The Second Messenger Cyclic Di-GMP
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Chapter 13

Roles of Diguanylate Cyclases and Phosphodiesterases in Motility and Biofilm Formation in *Vibrio fischeri*

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Cyclic di-GMP (c-di-GMP) plays important roles in lifestyle choices, notably in the decision to be motile and/or to form a biofilm (reviewed in chapter 8). This is true for *Vibrio fischeri,* a marine microorganism that encodes over 50 proteins predicted to synthesize, degrade, or bind c-di-GMP. A subset of these c-di-GMP metabolism genes are known to be involved in influencing flagellar biogenesis, while others have been shown to influence biofilm formation. Here, we will first describe the biology of *V. fischeri* and the environmental influences that may dictate its need for large numbers of proteins dedicated to c-di-GMP control and function. We then will present a bioinformatic analysis of these c-di-GMP-associated proteins. Next, we will provide a brief description of flagellar biogenesis and the roles played in controlling that process by both the magnesium cation (Mg^{2+}) and specific c-di-GMP-associated enzymes. We then will outline the current knowledge concerning the role of c-di-GMP in cellulose biosynthesis and biofilm formation in *V. fischeri*. Finally, we will present models for the roles of c-di-GMP in biofilm formation and flagellar biogenesis.

INTRODUCTION TO THE BIOLOGY OF *V. FISCHERI*

V. fischeri is a marine bioluminescent microorganism that exists both as a free-living organism in seawater and in associations with animals, including fishes and squids. For a long time, *V. fischeri* was best known for its ability to bioluminesce using a cell density detection system now termed quorum sensing. This fame was well deserved, as studies of *V. fischeri* bioluminescence resulted in the discovery of the canonical LuxI/LuxR quorum-sensing regulators now known to exist in most gram-negative bacteria studied to date (10).

In the past 20 years, *V. fischeri* has gained additional fame as an important model symbiont: this microbe forms an exclusive relationship with the small Hawaiian squid *Euprymna scolopes,* an association that is experimentally tractable and used as a natural model of bacterium-animal interactions (33, 46, 50, 53). Though not a pathogen itself, *V. fischeri* is closely related to important human pathogens, including *Vibrio cholerae* (which causes cholera), *Vibrio parahaemolyticus* (which causes gastroenteritis), and *Vibrio vulnificus* (which produces septicemia and is responsible for the majority of *Vibrio*-associated deaths in the United States) (29). Thus, studies of the *V. fischeri*-squid association have significant potential for illuminating both symbiotic and pathogenic associations.

Adult *E. scolopes* contain a symbiotic organ, termed a "light organ," where *V. fischeri* cells exist at a cell density greater than 10^{10} cells per cm^3 (32). The bioluminescence from *V. fischeri* is used by the squid to avoid detection by predators using a mechanism known as counterillumination (18, 55). In this phenomenon, the nocturnal animals direct the bacterial bioluminescence downward to avoid casting a shadow, ~~produced by~~ the down-welling moonlight, due to that can be detected by predators. In return, *E. scolopes* provides *V. fischeri* with nutrients, in the form of peptides, and a protected niche (13).

In contrast to the adult, newly hatched juveniles are not colonized. Within hours of hatching, however, the nascent light organs of these animals become colonized by *V. fischeri* (Fig. 1). Only *V. fischeri* cells colonize, despite the presence of numerous

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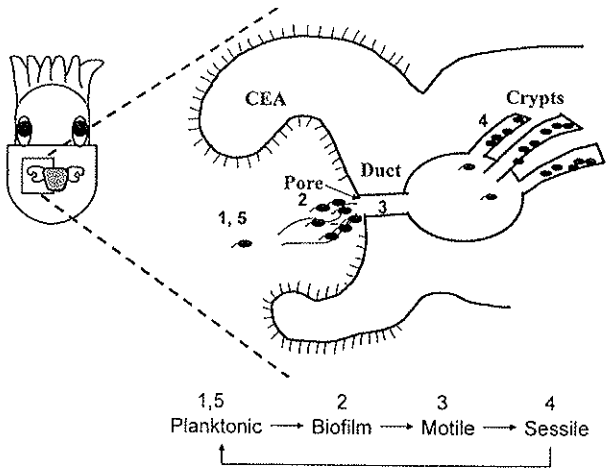


Figure 1. Lifestyle transitions during symbiosis. *V. fischeri* undergoes several switches between motile/planktonic and biofilm/sessile forms during symbiotic colonization of its host, *E. scolopes*. A cartoon of the juvenile squid with its light organ is depicted on the left. A portion of the light organ is enlarged on the right, representing the path to colonization of 1 of 6 crypts found in the juvenile light organ. The numbers represent different stages as follows: 1, motile, planktonic *V. fischeri* cells are present in seawater; 2, *V. fischeri* cells aggregate in squid-secreted mucus near the pores of the light organ; 3, motile bacteria enter the light organ; 4, the bacteria establish colonization in the crypts, where they lose their flagella; 5, an expulsion event releases *V. fischeri* cells back into the seawater, where they can become motile again. CEA, ●●●.

other bacteria in seawater. The factors accounting for this specificity remain under investigation. However, a number of genes and traits that are necessary for symbiotic colonization have been identified, and the process by which colonization occurs has been examined microscopically (33, 54). These studies have revealed that both flagellar motility and biofilm formation—traits known to be impacted by c-di-GMP—are critical to colonization by *V. fischeri*.

Indeed, the process of colonization (and release) appears to occur through a series of transitions between the motile and sessile forms of *V. fischeri*. Newly hatched juveniles ventilate seawater containing *V. fischeri* and other bacteria at a rapid rate: it has been estimated that a single *V. fischeri* cell enters and exits a juvenile squid every 0.3 seconds (53). Thus, there is little opportunity for *V. fischeri* to simply find its way into 1 of the 6 pores that lead into the light organ. Rather, it appears that the initial stage of colonization proceeds via a biofilm-like attachment to the surface of the light organ. Studies with green fluorescent protein-labeled cells revealed that *V. fischeri* cells first aggregate in a biofilm-like structure on the surface of the light organ by embedding in mucus secreted by the squid (34, 59). Other bacteria, such as the closely related *V. parahaemolyticus*, also can adhere to the light organ. In this respect, however, *V. fischeri* is superior: an equal mixture of *V. parahaemolyticus* and *V. fischeri* resulted in an aggregate of greater than 80% *V. fischeri* cells (31). This ability to aggregate depends upon a large polysaccharide locus, termed *syp:* loss of this locus disrupts aggregation and colonization, while overexpression of this locus leads to increased biofilm formation in culture and increased symbiotic aggregation (59, 60). The similar genetic requirements in vitro and in vivo support the conclusion that aggregation represents a form of biofilm formation.

Following aggregation, the bacteria migrate into the light organ through 1 of the 6 pores (Fig. 1). This stage of colonization requires motility. Nonmotile strains of *V. fischeri* successfully aggregate on the light organ surface but never migrate to the pores and, thus, fail to colonize (12, 26, 34). A number of hypermotile *V. fischeri* strains also colonize poorly; in this case, the strains fail to aggregate (25). Thus, control of motility by *V. fischeri* is an essential feature of symbiotic initiation.

The pores lead into ducts (Fig. 1), which contain mucus and cilia that beat outward (24, 53). Thus, bacterial motility also is likely to be required for combating these outwardly directed forces. Beyond the ducts lie the crypts (Fig. 1), the sites of bacterial colonization. Here, multiplication of *V. fischeri* occurs rapidly (47), coincident with the induction of bioluminescence. Surprisingly, flagella, which facilitate bacterial entry, appear to be superfluous inside the crypts. A majority of crypt-residing *V. fischeri* cells are nonflagellated within about 48 h (47); whether loss of flagella is required for efficient colonization by *V. fischeri* remains unknown. These now-sessile bacteria are embedded in a matrix of host and/or bacterial origin (32), making it likely that they exist in the form of a biofilm inside the light organ.

Finally, for the duration of the symbiosis, the bacteria face the daily challenge of a changing environment. Each dawn, the squid expels 90 to 95% of its symbionts from the light organ (20, 32) through a muscle-controlled contraction. At least some of the expelled bacteria can become flagellated again within 45 min of their release into seawater (47). These flagellated bacteria may be primed for entry into another host.

Less is known about the life cycle of *V. fischeri* outside its squid host. It can be found in the gut tracts of fishes as well as in a specific symbiosis with the fish *Monocentris japonica* (30, 48). In seawater, it is more abundant in areas near or adjacent to squid habitats than in distal regions, a finding that is consistent with the known expulsion behavior of the squid host (20). Outside the squid, the bacteria tend

to be found in the sediment: at the same sampling location, *V. fischeri* cells were much more abundant in sediment samples than in seawater (20). Given that small particles like those found in sediment are ideal surfaces for biofilm formation, these observations suggest that *V. fischeri* may form a host-independent, potentially abiotic, biofilm as part of its life cycle. Thus, *V. fischeri* represents an excellent model for understanding transitions between the motile/planktonic and sessile/biofilm forms of bacterial life, transitions known to correlate with c-di-GMP metabolism (see chapter 8).

BIOINFORMATICS-BASED INVESTIGATION OF c-di-GMP-SYNTHESIZING AND -DEGRADING PROTEINS

In 2001, M. Y. Galperin and colleagues inventoried complete prokaryotic genomes for the numbers and types of signaling domains (11) (see also chapter 3). In this study, they identified GGDEF, EAL, and HD-GYP domains, associated with the synthesis and degradation of c-di-GMP. Although *V. fischeri* was not part of their data set, the related microbe *V. cholerae* was. At that time, *V. cholerae* contained more predicted proteins with a GGDEF domain, 41, than any of the other 35 bacteria and archaea in the study. Although the numbers of the others varied from none to 33, most contained 11 or fewer. Similarly, *V. cholerae* contained the largest number of EAL domain proteins, with 22. Because some of the proteins contained both domains, the numbers for *V. cholerae* domains are as follows: 31 GGDEF, 12 EAL, and 10 GGDEF/EAL. We now known that *V. fischeri* contains a similarly large number of such proteins with 28 GGDEF, 9 EAL, and 11 GGDEF/EAL domains (Table 1; Fig. 2) (58). *V. fischeri* also contains 2 predicted HD-GYP domain proteins.

Table 1. Genes predicted to control c-di-GMP levels in *V. fischeri*

Domain(s)	No. of genes in:		Total no. of genes
	VF (chromosome 1)	VFA (chromosome 2)	
GGDEF	10	18	28
EAL	~~1~~ 2	~~8~~ 7	9
GGDEF/EAL	6	5	11
HD-GYP	1	1	2
Total	~~18~~ 19	~~32~~ 31	50

The sequenced vibrios each contain a larger chromosome that is relatively conserved among the different species and a smaller chromosome that is quite divergent. Thus, it is thought that the smaller chromosome is responsible for the differences in lifestyle and niche selection for the vibrio species (9). For *V. fischeri,* the sizes of the two chromosomes are 2.9 and 1.3 Mb, respectively (49). Interestingly, a recent examination of two-component response regulators encoded by *V. fischeri* revealed an uneven distribution of this regulatory class between the two chromosomes. Despite being twice as large, chromosome 1 contained 22, or slightly more than half, of the 40 response regulators, while the much smaller chromosome 2 contained almost as many (i.e., 18) response regulators (17). Similarly, there is an uneven distribution of genes with GGDEF and EAL domains (Table 1): the smaller chromosome accounts for 30 of the 48 genes. The two HD-GYP genes are split between the two chromosomes. The role(s) of all of these genes in niche selection for *V. fischeri* remains to be determined.

For each of the 28 proteins predicted to contain only a GGDEF domain, that domain resides at the C terminus, taking up about one-third of the protein (Fig. 2A). This organization is consistent with what is known for other GGDEF proteins. For 5 of the 9 EAL domain proteins, the EAL domain could be found in the N terminus or the C terminus (Fig. 2B). In the other 4 proteins, the EAL domain encompassed the majority of the protein; these proteins averaged less than 300 amino acids in length, with the EAL domain including about 200 of them. Finally, in 9 of 11 proteins that contained both domains, the EAL domain was located at the C terminus, with the GGDEF domain immediately adjacent (Fig. 2C). In the other 2 proteins, the EAL domain was at the N terminus while the GGDEF domain was at the C terminus. It will be interesting to determine whether the locations of the domains in the dual-domain proteins will be an accurate predictor of diguanylate cyclase (DGC) or phosphodiesterase (PDE) activity. In support of this possibility, the GGDEF domains in the dual-domain proteins tend to be less well conserved. If most dual-domain proteins function as PDEs, this would result in a more even balance between GGDEF proteins ~~(28)~~ 8 and EAL proteins ~~(9 and 11)~~ 8.

In many cases, GGDEF and/or EAL domains are combined with sensory domains such as HAMP (5), PAS (52), Cache (4), and CHASE (61) (Fig. 2) (see also chapter 3). Nine of the 28 GGDEF-only proteins contain sequences that are associated with other known or predicted domains, including CHASE (and

CHASE 3), Cache_1, CIA30, HAMP, and PAS, another 5 contain a portion of a conserved motif, and the remainder contain sequences dissimilar to any known (searchable) motif. Many of the 28 proteins appear to contain putative transmembrane domains, suggesting that the novel domains detect specific environmental signals that influence DGC activity. In one case, the GGDEF domain is combined with a response regulator receiver domain (REC), indicating that this protein responds to an upstream signaling event, presumably generated through a sensor kinase.

For the EAL-domain-only proteins that contain sequences other than the EAL domain (four of nine), only a single protein contains a fully conserved motif, a REC domain. Two contain truncated HDc superfamily motifs, and another contains a truncated PtsG superfamily motif. Finally, one contains a region with no similarity to known motifs.

For the GGDEF/EAL proteins, seven are combined with full motifs, including DUF1745, HAMP, Cache_1, PAS_4, Cache_2, GAF, and PRK11059, while the other four (including the two with the EAL domain located at the N terminus) contain no additional conserved sequences. As with the GGDEF proteins, many of these dual-domain proteins contain predicted transmembrane segments, indicating that the proteins may localize to the membrane where they would be positioned to receive an environmental signal that modulates their activity.

Of the 2 HD-GYP proteins, one contains two regions with similarity to periplasmic binding protein domains, while the other has a region with no similarity to known motifs (Fig. 2D). Each has putative transmembrane regions as well. Therefore, like the GGDEF and EAL proteins, the putative HD-GYP proteins in *V. fischeri* appear positioned to integrate signals.

In summary, the bioinformatics data show a wide variety of sensory domains associated with GGDEF and EAL domains, indicating that these proteins may recognize a variety of signal inputs that presumably modulate their activities. The truncation of such sensory domains or, in many cases, the absence of known sensory domains, however, suggests that the study of these proteins will reveal additional and novel sensory domains. This hypothesis is consistent with the finding that a high percentage of GGDEF and EAL domain genes reside on the less well conserved chromosome 2 of *V. fischeri*, the chromosome thought to be involved in niche selection. Finally, the potential association of many of these proteins with the inner membrane indicate that their positioning may play important roles in activity and/or signal exchange.

BIOINFORMATICS-BASED INVESTIGATION OF c-di-GMP RECEPTOR PROTEINS

Less is known about proteins predicted to be involved in c-di-GMP binding in *V. fischeri*. In 2006, Amikam and Galperin used bioinformatics, with sequenced bacterial genomes, to identify genes with a PilZ domain, one of several domains now known to bind c-di-GMP (3). In contrast to the abundant GGDEF and EAL domain genes, the *V. fischeri* genome was predicted to contain only 4 PilZ domain genes (a number similar to that seen for *V. cholerae*, which is 5). Two other c-di-GMP-binding domains are known: a degenerate GGDEF domain that retains the c-di-GMP inhibition site (see chapter 4), such as in PelD (21), and FleQ (16) (see also chapter 11). *V. fischeri* possesses at least one degenerate GGDEF that retains the inhibition site (VF0355) and one true homolog of FleQ, termed FlrA (VF1856). Both FleQ and FlrA are known to function as the master activator of the flagellar regulon (6, 26). FleQ is now known to directly bind to and repress transcription from the *pel* promoter, which is required for polysaccharide biosynthesis; c-di-GMP inhibits binding and relieves repression. Since a FleQ variant lacking its C-terminal domain still inhibits *pel* transcription, the c-di-GMP-binding site must be located in the C-terminal two-thirds of the protein (16). Much of this part of the FleQ is conserved in FlrA; thus, it is distinctly possible that FlrA also binds c-di-GMP. In addition, there are 11 other proteins with significant sequence similarity to FleQ, largely to the σ^{54} interaction domain, which is included in the truncated variant. Thus, other proteins could be sensitive to c-di-GMP..

Three of the four PilZ domain genes are located on the large chromosome. A bioinformatic examination of the genes and genomic context of the 4 PilZ domain genes (Fig. 3) immediately suggests a role for one, VFA0884, in cellulose biosynthesis and biofilm formation. The protein encoded by VFA0884 is similar to the cellulose synthase catalytic subunit protein, BcsA, that was originally identified as a c-di-GMP-binding protein (42, 45). Our recent evidence suggests that the protein encoded by VFA0884 does indeed play a role in cellulose biosynthesis (7) (see "Role for c-di-GMP in controlling cellulose biosynthesis" below). The genomic context of the other PilZ genes may also provide clues to function. For example, VF1838 is embedded in the part of the flagellar gene cluster dedicated to protein secretion,

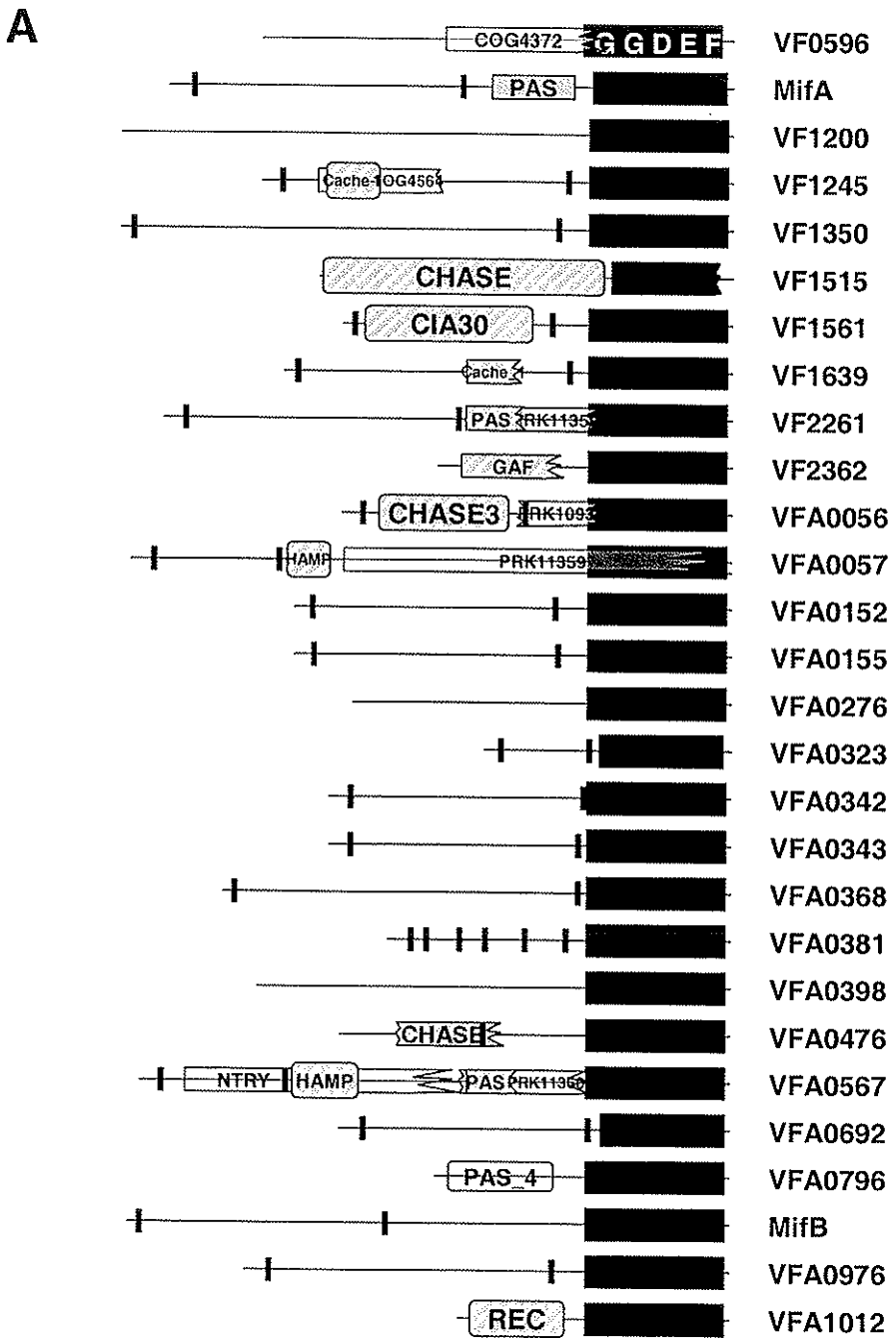
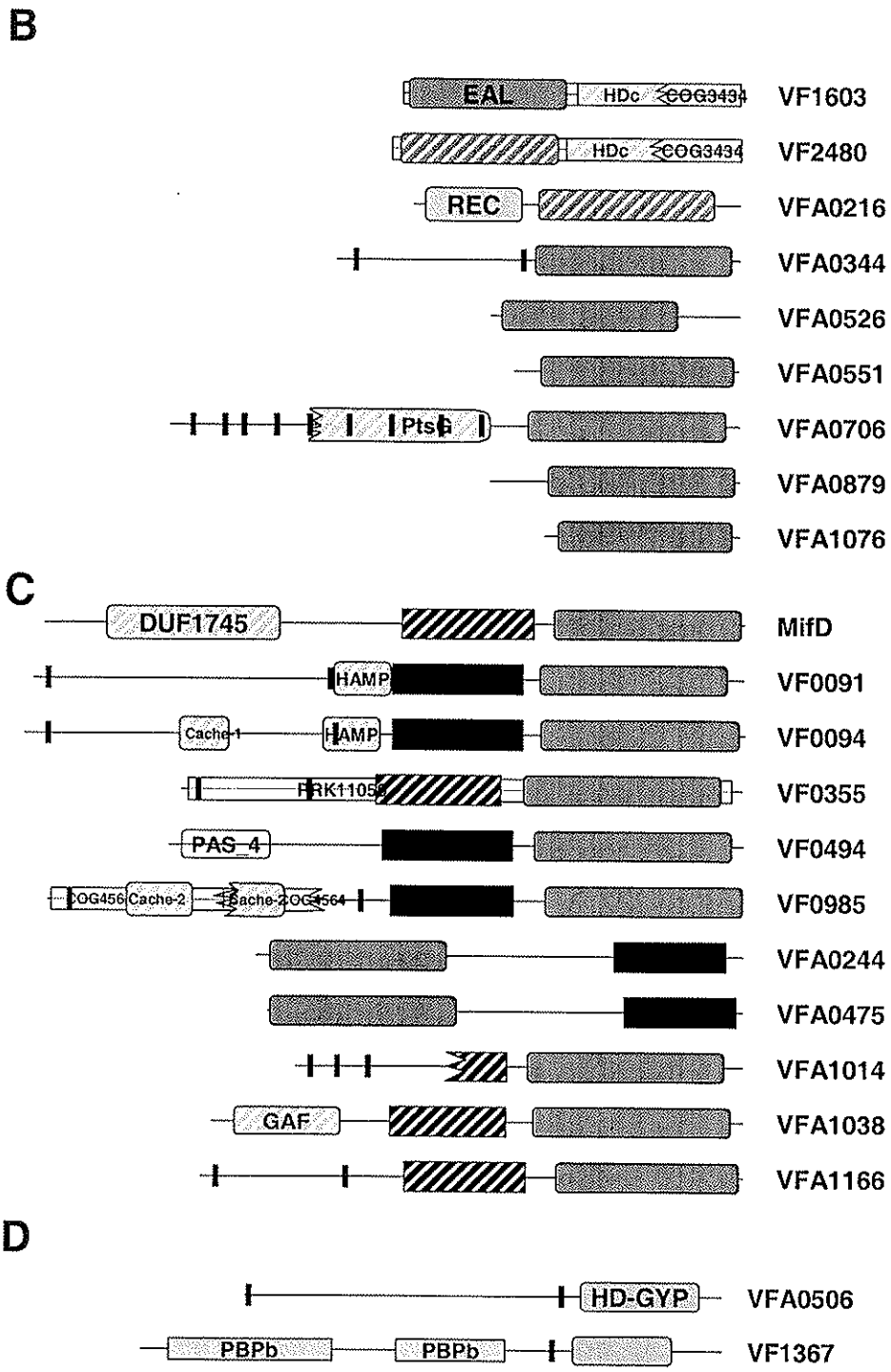


Figure 2. Bioinformatic analysis of the GGDEF, EAL, and HD-GYP domain proteins of *V. fischeri*. Domain structures of the 28 putative GGDEF proteins (A), 9 putative EAL proteins (B), 11 putative GGDEF/EAL proteins (C), and 2 putative HD-GYP proteins (D) encoded by *V. fischeri*. Each GGDEF domain is indicated by black box, while each EAL domain is indicated by a gray box; poorly conserved GGDEF and EAL domains contain white hatch marks within the black and gray boxes. Other domains are as indicated. Broken boxes indicate a truncated domain. Possible transmembrane segments are indicated by small black rectangles. The MifA, MifB, and MifD proteins are encoded by VF0989, VFA0959, and VF0087, respectively.

suggesting a role in flagellar biogenesis. VF0556 is flanked by genes that encode a putative transglycosylase-associated protein and a murein transglycosylase, suggesting a role in peptidoglycan remodeling. VF0527 may represent the first gene in an operon that also encodes a sensor kinase-response regulator pair, suggesting a role in signal transduction. Further work will be necessary to determine if these proteins actually bind c-di-GMP and, if so, to elucidate their precise functions.



NATURE OF THE FLAGELLAR AND CHEMOTAXIS APPARATUS OF *V. FISCHERI*

To appreciate the current understanding of the role played by c-di-GMP in controlling motility of *V. fischeri,* it is first necessary to review what we know concerning flagellar biosynthesis and regulatory control in this microbe. Motile *V. fischeri* cells contain a tuft of flagella at one pole (2, 30, 47). This arrangement contrasts with that of enterics (e.g., *Escherichia coli* and *Salmonella enterica*), which assemble flagella randomly across their surface, a distribution termed peritrichous (1). Furthermore, each *V. fischeri* flagellum is enclosed by a sheath, an extension of the outer

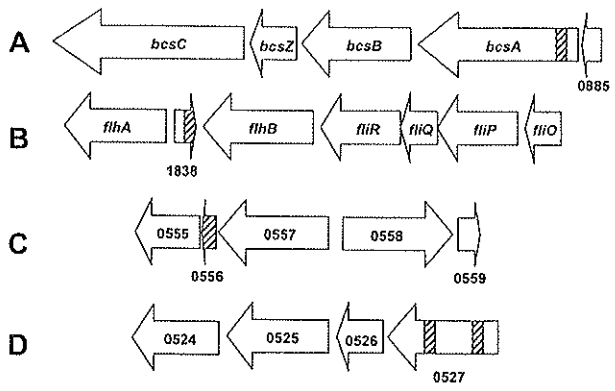


Figure 3. PilZ domain proteins of *Vibrio fischeri*. (A) VFA0884 *(bcsA)*, a member of the cellulose biosynthesis operon *(bcsABZC)*, encodes a glycosyltransferase that includes a PilZ domain (cross-hatching). (B) VF1838, embedded in the part of the large flagellar cluster devoted to flagellar protein secretion, is predicted to encode a PilZ domain protein whose function remains unknown. (C) VF0556, whose function also remains unknown, is flanked by genes predicted to encode a putative transglycosylase-associated protein (VF0555), a predicted ABC transporter (VF0557), a putative soluble lytic murein transglycosylase (VF0558), and a predicted transcription factor (VF0559). (D) VF0527, annotated as an ATP-dependent serine protease with two PilZ domains, is part of locus predicted to encode a two-component response regulator (VF0526) and 2 two-component sensor kinases (VF0525 and VF0524).

membrane (27, 30), while those of the enterics remain unsheathed.

V. fischeri carries on its large chromosome a single, large flagellar gene cluster that encodes proteins similar to well-characterized components of the flagellum, including basal body proteins, the hook, hook-associated proteins, and flagellins, as well as motility regulators such as FlrA and FlrCB (49). It has been hypothesized that *V. fischeri* regulates its flagellar genes in a hierarchy similar to that of *V. cholerae* (26, 38, 39). Consistent with this idea, the motility of *V. fischeri* absolutely depends on σ^{54}, encoded by *rpoN*, and FlrA, a σ^{54}-dependent regulator predicted to sit at the top of the hierarchy (26, 56). Motility also depends upon FlrC, a putative σ^{54}-dependent response regulator predicted to control a subset of flagellar genes (17).

Up to 6 different flagellins (FlaA to F) are assembled into the flagellar filament, although it is not known at this time whether a single filament can contain multiple flagellins (27). The flagellin FlaA appears to be an important component of the flagellum. In contrast to the loss of the FlaC flagellin, which did not noticeably impair motility or flagellation, the loss of FlaA greatly reduced motility. Fewer cells contained flagella, and those that were flagellated elaborated reduced numbers of flagella (26). These data suggest that while FlaA plays a key role, in its absence, other flagellins can be assembled to produce at least partially functional flagella.

The large flagellar gene cluster also includes genes that encode the chemotaxis machinery, including CheY. As is true for other organisms such as *E. coli* and *Salmonella*, loss of *cheY* results in smooth-swimming cells (17). Like its relative *V. cholerae*, *V. fischeri* also encodes a large number (about 40) of putative methyl-accepting chemoreceptors distributed to both chromosomes (8, 15, 49). This wealth of methyl-accepting chemoreceptors suggest that *V. fischeri* is capable of sensing and responding to many different environmental stimuli.

MOTILITY AND DIVALENT CATIONS

Experiments designed to examine the motility and chemotaxis of *V. fischeri* yielded a surprising result: the motility of *V. fischeri* depends upon the presence of divalent cations, and in particular, the magnesium cation (Mg^{2+}) (Fig. 4) (35). *V. fischeri* grows optimally in a growth medium that is similar to the standard *E. coli* LB medium. Termed LBS, this medium contains tryptone, yeast extract, sodium chloride (2%), and buffer. In this medium, however, the cells are largely nonmotile (Fig. 4). This is also true for a simplified version of LBS that lacks yeast extract and buffer, termed TBS. In contrast, growth of *V. fischeri* in the seawater-based, tryptone- and yeast extract-containing medium SWT permits substantial motility of *V. fischeri*, as monitored by migration of the cells through soft agar motility plates.

In addition to NaCl, SWT contains the following salts: $MgSO_4$, $CaCl_2$, and KCl. Introduction of each of these salts into TBS revealed that $MgSO_4$ is sufficient to induce migration, that $CaCl_2$ exerts a small positive effect on motility, and that KCl enhances growth of *V. fischeri* (35). Migration through soft motility agar occurred efficiently with the addition of as little as 0.2 mM $MgSO_4$. It was maximal in the presence of about 35 mM $MgSO_4$, a level that is similar to that which is present in seawater (about 50 mM). The effect was not limited to $MgSO_4$; $MgCl_2$ similarly exerted a strong positive effect on migration.

The fact that $CaCl_2$ could enhance migration suggested that the response is not specific to the Mg^{2+} cation but could encompass other cations. Indeed, 2 mM concentrations of salts containing either the Mg^{2+} or Ca^{2+} cations could promote similar rates of migration through soft agar. At concentrations greater than 10 mM, however, Ca^{2+} adversely affected the growth and, therefore, motility of *V. fis-*

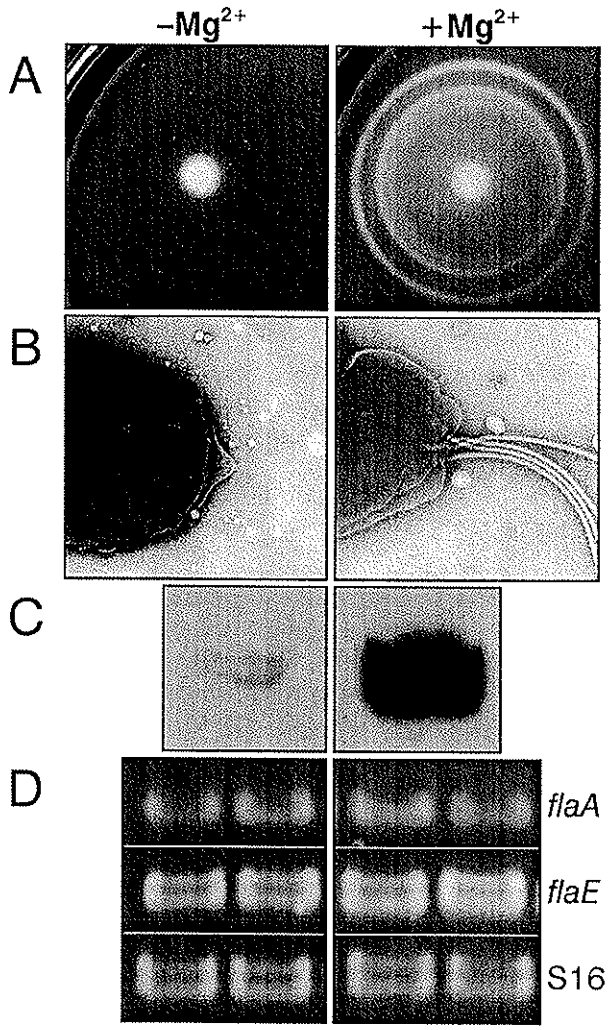


Figure 4. Mg^{2+}-dependent flagellation is controlled posttranscriptionally. In the presence (+) of Mg^{2+} (right), cells tend to migrate well in semisolid agar, forming dense bands of cells characteristic of chemotaxis (A), display a tuft of polar flagella (B), express large amounts of flagellins (C), and transcribe flagellin genes (D). In contrast, in the absence (−) of Mg^{2+} (left), cells tend to migrate poorly in semisolid agar (A), display no flagella (B), express small amounts of flagellins (C), but yet transcribe flagellin genes about as well as cells exposed to Mg^{2+} (D).

cheri, making it difficult to compare its relative efficacy to that of Mg^{2+}. The level of Ca^{2+} in seawater is about 10 mM; thus, higher levels of Ca^{2+} are unlikely to be physiologically relevant. Similarly, other divalent cations were toxic at the high concentrations that are optimal for Mg^{2+}-mediated induction of migration. Given the relevance of high Mg^{2+} concentrations to the biology of *V. fischeri* (in seawater), it seems likely that this cation is the primary cation sensed by the cell to control motility.

Swapping experiments, in which *V. fischeri* cells were grown in the presence or absence of Mg^{2+} and then inoculated onto the surface of soft agar motility plates in the presence or absence of Mg^{2+}, revealed that the impact of Mg^{2+} could be lasting. Cells grown with Mg^{2+} migrated sooner and farther on Mg^{2+}-free plates than those grown without Mg^{2+}. However, cells inoculated onto Mg^{2+}-containing plates exhibited the greatest rates of migration. Thus, exposure to Mg^{2+} at an early time provides an advantage, but continued Mg^{2+} exposure provides an even greater advantage.

Together, these data are consistent with the hypothesis that Mg^{2+} could impact the function and/or production of flagella. Indeed, subsequent analysis with transmission electron microscopy (Fig. 4B) revealed that while Mg^{2+}-grown *V. fischeri* cells display flagella, those grown without Mg^{2+} lack flagella. Mg^{2+}-grown cells carry on average 1 to 3 flagella and as many as 8 flagella, while the majority of those grown without Mg^{2+} possess no flagella (35). Similarly, Western analysis detected large amounts of flagellin protein from Mg^{2+}-grown cells but little from those grown in the absence of Mg^{2+} (Fig. 4C).

What is the significance of the role of Mg^{2+} in flagellar motility? Mg^{2+} can play both structural and signaling roles. Because of its unique size, hydration, and valence properties (reviewed in references 19, 22, 28, and 37), Mg^{2+} tends to play structural roles. Most commonly, it binds ATP or some other nucleotide triphosphate (e.g., c-di-GMP) in the catalytic pocket of an enzyme. Indeed, Mg^{2+} plays a significant structural role in c-di-GMP-regulated processes: it is required for both the DGC and PDE activities (42–44). However, Mg^{2+} also can function as an environmental signal. For example, it controls the activity of the PhoQP two-component signaling pathway (14). Whereas high Mg^{2+} conditions trigger PhoQP to upregulate one subset of genes and downregulate another, low Mg^{2+} conditions exert the opposite effect. It is hypothesized that this two-component system is used by *S. enterica* serovar Typhimurium to determine whether it is in an extracellular environment (where high millimolar concentrations of Mg^{2+} exist) or an intracellular one (where Mg^{2+} is limiting) and to control virulence gene expression accordingly.

We hypothesize that, in *V. fischeri* motility, Mg^{2+} plays a signaling role in addition to its required structural one. We propose that a scenario similar to that of *S. enterica* serovar Typhimurium *pho* control could occur for the marine microbe: the ability to sense Mg^{2+} could distinguish seawater with its high [50 mM] Mg^{2+} concentration from the interior of its host *E. scolopes*. If so, then this ability could help dictate the transition from the motile, free-living state to the sessile, host-associated state and vice versa. Little is known about Mg^{2+} levels in cephalopods, let

alone in *E. scolopes*. However, it is clear that these invertebrates can control the osmolarity and levels of inorganic ions in various body locations (40). Furthermore, recent evidence indicates that *E. scolopes* controls the osmolarity of its light organ environment (51). If symbiotic Mg^{2+} concentrations are reduced below 0.2 mM, then the mechanism controlling motility in response to Mg^{2+} may act to inhibit motility during colonization (Fig. 1).

ROLE FOR DGCs IN CONTROLLING MOTILITY

We hypothesized that Mg^{2+} could enhance the activity of a positive regulator of motility or inhibit the activity of a negative regulator. We further predicted that loss of the putative negative regulator would permit motility of *V. fischeri* even in the absence of Mg^{2+}. Using a random transposon mutagenesis, we isolated a number of mutants that migrated on soft agar plates despite the absence of Mg^{2+} (36). This screen repeatedly uncovered mutants with an insertion in VF0989, a gene predicted to encode a protein with a GGDEF domain and, thus, with DGC activity. It also revealed a mutant with an insertion in VFA0959, a second gene predicted to encode a GGDEF domain protein. These genes were subsequently termed *mifA* and *mifB*.

Loss of either *mifA* or *mifB* promotes migration on soft agar motility plates that lack Mg^{2+} (Fig. 5A). On this medium, the wild-type parent forms only fuzzy indistinct ring patterns, indicating poor motility. In contrast, both *mif* mutants form concentric ring patterns consistent with chemotaxis and, thus, considerably better motility. However, the addition of Mg^{2+} further enhances the motility of the *mif* mutants. One possible explanation is that the two putative DGCs carry out redundant functions. To test this hypothesis, a double *mifA mifB* mutant was constructed (36). In the absence of Mg^{2+}, this double mutant migrates at a slightly faster rate than do the two parental single mutants but, again, does not reach the rate that occurs in the presence of Mg^{2+}. Thus, at least in part, Mg^{2+} must operate through a c-di-GMP-independent pathway, or another Mg^{2+}-sensitive factor(s) remains to be identified.

Beyond identifying MifA and MifB as putative DGCs, bioinformatic analyses revealed few clues to function. They did, however, predict MifA to be a 631-amino-acid protein with 3 putative domains: a periplasmic domain, a cytoplasmic PAS domain, and a cytoplasmic GGDEF domain (Fig. 2). Hydrophobicity analysis predicted two transmembrane segments (amino acids 9 to 27 and 314 to 333). The

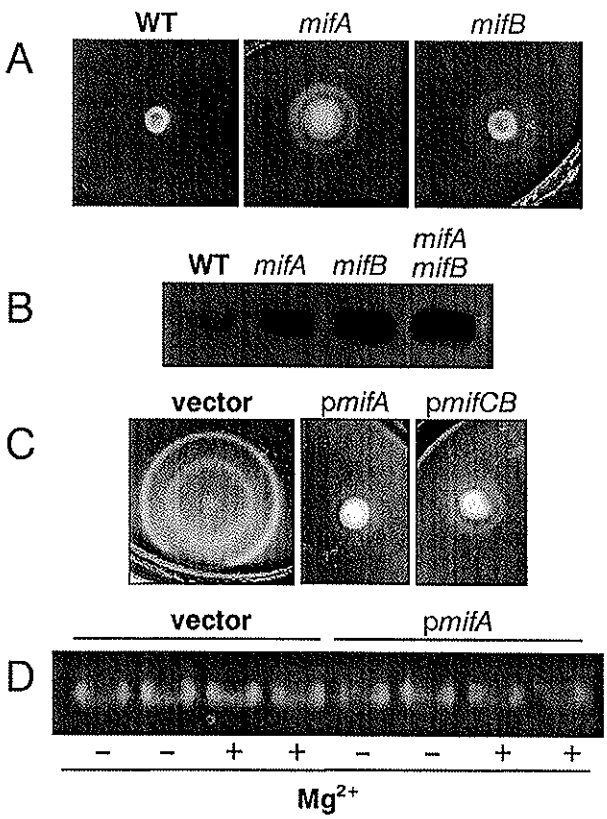


Figure 5. c-di-GMP-dependent flagellation is controlled postranscriptionally. (A) Relative to the wild-type (WT), in the absence of Mg^{2+}, loss of *mifA* or *mifB* increases motility. (B) Loss of *mifA*, *mifB*, or both increases flagellin protein levels relative to WT, as observed by Western immunoblot analysis. (C) Overexpression of *mifA* or *mifCB* inhibits motility, even in the presence of Mg^{2+}. (D) The levels of transcript of the major flagellin gene, *flaA*, are unaltered by either Mg^{2+} addition or *mifA* overexpression. +, present; –, absent.

intervening domain, therefore, would be predicted to project into the periplasm and thus be positioned to receive an environmental signal. The putative PAS domain is predicted to immediately follow the second transmembrane segment with a well-conserved GGDEF domain (with the signature amino acids being GGEEF) residing at the C terminus. These analyses yielded a similar arrangement for MifB, a putative 656-amino-acid protein predicted by hydrophobicity analysis to possess 2 transmembrane regions (amino acids 2 to 31 and 276 to 296); thus, the N-terminal portion may exist as a periplasmic domain that receives environmental signals. The approximately 200 amino acids that lie between these two putative domains do not exhibit similarity to known motifs in the databases.

Like MifA, MifB is predicted to possess a well-conserved GGDEF domain at its C terminus (Fig. 2). Unlike MifA, which is encoded by a single gene op-

eron, the MifB protein is encoded by the second gene of a two-gene operon. Because the two genes overlap by 1 bp, their protein products may have a common function. The first gene, VFA0960, encodes a protein with a putative Pbp domain (23), suggesting that it may function as a periplasmic binding protein. Neither the localization of this protein nor that of MifA and MifB has yet been determined.

Consistent with the role of MifA as a DGC, increased levels of c-di-GMP could be detected using two-dimensional thin-layer chromatography when the *mifA* gene was overexpressed in *E. coli,* and overexpression of *mifA* decreased motility in both *E. coli* and *V. fischeri* (36). Furthermore, alterations of specific amino acids within the GGEEF motif (for example, to AGEEF) disrupted the negative impact on motility generated by *mifA* overexpression (A. J. Wolfe and K. L. Visick, unpublished data). Finally, overexpression of *mifA* led to apparently nonspecific effects on biofilm formation (see cellulose biosynthesis section below), results that match well with those obtained with the overexpression of other DGCs (and PDEs, with opposite results) that have been studied (reviewed in reference 41).

Overexpression of the *mifCB* operon did not result in a detectable increase in c-di-GMP levels (36). Given the conservation of the GGDEF domain and the phenotype of the *mifB* mutant, it seems likely, however, that MifB indeed functions as a DGC. Consistent with this hypothesis, overexpression of *mifCB* in wild-type cells caused a substantial decrease in motility, regardless of exogenous Mg^{2+} concentration (Fig. 5C). Surprisingly, when *mifC* was deleted from the overexpression construct, the impact of *mifB* on motility became dependent on the presence of Mg^{2+} in the medium (36). When Mg^{2+} was present, overexpression of *mifB* exerted a weak influence over motility. In contrast, in the absence of Mg^{2+}, *mifB* overexpression reduced motility to levels similar to those obtained by overexpression of *mifCB*. These data suggest that MifB activity can be modulated by Mg^{2+}, at least in the absence of MifC. A deeper understanding of the interactions between MifC and MifB, and their roles in motility, await further investigation.

ROLE FOR A PDE IN CONTROLLING MOTILITY

Because DGCs (demonstrated and putative) controlled motility in the absence of Mg^{2+}, we hypothesized the existence of both c-di-GMP-associated PDEs and c-di-GMP-binding proteins. To identify the latter, we considered the 4 predicted PilZ genes to be an obvious place to start (Fig. 3). Indeed, as it is embedded in the flagellar gene locus, VF1838 seemed an excellent candidate to be involved in motility. However, under our conditions, we found no role for VF1838 in motility (Wolfe and Visick, unpublished). Similarly, our preliminary data also do not support major roles in motility for the other PilZ genes, VF0527, VF0556, and VFA0884 (Wolfe and Visick, unpublished). While it is possible that these proteins exhibit functional redundancy, it seems more likely that this pathway requires a protein(s) with a non-PilZ domain c-di-GMP-binding motif.

In contrast, we have succeeded in identifying a Mif-specific PDE. To search for this protein, we introduced a library of plasmid-borne chromosomal fragments into wild-type *V. fischeri* and evaluated motility (A. J. Wolfe, ●●● Zemaitaitis, ●●● Shibata, and K. L. Visick, unpublished data). Of 5 plasmids that appeared to increase motility, 3 encoded GGDEF and/or EAL proteins. The plasmid that exerted the greatest impact on motility encoded two GGDEF/EAL proteins, VF0087 and VF0091. Deletion of a portion of VF0091 did not exert a large impact on motility, while disruption of VF0087 largely abolished the increased motility caused by its parent plasmid. Thus, we focused our attention on VF0087.

Because it is well-known that overexpression of proteins that contain GGDEF and/or EAL domains causes nonphysiological effects (57), we sought verification of a role for VF0087 in *V. fischeri* motility by disrupting the gene in the chromosome, which resulted in decreased migration both in the absence of Mg^{2+} and in its presence. Because these motility phenotypes were consistent with our expectations for a *mif*-specific PDE, we designated this gene *mifD* (Fig. 2B) (Wolfe et al., unpublished). That this motility effect is relatively specific is suggested by the lack of motility defects exhibited by mutants defective for several other putative c-di-GMP PDE mutants, including VFA0216 (a REC/EAL domain protein) (17), and VFA1038, a putative GGDEF/EAL protein (●●● Anderson and K. L. Visick, unpublished data) (Fig. 2B and C).

We next evaluated the role of MifD in the Mif pathway by evaluating the motility of a double *mifD mifB* mutant. Because the two mutations exert opposite effects on motility-associated phenotypes (loss of *mifB* increases flagellation and thus migration in the absence of Mg^{2+}, while loss of *mifD* decreases both), we investigated the motility phenotypes of the double mutant. We found that flagellation and thus migration of the *mifD mifB* double mutant ~~mirrored~~ approached that of wild-type cells (Wolfe et al., unpublished). These data suggest that the loss of *mifD* compensates

for the loss of *mifB* and support our hypothesis that these two proteins function in the same pathway.

Our previous results, showing that the combined loss of *mifA* and *mifB* does not permit the same high rates of motility in the absence of magnesium as those observed with wild-type cells in the presence of magnesium, suggested the existence of additional regulators. We predicted that if a third DGC that modulates motility exists, then the overexpression of a PDE in the *mifA mifB* double mutant might further increase motility in the absence of Mg^{2+}. Thus, we overexpressed *mifD* ~~(and the other cloned PDE genes)~~ in the *mifA mifB* double mutant. However, none of these strains exhibited increased motility relative to the vector control (Wolfe et al., unpublished). Thus, either the overexpressed genes do not produce sufficient or correctly localized PDE activity or c-di-GMP cannot fully account for the impact of magnesium on motility of *V. fischeri*.

POSTTRANSCRIPTIONAL CONTROL OF MOTILITY

How do Mg^{2+} and c-di-GMP impact the motility of *V. fischeri*? Do they function in the same pathway or in parallel pathways? With the current data, it appears that Mg^{2+} exerts the greater impact on motility. To assemble its polar tuft of flagella, this marine symbiont requires 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 (Fig. 4C) (35). However, we have found that Mg^{2+} does not substantially impact transcription of a variety of flagellar genes, including those for flagellin, as assayed by reverse transcription-PCR (Fig. 4D) (36). Because Mg^{2+} does not seem to affect transcription but clearly influences steady-state protein levels (35, 36), we propose 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 (35), 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.

The impact of c-di-GMP on motility is not yet understood for *V. fischeri*. Like the addition of Mg^{2+}, loss of *mifA* or *mifB* appeared to increase the steady-state levels of flagellin protein (Fig. 5B) but not to substantially alter flagellin gene transcription (36). Of greater significance, overexpression of *mifA* substantially decreased motility but did not decrease transcription (evaluated by reverse transcription-PCR) (compare Fig. 5A and D) (36). Thus, like Mg^{2+}, the Mif pathway appeared to function somewhere after transcript synthesis and stability. Although some evidence supported the hypothesis that Mg^{2+} interacts with the Mif pathway, i.e., the observation that Mg^{2+} can influence MifB activity but only when MifB is overexpressed and thus present in excess over MifC (36), other evidence supports the hypothesis that Mg^{2+} works independently of the Mif pathway. When exposed to Mg^{2+}, each *mif* mutant dramatically increased the mean number of flagella per cell. Thus, the response to Mg^{2+} did not require each of the known Mif pathway components. Yet, even in the presence of Mg^{2+}, the impact of each *mif* mutation was still observed, e.g., *mifA* and *mifB* mutants displayed more flagella per cell than did their wild-type parent, which assembled more flagella per cell than did the *mifD* mutant (Wolfe et al., unpublished). On the basis of these data, we propose that Mif and Mg^{2+} work independently, at least in part, to exert posttranscriptional effects on motility. Thus, we propose to change the name of Mif from the original designation *m*agnesium-*i*nduced *f*lagellation to *m*agnesium-*i*ndependent *f*lagellation.

ROLE FOR c-di-GMP IN CONTROLLING CELLULOSE BIOSYNTHESIS

Overexpression of DGCs and PDEs tends to exert global impacts on cell physiology in various bacteria (41). This is true for *V. fischeri* as well. Overexpression of the DGC MifA not only inhibits motility but also results in an increase in biofilm formation (36). When MifA is overexpressed, the resulting colonies exhibit a wrinkled colony morphology, a trait that correlates with increased biofilm formation. Furthermore, those colonies exhibit increased dye-binding properties. Notably, when grown on plates that contain Congo red, a dye that is often used as an indicator of cellulose biosynthesis, the *mifA* overexpression strain shows an increase in red color relative to the vector control (36). In addition, when grown on plates that contain calcofluor, a fluorescent indicator of β-1,4 linkages like those in cellulose, and exposed to ultraviolet light, the *mifA* overexpression strain becomes distinctly fluorescent relative to the wild type, which remains dark. The increased Congo red dye binding property of the *mifA* overexpression cell depends upon the cellulose

locus, as its disruption eliminates that phenotype (●●● Darnell and K. L. Visick, unpublished data).

The cellulose locus contains a PilZ domain gene, VFA0884 (Fig. 3 and 6). The product of VFA0884 is predicted to bind c-di-GMP, permitting its activity in cellulose biosynthesis. The production of cellulose in *V. fischeri* appears to be under complex regulation. Overexpression of either of two regulators, the sensor kinase SypF (encoded by the *syp* polysaccharide locus) or the putative response regulator VpsR, causes an increase in cellulose biosynthesis (as measured by Congo red binding) and biofilm formation (as evaluated by a crystal violet-based assay of attachment to glass) (Fig. 6) (7). Loss of cellulose biosynthesis eliminates those phenotypes. In addition, a GGDEF/EAL protein, VFA1038, impacts cellulose biosynthesis: loss of VFA1038 increases Congo red binding and glass attachment measured by the crystal violet assay (~~Anderson~~ and Visick, unpublished). Like overexpression of MifA, SypF, and VpsR, disruption of the cellulose gene locus eliminates the increase in biofilm formation observed with the VFA1038 mutant. The roles for these genes remain uncertain, particularly in the case of overexpressed regulators. However, our data (particularly those for disruption of VFA1038 but also in the case of MifA overexpression) are consistent with a role for c-di-GMP in promoting cellulose biosynthesis, perhaps by binding to VFA0884, the PilZ protein. Because MifA is unlikely to be the cellulose-specific regulator, additional work is necessary to decipher the regulatory network controlling c-di-GMP production and cellulose biosynthesis (Fig. 6).

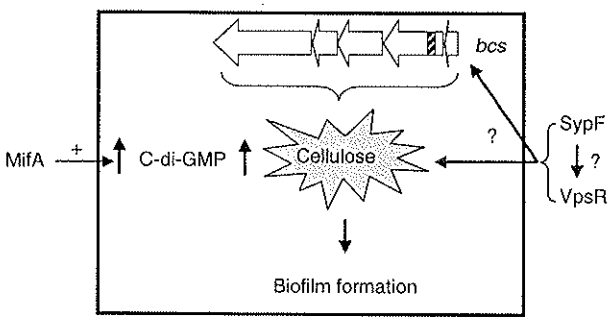


Figure 6. Model for c-di-GMP and cellulose. The *bcs* genes in *V. fischeri* (VFA0885 to 0881) encode the enzymes necessary for cellulose biosynthesis. The production of cellulose is enhanced by overexpression of either of the two-component regulators SypF and VpsR. It is also increased by overexpression of the DGC MifA. For MifA, it is likely that a rise in c-di-GMP levels increases the cellulose synthetic activity of the PilZ domain protein, BcsA. For SypF and VpsR, the level at which these proteins impact cellulose production is unknown, as indicated by the question marks. Increased production of cellulose results in enhanced biofilm formation.

MODEL FOR MOTILITY

We have developed a working model that takes into account all of our motility results to date (Fig. 7). Under conditions of low Mg^{2+}, flagellar biogenesis is impaired. MifA and MifB (perhaps in concert with MifC) catalyze the production of c-di-GMP, while MifD catalyzes its degradation. One or more (non-PilZ) c-di-GMP-binding proteins (X) bind the Mif-produced c-di-GMP. This causes a conformational change that interferes with flagellar biogenesis by impairing translation or protein stability, export, and/or assembly.

Under conditions of high Mg^{2+}, flagellar biogenesis proceeds. Several alternatives can be envisioned: Mg^{2+} could inhibit the DGC activity of MifA and MifB or enhance the PDE activity of MifD, by altering their expression, localization, and/or active conformation. Alternatively, Mg^{2+} could inhibit the c-di-GMP-binding protein X and/or override the c-di-GMP-mediated inhibition of translation or protein stability, export, and/or assembly. Finally, it is possible that Mg^{2+} acts through a Mif-independent pathway to promote flagellar biogenesis.

CONCLUDING REMARKS

There is now no doubt that c-di-GMP plays a key role in the transition of many bacterial species from the motile, planktonic form to the sessile, biofilm state—the so called "stick-or-swim" decision. Although much effort has been invested in understanding how c-di-GMP is synthesized and degraded and how c-di-GMP influences production of the extracellular polysaccharides that comprise the bulk of biofilm matrices, there exist only a limited number of studies that have explored the role of c-di-GMP in regulating flagellar biogenesis. Nonetheless, clear evidence suggests that the mechanisms used by c-di-GMP to impact motility are as diverse as the levels of control that are known to exist (57). The details of those mechanisms, however, remain unclear. The work described in this chapter is one of the few studies, along with those investigating YcgR (chapter 4) and PleD (chapter 9), where a detailed investigation of c-di-GMP in motility is being pursued.

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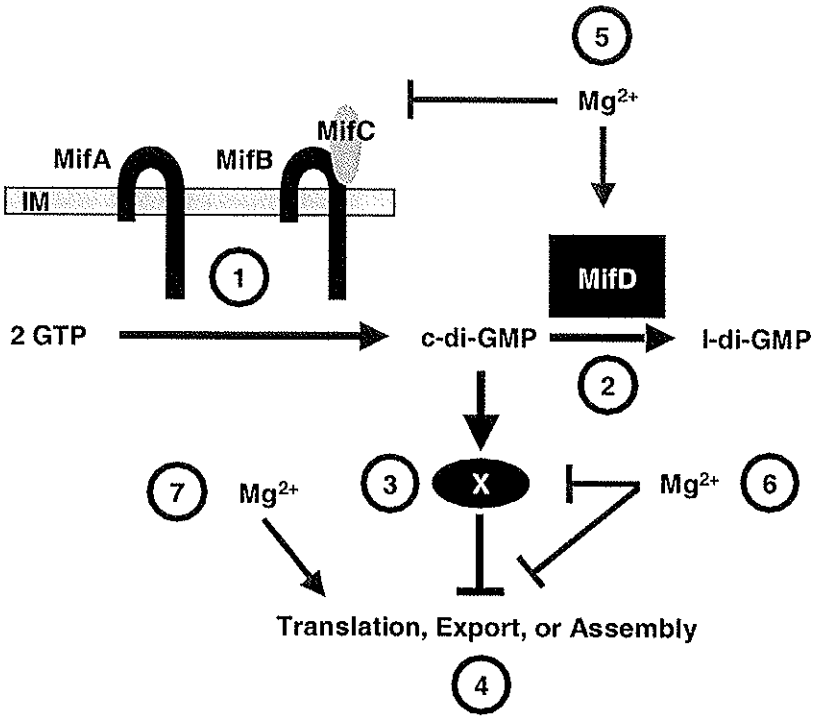

Figure 7. Model for c-di-GMP and motility. The DGCs MifA and MifB (in concert with MifC) (1) and the PDE MifD (2) set the steady-state levels of c-di-GMP, which binds to an unknown c-di-GMP-binding protein, X (3). This complex interferes with the translation, export, and/or assembly of very early flagellar components (4). Several possibilities exist for the role of Mg^{2+} in promoting motility. It could block this process upstream of c-di-GMP, either by inhibiting the DGCs MifA and MifB or by activating the PDE MifD (5). It could act downstream of c-di-GMP either by inhibiting the binding of c-di-GMP to its binding protein(s) X or by inhibiting the action of the c-di-GMP/X complex (6). Finally, Mg^{2+} could override the inhibitory action of c-di-GMP via an independent pathway (7). Combinations of these alternatives could operate. IM, ●●●; l-di-GMP, ●●●.

IM, inner membrane

l-di-GMP, linear-di-GMP

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