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The c-di-GMP Binding Protein YcgR Controls Flagellar Motor Direction and Speed to Affect Chemotaxis by a “Backstop Brake” Mechanism

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SUMMARY

We describe a mechanism of flagellar motor control by the bacterial signaling molecule c-di-GMP, which regulates several cellular behaviors. *E. coli* and *Salmonella* have multiple c-di-GMP cyclases and phosphodiesterases, yet absence of a specific phosphodiesterase YhjH impairs motility in both bacteria. *yhjH* mutants have elevated c-di-GMP levels and require YcgR, a c-di-GMP-binding protein, for motility inhibition. We demonstrate that YcgR interacts with the flagellar switch-complex proteins FliG and FliM, most strongly in the presence of c-di-GMP. This interaction reduces the efficiency of torque generation and induces CCW motor bias. We present a “backstop brake” model showing how both effects can result from disrupting the organization of the FliG C-terminal domain, which interacts with the stator protein MotA to generate torque. Inhibition of motility and chemotaxis may represent a strategy to prepare for sedentary existence by disfavoring migration away from a substrate on which a biofilm is to be formed.

INTRODUCTION

Cyclic or c-di-GMP has emerged as a key player in the decision between motile and sedentary forms of bacterial life (Hengge, 2009; Jenal and Malone, 2006; Romling et al., 2005; Schirmer and Jenal, 2009). c-di-GMP enhances biosynthesis of capsular and fimbrial components required for biofilm formation while inhibiting flagella and pili that allow movement (Simm et al., 2004; Wolfe and Visick 2008). Steady-state levels of c-di-GMP are thought to be maintained by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). DGC activity depends on a conserved GGDEF domain, while PDE activity requires an EAL or HD-GYP domain (Ryan et al., 2006). GGDEF and EAL domains are associated with many diverse input and output domains, suggesting that they receive a variety of signals and respond through a variety of different mechanisms. These two domains are the most abundant domains encoded in bacterial genomes, with many bacteria possessing multiple proteins with these domains (Galperin, 2005; Galperin et al., 2001). For example, *Salmonella* has five GGDEF proteins, eight EAL proteins, and seven proteins with both GGDEF and EAL domains (Simm et al., 2007; Solano et al., 2009). Similarly, *E. coli* has a large number of such proteins. Despite the

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SUPPLEMENTAL INFORMATION

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existence of multiple such proteins, the association of specific phenotypes with mutation of individual proteins suggests that c-di-GMP levels may be modulated in a spatially localized manner.

Bioinformatic analysis first suggested that PilZ domains may function as c-di-GMP effectors (Amikam and Galperin 2006). Binding and mutagenesis studies of several PilZ domain proteins have confirmed this observation and demonstrated that c-di-GMP binding depends on residues in RxxxR and D/NxSxxG sequence motifs. A crystal structure of a PilZ domain–c-di-GMP complex from *Vibrio cholerae* shows c-di-GMP contacting seven of nine strongly conserved residues (Benach et al., 2007).

In *E. coli* and *Salmonella*, YhjH is a PDE and YcgR a PilZ domain protein that binds c-di-GMP (Ryjenkov et al., 2006; Schmidt et al., 2005). Both proteins are members of the class 3 flagellar regulon (Frye et al., 2006; Ko and Park 2000a; Wang et al., 2006). Mutation of *yhjH* reduces motility, while that of *ycgR* does not. However, *ycgR* suppresses the motility defect of *yhjH*, suggesting that in the absence of YhjH, YcgR interacts with the resultant increased levels of c-di-GMP. A *yhjH* mutant in *E. coli* was shown to have a counterclockwise (CCW) bias, suggesting a role for the YcgR - c-di-GMP in motor switching (Girgis et al., 2007). Early evidence implicating YcgR at the motor comes from the work of Ko and Park, who found that absence of H-NS results in paralyzed flagella, a defect that could be suppressed either by a mutation in *ycgR* or by providing *yhjH* in multiple copies (Ko and Park 2000a). Since H-NS was shown to interact with FliG (Donato and Kawula, 1998; Marykwas et al., 1996), a protein that interacts with both the proton-conducting protein MotA that energizes rotation and the rotor protein FliM that controls the direction of rotation, it was proposed that the YcgR–c-di-GMP complex might interfere with motor rotation by interfering with the proper association of the Mot proteins with FliG (Wolfe and Visick 2008).

The flagellar motor consists of a rotating part (the rotor) and a membrane-embedded, nonrotating part (the stator). The rotor is formed from about 25 copies of FliG, 34 copies of FliM, and more than 100 copies of FliN, together forming an assembly called the C-ring or switch complex (Francis et al., 1992; Yamaguchi et al., 1986). The switch complex is located at the bottom of the basal body and functions in flagellar assembly, rotation, and clockwise/counterclockwise (CW/CCW) directional control. The stator is formed from the membrane proteins MotA and MotB. These proteins function to conduct ions across the membrane and harness ion flow to rotation (reviewed in Blair, 2003). Rotation depends upon electrostatic interactions between sites in the stator protein MotA and the rotor protein FliG (Zhou et al., 1998a). The direction of rotation is controlled by the switch complex, which changes to the CW state in response to the signaling protein phospho-CheY (CheY~P) (Welch et al., 1993).

Here we present evidence that YcgR interacts with the flagellar rotor proteins FliM and FliG in a c-di-GMP-dependent manner. In *E. coli*, regions of protein-protein interaction were mapped using mutations, and the effects of YcgR on flagellar protein organization were probed by targeted crosslinking. In *Salmonella*, these were followed by localization of a fluorescent YcgR-GFP fusion to flagellar basal bodies. We show that overexpression of wild-type YcgR, but not the YcgR^{R118D} mutant protein, alters the organization of the C-terminal domain of FliG, the rotor component most directly involved in generation of torque. The results are discussed in the framework of a model in which c-di-GMP-bound YcgR alters the orientation of the FliG C-terminal domain so that the rotor-stator interface is disrupted and the motor is biased toward CCW rotation.

RESULTS

Cyclic-di-GMP Acts through YcgR to Inhibit Swimming and Swarming

As has been noted previously, deletion of the c-di-GMP phosphodiesterase *yhjH* impairs swimming motility in *Salmonella*, and motility of the $\Delta yhjH$ strain is substantially rescued by deletion of the PilZ-domain protein YcgR (Ryjenkov et al., 2006) (Figure 1A). The inhibitory effect of the *yhjH* deletion on both swimming and swarming was made stronger by overexpression of YcgR from a plasmid (Figure 1B). Similar results have been obtained in experiments with *Escherichia coli* mutants (Ko and Park, 2000a; Ryjenkov et al., 2006) and cyclic-di-GMP was shown to bind directly to the purified YcgR protein of *E. coli* (Ryjenkov et al., 2006). Together, these observations indicate that cyclic-di-GMP acts through YcgR to inhibit motility and that this regulatory mechanism operates in both *Salmonella* and *E. coli*.

Motility Inhibition Is Due to Reduced Motor Speed and Increased CCW Bias

We hypothesized that the motility inhibition in $\Delta yhjH$ cells might be caused by a loss of chemotactic function, because nonchemotactic mutants of *Salmonella* or *E. coli* cannot swarm (Burkart et al., 1998; Harshey and Matsuyama, 1994; Mariconda et al., 2006; Wang et al., 2005) and a $\Delta yhjH$ mutant of *E. coli* was reported to have an aberrantly CCW motor bias (Girgis et al., 2007). To look more directly at motor performance in the $\Delta yhjH$ and YcgR-overexpressing (YcgR^{OE}) cells, we used a tethered-cell assay to measure motor speed and bias (Figure 1C). In a wild-type population, most cells rotated predominantly CCW but also displayed some intervals of CW rotation. When the *yhjH* gene was deleted, or when YcgR was overexpressed, the number of cells showing CW intervals was decreased, with a corresponding increase in cells turning exclusively CCW. Effects of *yhjH* deletion and YcgR overexpression were additive; most cells of the $\Delta yhjH$ /YcgR^{OE} strain turned exclusively CCW. Motor speeds were also reduced in the mutant strains. Rotation rates for the tethered cells (mean \pm SD, revs/min) were the following: wild-type, 146 ± 21 ; YcgR^{OE}, 132 ± 24 ; $\Delta yhjH$, 138 ± 20 , and $\Delta yhjH$ /YcgR^{OE}, 97 ± 25 . Thus, under conditions of elevated c-di-GMP and YcgR, motor torque was reduced by about 30%.

Motor bias is normally set by the chemotactic signaling protein CheY~P, which interacts with the flagellar switch to increase the probability of CW rotation. CheY~P level is governed by the relative activities of the kinase CheA and the phosphatase CheZ. CheA kinase activity is modulated by the chemoreceptors, which are in turn regulated by both the binding of chemoeffector and the level of methylation at specific glutamyl residues (Hazelbauer et al., 2008). Receptor methylation level is determined by the activities of the methyltransferase CheR and the methylesterase CheB, and is the basis of sensory adaptation: CheA transfers some of its phosphoryl groups to CheB, activating the methylesterase and bringing the receptors back to their “kinase-off” signaling state. The absence of either the methylesterase CheB or the phosphatase CheZ elevates the level of CheY~P in the cell, either by inducing the methylated, “kinase-on” state of the receptors or by slowing the dephosphorylation of CheY~P. Both $\Delta cheB$ and $\Delta cheZ$ strains display a strong CW motor bias. The effect of YcgR on motor switching was most clearly evident in these CW-biased backgrounds; deletion of *yhjH* coupled with overexpression of YcgR caused many of the $\Delta cheB$ and $\Delta cheZ$ cells to switch from CW to CCW bias (Figures 1D and 1E).

YcgR Interacts with Proteins of the Rotor

The direction of motor rotation is controlled by a “switch complex” on the rotor formed from the proteins FliG, FliM, and FliN (Berg, 2003). Torque is produced at the interface between FliG and the membrane protein MotA, which is a component of the stator (Lloyd et al., 1996; Zhou et al., 1998a). To identify the target(s) of YcgR in the motor, we used pull-down assays to examine the binding of YcgR to the switch-complex proteins and to a cytoplasmic

domain of MotA that is known to form the site of interaction with the rotor (Yakushi et al., 2006; Zhou et al., 1998a). The experiments used a fusion of GST (glutathione S-transferase) to the amino terminus of YcgR and were done using proteins of *E. coli*, which are very similar in sequence to those of *Salmonella*. Experiments were conducted in the presence of 5 μ M c-di-GMP. Neither MotA_C (a domain comprising residues 70–170 and so including residues that interact with the rotor) nor FliN was coisolated with the GST-YcgR fusion in this assay. The FliG and FliM proteins were reproducibly coisolated with the GST-YcgR fusion at levels significantly above GST-only controls (Figure 2A). In the experiments with FliM, some of the FliM was found in a low-speed pellet, consistent with the reported tendency of this protein to aggregate when expressed separately from the other switch-complex proteins (Mathews et al., 1998). Aggregation was decreased by coexpression of FliN, and because no direct YcgR-FliN interaction was detected, we included FliN in subsequent binding experiments with FliM. In experiments with both FliM and FliN present, FliM was coisolated with GST-YcgR at relatively high levels, and FliN was coisolated in smaller amounts, consistent with its known binding to FliM (Mathews et al., 1998) (data not shown).

Effect of c-di-GMP on YcgR Binding to FliM and FliG

Because c-di-GMP binds to YcgR and is important for the YcgR-mediated motility inhibition, we next measured the binding of YcgR to FliM and FliG in the presence of various concentrations of c-di-GMP. YcgR showed measurable binding to both in the absence of any externally added effector, and this binding was increased by roughly 2-fold when c-di-GMP was added to 5 μ M or higher (Figure 2B). Subsequent binding experiments were done in the presence of 5 μ M c-di-GMP.

In experiments in *E. coli*, the mutation R118D in YcgR was shown to prevent c-di-GMP binding to the purified protein and to eliminate the motility impairment in the *yjhH* deletion strain (Ryjenkov et al., 2006). To test binding of this mutant protein to the switch proteins, we introduced the R118D mutation into the GST-YcgR fusion construct and performed the pull-down experiment as before. Binding of the YcgR^{R118D} protein to FliM was weakened but was not prevented. Surprisingly, binding of the YcgR^{R118D} protein to FliM was stimulated by c-di-GMP despite the reported inability of this protein to bind the effector (Figure 2C). Binding of the YcgR^{R118D} protein to FliG was much weaker than that of wild-type YcgR, both in the presence and absence c-di-GMP (Figure 2C).

Localization of YcgR-GFP to the Flagellar Basal Body

To determine whether the interaction between YcgR and the switch proteins occurs in cells, we constructed a GFP fusion to the C terminus of *Salmonella* YcgR and examined the localization of the protein. The fusion protein behaved similarly to wild-type YcgR in causing motility impairment when overexpressed (data not shown). The YcgR-GFP construct displayed a punctate fluorescence in cells, unlike the diffuse fluorescence of GFP alone (Figure 2; compare Figures 2D and 2E). Punctate localization was more distinct in a $\Delta yjhH$ strain (Figure 2F) than in wild-type and was retained in a strain lacking all seven chemoreceptors (see Figure S1 available online). Puncta were eliminated in cells lacking either FliM (Figure 2G) or FliG (Figure 2H) but were still present in a strain lacking MotA (Figure 2I; an *E. coli motA* null strain was also punctate, as shown in Figure S1), confirming that localization is via the C-ring.

Surface on FliM Involved in Binding YcgR

To identify the regions of FliM most important for its interaction with YcgR, we used the pull-down assay and a collection of nonconservative FliM mutations in *E. coli* that sample various regions of the protein surface. Binding to YcgR was eliminated by the FliM mutations R156D and L160E and was weakened by the replacements V153D, N155E, D167R, and E176R (Figure 3A). These residues lie near each other on a surface region of FliM that overlaps the binding

site for FliG (Figure 3B). Binding to YcgR was not measurably weakened by the FliM mutations R45D, I56D, R63D, S76E, N95W, F131D, G133W, K140D, H148D, or T149D (Figures 3A and 3B).

To determine the importance of this binding site in cells, we examined localization of YcgR-GFP in *Salmonella* cells containing the N155E, L160E, D167R, and E176R mutations in FliM. Puncta were eliminated by the N155E and L160E FliM mutations, which lie near the center of the region identified in the pull-down experiments (Figure 4A). Localization was not affected by the D167R and E176R mutations that lie nearer the edge of the YcgR-binding region (data not shown). The YcgR^{R118D} variant was also examined and showed normal punctate localization (Figure 4B), consistent with the retention of FliM binding observed in this mutant (Figure 2C). The R118D mutation was reported to eliminate the YcgR-linked motility impairment in *E. coli* (Ryjenkov et al., 2006). To determine whether this is also the case in *Salmonella*, we used the soft agar swimming and tethered-cell rotation assays to examine motility of *Salmonella* cells overexpressing the YcgR^{R118D} protein. Consistent with the previous reports in *E. coli*, the mutation relieved the motility impairment in both assays (Figure 4C). Thus, the YcgR^{R118D} protein is able to bind FliM and localize to the basal body but does not affect motor bias or speed.

If the motility inhibition in the $\Delta yhjH$ strain requires binding of effector-stimulated YcgR to FliM, then FliM mutants defective in the YcgR interaction might show improved motility in the $\Delta yhjH$ background. To test this, plasmids expressing YcgR-interaction-defective FliM mutants (N155E, L160D, D167R, and E176R) were transformed into a $\Delta yhjH\Delta fliM$ *Salmonella* strain, and rates of migration on soft agar plates were measured (the V153D and R156D mutations were not included because they disrupt binding of FliM to FliG and cause a nonflagellate phenotype). The N155E and L160D mutations caused a reproducible but fairly small improvement in motility in the $\Delta yhjH$ background (Figure 4C, left; compare 3 and 5 with 4). These mutants by themselves had an altered motor bias, with a significant CW(r) fraction (Figure 4C, right). This bias remained unaltered in the presence of increased YcgR and may account for their smaller swim colony diameter. In parallel experiments in *E. coli*, similarly mild effects were observed (data not shown) but could be made stronger by further optimization of the experiment (described below).

FliG Mutations Relieve Motility Inhibition and Weaken the YcgR Interaction

Since FliG also interacted with YcgR in the pull-down assay, FliG mutants with defects in the YcgR interaction might relieve the motor from YcgR-mediated inhibition and show improved motility. To examine this, plasmids expressing a collection of *E. coli* FliG proteins with Trp replacements in various surface positions (obtained in a previous study; Brown et al., 2007) were transformed into a $\Delta yhjH\Delta fliG$ strain, and motility was examined on soft agar plates. Three Trp replacements in the C-terminal domain of FliG (Q252W, N292W, and P295W) showed significant motility improvement in the $\Delta yhjH$ background, while two others (D284W and L298W) showed smaller but reproducible improvements (Figure 3E). The motility inhibition was not relieved by FliG mutations at positions 170, 205, 217, 218, 243, or 280 (Figure 3; position 218 mutant not shown). Next, the mutant FliG proteins were tested in pull-down experiments to see if the YcgR-FliG binding was affected. Binding to YcgR was weakened significantly by the Q252W, N292W, and P295W mutations in FliG and was somewhat diminished by the D284W mutation (Figure 3C). The binding to YcgR was not affected by mutation of residues 126, 158, or 170 in the middle domain of FliG, or residue 225 in the hydrophobic patch of the C-terminal domain (Figures 3C and 3D). Residues 292 and 295 lie on the “top” of the domain near the charged ridge that interacts with the stator (Lloyd and Blair, 1997; Yakushi et al., 2006; Zhou et al., 1998a, 1998b), and residue 252 is on the side of the domain, adjacent to the charged ridge (Figures 3D). To test the importance of the

FliG binding for YcgR localization to the basal body, we examined the distribution of YcgR-GFP in *Salmonella* cells containing the P295W mutant FliG. Localization appeared not to be affected (Figure 4B).

Because YcgR interacts with both FliM and FliG, we hypothesized that the presence of a suitable mutation in FliG might reveal otherwise-subtle effects of FliM mutations. To test this proposal, we remeasured the effects of selected FliM mutations on the YcgR-mediated motility inhibition with a mutation also present in FliG (N292W) to decrease the affinity for YcgR. In the presence of this FliG mutation, the FliM mutations N155E and D160R both gave substantial relief from the motility inhibition (motility plates shown in Figure S2).

Point Mutations in YcgR Affecting Its Interactions with FliM and FliG

We next sought to identify the regions of YcgR protein important for its interactions with FliM and FliG. YcgR is composed of two domains, an N-terminal PilZ_N domain (sometimes called the YcgR domain) whose function is not precisely known, and a C-terminal domain that has been associated with the binding of c-di-GMP (Christen et al., 2007; Merighi et al., 2007; Pratt et al., 2007; Ryjenkov et al., 2006). A crystal structure is known for a YcgR-like protein of *Vibrio cholerae* (VCA0042/PilZD), bound to its effector c-di-GMP (Benach et al., 2007). Using this structure and a sequence alignment with *E. coli* YcgR, a homology model for the *E. coli* protein was constructed (Figure 5A). The structure consists of two β barrel domains with c-di-GMP bound in a region between the domains where it could regulate their relative positions or orientation. Additional features include an α helix followed by an antiparallel β hairpin near the C terminus (at the top of the molecule in the orientation shown). Residues in this α helix are relatively well conserved. Further, in the case of the YcgR-like protein DrgA of *Caulobacter crescentus* the C-terminal domain has been suggested to be involved in interaction with downstream target(s) (Christen et al., 2007). We hypothesized that the α helix in YcgR_C might form the site of interaction with FliM and accordingly targeted it for mutagenesis.

Mutations were made at five positions in YcgR, four on the α helix (R222W, Q223W and Q223P, R226W, and I227W) and one at a nearby position on the surface (H183D) (Figure 5A). The mutant *ycgR* genes were fused to sequences encoding an HA tag to facilitate detection of the proteins. Control blots using anti-HA antibody showed that the mutant proteins were expressed at levels similar to HA-tagged wild-type YcgR (Figure S3). The mutant YcgR proteins were expressed from plasmids in the $\Delta yhjH \Delta ycgR$ strain to measure their effects on motility. Proteins with the H183D, R222W, or R226W replacements caused motility impairments similar to wild-type. The Q223W, Q223P, and I227W mutations gave substantial relief from the motility impairment, suggesting a role for these residues in interaction with the motor (Figure 5D).

To measure the effects of the YcgR mutations on the binding to FliM, the Q223P and I227W mutations, and the H183D replacement as a negative control, were transferred into the GST-YcgR-HA fusion protein and used in pull-down experiments. The GST-YcgR protein with the H183D mutation bound FliM as strongly as wild-type, whereas the Q223P and I227W mutants showed no binding even in the presence of 5 μ M c-di-GMP (Figure 5B). The Q223W mutation was then introduced into the YcgR-GFP fusion construct to examine effects on localization. Punctate localization was eliminated in the Q223W YcgR mutant (Figure 5C). Finally, the tethered-cell assay was used to measure motor performance in $\Delta yhjH$ cells overexpressing the Q223W mutant protein. Motor speed and bias were normal (motor speed 141 ± 27 ; bias data provided in Figure S4). We conclude that the α helix near the C terminus of YcgR is important for the binding of YcgR to the flagellar switch and the resulting alteration of motor function.

YcgR proteins with mutations in the α helix were also tested in pull-down assays with FliG but were found to have no effect on the YcgR-FliG interaction (Figure S5A). We hypothesized

that binding to FliG might occur through the N-terminal domain of YcgR and accordingly mutagenized several moderately well-conserved surface residues in this domain. The mutant proteins were expressed in a $\Delta fliG\Delta yhjH$ strain, and motility was measured in soft agar plates. Of five mutations studied, three (K42D, N62W, and K81D) showed a substantial motility improvement in the $\Delta yhjH$ background, and two others (N12W and E83K) showed mild improvements (Figure 5D). To determine whether the motility improvement was due to a weakened interaction with FliG, three of the YcgR mutations (N12W, N62W, and K81D) were transferred into the GST-YcgR fusion construct and used in a pull-down experiment. Binding to FliG was weakened in the N62W and K82W mutants but was not affected in the N12W mutant (Figure 5B). Binding to FliM was normal for all three of these YcgR mutant proteins (Figure S5). Together with the motility enhancements (Figure 5D), these results implicate the N-terminal domain of YcgR in binding to FliG.

Improved Motility upon Overexpression of Stator Components

Because the motility defect in the $\Delta yhjH$ strain is due at least in part to CCW motor bias, we hypothesized that overexpression of the CW-signaling protein CheY might reverse this bias defect and improve motility of the $\Delta yhjH$ strain. However, overexpression of CheY alone did not improve motility of the $\Delta yhjH$ null strain (Figure 6A). In a study of Δhns mutants of *E. coli*, Ko and Park found that certain defects associated with the rotor can be suppressed by overexpression of the stator proteins (Ko and Park, 2000b). To see if this is true of the $\Delta yhjH$ mutant, we introduced plasmids expressing MotA, MotB, or MotA and MotB together and retested motility in the soft agar assay. Motility of the $\Delta yhjH$ mutant was markedly improved by overexpression of MotA, either by itself or with MotB (Figure 6A). In the strains overexpressing MotA, migration in soft agar was enhanced further by overexpression of CheY (Figure 6A). These findings are in accordance with the proposal that YcgR affects both motor bias and torque generation. Further, they suggest that the defect involves a rearrangement at the rotor-stator interface.

Effect of YcgR on Organization of the FliG C-Terminal Domain

The C-terminal domain of FliG is at the interface with the stator and contributes directly to generation of torque (Lloyd et al., 1996; Zhou et al., 1998a). In a current structural model of the switch complex, FliG is at the “top” (the membrane-proximal part) of the C-ring, to allow interactions with the stator, while the major part of FliM (the middle domain, FliM_M) is located just below FliG_C and positions this domain in its proper relationship to the stator (Yakushi et al., 2006; Zhou et al., 1998a). We previously examined the organization of FliG in the rotor using targeted disulfide cross-linking, identifying cysteine residues capable of forming FliG-FliG crosslinks in the wild-type motor (Lowder et al., 2005). To examine the effects of YcgR upon the organization of FliG, we examined patterns of FliG-FliG crosslinking in the $\Delta yhjH$ mutant and in the $\Delta yhjH$ mutant overexpressing YcgR. To facilitate detection of the proteins, an HA tag was fused to the FliG C terminus. Disulfide crosslinking was induced with iodine, and products were examined on immunoblots using anti-HA antibody. The HA-fusion construct was functional and, when expressed in a wild-type background, gave crosslinking results similar to those reported previously (data not shown).

Cys residues at positions 117 and 166 in the middle domain of FliG were previously shown to crosslink efficiently to yield dimers and larger multimers (Lowder et al., 2005). The crosslinking occurred in flagellate cells, but not in a nonflagellate $\Delta flhDC$ mutant, indicating that it occurs between protein subunits in the flagellum (rather than by collision in the cytosol). Patterns of crosslinking in the 117/166 double-Cys mutant were not substantially affected either by the deletion of *yhjH* or by overexpression of YcgR (Figure 6B). Thus, YcgR does not appear to affect the organization of the FliG middle domain.

A FliG protein with a Cys replacement of residue 297 in the C-terminal domain was shown previously to crosslink efficiently into dimers, again only when the protein was present in assembled motors (Lowder et al., 2005). Crosslinking in a single-Cys mutant presumably requires significant motion of the domain because equivalent positions in adjacent subunits should be separated by about 4 nm (assuming uniform spacing) (Thomas et al., 2006). The yield of the 297-297 crosslink was somewhat decreased in the $\Delta yhjH$ mutant and was decreased further (to about 50% of wild-type) in the mutant that also overexpressed YcgR (Figure 6C). Thus, the binding of YcgR to the motor alters the orientation and/or motional freedom of the FliG C-terminal domain. The effect was prevented by the R118D mutation in YcgR (Figure 6C) and so appears to depend on normal interactions with c-di-GMP.

DISCUSSION

In *E. coli* and *Salmonella*, YcgR is dedicated to c-di-GMP-dependent regulation of flagellum-based motility and does not appear to influence other c-di-GMP-associated phenotypes (Ryjenkov et al., 2006). YcgR is composed of two domains: an N-terminal domain of unknown function and a C-terminal PilZ domain. The PilZ domain binds c-di-GMP in vitro with an affinity that is high enough to allow it to respond to small changes in intracellular c-di-GMP levels. This domain organization is conserved in a large number of proteobacteria (Amikam and Galperin 2006). Therefore, the function of YcgR in motility control is likely to be conserved. In this work, we show that elevated c-di-GMP and YcgR slow motor speed and inhibit chemotaxis by inhibiting CW rotation. We provide evidence that YcgR, when bound to c-di-GMP, interacts strongly with the flagellar motor proteins FliG and FliM. We present a model showing how both effects can result from disrupting the organization of the C-terminal domain of FliG, altering the rotor-stator interface in a way that reduces the efficiency of torque generation and induces a CCW motor bias.

“Backstop Brake” Model for Torque Reduction and Promotion of CCW Bias by YcgR

By use of mutations, we identified the region of FliM involved in the binding to YcgR and found that this binding surface overlaps the region shown previously to interact with FliG (Figure 3). This suggests that binding of YcgR could weaken the FliM-FliG inter-action, possibly displacing FliG_C and allowing it to adopt an alternative conformation. Physiological and binding experiments with FliG mutants show, furthermore, that YcgR interacts directly with FliG_C. Several Trp replacements in FliG_C relieved the YcgR-mediated motility inhibition, and when some of these were tested further in pull-down experiments they showed reduced binding to YcgR (Figure 3). Most such FliG mutations occur near the charge-bearing ridge that interacts with the stator (Lloyd et al., 1996; Yakushi et al., 2006; Zhou et al., 1998a). Determinants for YcgR binding thus lie near the “top” of the FliG_C domain, i.e., in the region that must point toward the stator in the functioning motor. In our current working model for the switch-complex structure, the charged ridge of FliG_C is quite distant from the YcgR-binding region on FliM (the distance between FliG-295 and FliM-160, for example, is approximately 55 Å). Based on the structure of the YcgR homolog of *Vibrio* (Benach et al., 2007), the YcgR molecule would be unable to span this distance. Accordingly, we propose that binding of c-di-GMP-loaded YcgR to the motor disrupts the interaction between FliM and FliG_C and causes FliG_C to be reoriented with its charged ridge downward to interact with YcgR. A conserved Gly-Gly linker at the base of the FliG_C domain, which has been proposed to function as a hinge during CW/CCW switching (Brown et al., 2002), could function in this context to allow this reorientation of the domain (Figure 7).

Torque generation in the motor is known to depend upon the proper relationship between FliG_C and MotA (Garza et al., 1996; Yakushi et al., 2006; Zhou et al., 1998a), and so the hypothesized domain movement should reduce the torque of the motor, roughly in proportion

to the number of domains that are flipped. Further, given the dimensions of the *Vibrio* YcgR-homolog (Benach et al., 2007) and the FliG_C domain (Brown et al., 2002), and assuming a 4 nm spacing between proteins in the outer C-ring (Thomas et al., 2006; Young et al., 2003), a YcgR molecule interacting with one FliG_C domain could easily inhibit the movements of neighboring domains, even those that remain unflipped. Thus, even the FliG_C domains that remain correctly oriented for torque generation could be prevented from undergoing movements needed for direction switching, accounting for the CCW bias of the YcgR-inhibited motors. YcgR could thus function as a “backstop brake,” both slowing the motor and inhibiting preferentially its rotation in one direction.

The model can account also for the effects of stator overexpression. Installation of the stator complexes is thought to depend in part on the FliG_C-MotA interactions, which are mostly attractive. Loss of the rotor-stator interactions, as occurs when FliG_C reorients, should then reduce the average number of stator complexes present in the motor. Overexpression of MotA (or MotA and MotB together) would counter this effect, increasing the number of stator units present and (because such stabilizing effects must be mutual) favoring the normal FliG_C conformation. Alternatively, the Mot-overexpression effects might reflect the occurrence of a direct interaction between YcgR and MotA. While our results do not rule out any YcgR-MotA interaction, they suggest that if such an interaction occurs it is weaker than the binding to FliM and FliG (Figure 2A). It is important to point out, however, that YcgR-GFP localization is completely eliminated in single point mutants of FliM (N155E and N160E) (Figure 4A), while localization remains unaffected in a *motA* null strain (Figure 2I and Figure S1). Thus, YcgR-dependent motility control is primarily through interaction with the switch complex.

The YcgR^{R118D} mutant was reported to be completely defective in c-di-GMP binding and does not impair motility when expressed in the $\Delta yhjH$ strain (Ryjenkov et al., 2006). We found, however, that the YcgR^{R118D} protein retains the ability to bind FliM (Figure 2C), and that this binding is responsive to c-di-GMP in the same micromolar concentration range as wild-type YcgR (Figure 2). This indicates that in the binding experiment here, with FliM present, the YcgR^{R118D} protein retains some c-di-GMP binding ability not observed with the purified protein (Ryjenkov et al., 2006). This is not unexpected: if the c-di-GMP-bound form of YcgR binds more tightly to FliM, then it must also be true that the FliM-bound form of YcgR binds more tightly to c-di-GMP, and the binding of c-di-GMP in this case may no longer be sensitive to mutation of Arg118. The R118D mutation clearly does alter YcgR function, eliminating both the motility impairment (Figure 4) and the FliG_C domain reorganization (Figure 6C) and weakening the binding to FliG (Figure 2C). These results can be accounted for if the mutation affects the rearranged state of the motor, in which YcgR interacts with FliG_C to displace this domain from its normal position. Based on structure of the *Vibrio* YcgR homolog, Arg118 lies in a hinge region between the two domains of YcgR, where it might influence the relative position of the domains. Accordingly, we propose that Arg118 is important in modulating the conformational change that occurs upon binding c-di-GMP, and that while it is also important for c-di-GMP binding affinity in the purified protein (Ryjenkov et al., 2006), this may not be the case in vivo.

Motility Control by Other YcgR-like Proteins

c-di-GMP-mediated motility control is not restricted to enterics, and can be mediated through alterations in flagellar gene expression, assembly, or function (Wolfe and Visick, 2008). The PilZ domain proteins YcgR in *E. coli/Salmonella* and DgrA in *Caulobacter crescentus* are the only known examples of c-di-GMP-binding proteins that do not affect expression of flagellar genes but directly interfere with the function of fully assembled flagella (Christen et al., 2007; Girgis et al., 2007). In *Caulobacter*, c-di-GMP levels control flagellar function by two different mechanisms acting through the protein FliL (Christen et al., 2007). FliL is required

for flagellar rotation in *Caulobacter*. Elevated c-di-GMP levels, when coupled with elevated expression of DgrA, apparently inhibit FliL synthesis and consequently paralyze the flagella. FliL is also required for the developmentally programmed ejection of the flagellum during the swimmer-to-stalk cell transition in this dimorphic bacterium. Flagellar ejection is dependent on the response regulator PleD, whose C-terminal output domain has DGC activity (Paul et al., 2004). In *C. crescentus*, DgrA-mediated downregulation of flagellar activity is probably an early step in the attachment of a swarmer cell to a surface, and is followed by flagellar-to-stalked cell pole differentiation. In *E. coli* and *Salmonella*, FliL is important for surface swarming, but not for swimming (Attmannspacher et al., 2008). However, the YcgR phenotype is independent of FliL in *Salmonella*, and overexpression of FliL did not improve motility in the *E. coli* $\Delta yhjH$ null strain (our unpublished data), suggesting that FliL is not a major target of YcgR action in these species.

Logic of Regulating Motility after a Flagellum Is Fully Assembled

Regulating motility by controlling the function of the fully assembled flagellum has a distinct advantage over transcriptional control of flagellar gene expression. In *Bacillus subtilis*, a related mechanism has been demonstrated for the protein EpsE, which functions as a “clutch” by interacting with the C-terminal domain of FliG to disengage the rotor from the stator (Blair et al., 2008). The biological function of the clutch in *B. subtilis* is intimately associated with biofilm formation, because *epsE* is encoded within an operon devoted to biosynthesis of extracellular poly-saccharides (EPS). In both the EpsE case and the YcgR mechanism proposed here, motor control is more rapid, and more readily reversible, than mechanisms based on gene expression or assembly. Cells that have been partially immobilized by the action of YcgR and c-di-GMP presumably remain ready to move if the biofilm disperses.

While the targets of YcgR and EpsE are similar, involving proteins of the rotor in both cases, their modes of action show some important differences. Unlike EpsE, YcgR does not appear to disengage the rotor completely from the stator but leaves most rotor elements in position to interact with the stator, though with reduced ability to switch. This difference might reflect the different stages at which the proteins act: Eps functions after the commitment to make a biofilm has already been made and the bacteria are likely to be assured of a suitable substrate for biofilm development, whereas YcgR could facilitate an earlier step in surface attachment. Inhibition of tumbling together with the reduction in motor torque could provide cells with a longer window of opportunity to interact with the surface, setting up the platform for early stages of biofilm formation. Inhibition of chemotaxis has been reported to reduce surface hydration and inhibit swarming in *Salmonella* and *E. coli* (Mariconda et al., 2006; Wang et al., 2005). *P. aeruginosa* also adjusts its motility during the transition to surface living, but using yet another strategy: in this species, c-di-GMP accumulation enhances biofilm formation by increasing EPS synthesis while simultaneously inhibiting chemoreceptor methylation to decrease the frequency of flagellar reversals (Caiazza et al., 2007; Kuchma et al., 2007). When circumstances call for less-vigorous motility, flagellar function can evidently be modulated by a variety of molecular mechanisms that leave the flagellar structure intact and so capable, presumably, of subsequent revival.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, Mutagenesis, and Motility Assays

Strains, growth conditions, mutagenesis, and motility assays are described in the Supplemental Experimental Procedures and Table S1.

Cell Tethering Assay

Tethering was performed as described previously (Mariconda et al., 2006). Bacteria were observed through phase-contrast microscopy and recorded on an external Sony video recording device. Average rotation speed per minute was calculated using a stopwatch as described (Attmannspacher et al., 2008).

Fluorescence Microscopy

Cells containing GFP-tagged fusion proteins were grown in the presence of 0.005% L-arabinose as described previously (Sourjik and Berg, 2000). Cells were visualized through a Nikon Eclipse 50i upright microscope using a B-2E/C filter. Fluorescent images were obtained using an attached Nikon digital camera. Images were archived using Nis-Elements D 3.0 software and subsequently cleaned and colorized using Adobe Photoshop CS3.

Binding Assays

Binding of FliG, FliM, FliN, and MotA_C to YcgR was measured using a pull-down assay with GST fusions to HA-tagged YcgR. Proteins were expressed separately in two strains, using plasmid pHT100 (Tang et al., 1996) derivatives to express the GST fusions to YcgR-HA (or its variants) and pHT53 (Tang et al., 1996) to express FliG (or its variant), pDB72 (Tang et al., 1996) to express FliM (or its variants), pHT39 (Lloyd et al., 1996) to express FliN, and pSB1 (this study) to express the cytoplasmic domain of MotA (MotA_C; comprising residues 70–170). For most experiments, FliN was coexpressed with FliM because FliM alone is prone to aggregation and did not accumulate readily in cells (Mathews et al., 1998). Control experiments used GST only, expressed from plasmid pHT100. Most binding experiments used strain RP3098, a Δ *fliHDC* mutant that expresses no flagellar genes from the chromosome (Tang et al., 1996).

Cells were cultured overnight at 32°C in 40 ml TB containing appropriate antibiotics and 400 μ M IPTG for expressing GST (pHT100), FliG (pHT53), FliN (pHT39), and MotA_C (pSB1). FliM and its mutant variants (pDB72) and FliN (pKP41) were cultured at the same condition containing appropriate antibiotics. IPTG (40 μ M) was used to induce expression of FliM and 10 μ M Na-salicylate to induce expression of FliN. Cells expressing the GST-YcgR-HA fusion or its mutant variants were cultured as described by Ryjenkov et al. (2006) and were harvested and resuspended in lysozyme-containing phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 5 mM EDTA, 0.2 mM APMSF [4-amidinophenylmethanesulfonyl fluoride], and 0.1% CHAPS [3-(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate). Following a 1 hr incubation on ice, the cells were further disrupted by sonication, debris was pelleted (16,000 g, 40 min, 4°C), and 50 μ l of the supernatant was saved for use in estimating the amount of FliG, FliM, FliN, and MotA_C present before addition of affinity beads. The rest (~1 ml) was transferred to a clean tube, mixed with 150 μ l of a 50% slurry of glutathione Sepharose 4B (Pharmacia) prepared according to the manufacturer's directions, and incubated for 1 hr at room temperature along with cyclic-di-GMP (Sigma) and with gentle rotation to allow binding. The Sepharose beads were then pelleted by a 1 min microcentrifuge spin, washed with 1 ml of phosphate-buffered saline, and pelleted again by a brief spin. FliG was washed slightly differently with phosphate-buffered saline containing 1% BSA and 0.1% Triton-X. The beads were then incubated with 50 μ l of elution buffer (50 mM reduced glutathione in 50 mM Tris-HCl [pH 8.0]) for 10 min at room temperature with gentle rotation to release the GST-YcgR-HA and associated proteins. Beads were then pelleted and the supernatant was collected for analysis by SDS-PAGE and immunoblotting using corresponding antibodies.

The expression of GST-YcgR-HA and its variants were also examined by anti-HA antibody (Covance, USA); these experiments used strain MS1280 (Table S1) and the culturing procedures described by Ryjenkov et al. (2006).

Crosslinking

Crosslinking experiments were carried out as described by Lowder et al. (2005). Products of crosslinking were examined on immunoblots using anti-HA antibody (Covance, USA).

SDS-PAGE and Immunoblotting

Protein samples were separated on 12% SDS-PAGE minigels and transferred to nitrocellulose using a semidry transfer apparatus (Bio-Rad). Rabbit poly-clonal antibodies against FliG, FliM, FliN, and MotA were prepared as described previously (Kim et al., 2008; Tang et al., 1995; Tang and Blair, 1995) and were used at 1500-fold dilution. YcgR-HA tagged proteins were detected using mouse anti-HA antibody at 1000-fold dilution (Covance, USA). Bands were visualized using the Super Signal West Picoluminol system (Pierce) and X-ray film.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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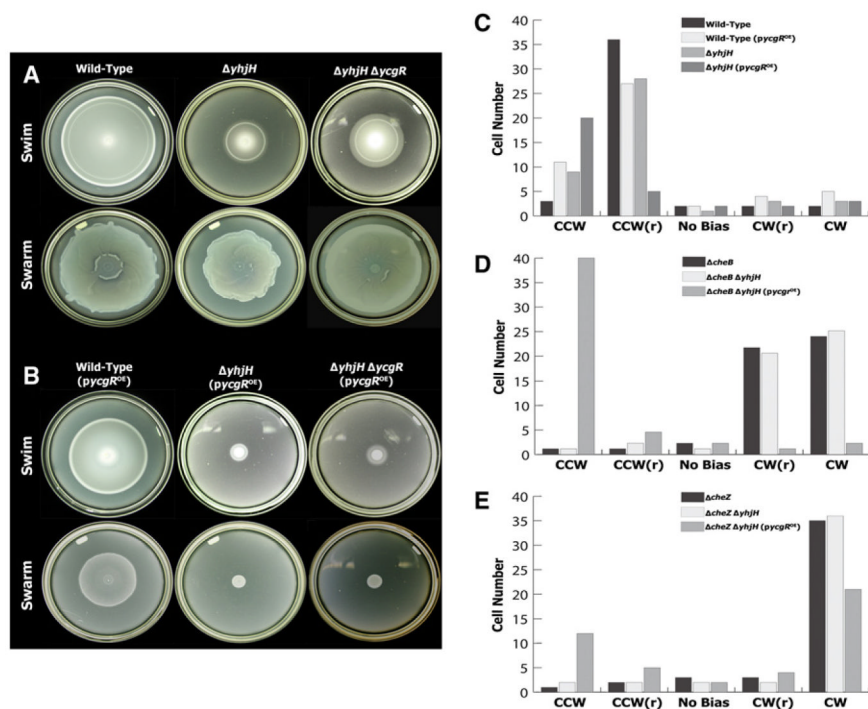


Figure 1. Swimming, Swarming, and Flagellar Rotational Bias of *Salmonella* Strains with Altered c-di-GMP Levels

(A) Motility of wild-type, $\Delta yhjH$, and $\Delta yhjH \Delta ycgR$ strains. Swim plates contain 0.3% agar and swarm plates 0.6% agar in addition to 0.5% glucose. Plates were incubated at 37°C for 6–8 hr.

(B) Motility of strains shown in (A) transformed with a plasmid directing overexpression (OE) of YcgR. Plasmid gene expression was induced with 0.2% arabinose.

(C–E) Histograms display flagellar rotation bias of tethered cells in the absence of chemotactic stimuli. Forty-four tethered cells were observed for 60 s each as described in the Experimental Procedures. Cells were classified into five categories (from left to right): exclusively CCW, CCW biased with reversals (r), frequent reversals with no bias, CW biased with reversals, and exclusively CW.

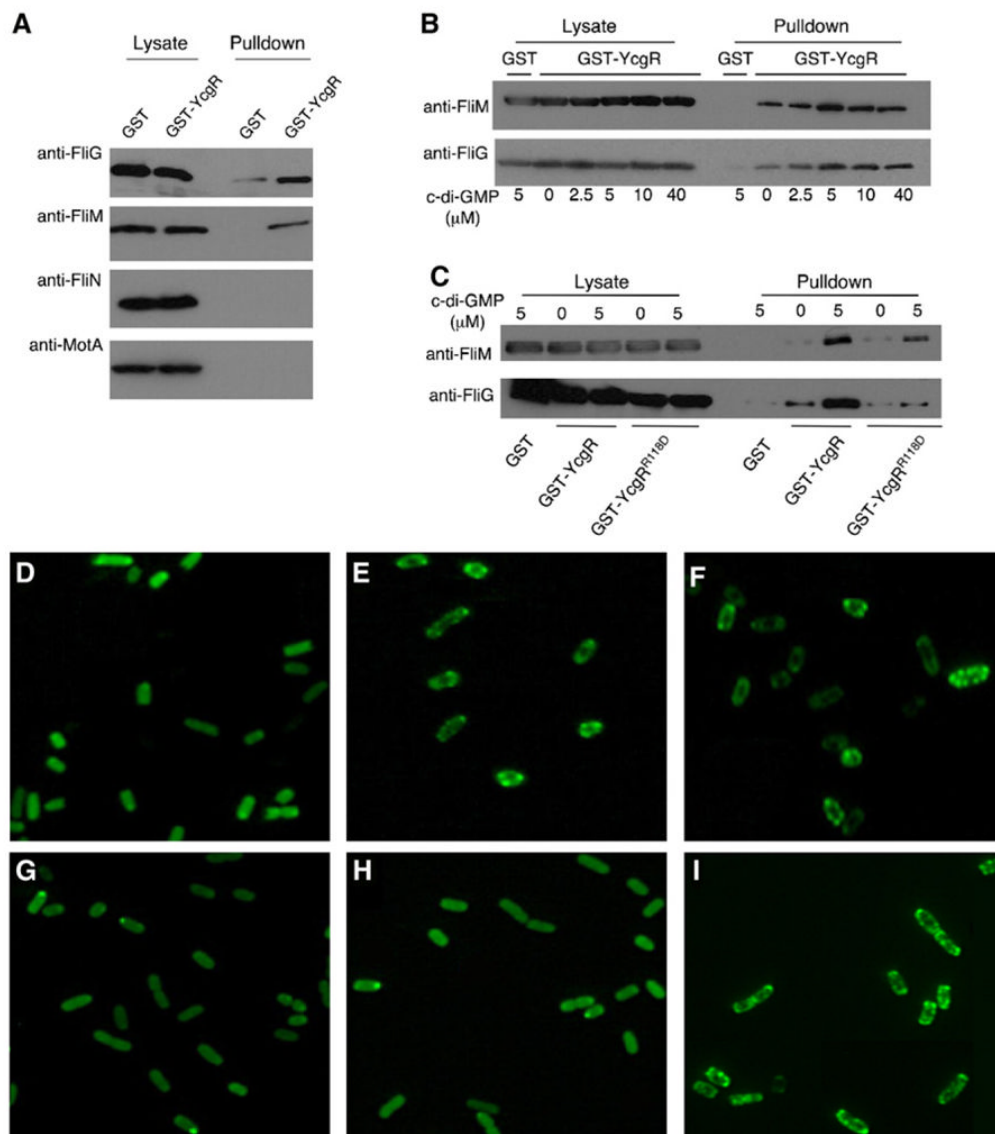


Figure 2. YcgR Interaction with Flagellar Rotor Proteins and YcgR-GFP Localization in Cells
 (A) Coisolation of YcgR with FliG and FliM but not with FliN or MotA_C (a cytoplasmic domain of MotA). GST-YcgR and the flagellar proteins were expressed in cells of *E. coli* strain RP3087 (Δ *fliHDC*). Cell lysates were incubated with Sepharose beads and 5 μ M c-di-GMP. Pull-down assays were carried out as described in the Experimental Procedures. Lysate controls show protein levels in samples prior to treatment with beads.
 (B) c-di-GMP dependence of YcgR binding to FliM and FliG. Samples for pull-down assays were supplemented with c-di-GMP at the concentrations indicated.
 (C) Induction by c-di-GMP of binding of the mutant GST-YcgR^{R118D} protein to FliM (top), and weakened binding of GST-YcgR^{R118D} to FliG (bottom).
 (D–I) Localization of YcgR-GFP in various *Salmonella* strains. Strains expressing either GFP or YcgR-GFP were grown in the presence of arabinose and analyzed by fluorescence microscopy as described in the Experimental Procedures. (D) Wild-type (*pgfp*^{OE}). (E) Wild-type (*pycgR*^{OE}). (F) Δ *yhjH* (*pycgR*^{OE}). (G) Δ *yhjH* Δ *fliM* (*pycgR*^{OE}). (H) Δ *yhjH* Δ *fliG* (*pycgR*^{OE}). (I) Δ *yhjH* Δ *motA* (*pycgR*^{OE}).

(D) Binding results with mutants, mapped onto the FliG structure (with colors as in B). A mutation that gave nonflagellate phenotype (225W) (which was not included in the swimming assays) is indicated by the black sphere.

(E) Effects of FliG mutations on the motility impairment caused by elevated levels of c-di-GMP. Mutant FliG proteins were expressed from plasmids in a $\Delta yjH\Delta fliG$ strain. Fresh transformants were picked onto swim plates containing tryptone and 0.27% agar and incubated at 32°C for 10 hr.

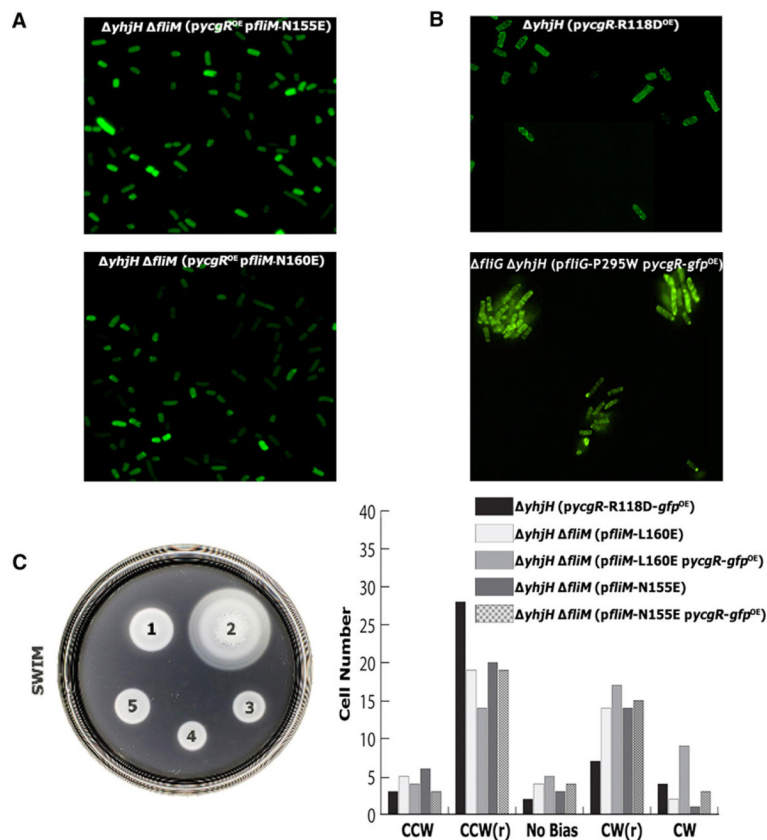


Figure 4. YcgR Localization and Phenotypic Analysis of Mutants in *Salmonella*

(A) Diffuse localization of YcgR-GFP in cells with FliM mutations N160E and N155E.

(B) Normal punctate localization of YcgR in the YcgR R118D mutant and the FliG P295W mutant.

(C) Swimming motility (left) and rotational bias (right) of mutant strains. Strains on the swim plate are as follows: (1) $\Delta yhjH$ /*pycgr*-*gfp*^{OE}; (2) $\Delta yhjH$ /*pycgr*(R118D)-*gfp*^{OE}; (3) $\Delta yhjH \Delta fliM$ /*pfliM*(L160E), *pycgr*-*gfp*^{OE}; (4) $\Delta yhjH \Delta fliM$ /*pfliM*, *pycgr*-*gfp*^{OE}; (5) $\Delta yhjH \Delta fliM$ /*pfliM* (N155E), *pycgr*-*gfp*^{OE}.

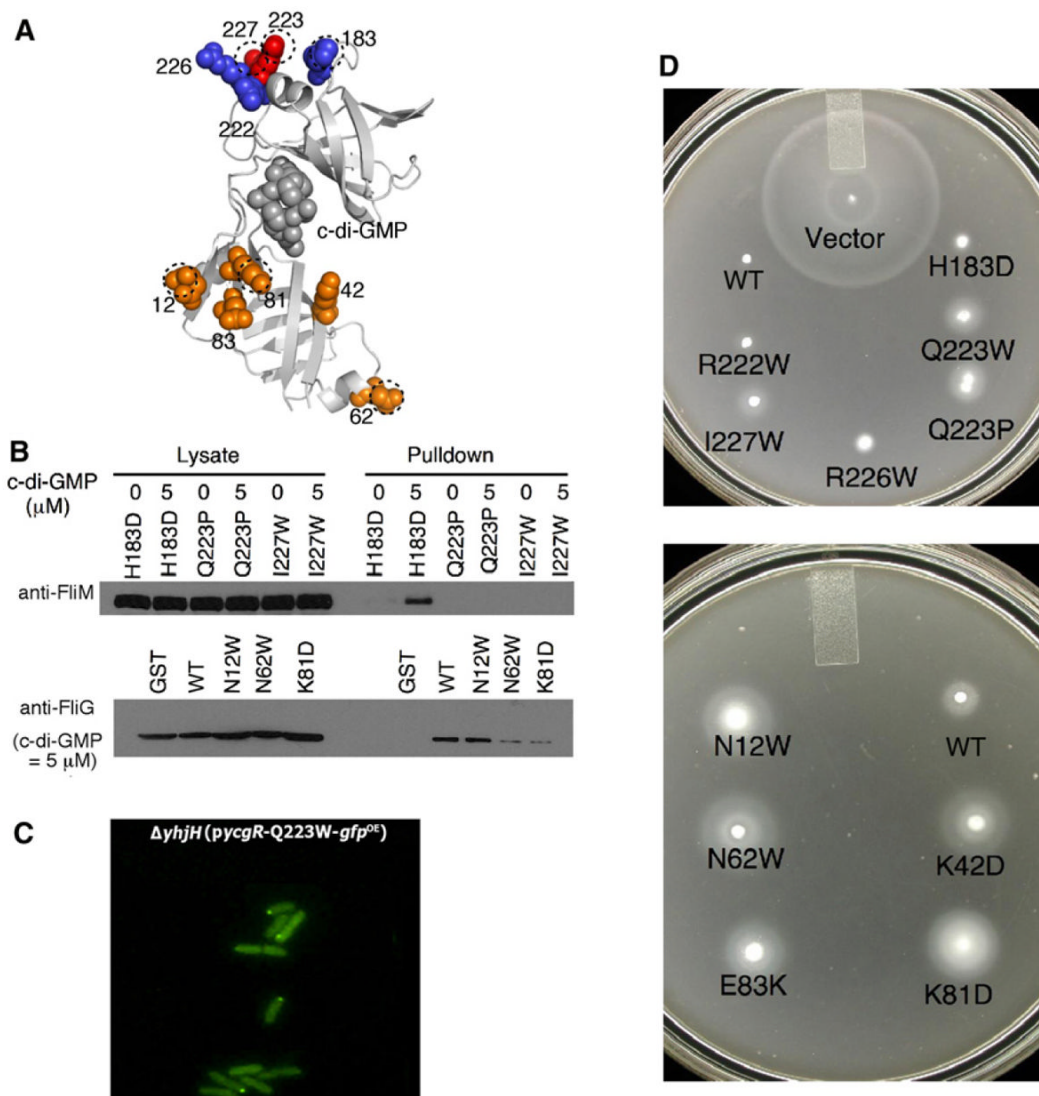


Figure 5. Effects of YcgR Mutations on Motility and Binding

(A) Homology model of *E. coli* YcgR, based on the crystal structure of the *Vibrio cholerae* protein VCA0042 (Protein Data Bank ID code 2RDE). YcgR mutations characterized in this study are indicated. Mutations that relieved the motility inhibition are colored red (in the C-terminal domain) or orange (in the N-terminal domain); mutations that did not relieve the motility inhibition are colored blue. Mutations characterized further in pull-down assays with FliM and FliG are indicated by dotted lines.

(B) Effects of YcgR mutations on the binding to FliM and FliG in *E. coli*.

(C) Diffuse localization of the Q223W YcgR mutant in *Salmonella*.

(D) Effects of YcgR mutations on the motility impairment caused by YcgR overexpression. Mutant YcgR proteins were overexpressed from plasmids in the $\Delta yhjH \Delta ycgR$ background in *E. coli* by addition of 2.5 μM Na-salicylate. Soft agar plates were incubated at 32°C for 10 hr. The upper and lower plates show, respectively, the behaviors of C- and N-terminal domain mutants.

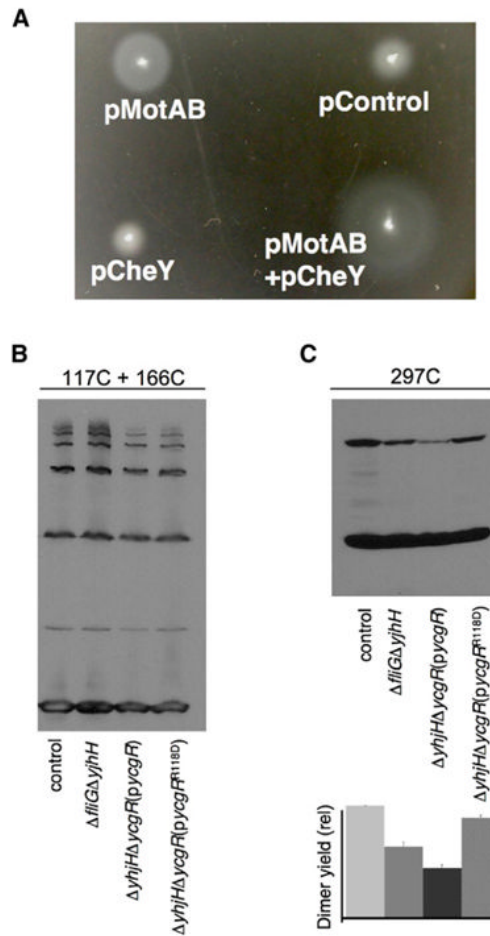


Figure 6. Effects of Overexpressing Stator Proteins, and CheY, in $\Delta yhjH$ *E. coli* Cells
 (A) Motility of $\Delta yhjH$ cells transformed with plasmids expressing the stator proteins MotA and MotB, the CW-signaling protein CheY, or all three proteins. Parent vectors pRR48 and pKG116 were used as controls. Mot proteins were expressed from the *trp* promoter, with induction by 100 μ M indole acrylic acid. CheY expression was induced using 500 μ M arabinose. Fresh transformants were picked onto a soft agar tryptone plate and incubated at 32°C for 10 hr. (B and C) Effects of *yhjH* deletion and *ycgR* overexpression on FliG domain organization. Iodine was used to induce crosslinking of FliG proteins with Cys replacements at positions 117 and 166 in the middle domain (B) or position 297 in the C-terminal domain (C). YcgR was wild-type or with the mutation R118D, as indicated. Protein expression was induced with 50 μ M IPTG (YcgR) or 10 μ M sodium salicylate (FliG). FliG proteins were HA tagged, and products of crosslinking were visualized on anti-HA immunoblots. Relative crosslinking yields for the position 297 experiments are shown at the bottom of (C). In (C), error bars indicate standard variation in three experimental repeats.

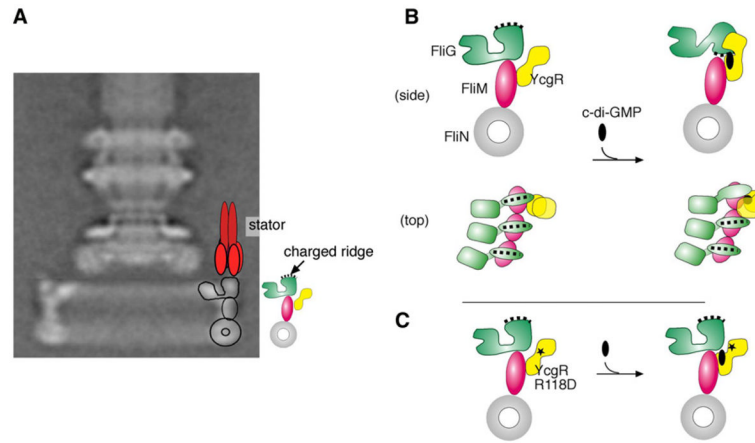


Figure 7. Model for YcgR-Mediated Motility Control

(A) Overall disposition of stator and rotor proteins. A single stator complex (of approximately 11 present in the motor) is shown in red. Proteins of the rotor are shown in outline on the basal-body C-ring and are also shown to the side: FliG (green), FliM (magenta), and FliN (gray). YcgR (yellow) is shown attached to FliM. The charge-bearing ridge indicated on the C-terminal domain of FliG forms the site of interaction with the stator. The electron micrograph was reproduced with permission from the American Society for Microbiology (Brown et al., 2007).

(B) Hypothesis for c-di-GMP-induced rearrangements. Binding of c-di-GMP to YcgR induces movement of the YcgR N-terminal domain, which allows it to bind to the C-terminal domain of FliG. Interaction with YcgR causes the FliG C-terminal domain to reorient so that the charged ridge is no longer able to interact with the stator. YcgR_N might also be expected to constrain the movement of the neighboring FliG_C domain (top view), preventing movements needed for direction switching.

(C) Hypothesis for the effect of the R118D mutation in YcgR^{R118D} (indicated by *). The R118D mutant protein retains the ability to bind to FliM. When bound to FliM, it can also bind c-di-GMP, but this interaction is altered so that it no longer undergoes the c-di-GMP-induced domain rearrangement and does not interact with FliG_C to impair motility.