activity, and the evidence supports this hypothesis (72, 73). Furthermore, this phosphorylation controls localization. PleD localizes to the stalked pole at the swarmer cell-to-stalked cell transition (73). This localization depends upon activation of PleD: PleD fails to localize to the poles when it is unable to be phosphorylated, either through mutation of the site of phosphorylation or by disruption of the two kinases (DivJ and PleC) known to control the phosphorylation status of PleD (4, 73). In contrast, localization is independent of the GGDEF domain. A dominant negative allele of *pleD*, which produces a protein (PleD*) with four substitutions, exhibited substantially reduced motility (4). PleD* functions independently of phosphorylation signals, as disruption of the site of phosphorylation (Asp53) did not impact its activity. In contrast, the addition of a mutation that prevents PleD dimerization (Y26A) disrupted localization of PleD* (72), thus supporting the importance of dimerization for localization of PleD. In summary, the activity of PleD is controlled by phosphorylation, which promotes formation of a dimer, the form of PleD associated with DGC activity (Fig. 7) (72, 73). Thus, it is clear from the investigation of this one DGC that cells can tightly control the activity of a DGC to ultimately regulate motility. In this case, the posttranscriptional mechanisms are phosphorylation and localization, which are further controlled in *Caulobacter* by links to the cell cycle (59). In other organisms under different environmental and developmental scenarios, the mechanisms will likely differ.

Concluding remarks. Many studies link c-di-GMP and motility. However, to understand the specific roles of c-di-GMP in controlling motility, it is necessary to look beyond studies that rely on DGC or PDE overexpression, as the observed impacts on motility may, in many cases, turn out to be nonspecific, artifactual consequences of high c-di-GMP levels on cellular physiology. Rigorous proof that a particular pathway exerts a specific effect on motility requires the construction of nonpolar chromosomal mutations, verification by single-copy complementation, and, where appropriate, epistasis tests to evaluate relationships within and between pathways.

Despite the limited number of studies in which such experiments have been completed, there is nonetheless clear evidence that c-di-GMP exerts specific effects on flagellar biogen-

esis. The mechanisms by which c-di-GMP appear to impact motility are as diverse as the levels of control that are already known to exist. A better understanding of the role of c-di-GMP in controlling motility, however, will be advanced substantially through additional efforts to fence in the level at which the c-di-GMP-dependent effect occurs. While it is relatively simple to investigate motility by using soft agar assays (108), too many studies appear to rely solely upon evaluating single time point data. This is particularly problematic because subtle phenotypes, such as the reduced motility reported in many investigations of c-di-GMP-dependent pathways, can be caused by a number of reasons. These include delayed migration due to a failure to express flagella at the time of inoculation, a subtle chemotactic defect, reduced numbers of flagella, and mildly defective flagella. Detailed knowledge of migration history can help distinguish among these diverse possibilities (for examples, see references 69 and 70). To complete the evaluation, other assays must be employed, including light microscopy to examine swimming behavior and motor rotation, electron microscopy to observe flagella and/or incomplete flagellar structures, reporters to test gene and protein expression, and biochemical studies (e.g., immunoblot analysis) to verify the conclusions.

Once it becomes clear that c-di-GMP is involved in controlling motility, the next goal should be to identify the effector. The discovery of the PilZ domain has provided insights and promise for identifying these effectors and elucidating their pathways. For the few PilZ proteins whose roles have been ascertained, however, their functions have been revealed only by their overexpression or when either a DGC has been overexpressed or a PDE disrupted. Are there circumstances in which these proteins play a role in the absence of high levels of c-di-GMP? One possibility is that the PilZ domain proteins do not become important except under conditions not typically assayed in the laboratory (e.g., swarming across surfaces or survival in a host). Alternatively, or in addition, the activity of certain DGCs and PDEs may be limited to specific locations and times. This certainly is the case for *Caulobacter* PleD, whose impact on flagellar ejection depends upon a number of factors, including the receipt of specific cell cycle-dependent signals (59), its status relative to phosphorylation and the monomer-dimer transition, and its localization. Other signals in other organisms are known or suspected to act upon the levels of c-di-GMP or its downstream output. These include Mg^{2+} (*V. fischeri*) (69, 70), norspermidine (*V. cholerae*) (44), phosphate (*P. fluorescens*) (67), and aminoglycosides (*P. aeruginosa*) (35). Even with evidence of specific signals that induce various c-di-GMP-dependent pathways, it would seem surprising if the very limited numbers of PilZ domain proteins identified by bioinformatics (5) alone can carry out all of the downstream consequences of c-di-GMP signaling. Perhaps some PilZ domain proteins are not highly conserved and, thus, have not been identified as such. Another possibility is that some of the proteins that carry less well-conserved EAL/GGDEF signature motifs will turn out to be effectors of the target rather than controllers of the signal. Moreover, other c-di-GMP-binding domains may yet exist. Alternatively, or additionally, c-di-GMP might bind directly to specific flagellar components.

In writing this review, two common themes we noticed were

the connection between c-di-GMP and FliL in controlling motility and the importance of c-di-GMP in swarming motility of multiple organisms. FliL, although part of the flagellar regulon, does not seem to be an integral component of the flagellum in the enterics. In *Caulobacter*, FliL also is not required to make a flagellum, but it is required for flagellar function, e.g., rotation. Furthermore, the levels of FliL are decreased by overexpression of the c-di-GMP receptor protein DgrA, which also causes the production of paralyzed flagella. It would be interesting to know whether the dual overexpression of FliL can restore some motility to cells that overexpress DgrA. Intriguingly, the loss of either FliL or PleD increases the stability of FliF, a phenotype that occurs coincident with flagellar ejection. Together, these data lead us to wonder about the role of FliL. Perhaps it is a sensor that recognizes that a cell is touching a surface and thereby needs to induce lateral flagella for swarming or that a flagellum is no longer required and thereby signals its ejection. That a protein such as FliL might be involved in relaying information about surface recognition is not too far-fetched. Indeed, for *Salmonella*, the flagellum has been shown to sense surface wetness and, in response, control filament length of swarming cells (106). Interestingly, the ability to reverse flagellar rotation seems to be all that is required to enhance surface wetness and restore filaments to their maximal length (56). The latter phenomenon could provide an alternative explanation for the swarming and biofilm phenotypes observed by O'Toole and colleagues. *P. aeruginosa sadC* mutants switch rapidly, potentially increasing surface hydration and promoting swarming. In contrast, *bifA* mutants reverse less frequently and would not hydrate their surfaces. The result would be reduced swarming and enhanced biofilm formation.

Finally, the variety of levels at which c-di-GMP can impact motility in the relatively few organisms studied begs the following question: how universal are these mechanisms? Given the evolutionary relationship between flagellar and nonflagellar TTS systems (organelles through which pathogens inject their virulence factors), we might expect to shortly see evidence that c-di-GMP controls virulence through impacting the transcription, translation, assembly, and function of such organelles. The groundwork that we lay down in understanding the impact of c-di-GMP on flagellar biogenesis and function, in that case, will be far-reaching.

ADDENDUM IN PROOF

Recent work studying ScrC suggests that this protein can function as both a PDE and a DGC (R. B. R. Ferreira, L. C. M. Antunes, E. P. Greenberg, and L. L. McCarter, J. Bacteriol., in press). ScrC may thus be the "unknown DGC" shown in Fig. 4A, and ScrB may modulate these two activities.

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