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A Polar Flagellar Transcriptional Program Mediated by Diverse Two-Component Signal Transduction Systems and Basal Flagellar Proteins Is Broadly Conserved in Polar Flagellates

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ABSTRACT Bacterial flagella are rotating nanomachines required for motility. Flagellar gene expression and protein secretion are coordinated for efficient flagellar biogenesis. Polar flagellates, unlike peritrichous bacteria, commonly order flagellar rod and hook gene transcription as a separate step after production of the MS ring, C ring, and flagellar type III secretion system (fT3SS) core proteins that form a competent fT3SS. Conserved regulatory mechanisms in diverse polar flagellates to create this polar flagellar transcriptional program have not been thoroughly assimilated. Using *in silico* and genetic analyses and our previous findings in *Campylobacter jejuni* as a foundation, we observed a large subset of Gram-negative bacteria with the FlhF/FlhG regulatory system for polar flagellation to possess flagellum-associated two-component signal transduction systems (TCSs). We present data supporting a general theme in polar flagellates whereby MS ring, rotor, and fT3SS proteins contribute to a regulatory checkpoint during polar flagellar biogenesis. We demonstrate that *Vibrio cholerae* and *Pseudomonas aeruginosa* require the formation of this regulatory checkpoint for the TCSs to directly activate subsequent rod and hook gene transcription, which are hallmarks of the polar flagellar transcriptional program. By reprogramming transcription in *V. cholerae* to more closely follow the peritrichous flagellar transcriptional program, we discovered a link between the polar flagellar transcription program and the activity of FlhF/FlhG flagellar biogenesis regulators in which the transcriptional program allows polar flagellates to continue to produce flagella for motility when FlhF or FlhG activity may be altered. Our findings integrate flagellar transcriptional and biogenesis regulatory processes involved in polar flagellation in many species.

IMPORTANCE Relative to peritrichous bacteria, polar flagellates possess regulatory systems that order flagellar gene transcription differently and produce flagella in specific numbers only at poles. How transcriptional and flagellar biogenesis regulatory systems are interlinked to promote the correct synthesis of polar flagella in diverse species has largely been unexplored. We found evidence for many Gram-negative polar flagellates encoding two-component signal transduction systems with activity linked to the formation of flagellar type III secretion systems to enable production of flagellar rod and hook proteins at a discrete, subsequent stage during flagellar assembly. This polar flagellar transcriptional program assists, in some manner, the FlhF/FlhG flagellar biogenesis regulatory system, which forms specific flagellation patterns in polar flagellates in maintaining flagellation and motility when activity of FlhF or FlhG might be altered. Our work provides insight into the multiple regulatory processes required for polar flagellation.

KEYWORDS *Campylobacter jejuni*, FlhF, FlhG, MS ring, *Pseudomonas aeruginosa*, T3SS, *Vibrio cholerae*, flagellar biogenesis, motility, polar flagellation, two-component signal transduction

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Many bacteria synthesize flagella for swimming motility. Each species produces a specific flagellation pattern defined by the spatial arrangement and number of flagella presented on the cell surface. Peritrichous flagellates construct many flagella across the surface, whereas polar flagellates generate flagella only at polar regions. Polar flagellates are further categorized by the number of flagella per cell: monotrichous (one flagellum at one pole), amphitrichous (one flagellum at each pole), and lophotrichous (a tuft of a few flagella at one pole).

Flagellar placement and number in many polar flagellates are controlled by the FlhF GTPase and FlhG/FleN ATPase (1, 2). FlhF is hypothesized to function in some polar flagellates in a GTP-bound "on" state to perform an unknown essential step for flagellar biogenesis at a pole. Transitioning to a GDP-bound "off" state upon GTP hydrolysis may limit the production of additional polar flagella. In some polar flagellates, FlhG stimulates FlhF GTPase activity *in vitro*, which has been proposed to influence flagellum numbers by controlling *in vivo* FlhF-dependent polar flagellation activities (3–6). However, FlhG orthologs in other species control polar flagellar number by repressing the activity or expression of a specific master transcriptional regulator so that an ideal level of flagellar genes sufficient to produce the correct number of flagella are expressed (7–9). Many molecular details for how FlhF and FlhG control polar flagellation remain elusive. It is anticipated that FlhF and FlhG activities vary among species, resulting in different flagellation patterns in polar flagellates.

Despite different flagellation patterns, many peritrichous and polar flagellates possess some conserved strategies to coordinate flagellar gene transcription with stages of flagellar assembly (10–13). These strategies allow for tight regulation of ordered flagellar protein secretion that is conducive to flagellar motor biogenesis. Stages of flagellar assembly can be marked by distinct cues or regulatory checkpoints that are detected by different mechanisms to stimulate gene transcription and protein production to complete the next stage of assembly. Flagellar biogenesis begins by activating the transcription of genes encoding components essential for the initial steps in assembly, which include the flagellar type III secretion system (fT3SS), MS ring, and C ring rotor and switch proteins (14–20). MS and C ring formation around the fT3SS core completes biogenesis of a competent fT3SS for export and assembly of rod and hook components (21–25). Up to this point, the alternative σ factor σ^{28}, which is required for transcription of flagellins and other motility genes, is inhibited by the anti-σ factor FlgM (11, 13, 26–29). Hook biogenesis completes a regulatory checkpoint that facilitates an fT3SS substrate specificity switch to secrete FlgM out of the cell via the fT3SS (11, 13, 30). Derepression of σ^{28} allows for transcription of genes that complete flagellar filament polymerization and motor assembly.

We previously explored how the amphitrichous polar flagellate *Campylobacter jejuni* coordinates the transcription of flagellar genes with flagellar assembly (Fig. 1) (31–33). We discovered that fT3SS core proteins (FlhA, FlhB, FliP, FliQ, and FliR), the FliF MS ring, and FliG rotor of the C ring assemble into the MS ring-rotor-fT3SS complex to form a regulatory checkpoint monitored by *C. jejuni*. We found the flagellum-associated FlgSR two-component signal transduction system (TCS) detects MS ring-rotor-fT3SS formation to directly activate σ^{54}-dependent flagellar rod and hook gene expression (Fig. 1) (31–33). As a means of signal detection, we observed that the cytoplasmic FlgS sensor kinase of the FlgSR TCS physically interacted with FliF and FliG only after these proteins multimerized into the MS ring and rotor around the fT3SS core (Fig. 1) (32). In mutants defective for MS ring-rotor-fT3SS complex assembly, FlgS did not interact with FliF and FliG. Currently, it is not known whether FlgS interacts with surfaces of adjacent FliF subunits, FliG subunits, or FliF-FliG complexes of the MS ring-rotor structure surrounding the fT3SS core. Detection of this regulatory checkpoint by an orthologous FlgSR TCS also may occur in *Helicobacter pylori*, a lophotrichous epsilonproteobacterium closely related to *C. jejuni*, for transcription of σ^{54}-dependent rod and hook genes (34–37). Genetic analyses indicate that *H. pylori* FlgSR TCS activity for rod and hook gene transcription is also dependent on fT3SS, MS ring, and C ring proteins, suggesting that this TCS in *H. pylori* also senses the formation of a competent fT3SS (38–43).

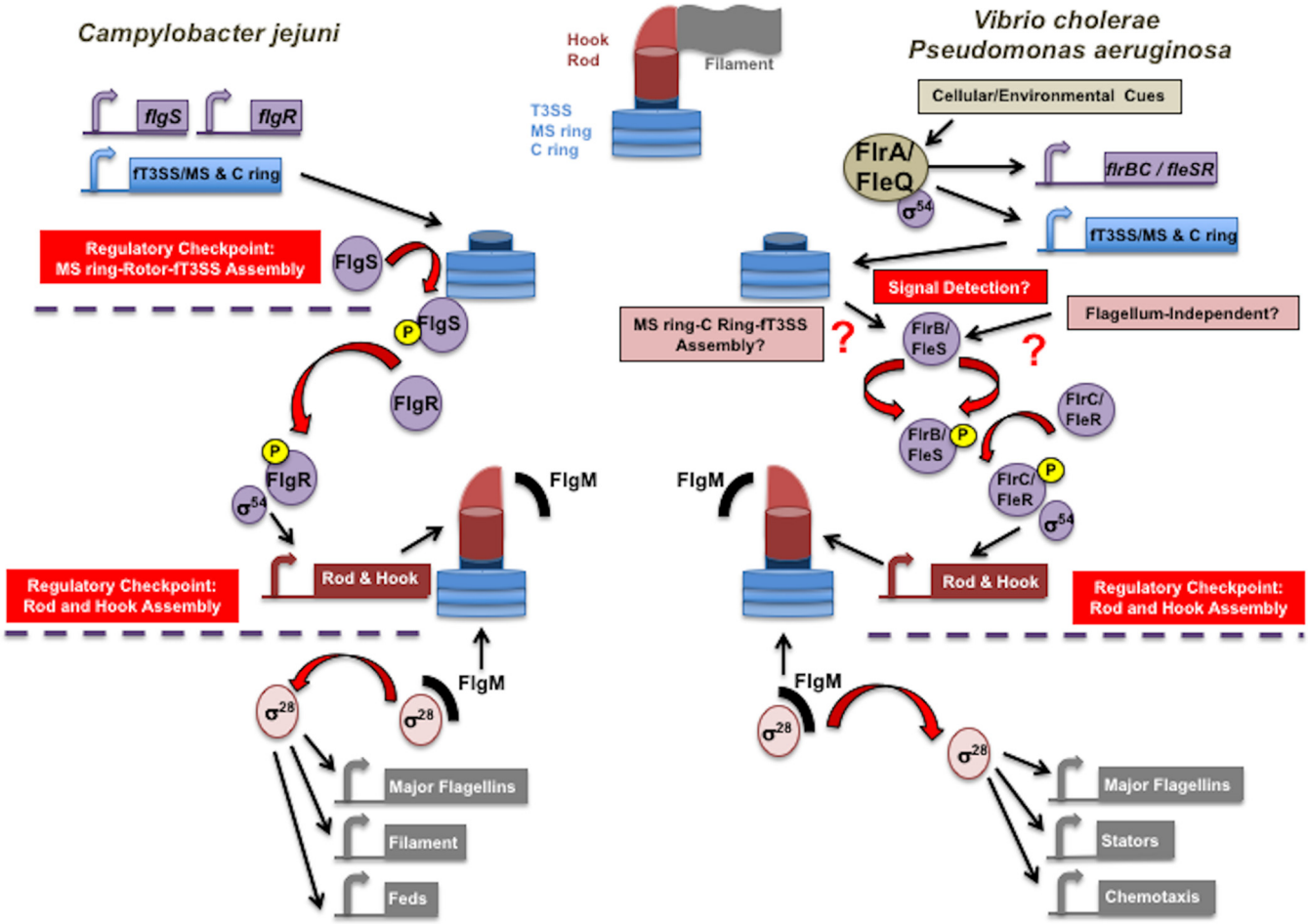


FIG 1 Defined and unknown regulatory steps of the polar flagellar transcriptional program of *C. jejuni*, *V. cholerae*, and *P. aeruginosa*. Simplified models of transcriptional regulation of different classes of flagellar genes in the Gram-negative polar flagellates *C. jejuni*, *V. cholerae*, and *P. aeruginosa*. *V. cholerae* and *P. aeruginosa* (right) initiate flagellar gene transcription via master transcriptional regulators (FlrA and FleQ, respectively) to transcribe an initial class of flagellar genes; *C. jejuni* lacks a master transcriptional regulator and expression of this class of flagellar genes appears to be constitutive (left). These initial flagellar genes encode the fT3SS, MS ring, C ring, and a flagellum-associated TCS (FlgSR in *C. jejuni*, FlrBC in *V. cholerae*, and FleSR in *P. aeruginosa*). Expression of the *C. jejuni* flagellar rod, ring, and hook genes is dependent upon σ^{54}, the FlgSR TCS, and the fT3SS, FliF MS ring, and FliG C ring rotor proteins (31–33). The formation of the MS ring-rotor-fT3SS complex is an early regulatory checkpoint sensed by the FlgS sensor kinase to initiate phosphotransfer to the FlgR response regulator, which allows FlgR to function with σ^{54} for rod and hook gene expression. (Right) Exploration of factors that may form a similar regulatory checkpoint during flagellar assembly that are required for the activity of the *V. cholerae* FlrBC TCS and the *P. aeruginosa* FleSR TCS addressed in this work. A regulatory checkpoint during late flagellar assembly occurs upon formation of the flagellar rod and hook in all of these organisms and many other peritrichous and polar flagellates. Completion of rod and hook assembly promotes a substrate specificity switch that facilitates secretion of the anti-σ factor FlgM from the cytoplasm and derepression of σ^{28} for transcription of flagellins and other proteins that complete flagellar biogenesis for motor function. *C. jejuni* also contains *fed* genes that are dependent on σ^{28} for expression and are not involved in motility but are required for the colonization of avian species and some virulence processes (92, 93).

Vibrio cholerae and *Pseudomonas aeruginosa* are monotrichous polar flagellates that also produce a flagellum-associated TCS. The *V. cholerae* FlrBC and *P. aeruginosa* FleSR TCSs, like the *C. jejuni* FlgSR TCS, are directly required with σ^{54} for flagellar rod and hook gene expression (17–19, 44, 45). As with *C. jejuni* FlgSR, signal transduction through *V. cholerae* and *P. aeruginosa* TCSs results in phosphorylation of the cognate FlrC and FleR response regulators to promote their binding to rod and hook gene promoters and directly assist σ^{54} in activating transcription of rod and hook genes (44–47). Although transcription of the *V. cholerae* and *P. aeruginosa* FlrBC and FleSR TCSs occurs simultaneously with fT3SS, MS ring, and C ring genes by a master transcriptional regulator (18, 19), it is not known whether these TCSs sense cues associated with flagellar assembly or flagellum-independent cellular cues to initiate and coordinate flagellar rod and hook gene expression with a stage of flagellar assembly (Fig. 1). Transcription of rod and hook genes after expression of MS ring, C ring, and fT3SS components appears

to comprise a polar flagellar transcriptional program not observed in peritrichous flagellates.

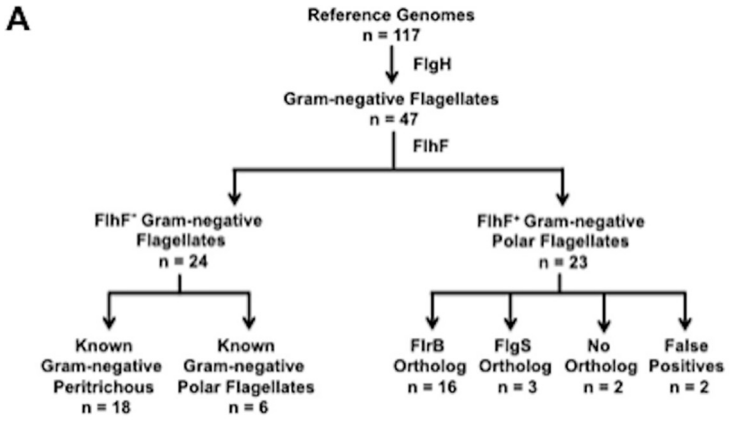
Noticeably, many peritrichous flagellates appear to ignore the formation of the MS ring-rotor-fT3SS complex as a regulatory checkpoint that we discovered in *C. jejuni* and is likely present in *H. pylori*. The model peritrichous bacteria *Escherichia coli* and *Salmonella* lack a flagellum-associated TCS and do not employ σ^{54} for flagellar gene transcription. These bacteria also do not require the MS ring, C ring, and fT3SS proteins for rod and hook gene expression (10, 15, 48). Instead, these bacteria express fT3SS, MS ring, and C ring proteins simultaneously with flagellar rod and hook genes, which we designate, for the purposes of this report, the peritrichous flagellar transcriptional program. Hence, a competent fT3SS is not required for expression of rod and hook genes in peritrichous flagellates, in contrast to *C. jejuni* and *H. pylori*. These differences raise intriguing questions for flagellar biogenesis in polar flagellates. (i) How common is it for polar flagellates to possess flagellum-associated TCSs? (ii) Do polar flagellates with flagellum-associated TCSs broadly require MS ring, C ring, and fT3SS proteins for activity to result in rod and hook gene expression? (iii) Do polar flagellates employ the respective sensor kinases to detect a regulatory checkpoint formed by the MS ring, C ring, and/or fT3SS? (iv) Do some TCSs detect flagellum-independent cues to activate rod and hook gene expression? (v) Is the polar flagellar transcriptional program that separates production of fT3SS, MS ring, and C ring proteins from rod and hook proteins required for or beneficial to a specific process in polar flagellates to build flagella?

We evaluated the conservation of regulatory systems that order gene expression for a polar flagellar transcriptional program in diverse polar flagellates. Our results, combined with previous findings in alphaproteobacteria, indicate a broad, common theme in polar flagellates, whereby different mechanisms are employed to coordinate rod and hook protein production with a stage of flagellar assembly involving the formation of a competent fT3SS. We found that a large subset of polar flagellates with FlhF/FlhG flagellar biogenesis regulatory systems encode orthologous flagellum-associated TCSs. Additional evidence combined with our previous findings suggests that these TCSs have a conserved function in detecting a similar regulatory checkpoint centered around MS ring-rotor-fT3SS complex formation. Our findings suggest that the polar flagellar transcriptional program, rather than a peritrichous one, allows polar flagellates to sustain flagellation and motility if FlhF and/or FlhG activity is altered and provide speculation into the evolution of polar flagellates. Our work provides insight into connections between flagellar transcriptional and biogenesis regulatory systems involved in polar flagellation.

RESULTS

A large subset of gram-negative polar flagellates possesses flagellum-associated TCSs. We followed a bioinformatic strategy to evaluate the prevalence of flagellum-associated TCS in a broad range of Gram-negative polar flagellates. We limited our analysis to Gram-negative bacteria to enable comparisons with previous studies in bacteria that employ flagellum-associated TCSs for direct activation of σ^{54}-dependent flagellar rod and hook gene expression, such as *C. jejuni*, *H. pylori*, *V. cholerae*, and *P. aeruginosa*. For this approach, we performed reciprocal best hit sequence alignments with a set of 117 reference bacterial genomes available at NCBI Assembly to identify Gram-negative flagellates. Genomes included in this reference set were curated by NCBI to represent high-quality, community-standard bacterial genomes or genomes of medically relevant bacteria. Although not every sequenced bacterial genome is included in this reference set, it is sufficient to survey and acquire information regarding features present among phylogenetically diverse bacteria.

We first used *E. coli* FlgH as a marker for Gram-negative bacteria producing flagella (Fig. 2A). FlgH forms the L-ring required for external flagella to penetrate the outer membrane barrier in Gram-negative flagellates (49–51). Since FlhF is involved in polar flagellation, we then used *V. cholerae* FlhF as a marker to predict polar flagellates within the Gram-negative flagellates (Fig. 2A) (1, 2, 7). Our results initially identified 23 of 47



B

Polar Flagellate	FlrB Ortholog	FlgS Ortholo
Vibrio cholerae	FlrB	-
Vibrio fishcheri	FlrB	-
Vibrio parahaemolyticus	FlaL	-
Pseudomonas aeruginosa	FleS	-
Pseudomonas putida	FleS	-
Pseudomonas syringae pv syringae	FleS	-
Pseudomonas syringae pv tomato	FleS	-
Aeromonas hydrophila	FlrB	-
Shewanella oneidensis	FlrB	-
Legionella pneumohila	FleS	-
Aquifex aeolicus	HksP4	-
Desulfovibrio vulgaris str Hildenborough	DVU1418	-
Geobacter sulfurreducens	GSU0471	-
Rhodospirillum rubrum	NtrY	-
Rhodopirellula baltica	RB10322	-
Salinibacter ruber	SRU_0472	-
Campylobacter jejuni	-	FlgS
Helicobacter pylori	-	FlgS
Burkholderia pseudomallei	-	BPSL0127
Thermanaerovibrio acidaminovorans	-	-
Xanthamonas campestris pv *campestris*	-	-

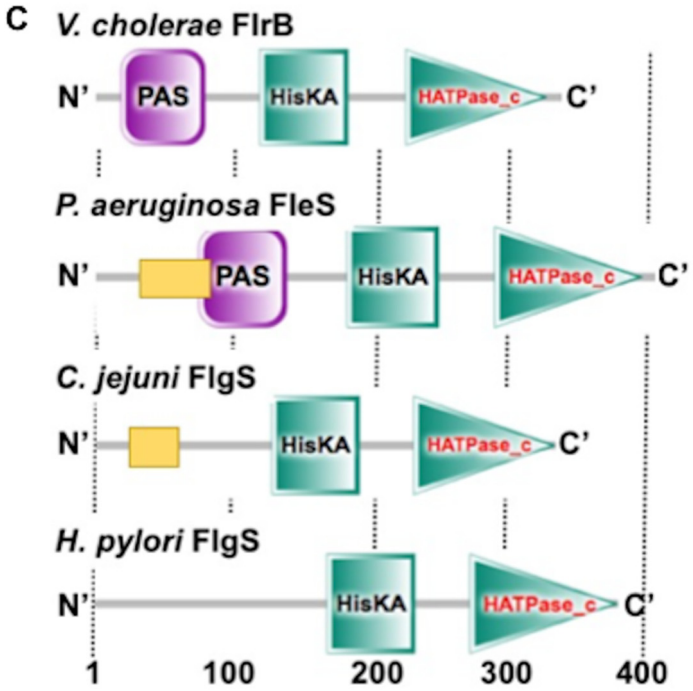


FIG 2 Bioinformatic analysis of predicted Gram-negative polar flagellates and flagellum-associated TCSs. (A) Flowchart of the strategy and outcomes of tBLASTn reciprocal best hit sequence alignments of

(Continued on next page)

putative Gram-negative flagellates within the reference set as polarly flagellated species (Fig. 2B), with two clear false positives: *Bordetella bronchiseptica*, which encodes a predicted FlhF (BN112_0372) but is a known peritrichous organism (52), and *Burkholderia mallei*, which harbors mutations in multiple flagellar genes and is likely undergoing reductive genome evolution (53). Twenty of 21 predicted Gram-negative polar flagellates encoded a predicted FlhG ortholog immediately downstream of *flhF*, indicating that an FlhF/FlhG flagellar biogenesis regulatory system was intact; only *Rhodospirillum rubrum* lacked an *flhG* ortholog organized with *flhF*. Of the 24 predicted Gram-negative flagellates lacking FlhF, 18 are known peritrichous organisms. The remaining six are actually polar flagellates with five species from alphaproteobacteria, best represented by *Caulobacter crescentus*, which employs factors other than FlhF for polar flagellation. Thus, FlgH and FlhF were relatively robust predictors of Gram-negative polar flagellates within the reference set except for alphaproteobacterial species.

From the 21 remaining predicted Gram-negative polar flagellates with FlhF orthologs, we performed reciprocal best hit sequence alignments using the *V. cholerae* FlrB sensor kinase of the flagellum-associated FlrBC TCS to identify 16 species with flagellum-associated TCSs (Fig. 2A and B). Although this approach identified putative flagellum-associated TCS sensor kinases in many polar flagellates (including FleS of the FleSR TCS of different *Pseudomonas* species), *C. jejuni* FlgS was not identified. We repeated our reciprocal best hit sequence alignment analysis using *C. jejuni* FlgS. *C. jejuni* FlgS did not identify FlrB or FleS as orthologs but did identify flagellum-associated sensor kinases in two of the four predicted polar flagellates without FlrB orthologs, *H. pylori* FlgS and *Burkholderia pseudomallei* BPSL0127 (Fig. 2A and B). These observations suggest that sensor kinases of flagellum-associated TCSs in polar flagellates are divided into distinct FlrB-like or FlgS-like groups. Overall, our bioinformatic analysis indicated that 19 of 21 predicted Gram-negative polar flagellates from the reference set encode both an FlhF/FlhG regulatory system and a putative flagellum-associated TCS. Thus, our results suggest a high degree of cooccurrence between the two regulatory systems exist in bacterial species that produce polar flagella. The only two Gram-negative FlhF/FlhG-positive species in the reference set that did not encode a flagellum-associated TCS were the known polar flagellate *Xanthamonas campestris* pv. *campestris*, previously noted to lack a respective TCS, and *Thermanaerovibrio acidaminovorans,* whose prediction as a polar flagellate may be dubious, as it produces lateral flagella on its concave surface (54, 55) (Fig. 2B).

To ensure that our FlrB homologs are flagellum-associated sensor kinases rather than conserved unrelated sensor kinases involved in other processes, we examined the correlation of *V. cholerae* sensor kinases with the presence of FlhF across genomes in the reference set (see Fig. S1 in the supplemental material). If non-flagellum-associated sensor kinases are within our predicted FlrB orthologs, we would expect a weak to negative correlation of these kinases with FlhF that is indistinguishable from the correlation of a random *V. cholerae* sensor kinase to FlhF. In contrast, if FlrB orthologs are flagellum-associated TCS kinases, we would expect *V. cholerae* FlrB to be one of the kinases most highly correlated with FlhF. We found that predicted FlrB orthologs had a stronger positive correlation to *V. cholerae* FlhF than all but two of 51 *V. cholerae* sensor kinases (Fig. S1). One of these kinases is *V. cholerae* CheA, which is the major sensor kinase in the chemotaxis system that influences flagellar rotation and motility in

FIG 2 Legend (Continued)
predicted Gram-negative polar flagellates from a reference set of bacterial genomes and those with *V. cholerae* FlrB or *C. jejuni* FlgS orthologs. (B) Sensor kinases of putative flagellum-associated TCSs of predicted Gram-negative polar flagellates. For each polar flagellate, the annotated or predicted kinase is indicated as an FlrB or FlgS ortholog depending on the resultant score. (C) Domain analysis of *V. cholerae* FlrB, *P. aeruginosa* FleS, *C. jejuni* FlgS, and *H. pylori* FlgS as predicted by SMART. The conserved HisKA and HATPase_C domains of bacterial sensor kinases for histidine autophosphorylation, dimerization, and ATPase activity are shown in turquoise squares and triangles, respectively. Putative predicted PAS domains are shown as purple squares, and predicted coiled-coil domains are shown as gold rectangles. Numbers below indicate approximate positions of amino acids.

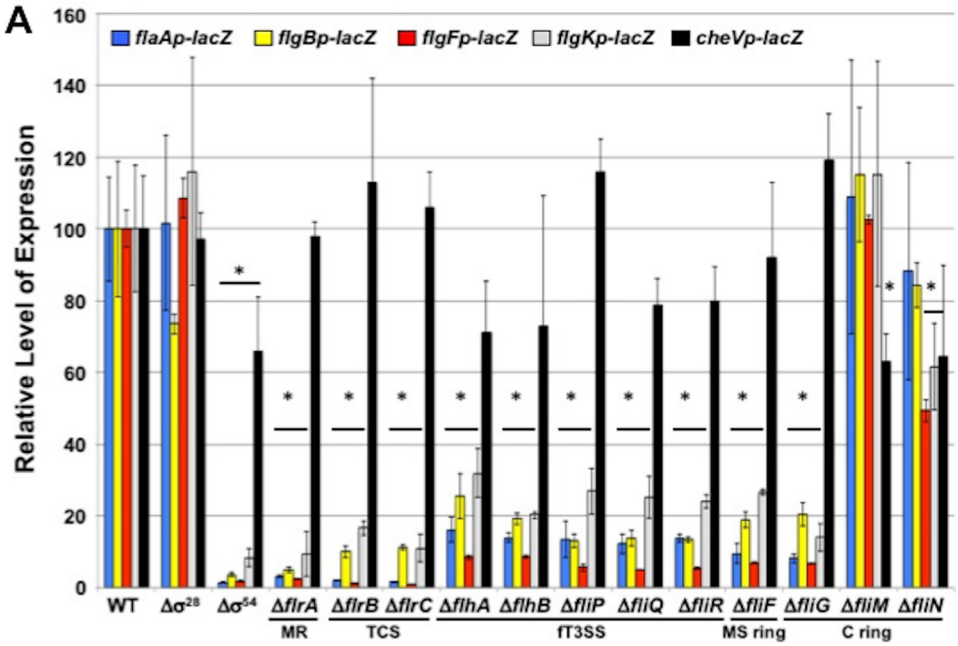
many bacterial species (56, 57). The other *V. cholerae* kinase is VCA0851, an uncharacterized kinase. These results support our flagellum-associated TCS predictions and further emphasize the correlation between the FlhF/FlhG flagellar biogenesis system and the flagellum-associated TCSs.

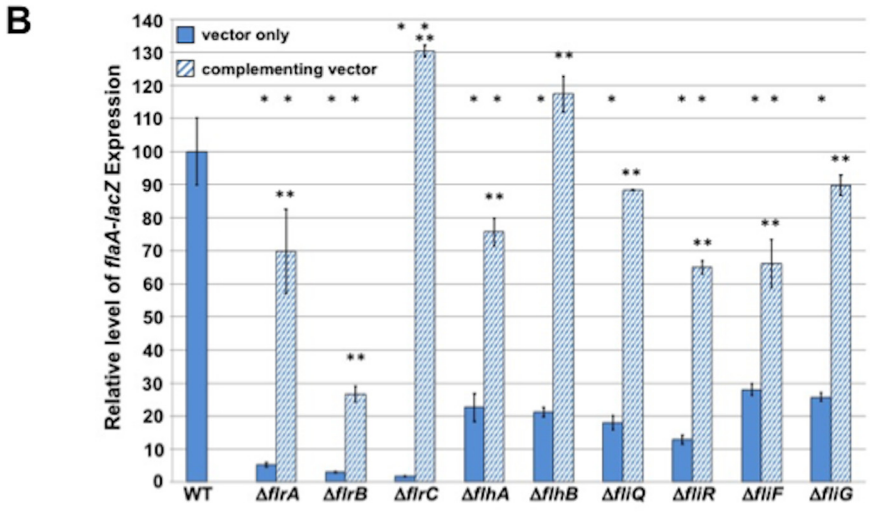
Our bioinformatic analysis suggested that the sensor kinases in flagellum-associated TCS systems of Gram-negative polar flagellates belong to two or more unrelated groups and possess different features, since *C. jejuni* FlgS and *V. cholerae* FlrB did not identify each other as orthologs. Many of these sensor kinases are predicted to be cytoplasmic kinases lacking transmembrane domains. Representative kinases such as *C. jejuni* FlgS, *H. pylori* FlgS, *V. cholerae* FlrB, and *P. aeruginosa* FleS contain similar HisKA and HATPase_c domains that function in histidine autophosphorylation, dimerization, and ATPase activity but have divergent N-terminal sensor domains (Fig. 2C) (58, 59). Both *V. cholerae* FlrB and *P. aeruginosa* FleS contain a predicted PAS domain that is a common sensing domain in sensor kinases; FleS also has a predicted coiled-coil domain for potential protein interactions (Fig. 2C) (59). However, the only predicted domain within the *C. jejuni* FlgS sensor region is a coiled-coil domain, and no predicted structural domain was identified in *H. pylori* FlgS (Fig. 2C). These observations suggest that flagellum-associated sensor kinases within FlhF/FlhG-containing polar flagellates detect different cellular signals or detect similar signals by different mechanisms to initiate signal transduction for flagellar gene expression and polar flagellar biogenesis.

The *V. cholerae* FlrBC TCS requires the fT3SS, MS ring, and rotor for activity. As described above, we previously discovered that *C. jejuni* FlgS detects MS ring-rotor-fT3SS assembly by a direct interaction as a regulatory checkpoint to initiate signal transduction for FlgSR- and σ^{54}-dependent transcription of rod and hook genes for the polar flagellar transcriptional program (Fig. 1) (32). The *V. cholerae* and *P. aeruginosa* flagellum-associated FlrBC/FleSR TCSs are expressed simultaneously with the fT3SS, MS ring, and C ring genes. However, investigations to identify what signals are detected by these TCSs to initiate signal transduction for TCS- and σ^{54}-dependent flagellar rod and hook gene expression that occurs in the subsequent tier of the polar flagellar transcriptional program are lacking (Fig. 1) (18, 19). Considering that the sensor domains of *V. cholerae* FlrB and *P. aeruginosa* FleS differ from that of *C. jejuni* FlgS, we hypothesized that the FlrB and FleS kinases detect a similar regulatory checkpoint formed by fT3SS, MS ring, and/or C ring components but by different means, or they detect a signal independent of flagellar biogenesis (Fig. 1). Thus, we first investigated whether disruption of the fT3SS, MS ring, and/or C ring impacted the activity of the *V. cholerae* FlrBC TCS for σ^{54}-dependent rod and hook gene expression.

Transcriptional fusions of the promoters of four *V. cholerae* FlrBC- and σ^{54}-dependent operons, *flgBCDE*, *flgFGHIJ*, *flgKL*, and *flaA*, to a promoterless *lacZ* gene were created in WT *V. cholerae* and isogenic flagellar mutants. The former three operons encode rod and hook genes, whereas *flaA* encodes the major flagellin. *V. cholerae* is different from many other flagellates in that the major flagellin is not expressed from a σ^{28}-dependent promoter; instead, minor flagellins are expressed from a σ^{28}-dependent promoter in this bacterium (60). Of note, the *flgFGHIJ* rod operon was previously proposed to be cotranscribed with the *flgBCDE* rod and hook operon from the *flgB* promoter (18). However, we identified a putative σ^{54} binding site between *flgE* and *flgF* and constructed an *flgFp-lacZ* transcriptional fusion for analysis. A *cheVp-lacZ* transcriptional fusion served as a control, as *cheV* expression is independent of FlrBC TCS and σ^{54} (18).

We verified previous findings that *V. cholerae* Δ*flrA*, Δσ^{54} Δ*flrB*, and Δ*flrC* (but not Δσ^{28}) mutants were defective for *flaAp-*, *flgBp-*, and *flgKp-lacZ* expression (Fig. 3A) (18, 45); expression of these reporters was reduced 6- to >50-fold in these mutants relative to that of WT *V. cholerae*. The defect in Δ*flrA* is due to FlrA functioning as a master transcriptional regulator required for *flrBC* expression (Fig. 1) (17, 18). We also confirmed that an FlrBC- and σ^{54}-dependent promoter upstream of *flgF* drives expression





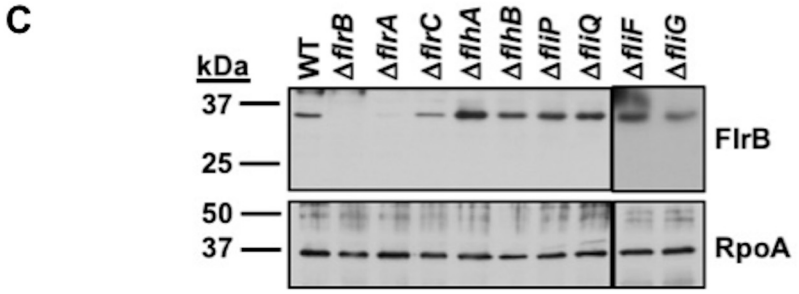


FIG 3 Analysis of TCS- and σ^{54}-dependent flagellar gene expression in flagellar mutants of *V. cholerae*. (A) Expression of flagellar rod and hook operons in WT *V. cholerae* C6706 and isogenic flagellar mutants. *flgBp-*, *flgFp-*, *flgKp-*, *flaAp-*, and *cheVp-lacZ* transcriptional reporters were maintained on plasmids in *V. cholerae* strains. The level of expression of each transcriptional reporter in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. MR, master regulator; TCS, two-component signal transduction system. (B) Expression of *flaAp-lacZ* transcriptional fusion in WT *V. cholerae* and an isogenic mutant containing vector alone (solid blue bars) or vectors to express genes from a constitutive promoter for complementation (hatched blue bars). The level of expression of transcriptional reporters in each mutant is relative to the level of expression in WT *V. cholerae* with vector alone, which was set to 100 U. For panels A and B, results are from a representative assay with each sample analyzed in triplicate. Error bars indicate standard deviations of the average level of expression from three samples.

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of *flgFGHIJ* independently of *flgBp*; *flgFp-lacZ* expression was decreased 50- to 100-fold in FlrBC TCS or σ^{54} mutants (Fig. 3A).

We next discovered that *V. cholerae* mutants lacking fT3SS core proteins (including FlhA, FlhB, FliP, FliQ, and FliR) and the FliF MS ring protein were defective for FlrBC activity and rod and hook transcription, as expression of the respective transcriptional reporters was reduced 3- to 20-fold (Fig. 3A). The C ring FliG rotor protein was also required for FlrBC- and σ^{54}-dependent flagellar gene expression, but the C ring FliM and FliN switch proteins were not (Fig. 3A). These results are similar to our findings for requirements for *C. jejuni* FlgSR TCS directly activating σ^{54}-dependent flagellar rod and hook gene expression (Fig. S2) (31–33). Either no reductions or only modest reductions in *cheVp-lacZ* expression were observed in these flagellar mutants. *In trans* complementation of the *V. cholerae* fT3SS, *fliF*, and *fliG* mutants with the respective gene restored expression of *flaAp-lacZ* to a significantly higher level than that of each mutant with vector alone (and to at least 60% of the level observed in the WT; Fig. 3B). We were unable to construct a complementing vector for the *fliP* mutant due to toxicity during attempted construction. *flaAp-lacZ* expression was partially or fully restored in *flrA*, *flrB*, and *flrC* mutants with complementation relative to the mutants with vector alone. Additionally, we verified that FlrB production was unaffected in mutants lacking the fT3SS, FliF, or FliG, which eliminated the possibility that the FlrBC TCS was unstable or not expressed in these mutants as an explanation of their reduction in rod and hook gene expression (Fig. 3C). The finding that a lack of individual MS ring, rotor, and fT3SS proteins abolishes FlrBC-dependent gene expression is consistent with our hypothesis that *V. cholerae* flagellar components form a regulatory checkpoint, possibly involving functional fT3SS assembly, required for FlrBC to activate rod and hook gene expression, as we previously demonstrated in *C. jejuni* (31–33).

The *P. aeruginosa* FleSR TCS requires the fT3SS, MS ring, and rotor for activity. We next investigated whether *P. aeruginosa* FleSR-dependent expression of flagellar rod and hook genes was influenced by fT3SS, MS ring, and C ring protein production and possibly assembly into an MS ring-rotor-fT3SS complex. We generated complete or partial in-frame deletion mutants of flagellar genes in *P. aeruginosa* PA14 and then integrated transcriptional fusions of the promoters of the *flgB* rod and hook operon and *fliA* (encoding σ^{28}) to *lacZ* in the *att* site on the chromosome. We verified that transcription of the *P. aeruginosa flgB* rod and hook operon is dependent on σ^{54} and the FleSR TCS, in addition to the FleQ master transcriptional regulator that is required for *fleSR* transcription (Fig. 1 and 4) (19, 20). The *fliAp-lacZ* reporter served as a control, as *fliA* expression is independent of σ^{54} or the FleSR TCS (19). Deletion of σ^{28} did not affect expression of either reporter. We discovered that FleSR- and σ^{54}-dependent expression of *flgBp-lacZ* in *P. aeruginosa* required the fT3SS, FliF MS ring, and the FliG C ring proteins, but not FliM or FliN, for full expression, similar to our analysis of the *V. cholerae* FlrBC and *C. jejuni* FlgSR TCSs (Fig. 3A and 4 and Fig. S2) (31–33). Expression of *fliAp-lacZ* was unaffected in these mutants. Thus, our data continue to support that many polar flagellates with the FlhF/FlhG flagellar biogenesis regulatory system contain flagellum-associated TCSs that require flagellar-dependent cues to stimulate expression of σ^{54}-dependent flagellar rod and hook genes as a discrete step for the polar flagellar transcriptional program.

Requirements of FliF and FliG for flagellum-associated TCS activity. Having established that deletion of individual MS ring, rotor, and fT3SS proteins impeded FlrBC/FleSR TCS activity, we assessed whether these proteins alone or as a part of the MS ring-rotor-fT3SS complex were required for the activity of *V. cholerae* and *P.*

FIG 3 Legend (Continued)
An asterisk indicates significant difference in expression from the WT (A) or WT containing vector alone (B) ($P < 0.05$). Two asterisks indicate significant increase in expression from the respective mutant containing vector only ($P < 0.05$). (C) Immunoblot analysis of the FlrB sensor kinase levels in whole-cell lysates of WT *V. cholerae* and isogenic mutants. Specific antiserum to FlrB was used to detect the protein. Detection of RpoA served as a control to ensure equal loading of proteins across strains.

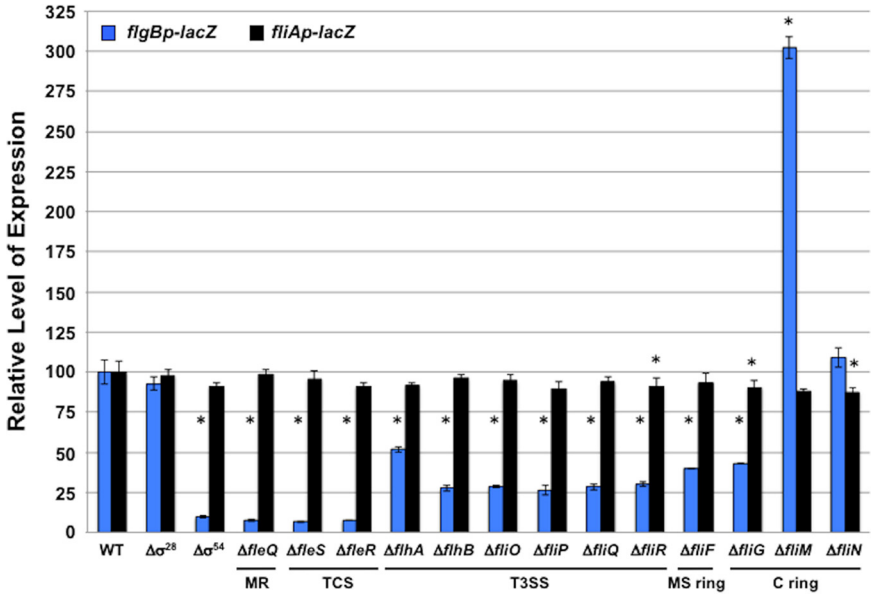

FIG 4 Analysis of TCS- and σ^{54}-dependent flagellar gene expression in flagellar mutants of *P. aeruginosa*. Expression of the flagellar rod and hook operon in WT *P. aeruginosa* PA14 and isogenic flagellar mutants is shown. *flgBp-lacZ* and fliAp-*lacZ* transcriptional reporters were integrated at the *att* site on the chromosome of *P. aeruginosa* strains. The level of expression of transcriptional reporters in each mutant is relative to the level of expression in WT *P. aeruginosa*, which was set to 100 U. Results are from a representative assay, with each sample analyzed in triplicate. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates the mutant had a significantly increased or decreased reporter expression relative to that of the WT strain ($P < 0.05$). MR, master regulator; TCS, two-component signal transduction system.

aeruginosa TCSs to stimulate flagellar rod and hook gene transcription. Because FlrB and FleS are predicted to be cytoplasmic kinases, we hypothesized that they detect signals within the cytoplasm, similar to *C. jejuni* FlgS sensing formation of the MS ring and rotor by FliF and FliG via a direct interaction with the cytoplasmic domains of these structures (32). FliF is predicted to contain two transmembrane domains with a large central periplasmic domain and smaller N- and C-terminal cytoplasmic domains. A conserved periplasmic ASVXV motif in FliF is required for flagellation in *Salmonella* (61). This motif has been hypothesized to promote recruitment of FliF to the fT3SS core via interactions with FlhA and/or FliF multimerization into the MS ring around the fT3SS core. Alteration of the *C. jejuni* FliF ASVXV motif eliminated FlgS interactions with FliF and FliG and abolished FlgSR TCS signal transduction for rod and hook gene expression (32), supporting the hypothesis that FliF multimerization into the MS ring (and simultaneous rotor formation by FliG) around the complete fT3SS core is required to form a signal directly detected by FlgS.

The *V. cholerae* FliF MS ring protein contains a motif in its periplasmic region (ASASVXL from residues 200 to 206) similar to that of *Salmonella* and *C. jejuni* FliF. We expressed WT FliF, $FliF_{\Delta AS200\text{-}201}$, and $FliF_{\Delta AS202\text{-}203}$ from plasmids in *V. cholerae* Δ*fliF* and monitored expression of *flaAp-lacZ* to assess FlrBC TCS activity. WT FliF restored *flaAp-lacZ* expression to the Δ*fliF* mutant, but $FliF_{\Delta AS200\text{-}201}$ and $FliF_{\Delta AS202\text{-}203}$ did not (Fig. 5A). Consistent with these observations, WT FliF restored flagellation and motility, but the mutant FliF proteins did not (data not shown). Immunoblot analysis verified that the WT FliF and FliF mutant proteins were produced at similar levels, as were the FliG rotor proteins in these strains (Fig. 5B). Of note, we did observe that FliG is dependent upon FliF for stability, as FliG levels were reduced in the *V. cholerae* Δ*fliF* mutant with or without empty vector (Fig. 5B). If the ASVXV motif in *V. cholerae* FliF is required for recruitment to the fT3SS core and/or FliF multimerization into the MS ring around the fT3SS core, as in other flagellates, these data, along with our analysis of mutants lacking FliF, FliG, and fT3SS core proteins, support a model that the formation

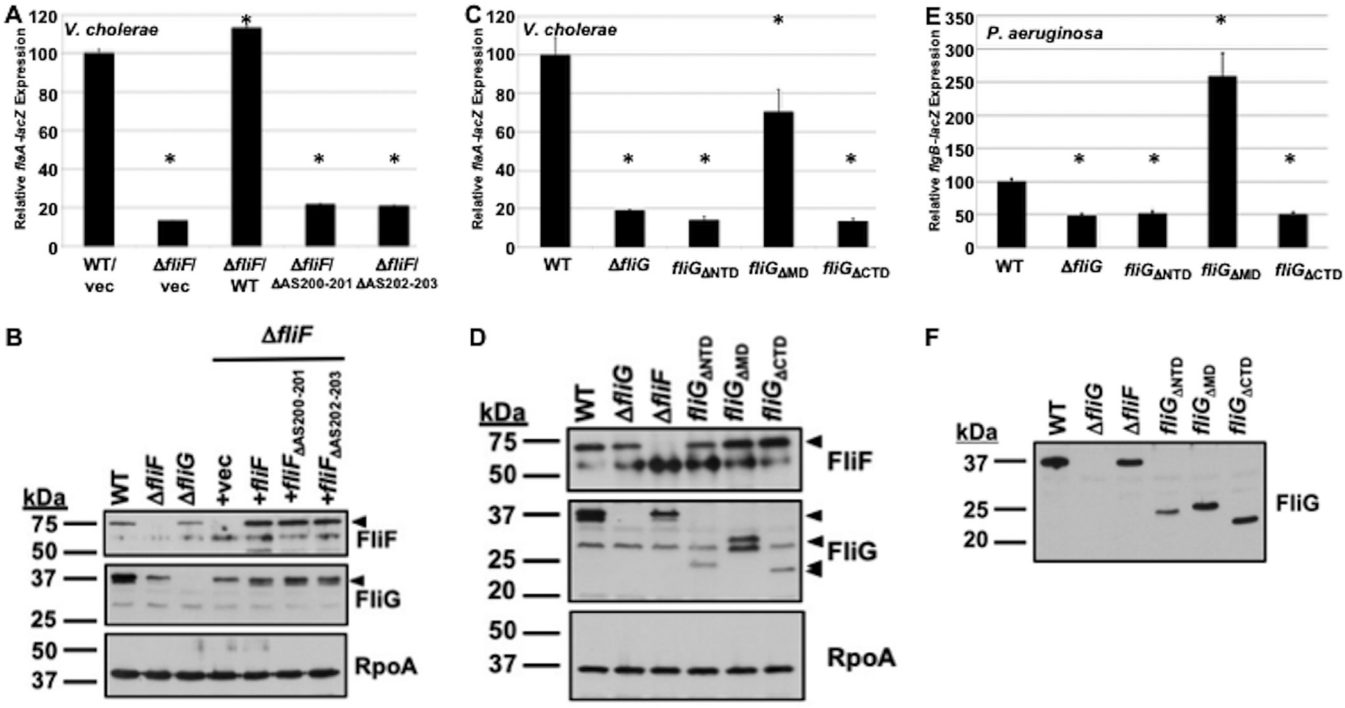

FIG 5 Requirements of *V. cholerae* and *P. aeruginosa* for TCS activation and flagellar gene expression. (A) Expression of *flaAp-lacZ* transcriptional fusion in WT *V. cholerae* and isogenic Δ*fliF* mutant. Both the WT and Δ*fliF* mutant strain contained vector alone (vec). The Δ*fliF* mutant also contained vectors to express WT *fliF* (WT) or *fliF* containing in-frame deletions within the ASASVXL motif (depicted as deletion of the AS residues from positions 200 and 201 or positions 202 and 203). (B) Immunoblot analysis of FliF and FliG in whole-cell lysates of WT *V. cholerae* and isogenic mutants. Specific antiserum to FliF or FliG was used to detect each protein. Detection of RpoA served as a control to ensure equal loading of proteins across strains. (C) Expression of *flaAp-lacZ* transcriptional fusion in WT *V. cholerae* and isogenic *fliG* mutants lacking the N-terminal domain (NTD), middle domain (MD), or C-terminal domain (CTD). (D) Immunoblot analysis of FliF and FliG in whole-cell lysates of WT *V. cholerae* and isogenic mutants. Specific antiserum to FliF or FliG was used to detect each protein. Detection of RpoA served as a control to ensure equal loading of proteins across strains. (E) Expression of *flgBp-lacZ* transcriptional fusion in WT *P. aeruginosa* and isogenic *fliG* mutants lacking the N-terminal domain (NTD), middle domain (MD), or C-terminal domain (CTD). (F) Immunoblot analysis of FliG in whole-cell lysates of WT *P. aeruginosa* and isogenic mutants. Specific antiserum to FliG was used to detect the protein. For panels A, C, and E, the level of expression of the transcriptional reporter in each strain is relative to the level of expression in WT *V. cholerae* or *P. aeruginosa*, which was set to 100 U. Results from a representative assay, with each sample analyzed in triplicate, are shown. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates increased or decreased reporter activity of a mutant relative to that of the WT strain ($P < 0.05$).

of the *V. cholerae* MS ring-rotor-T3SS complex, rather than the production of unassembled fT3SS, MS ring, and rotor proteins, is a cue and regulatory checkpoint influencing FlrBC activity. We were unable to perform a similar analysis in *P. aeruginosa*, as we could not construct mutations in the FliF ASVXV motif.

We next explored whether differences in the requirements of FliG rotor domains for activity of the *V. cholerae*, *P. aeruginosa*, and *C. jejuni* flagellum-associated TCSs existed. Typical FliG proteins possess three major domains: an N-terminal domain (NTD) that interacts with the cytoplasmic C-terminal domain (CTD) of FliF and is required for multimerization of the MS ring and rotor; a middle domain (MD) to interact with FliM for assembly of the switch complex and the lower portion of the C ring; and a CTD to interact with stator proteins that generate torque for flagellar rotation (62–64). In *C. jejuni*, only the FliG NTD was required with FliF to form a signal detected by the FlgSR TCS for flagellar rod and hook gene expression (32). We generated *V. cholerae* and *P. aeruginosa fliG* mutants with deletions of the NTD, MD, or CTD and then assessed TCS- and σ^{54}-dependent flagellar gene expression.

FliG mutants lacking the NTD or CTD were unable to restore FlrBC- and σ^{54}-dependent flagellar gene expression in *V. cholerae* (Fig. 5C). However, these proteins were produced at reduced levels compared to those of WT FliG (Fig. 5D), tempering interpretations that these domains are essential for FlrBC TCS activity. In contrast, the expression of $FliG_{\Delta MD}$ restored gene expression to ~70% of the WT level (Fig. 5C), suggesting that at least the middle domain of FliG is not required for FlrBC TCS-dependent flagellar gene expression.

For analysis of *P. aeruginosa* FliG, WT *fliG* was replaced on the chromosome with *fliG* mutants encoding in-frame deletions of the NTD, MD, or CTD. FliG levels were reduced only modestly with removal of the NTD, but levels of $FliG_{\Delta MD}$ and $FliG_{\Delta CTD}$ were comparable to those of WT FliG (Fig. 5F). Expression of the FleSR- and σ^{54}-dependent *flgB-lacZ* reporter was reduced to the same level in the $fliG_{\Delta NTD}$ and $fliG_{\Delta CTD}$ mutants as in the Δ*fliG* mutant (Fig. 5E). However, a 2.5-fold increase in *P. aeruginosa* FleSR activity and flagellar gene expression was observed with $FliG_{\Delta MD}$. These observations suggest that at least the *P. aeruginosa* FliG CTD is required for FleSR TCS activity, whereas the requirement for the NTD is less clear. Considering that only the *C. jejuni* FliG NTD was required to activate flagellar gene expression (32), our findings indicated different FliG domains, along with FliF, are required in *P. aeruginosa* (and possibly *V. cholerae*) for flagellum-associated TCS activity. While we did not analyze MS ring-rotor-fT3SS complex formation directly in *V. cholerae* and *P. aeruginosa*, these differences in FliF and FliG domains required for activity of the flagellar TCSs suggest requirements for the assembly of functional fT3SSs vary in these organisms. Additionally, the different sensor domains within FleS and FlrB relative to *C. jejuni* FlgS may be needed to detect the distinctive signal composed by the different FliF and FliG domains from the respective bacteria.

A functional link between the polar flagellar transcriptional program and the FlhF/FlhG flagellar biogenesis regulatory system. Our analysis presented above suggested a connection with many polar flagellates possessing (i) a FlhF/FlhG regulatory system, for spatial and numerical control of polar flagellar biogenesis; (ii) a flagellum-associated TCS whose activity is dependent on MS ring, rotor, and fT3SS proteins; and (iii) a polar flagellar transcriptional program that requires MS ring-rotor-fT3SS protein production and possibly assembly for subsequent flagellar rod and hook gene expression. The FlhF/FlhG flagellar biogenesis regulatory systems and flagellum-associated TCSs are absent from peritrichous bacteria (with *Bacillus subtilis* as an exception for FlhF and FlhG). In these peritrichous bacteria, a master transcriptional regulator promotes the peritrichous flagellar transcriptional program by expressing MS ring, C ring, rod, and hook genes simultaneously to result in efficient creation of multiple flagella across the surface (Fig. S3) (10, 12, 14, 48). The cooccurrence of the FlhF/FlhG system, flagellum-associated TCSs, and a transcriptional program that separates MS ring-C ring-fT3SS complex gene expression from that of rod and hook genes raises interesting questions. (i) Do polar flagellates require the specific polar flagellar transcriptional program to build flagella in general or to specifically construct polar flagella? (ii) Is ordering rod and hook gene transcription after MS ring-C ring-fT3SS assembly required for an FlhF/FlhG-dependent activity for flagellation? (iii) Can polar flagellates produce flagella (polar or otherwise) if reprogrammed to transcribe flagellar genes similarly to a peritrichous organism in the presence or absence of the FlhF/FlhG flagellar biogenesis regulatory system?

For these analyses, we developed a transcriptional reprogramming strategy in *V. cholerae* so that expression of one, two, or all three FlrBC- and σ^{54}-dependent flagellar rod and hook operons (*flgBCDE*, *flgFGHIJ*, and *flgKL*) were under the control of the FlrA master transcriptional regulator that normally only controls expression of MS ring, C ring, fT3SS, and FlrBC TCS genes (Fig. S3). By replacing the FlrBC- and σ^{54}-dependent *flgB*, *flgF*, and *flgK* promoters with the FlrA-dependent *fliE* promoter at the native locations on the *V. cholerae* chromosome (Fig. S3) (18), the requirement to detect the regulatory checkpoint centered around MS ring-rotor-fT3SS protein production for rod and hook gene expression would be bypassed. Thus, *V. cholerae* would produce some or all rod and hook proteins earlier than normal and at the same time as the MS ring, C ring, and fT3SS proteins, which shifts the normal *V. cholerae* polar flagellar transcriptional program to one more closely following the peritrichous transcriptional program that normally exists in *E. coli* and *Salmonella* species (Fig. S3) (10, 15).

In these experiments, we retained *flaA* expression under the control of its natural FlrBC- and σ^{54}-dependent promoter. By doing so, the FlaA major flagellin in these *V. cholerae* transcriptional reprogramming mutants was produced either simultaneously

TABLE 1 Measurement of flagellation in WT *V. cholerae*, *V. cholerae* Δ*flhG* mutant, and transcriptional reprogramming mutants

Strain	% Of population[a]			Strain	% Of population[a]		
	Flagellated	Aflagellated	% Hyperflagellated		Flagellated	Aflagellated	% Hyperflagellated[b]
WT	54.2 ± 2.5	45.9 ± 2.5	1.4 ± 2.5	Δ*flhG*	67.5 ± 11.1	32.5 ± 11.1	61.4 ± 11.6
fliEp-flgB operon	56.6 ± 4.3	43.4 ± 4.3	0 ± 0	Δ*flhG fliEp-flgB* operon	8.3 ± 4.1[c]	91.7 ± 4.1[c]	0 ± 0[c]
fliEp-flgF operon	48.2 ± 4.0	51.8 ± 4.0	0.8 ± 1.3	Δ*flhG fliEp-flgF* operon	0 ± 0[c]	100 ± 0[c]	0 ± 0[c]
fliEp-flgK operon	53.4 ± 5.6	46.6 ± 5.6	0.9 ± 0.9	Δ*flhG fliEp-flgK* operon	0 ± 0[c]	100 ± 0[c]	0 ± 0[c]
fliEp-flgB operon *fliEp-flgF* operon	48.1 ± 6.3	51.9 ± 6.3	0.6 ± 0.5	Δ*flhG fliEp-flgB* operon *fliEp-flgF* operon	13.9 ± 2.1[c]	86.1 ± 2.1[c]	9.5 ± 4.8[c]
fliEp-flgB operon *fliEp-flgK* operon	55.6 ± 2.0	44.4 ± 2.0	2.0 ± 0.6	Δ*flhG fliEp-flgB* operon *fliEp-flgK* operon	0 ± 0[c]	100 ± 0[c]	0 ± 0[c]
fliEp-flgF operon *fliEp-flgK* operon	50.9 ± 6.4	49.1 ± 6.4	4.4 ± 6.3	Δ*flhG fliEp-flgF* operon *fliEp-flgK* operon	0.4 ± 0.6[c]	99.6 ± 0.6[c]	0 ± 0[c]
fliEp-flgB operon *fliEp-flgF* operon *fliEp-flgK* operon	53.0 ± 3.5	47.0 ± 3.5	2.1 ± 1.9	Δ*flhG fliEp-flgB* operon *fliEp-flgF* operon *fliEp-flgK* operon	0 ± 0[c]	100 ± 0[c]	0 ± 0[c]

[a]For each strain, three independent samples with at least 100 bacterial cells per sample were analyzed. Values are presented as averages ± standard deviations.
[b]The values for the hyperflagellated population represent the percentages of the flagellated population that had at least two or more flagella at one pole of the cell.
[c]The level of flagellation, aflagellation, or hyperflagellation of individual *V. cholerae* Δ*flhG* transcriptional reprogramming mutants was statistically significantly different from the *V. cholerae* Δ*flhG* mutant with normal flagellar gene transcription ($P < 0.05$). No significant differences were noted among WT *V. cholerae* and the respective transcriptional reprogramming mutants.

with some rod and hook proteins as normal (if the mutant contained only one or two promoter alterations) or after all rod and hook proteins (if the mutant contained all three promoter alterations) (Fig. S3). Transcription of *flaA* after rod and hook protein production in these *V. cholerae* mutants would be temporally similar to how most polar and peritrichous flagellates naturally express major flagellins from a σ^{28}-dependent promoter after formation of the flagellar rod and hook (Fig. 1 and Fig. S3). As shown below, maintaining *flaA* expression under its natural FlrBC- and σ^{54}-dependent promoter allowed for sufficient flagellin production for filament assembly and motility in many transcriptionally reprogrammed mutants.

We also deleted *flhF* and *flhG* from these *V. cholerae* transcriptional reprogramming mutants to examine any potential link between the activity of the FlhF/FlhG regulatory system for monotrichous flagellation in *V. cholerae* and alteration in the timing of rod and hook gene transcription relative to MS ring, C ring, and fT3SS expression. As reported previously, FlhF is required for flagellar biogenesis in *V. cholerae*, and we confirmed that *V. cholerae* Δ*flhF* lacked flagella (7 and data not shown); thus, we could not analyze flagellation in our transcriptional reprogramming mutants in the Δ*flhF* background. However, deletion of *flhG* from the classical *V. cholerae* O395 strain allowed for the production of polar flagella but with hyperflagellation due to the lack of proper numerical control of flagellar biogenesis (7). Hyperflagellation was proposed to be due to increased *flrA* expression to cause overexpression of all flagellar genes, resulting in multiple flagella. However, this hyperflagellated phenotype was unstable in *V. cholerae* O395 Δ*flhG*; after subsequent *in vitro* passaging, flagellar gene expression was reduced and the monotrichous phenotype returned (7).

We verified the hyperflagellation phenotype of a Δ*flhG* mutant in *V. cholerae* C6706 (the strain used throughout this study). In our analysis, 54% of the WT population produced exclusively monotrichous flagella, with 46% lacking a flagellum (Table 1 and Fig. 6A). Only a small minority of the WT flagellated population was hyperflagellated (1.4%). *V. cholerae* Δ*flhG* cells produced flagella in a higher percentage of the population (67.5% versus 54.2% for the WT). Furthermore, 61% of flagellated Δ*flhG* cells were hyperflagellated by producing 2 to 7 polar flagella at a single pole (Table 1 and Fig. 6A). The flagella of *V. cholerae* Δ*flhG* cells occasionally appeared thinner in structure with possible defects in flagellar sheath formation compared to the monotrichous flagellum of WT *V. cholerae*. Despite hyperflagellation, the Δ*flhG* mutant was motile, although modestly less so than the WT (Fig. 6B). Contrary to a previous report, hyperflagellation was stable in the *V. cholerae* C6706 Δ*flhG* strain (7). Expression of *lacZ* transcriptional reporters linked to promoters from different classes of flagellar genes were generally

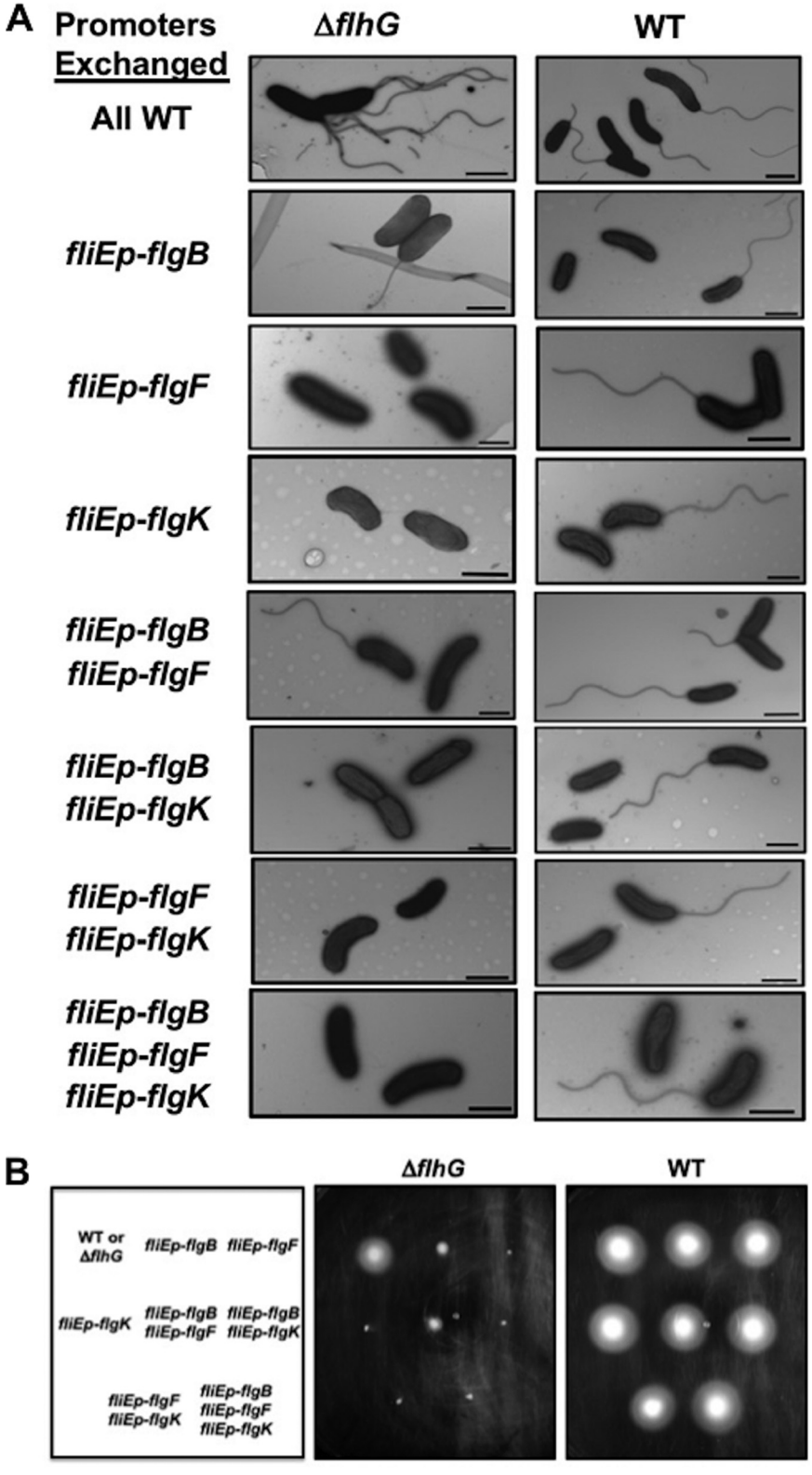

FIG 6 Effect of transcriptional reprogramming of flagellar genes on *V. cholerae* flagellation. Shown are electron micrographs (A) and motility phenotypes (B) of WT *V. cholerae*, the *V. cholerae* Δ*flhG* mutant, or isogenic transcriptional reprogramming mutants in which one or more operons with an FlrBC TCS- and σ^{54}-dependent promoter was replaced with the *fliE* promoter (*fliEp*), as indicated, to transition flagellar gene transcription toward the peritrichous flagellar program. All combinations of transcriptional reprogramming mutants were made in *V. cholerae* Δ*flhG* and WT *V. cholerae* strains (left and right columns, respectively). In panel A, the bar represents 1 μm. In panel B, motility was assessed after inoculating

(Continued on next page)

altered in the Δ*flhG* strain but with either increased or decreased expression depending on the promoter examined (Fig. 7A). Thus, we could not link hyperflagellation to gross overexpression of flagellar genes in the Δ*flhG* strain, as previously hypothesized (7), suggesting that FlhG regulates flagellar number by another means, such as controlling *in vivo* FlhF activity by modulating its GTP-binding state, as postulated for *Vibrio alginolyticus* and *C. jejuni* (3–5). In summary, the *V. cholerae* Δ*flhG* mutant with a WT polar flagellar transcriptional program efficiently produced polar flagella (possibly due to dysregulated FlhF activity), as observed by an increase in polarly flagellated cells and in the number of polar flagella per cell (hyperflagellation).

Upon alteration of the polar flagellar transcriptional program in the *V. cholerae* Δ*flhG* mutant to more closely resemble a peritrichous transcriptional program by replacing the FlrBC- and σ^{54}-dependent promoter for a single rod and hook operon with the FlrA-dependent *fliE* promoter (Fig. S3), we observed almost complete elimination of polar flagellar biogenesis and the prominent Δ*flhG* hyperflagellation phenotype (Table 1 and Fig. 6A). Further shifts toward the peritrichous transcriptional program in which two or all three rod and hook promoters were replaced with *fliEp* were also mostly or completely aflagellated. The only Δ*flhG* transcriptional reprogramming mutants that were flagellated were those with *fliEp* expressing the *flgB* operon alone or both the *flgB* and *flgF* operons, but a significantly smaller population of cells were flagellated than those of the Δ*flhG* mutant with the normal polar flagellar transcriptional program (8.3% to 13.9%) (Table 1 and Fig. 6A). These mutants also were severely reduced for motility relative to *V. cholerae* WT and Δ*flhG* strains (Fig. 6B). The flagella of the Δ*flhG fliEp-flgBCDE* mutant that were produced tended to be shorter than those of WT *V. cholerae* and the Δ*flhG* mutant (Fig. 6A).

In select Δ*flhG* transcriptional reprogramming mutants, we did not observe gross decreases in FlrA activity, which was driving rod and hook gene expression in these mutants, as monitored by *fliEp-lacZ* expression relative to that of WT *V. cholerae* (Fig. 7B). Instead, FlrA activity was comparable to or modestly greater in Δ*flhG* than in WT strains. Thus, reduced or absent flagellation in the Δ*flhG* transcriptional reprogramming mutants was not due to impaired FlrA activity and expression of rod and hook genes from the *fliE* promoter. Our data indicate that polar flagellar biogenesis efficiently occurs in *V. cholerae* with altered activity of the FlhF/FlhG flagellar biogenesis system (as in a Δ*flhG* mutant), albeit with hyperflagellation, as long as the WT polar flagellar transcriptional program is maintained by the FlrBC TCS to order rod and hook gene transcription after MS ring-C ring-fT3SS production. Any alteration toward a peritrichous flagellar transcriptional program without an intact FlhF/FlhG polar flagellar regulatory system leads to severe reduction or loss of flagellation.

We next addressed whether flagellar biogenesis was affected in a *V. cholerae* hybrid that contained an intact FlhF/FlhG flagellar biogenesis regulatory system but with alterations to follow more closely a peritrichous flagellar transcriptional program. Contrary to the *V. cholerae* Δ*flhG* transcriptional reprogramming mutants, the level of monotrichous flagellation in any population of transcriptional reprogramming mutants with a WT FlhF/FlhG regulatory system did not change relative to that of WT *V. cholerae* (Table 1 and Fig. 6A), even in the *V. cholerae* mutant with all three *fliE* promoter substitutions (*fliEp-flgB*, *fliEp-flgF*, and *fliEp-flgK*), to most closely resemble a peritrichous flagellar transcriptional program. We also did not detect differences in flagellar filament length, appearance, or function in motility *in vitro* (Fig. 6A and B). Thus, *V. cholerae* with a peritrichous flagellar transcriptional program produces polar flagella normally during *in vitro* growth, as long as the FlhF/FlhG flagellar biogenesis regulatory system is fully operational and intact. Disruption of the FlhF/FlhG system in such a transcriptionally reprogrammed *V. cholerae* cell abolishes or greatly reduces flagellation and motility.

FIG 6 Legend (Continued)
overnight cultures in LB with 0.3% agar and incubation at 37°C for 8 h. The box on the left in panel B is a map depicting how the Δ*flhG* or WT strain and their corresponding transcriptionally reprogrammed mutants were inoculated into motility agar in the center and right panels.

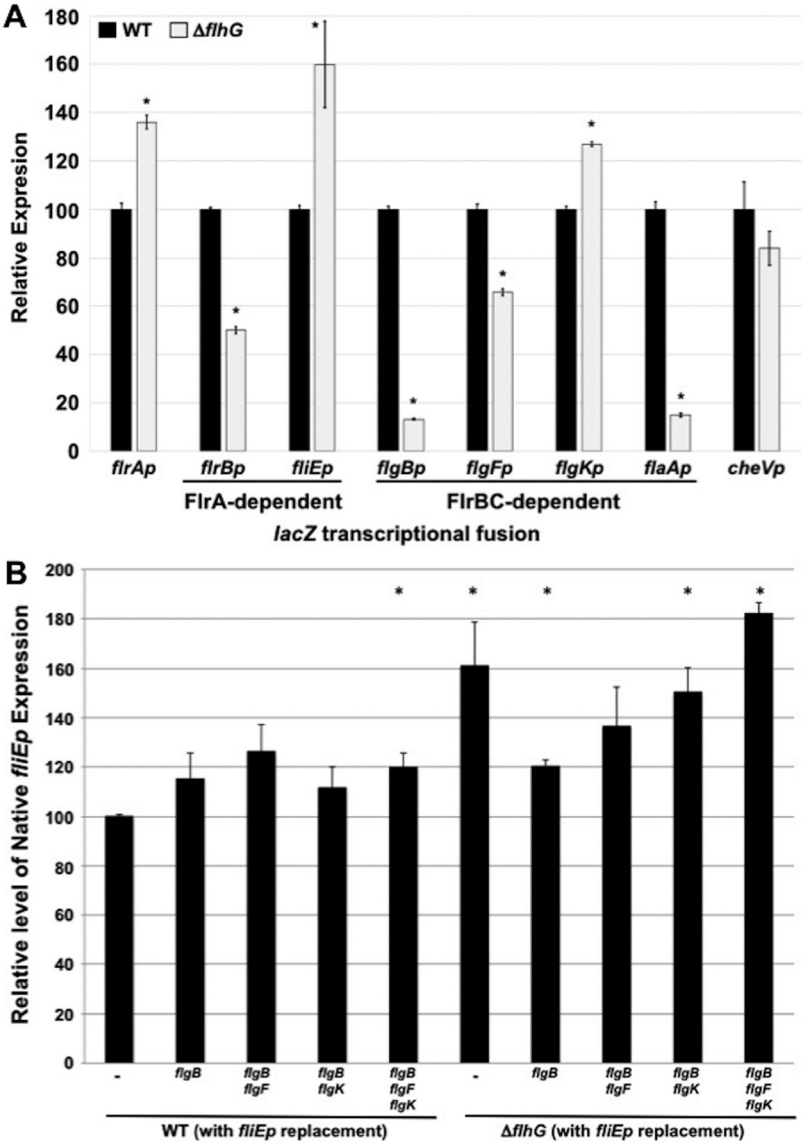

FIG 7 Activity of flagellar promoters in WT *V. cholerae* and Δ*flhG* mutant strains. (A) Expression of *lacZ* transcriptional fusions from different flagellar promoters in WT *V. cholerae* C6706 and an isogenic Δ*flhG* mutant. *flrAp-*, *flrBp-*, *fliEp-*, *flgBp-*, *flgF-*, *flgKp-*, *flaAp-*, and *cheVp-lacZ* transcriptional reporters were maintained on plasmids in *V. cholerae* strains. The level of expression of each transcriptional reporter in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. Results from a representative assay with each sample analyzed in triplicate are shown. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates significant difference in expression from the WT containing vector alone ($P < 0.05$). (B) *fliE* promoter activity in WT *V. cholerae* and the Δ*flhG* mutant with either a normal polar flagellar transcriptional program or transcriptionally reprogrammed toward a peritrichous pattern. An *fliEp-lacZ* transcriptional reporter was introduced on a plasmid in WT *V. cholerae* or the Δ*flhG* mutant with a normal polar flagellar transcriptional program (indicated by a dash) and select transcriptional reprogramming mutants. In the transcriptional reprogramming mutants analyzed, the promoter for one or more rod and hook operons that was replaced with the *fliE* promoter is indicated. The level of expression of the *fliEp-lacZ* transcriptional reporter in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. Results from a representative assay, with each sample analyzed in triplicate, are shown. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates significant difference in expression relative to that of WT *V. cholerae* ($P < 0.05$).

Thus, we propose that flagellum-associated TCSs of polar flagellates mediate the polar flagellar transcriptional program, characterized by the ordered transcription of flagellar rod and hook genes after the MS ring-rotor-fT3SS regulatory checkpoint during flagellar assembly, to allow polar flagellates flexibility in producing flagella and retaining

motility (even with hyperflagellation) when the FlhF/FlhG system may not function properly.

DISCUSSION

Polarly flagellated bacteria are present in a wide range of proteobacterial classes. To achieve species-specific flagellation patterns for optimal motility, each polar flagellate must have transcriptional mechanisms to correctly control flagellar gene expression and biogenesis regulators to create specific flagellation patterns composed of the correct number of flagella at one or both poles. However, little is known regarding how broadly conserved these transcriptional and biosynthetic regulatory mechanisms are and how they may be intertwined for correct biogenesis of polar flagella and polar flagellation patterns.

To explore what may contribute to the conserved polar flagellar transcriptional program for creating a specific ordering of transcription of different classes of flagellar genes, we showed by *in silico* analysis that Gram-negative polar flagellates can be divided into two distinct groups. One group produces an FlhF/FlhG flagellar biogenesis regulatory system with a flagellum-associated TCS, and another is composed of alphaproteobacteria that lack an FlhF/FlhG system. We found that FlhF/FlhG- and flagellum-associated TCS-positive polar flagellates may be subdivided into two or more groups based on the sensory domains of the TCS kinases. The different kinases are best represented by *C. jejuni* FlgS, which contains a predicted coiled-coil domain in the sensory region, and *V. cholerae* FlrB/*P. aeruginosa* FleS, which contain PAS domains.

Despite their identification years ago, the actual signals detected by the *V. cholerae* FlrB and *P. aeruginosa* FleS sensor kinases had not been analyzed. We previously discovered that the amphitrichous polar flagellate *C. jejuni* detects a regulatory checkpoint formed by the MS ring and rotor assembling around the fT3SS core by FlgS of its flagellum-associated FlgSR TCS (32). In our current work, we found that MS ring, rotor, and fT3SS proteins are broadly required for the activity of the *V. cholerae* FlrB and *P. aeruginosa* FleS sensor kinases to result in rod and hook gene transcription. Additionally, we found that *V. cholerae* FliF mutants that likely fail to form an MS ring were defective in FlrBC TCS activity. These findings are similar to our previous studies in *C. jejuni* and what others have reported in *H. pylori* (32, 40, 41, 43). Furthermore, we accumulated evidence that for at least *P. aeruginosa* (and possibly *V. cholerae*), more domains of the FliG rotor were required for flagellum-associated TCS activity than in *C. jejuni*. These combined data build support for a general conserved mechanism in which flagellum-associated TCSs of polar flagellates broadly detect a regulatory checkpoint centered around MS ring-rotor-fT3SS assembly as a signal to facilitate the polar flagellar transcriptional program that orders rod and hook gene expression as a subsequent step for the progression of flagellar biogenesis. We suspect that the FliF MS ring, FliG rotor, and fT3SS core proteins (FlhA, FlhB, FliP, FliQ, and FliR) would be required for the activity of flagellum-associated TCSs for rod and hook gene expression in many other polar flagellates that have yet to be explored.

A caveat to our work presented here is that we have not yet been able to detect a direct interaction between the FlrB or FleS kinase with the FliF MS ring or FliG rotor proteins, like we observed with *C. jejuni* FlgS, as a mechanism to monitor formation of the MS ring and rotor around the fT3SS core (32). This indicates that the interactions between the kinases and flagellar proteins are weaker or more transient than in *C. jejuni* or that FlrB and FleS monitor fT3SS assembly indirectly through an unidentified factor. Differences in FliG domains potentially required for signal formation and detection, the abilities of the kinases to interact with MS ring and rotor components, and potential sensory domains within the kinases may reflect varied mechanisms for how these flagellum-associated TCSs monitor the formation of the regulatory checkpoint. Regardless, our findings continue to support that bacteria have mechanisms to monitor the formation of intracellular macromolecular structures and to relay information that influences behavior. Verification and deeper investigation of how each flagellum-

associated TCS might detect MS ring-rotor-fT3SS assembly will be insightful for how these regulatory systems function.

When also considering the alphaproteobacterial polar flagellates that lack the FlhF/FlhG flagellar biogenesis regulatory system, monitoring formation of a competent fT3SS by different systems to influence subsequent rod and hook gene expression emerges as a common strategy across Gram-negative polar flagellates for the development of the conserved polar flagellar transcriptional program. Of the alphaproteobacterial polar flagellates in the reference collection, the flagellar system of *C. crescentus* is the best characterized (reviewed in references 65). *C. crescentus* executes the polar flagellar transcriptional program so that transcription of MS ring, C ring, and fT3SS genes occurs prior to σ^{54}-dependent transcription of rod and hook genes (66). *C. crescentus* σ^{54} requires FlbD, an enhancer-binding protein similar to the *C. jejuni* FlgR, *V. cholerae* FlrC, and *P. aeruginosa* FleR response regulators of the flagellum-associated TCSs, to activate flagellar rod and hook gene expression (67–71). MS ring, C ring, and fT3SS proteins are also required for FlbD activity and FlbD- and σ^{54}-dependent rod and hook gene expression (69, 71). Thus, FlbD activity is linked to MS ring-C ring-fT3SS complex formation, yet FlbD lacks a cognate sensor kinase like FlgS, FlrB, or FleS to monitor flagellar assembly and control its activity. The FliX transactivating factor has been identified as the link that relays the status of fT3SS assembly to positively or negatively control the activity of FlbD to bind to target flagellar rod and hook promoters (72–75). Although its regulatory mechanism is not understood, FliX does not function as a kinase to transduce signals regarding fT3SS assembly.

As diverse polar flagellates have evolved different mechanisms to create and maintain the polar flagellar transcriptional program so that rod and hook gene expression occurs after formation of the regulatory checkpoint at fT3SS assembly, we hypothesized that this program is beneficial for biogenesis of polar flagella. *E. coli* and *Salmonella*, as models with the peritrichous flagellar transcriptional program, do not recognize this checkpoint and transcribe most basal, rod, and hook genes simultaneously to efficiently build peritrichous flagella (10, 14, 48). Since, in this study, we show a *V. cholerae* cell containing the WT FlhF/FlhG regulatory system engineered with a peritrichous flagellar transcriptional program produced a monotrichous flagellum efficiently, the type of flagellar transcriptional program itself does not seem to determine the peritrichous or polar flagellation pattern of the species.

Instead, we discovered that possessing a polar transcriptional program and the FlhF/FlhG flagellar biogenesis regulatory system allows polar flagellation while retaining motility to a modest extent when perturbations to FlhF and FlhG activity occur. For example, the *V. cholerae* Δ*flhG* mutant with a polar flagellar transcriptional program was hyperflagellated, indicating high proficiency in producing flagella. However, the Δ*flhG* mutant was less motile than the WT monotrichous strain, likely due to the inability of the Δ*flhG* mutant to coordinate multiple rotating polar flagella for optimal swimming motility. In contrast, flagellation was severely diminished or even abolished in *V. cholerae* mutants that more closely resembled most peritrichous bacteria by lacking a properly functioning FlhF/FlhG flagellar biogenesis system (through deletion of *flhG*) and engineered with a peritrichous flagellar transcriptional program. Thus, the peritrichous transcriptional program is much more affected by alterations in FlhF/FlhG activity in a polar flagellate, resulting in greatly decreased flagellation, motility, and, likely, fitness in nature. Currently, it is unknown whether FlhF/FlhG activity is naturally regulated or altered by extrinsic factors or metabolic capacity. We have observed hyperflagellation in WT polarly flagellated systems in a small minority of cells (in *V. cholerae* in this work and previously in *C. jejuni* [3, 76]). Thus, the FlhF/FlhG flagellar biogenesis regulatory system is likely affected by stochastic influences on a cell-to-cell basis that may at least alter FlhG activity and likely its ability to regulate FlhF. It remains to be determined whether the polar flagellar transcriptional program also provides an advantage to *C. crescentus* that has a different collection of determinants to produce polar flagella during developmental stages and asymmetrical division (77–80).

Our analysis of *V. cholerae* flagellar transcriptional reprogramming mutants provided

some intriguing observations, but many questions remain. One such question is what advantage exactly does the polar flagellar transcriptional program provide for the FlhF/FlhG regulatory system to enable efficient flagellation when FlhF or FlhG activity is altered that the peritrichous flagellar transcriptional program does not provide. It is currently unclear how FlhG controls flagellum numbers in *V. cholerae*. FlhG orthologs regulate flagellum number by at least two different processes, including influencing the activity of FlhF or a master regulator of flagellar gene transcription, such as FlrA (1–5, 7–9, 81). In contrast to a previous report, we did not observe a broad increase in transcription across classes of flagellar genes in the *V. cholerae* Δ*flhG* mutant that would explain the consistent hyperflagellation phenotype we observed (7). Thus, hyperflagellation in the *V. cholerae* Δ*flhG* mutant may be due to a dysregulated, hyperactive FlhF, which has been proposed in *C. jejuni* and other *Vibrio* species (3–5).

The molecular mechanism by which the FlhF GTPase influences polar flagellation has not been determined in many bacteria. One hypothesis includes that FlhF localizes MS ring, C ring, and fT3SS core proteins at a pole or facilitates interactions between these proteins to create a new flagellum (1, 2, 82). FlhG presumably functions in some polar flagellates to transition FlhF from a GTP-bound "on" state competent for a function to initiate flagellation to a GDP-bound "off" state. Tight control of FlhF by FlhG may be required so that FlhF can organize flagellar proteins properly for MS ring-C ring-fT3SS assembly either with the stepwise production of MS ring, C ring, fT3SS, rod, and hook proteins provided by the polar flagellar transcriptional program or with their simultaneous production facilitated by the peritrichous flagellar transcriptional program. However, a dysregulated FlhF in the Δ*flhG* mutant may be unable to perform its natural function in flagellation when MS ring, C ring, fT3SS, rod, and hook proteins are produced at the same time. Consistent with this, we observed a great reduction or abolishment of flagellation when some or all rod and hook proteins were simultaneously produced with MS ring, C ring, and fT3SS proteins in the Δ*flhG* mutant engineered to follow more closely a peritrichous transcriptional flagellar program. Our findings may point toward a more expansive role for FlhF: in addition to its hypothesized role in assisting polar assembly of the MS ring-C ring-fT3SS complex, FlhF may also organize flagellar proteins, such as the rod and hook proteins, for secretion via the fT3SS. If so, production of multiple FlhF-interacting proteins (fT3SS complex proteins and their secretion substrates) simultaneously may overwhelm a dysregulated FlhF so that flagellar biogenesis does not occur. Other possibilities exist, including that the peritrichous flagellar transcriptional program in a Δ*flhG* mutant disrupts flagellar protein stoichiometry. In this case, there may not be enough rod and hook proteins produced for the multiple fT3SSs that may form in the Δ*flhG* mutant. Undoubtedly, there are functions for FlhF and FlhG that are not yet adequately understood to reveal how the polar flagellar transcriptional program contributes to the FlhF/FlhG flagellar biogenesis system for efficient polar biogenesis. Continued exploration will likely further reveal how transcriptional and biosynthetic processes are integrated in polar flagellates to construct the ideal number and positioning of these macromolecular machines for motility in bacterial cells.

Our findings raise some questions regarding how different flagellar transcriptional programs formed across flagellated species. One prominent question is whether polar and peritrichous flagellar transcriptional programs developed independently of each other or if one evolved from the progenitor of another. It is clear that flagellar structural components are largely conserved across bacterial species. Even the mechanism to detect rod and hook formation as a late regulatory checkpoint required for activation of σ^{28} and expression of terminal flagellar genes is widely conserved (11, 13, 30). However, regulatory factors and mechanisms required for expression of flagellar components required for formation of the fT3SS, rod, and hook differ in peritrichous and polar flagellates. Most peritrichous bacteria (albeit with *B. subtilis* as a Gram-positive exception) have a seemingly less complex flagellar transcriptional program so that MS ring, C ring, fT3SS core, rod, and hook proteins are produced simultaneously by the

activity of a flagellar master regulator, and these bacteria do not require FlhF or FlhG to efficiently construct multiple flagella across their surfaces.

Polar flagellates may have originated from a peritrichous progenitor but also could have developed independently. Comparisons between *C. crescentus* and many other Gram-negative polar flagellates as discussed above clearly show that different polar flagellar biogenesis systems exist in the presence of somewhat conserved regulatory mechanisms to facilitate the polar flagellar transcriptional program, indicating convergent evolution of polar flagellates. Regardless, our findings suggest that a species needs to acquire a polar flagellar biogenesis system (such as the FlhF/FlhG system) and a mechanism to order flagellar genes for the polar flagellar transcriptional program (such as a flagellum-associated TCS) to become an efficient polar flagellate. Possessing only the FlhF/FlhG system with the peritrichous program does not guarantee optimal flagellation and motility if FlhF/FlhG activity is affected by extrinsic, stochastic factors.

It is unknown which came first in a polar flagellate, the FlhF/FlhG polar flagellar biogenesis system or the flagellum-associated TCSs, to drive the polar flagellar transcriptional program. Both the FlhF GTPase and FlhG ATPase are members of the SIMIBI class of nucleotide-binding proteins that commonly function in cellular organization and protein targeting (83, 84). FlhF is related to the Ffh GTPase of the signal recognition particle system, whereas FlhG is closely associated with the MinD and ParA ATPases that generally perform partitioning functions related to division and DNA segregation (6, 83–85). Development of the FlhF/FlhG flagellar biogenesis regulatory system, perhaps from Ffh and MinD/ParA superfamilies, could have caused the emergence of a polar flagellate in a Gram-negative organism. The motile, monotrichous *V. cholerae* strain we engineered with an intact FlhF/FlhG system and a peritrichous flagellar transcriptional program might resemble this ancestor. As revealed in this work, this bacterium is heavily reliant on a precisely functioning FlhF/FlhG system to form any flagella and retain some level of motility; perturbations to FlhF or FlhG activity severely reduce or completely abolish flagellation. By possessing a mechanism mediated by the flagellum-associated TCSs to order rod and hook gene transcription after production of MS ring, rotor, and fT3SS proteins (and possibly assembly of a functional fT3SS), a bacterium can produce polar flagella with some alterations to FlhF/FlhG activity. In this bacterium, an optimally functioning FlhF/FlhG system allows for the correct number and placement of polar flagella and WT motility; an impaired FlhF/FlhG system (at least by altering FlhG) results in polar flagellation with extra flagella produced and at least modest motility. This hyperflagellated bacterium has an advantage over one with the FlhF/FlhG system and a peritrichous program that cannot maintain flagellation and motility with perturbations to the FlhF/FlhG system.

Modulations in FlhF and FlhG activity in different species with flagellum-associated TCSs to maintain the polar flagellar transcriptional program and flagellar biogenesis may have facilitated the emergence of different polar flagellation patterns, amphitrichous, lophotrichous, and monotrichous. An example of this is *C. jejuni* and *H. pylori*, which, while closely related, produce amphitrichous and lophotrichous flagella, respectively, yet have the FlhF/FlhG flagellar biogenesis regulatory system and similar flagellum-associated FlgSR TCSs. A study comparing FlhF and FlhG biochemical activity and biological function between these two bacterial species has not been conducted. Although many details remain to be discovered for how FlhF and FlhG function in polar flagellates, our results indicate regulatory links between the FlhF/FlhG flagellar biogenesis regulatory systems and the order of flagellar protein production controlled by the flagellum-associated TCSs for polar flagellar biogenesis.

MATERIALS AND METHODS

General growth and storage conditions of bacteria. *C. jejuni* 81-176 strains were stored at −80°C as frozen stocks in a solution of 85% Mueller-Hinton (MH) broth and 15% glycerol. All *C. jejuni* strains were grown from frozen stocks on MH agar for 48 h under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) at 37°C and then restreaked on MH agar and grown for another 16 h under microaerobic conditions at 37°C. As required, antibiotics were added to MH medium at the following concentrations:

10 μg/ml trimethoprim (TMP), 15 μg/ml chloramphenicol, 50 or 100 μg/ml kanamycin, or 0.5, 1, 2, or 5 mg/ml streptomycin.

V. cholerae C6706 *lacZ*, a spontaneous *lacZ* derivative of the WT El Tor C6706 strain, and isogenic mutants were used for all analyses involving *V. cholerae* strains (86). *Pseudomonas aeruginosa* PA14 and isogenic mutants were used for all analyses involving *P. aeruginosa* strains (87, 88). *E. coli*, *V. cholerae,* and *P. aeruginosa* strains were routinely grown in LB broth at 37 or 30°C and stored as frozen stocks at −80°C in a solution of 80% LB and 20% glycerol. As required, antibiotics or growth inhibitors were added to LB broth or agar at the following concentrations: 100 μg/ml ampicillin, 10 μg/ml chloramphenicol, 100 μg/ml kanamycin, 100 μg/ml streptomycin, 15 μg/ml gentamicin, 12.5 μg/ml tetracycline, and 10% sucrose.

Bacterial strains and plasmid construction. All methodologies to construct plasmids and *C. jejuni*, *V. cholerae*, and *P. aeruginosa* mutants are described in Text S1 of the supplemental material. All bacterial strains and plasmids used in this study are listed in Tables S1 and S2.

Bioinformatic analyses. Complete reference bacterial genomes were acquired from www.ncbi.nlm.nih.gov/assembly to form a database containing 117 genomes. tBLASTn was run against the genome database to identify the top-scoring hit for each genome in the database for the following protein sequences in FASTA format from the UniProt database: *E. coli* FlgH (P0A6S0), *V. cholerae* FlhF (C3LP19) or *C. jejuni* FlhF (A0A0H3P9N0), and *V. cholerae* FlrB (C3LPE1) or *C. jejuni* FlgS (A0A0H3PDD6). To perform a reciprocal best hit sequence alignment, we performed another tBLASTn search with each top-scoring hit from each genome against the genome containing each protein query and only considered positive hits as those that were able to identify the protein query as the top-scoring hit in the respective genome. We additionally acquired 51 sensor histidine kinase sequences from the *V. cholerae* N16961 proteome from UniProt. We performed reciprocal best hit sequence alignments against the reference bacterial genome database with these 51 *V. cholerae* sensor kinases and calculated the Pearson correlation coefficient between each of the 51 sets of sensor kinase hits and *V. cholerae* FlhF hits in the reference bacterial genomes. For completion of all bioinformatics analysis, the following software was used: tBLASTn (www.ncbi.nlm.nih.gov) for reciprocal best hit sequence alignments, Biopython (www.biopython.org) to read XML files and prepare command lines, Python 2.7.6 (www.python.org) to run scripts, and NumPy (www.numpy.org) to calculate Pearson correlation coefficients.

Arylsulfatase assays. Arylsulfatase assays were used to measure the level of expression of the *flaB*::*astA* transcriptional fusion on the chromosome of *C. jejuni* Δ*astA* strains as previously described (31, 89, 90). Each strain was analyzed in triplicate, and each assay was performed three times. The level of expression of the transcriptional fusion in each strain was calculated relative to the expression in the wild-type *C. jejuni* Δ*astA* strain, which was set to 100 U.

β-Galactosidase assays. The level of gene expression in *V. cholerae* and *P. aeruginosa* strains was compared by monitoring the β-galactosidase activity of strains harboring *lacZ* transcriptional fusions to specific promoters by standard procedures (91). Strains were grown in LB at 37°C with shaking to an optical density at 600 nm (OD_{600}) of approximately 0.8 prior to the start of the assays. Each strain was analyzed in triplicate, and each assay was performed three times. The level of expression of the transcriptional fusion in each strain was calculated relative to the expression in wild-type *V. cholerae* C6706 *lacZ* or PA14, which was set to 100 U.

Antiserum production. All use of animals in experimentation has been approved by the IACUC at the University of Texas Southwestern Medical Center. Recombinant protein for antiserum production was produced by first cloning the coding sequences from codon 2 to the stop codon of *V. cholerae flrB*, *fliG*, and *rpoA* into the SmaI site, the BamHI site, or the BamHI and SalI sites of pGEX4T-2 to create N-terminal fusions of glutathione *S*-transferase. For recombinant *V. cholerae* FliF, the region of *fliF* encoding the predicted periplasmic domain, from codons 45 to 473, was cloned into the BamHI and SalI sites of pQE30 to create an N-terminal fusion of 6× His tag. For recombinant *P. aeruginosa* FliG, the coding sequence from codon 2 to the stop codon was cloned into the BamHI and SmaI sites of pQE30 to create an N-terminal fusion of a 6× His tag. Resultant plasmids were transformed into BL21(DE3) or XL1-Blue and then induced in LB broth with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Recombinant protein was purified from the soluble fractions by affinity chromatography according to the manufacturer's instructions. Purified recombinant protein was used to immunize guinea pigs by standard procedures for antiserum generation via a commercial vendor (Cocalico Biologicals).

Immunoblot analysis. Whole-cell lysates (WCLs) of *V. cholerae* and *P. aeruginosa* strains for immunoblot analysis were prepared by first inoculating 5 ml LB with a 1:50 dilution of overnight cultures. Cultures were grown at 37°C with shaking to an OD_{600} of 0.8. One-milliliter aliquots of each culture were recovered by centrifugation in microcentrifuge tubes, washed once with phosphate-buffered saline (PBS), and then resuspended in 50 μl of 1× SDS-loading buffer. Samples were boiled for 5 min prior to separation by SDS-PAGE and transferred to membranes for immunoblotting by standard procedures. For specific detection of proteins in WCLs, 10 μl of WCLs was analyzed to detect FliF, FliG, and RpoA, and 25 μl of WCLs was analyzed to detect FlrB. Proteins were detected with specific guinea pig antisera generated as described above. Primary antisera were applied to immunoblots for 1 to 2 h and used at the following concentrations: *V. cholerae* FliF UTGP151 (1:1,000), *V. cholerae* FliG UTGP198 (1:1,000), *V. cholerae* FlrB UTGP151 (1:2,000), *V. cholerae* RpoA UTGP197 (1:2,000), and *P. aeruginosa* FliG UTGP145 (1:1,000). A 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-guinea pig antibody was then applied for detection of proteins.

Electron microscopy analysis. Overnight cultures of *V. cholerae* strains were inoculated into 5 ml LB at a 1:50 dilution and grown at 37°C with shaking to an OD_{600} of approximately 0.8. One milliliter of each culture was pelleted for 3 min at 13,200 rpm in a microcentrifuge, resuspended in 2% glutaraldehyde in

0.1 M cacodylate, and then incubated on ice for 1 h. Copper-coated Formvar grids were negatively glow discharged, and bacterial samples then were applied to the grids. The samples were stained with 2% uranyl acetate and visualized with an FEI Technai G2 Spirit Bio TWIN transmission electron microscope. Flagellum numbers were counted from at least 100 individual cells and averaged from three biological replicates to determine the proportion of bacterial populations producing different flagellation phenotypes: hyperflagellated (producing at two or more flagella at least at one pole), wild-type (producing a single flagellum at one pole), or aflagellated (lacking a flagellum). After averaging, the standard deviations for each population were calculated.

Motility analysis. *V. cholerae* strains were grown from freezer stocks in 5 ml LB overnight at 37°C with shaking. After growth, each strain was inoculated into LB motility agar (containing 0.3% agar) with an inoculation needle. Agar plates then were incubated for 8 h at 37°C.

Statistical analysis. Tests for significance in differences in expression of transcriptional reporter assays were conducted using the Student's *t* test (two-tailed distribution with two-sample, equal variance calculations). For analysis of flagellation of *V. cholerae* populations, a Student's *t* test (two-tailed distribution with two-sample, equal variance calculations) was used to evaluate statistical significance of monotrichous flagellation, aflagellation, or hyperflagellation between WT *V. cholerae* and transcriptional reprogramming mutants in the WT background and between the *V. cholerae* Δ*flhG* strains and transcriptional reprogramming mutants in the Δ*flhG* background. As indicated in the tables, figures, or figure legends, statistically significant differences between relevant strains possessed *P* values of <0.05.

Data availability. All data and methodologies are available upon request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, PDF file, 0.2 MB.

FIG S1, TIF file, 1.1 MB.

FIG S2, TIF file, 1.1 MB.

FIG S3, TIF file, 1.1 MB.

TABLE S1, PDF file, 0.2 MB.

TABLE S2, PDF file, 0.1 MB.

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