A Critical Region in the FlaA Flagellin Facilitates Filament Formation of the *Vibrio cholerae* Flagellum

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ABSTRACT *Vibrio cholerae* is a Gram-negative bacterium with a monotrichous flagellum that causes the human disease cholera. Flagellum-mediated motility is an integral part of the bacterial life cycle inside the host and in the aquatic environment. The *V. cholerae* flagellar filament is composed of five flagellin subunits (FlaA, FlaB, FlaC, FlaD, and FlaE); however, only FlaA is necessary and sufficient for filament synthesis. *flaA* is transcribed from a class III flagellar promoter, whereas the other four flagellins are transcribed from class IV promoters. However, expressing *flaA* from a class IV promoter still facilitated motility in a strain that was otherwise lacking all five flagellins ($\Delta flaA-E$). Furthermore, FlaA from *V. parahaemolyticus* (FlaA^{VP}; 77% identity) supported motility of the *V. cholerae* $\Delta flaA-E$ strain, whereas FlaA from *V. vulnificus* (FlaA^{VV}; 75% identity) did not, indicating that FlaA amino acid sequence is responsible for its critical role in flagellar synthesis. Chimeric proteins composed of different domains of FlaA^{VC} and FlaD or FlaA^{VV} revealed that the N-terminal D₁ domain (D_{1N}) contains an important region required for FlaA function. Further analyses of chimeric FlaA^{VC}-FlaD proteins identified a lysine residue present at position 145 of the other flagellins but absent from FlaA^{VC} that can prevent monofilament formation. Moreover, the D_{1N} region of amino acids 87 to 153 of FlaA^{VV} inserted into FlaA^{VC} allows monofilament formation but not motility, apparently due to the lack of filament curvature. These results identify residues within the D_{1N} domain that allow FlaA^{VC} to fold into a functional filament structure and suggest that FlaA^{VC} assists correct folding of the other flagellins.

IMPORTANCE *V. cholerae* causes the severe diarrheal disease cholera. Its ability to swim is mediated by rotation of a polar flagellum, and this motility is integral to its ability to cause disease and persist in the environment. The current studies illuminate how one specific flagellin (FlaA) within a multiflagellin structure mediates formation of the flagellar filament, thus allowing *V. cholerae* to swim. This knowledge can lead to safer vaccines and potential therapeutics to inhibit cholera.

KEYWORDS cholera, filament, flagella, flagellar motility, swimming

Vibrio cholerae causes the devastating diarrheal disease cholera. The Gram-negative bacteria are ingested through contaminated water and colonize the human small intestine. Within the intestine, *V. cholerae* expresses cholera toxin (CT), an ADP-ribosylating toxin that induces severe water loss that causes the rice water stool which is the hallmark of this disease (1). *V. cholerae* synthesizes a single polar flagellum which facilitates chemotactic motility. Motility has been linked to *V. cholerae* virulence in several different animal models, including in humans (2), and motility is also linked to biofilm formation, which is responsible for environmental persistence (3). Nonmotile mutants are defective for intestinal colonization in mice, and some intestinal adhesion factors have been shown to be coregulated with the flagellum (4). The flagellin subunits of the flagellar filament are responsible for inflammatory responses experienced by

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humans during *V. cholerae* infection (5). Moreover, protective antibody responses specifically inhibit flagellum-mediated motility (6), emphasizing the important role motility plays during *V. cholerae* infection. Flagellum-mediated chemotaxis is suppressed during infection, which leads to an increase in intestinal colonization and a transient hyperinfectivity that is thought to enhance transmission and stimulate epidemic spread (7, 8). Some virulence genes are inversely regulated with flagellar genes, illuminating the interrelated nature of flagellar synthesis and motility with *V. cholerae* pathogenesis (4, 9).

One unique aspect of the *V. cholerae* flagellum is the presence of a sheath, which is an extension of the outer membrane (10) that coats the flagellar filament. The filament is composed of five distinct flagellins, FlaA, FlaB, FlaC, FlaD, and FlaE, that share 61 to 82% identity (11). Interestingly, only FlaA is required for filament synthesis and motility, whereas the other four flagellins are dispensable (5, 11). Inactivation of *flaB*, *flaC*, *flaD*, and/or *flaE*, singly or in combination, results in motile bacteria that still synthesize a filament, whereas inactivation of *flaA* results in nonmotile bacteria without a flagellar filament (5, 11). *V. cholerae* strains lacking *flaA* still express *flaBCDE* (11), but the flagellins are secreted into the supernatant rather than assembling into a filament (12, 13). The *V. cholerae* flagellar genes are transcribed in a four-tiered temporal hierarchy (4, 14, 15). *flaA* is a class III gene that is transcribed by a phospho-FlrC-stimulated σ^{54} -dependent promoter (11, 16, 17). In contrast, *flaB*, *flaC*, *flaD*, and *flaE* are class IV genes, which are transcribed by σ^{28} -dependent promoters (11, 18). Class III gene expression precedes class IV gene expression (14).

The structures of several bacterial flagellins have been solved by X-ray crystallography (19–21), which involved removal of the N- and C-terminal D₀ domains. The structure of the complete *Salmonella enterica* serovar Typhimurium flagellin (i.e., also containing the D₀ domains) has also been solved by cryoelectron microscopy (22). The bacterial flagellin contains two highly conserved predominantly α -helical domains at both the N and C termini, D₀ and D₁, that mediate assembly of the filament, and a variable domain, D₂ (and sometimes D₃), in the central portion of the protein. The linear arrangement within the protein sequence is D_{0N}-D_{1N}-D₂-D_{1C}-D_{0C}, but the N- and C-terminal D₀ and D₁ domains are spatially adjacent within the filament. The crystal structure of flagellin bound to the flagellin-specific Toll-like receptor TLR5 has also been determined (23), which revealed that the primary recognition contacts are amino acid stretches within the D_{1N} and D_{1C} domains that are accessible in monomeric flagellin but buried in polymerized flagellin within the filament (24). Eleven flagellin subunits assemble into the filament per helical turn, with the D₀ domains lining the inner channel of about 20 Å and the D₁ domains forming an outer tube surrounding this; the D₂ (and D₃) domains radiate out from the inner and outer tubes like spokes on a wheel but do not show the tight packing present between the D₀ and D₁ domains (22). The unfolded flagellins are secreted into the filament channel through the basal body, and they fold into their 3-dimensional structure at the distal end under the flagellar cap (25).

It has been unclear why there is a strict requirement for FlaA for filament formation but not for the other four flagellins in *V. cholerae*. In the present study, we confirm that only FlaA is capable of forming a monoflagellin filament and demonstrate that the amino acid sequence of FlaA, rather than its temporal expression, facilitates filament formation. Specifically, substitution of a lysine residue (K145) present in the D_{1N} domain of the other *V. cholerae* flagellins into *V. cholerae* FlaA (FlaA^{VC}) inhibits monofilament formation, and insertion of amino acids (aa) 87 to 152 from *V. vulnificus* FlaA into FlaA^{VC} inhibits motility due to lack of curvature within the filament. We predict residue 145 lies in an interaction surface that is required for FlaA's ability to facilitate functional filament formation.

RESULTS

Class III gene expression is not required for the essential role of FlaA in filament formation. Inactivation of the *flaA* gene prevents flagellar synthesis in *V. cholerae* despite the expression of the four other flagellin subunits, indicating that *flaA*

FIG 1 The FlaA gene expressed as a class IV gene stimulates motility of *V. cholerae*. *V. cholerae* strains were inoculated into motility agar at 30°C; motility is visualized by swarm diameter. Shown is plasmid construction with promoter swap of class III (*flaAp*) and class IV (*flaDp*) promoters, indicated schematically to the left. Strains shown (see Table S2 in the supplemental material) are O395 (wild type), KKV2431 (Δ *flaA-E*), and KKV2431 carrying pKEK1340 (*flaAp-flaA*), pKEK1342 (*flaDp-flaA*), pKEK1927 (*flaAp-flaD*), and pKEK1341 (*flaDp-flaD*). Results were similar when this assay was performed at 37°C (not shown).

plays some essential role in filament formation (11). *flaA* is transcribed from a class III FlrC- and σ^{54} -dependent promoter, whereas the other four flagellin genes are transcribed from class IV σ^{28} -dependent promoters (4, 11, 14). To determine if this position within the flagellar hierarchy was responsible for the essential role of FlaA in filament formation, plasmids were constructed in which either the *flaA* gene or one of the other flagellin genes (*flaD*) was transcribed from a class III (*flaAp*) promoter or a class IV (*flaDp*) promoter (Fig. 1). *flaD* was chosen because it is transcribed at one of the highest levels of all the other flagellin genes and because FlaD shares the closest homology with FlaA (11). The plasmids were transformed into *V. cholerae* strain KKV2431, from which all flagellin coding sequences have been deleted (Δ *flaABCDE*; termed the Δ *flaA-E* strain), and the resulting strains were analyzed for swimming behavior in motility agar.

V. cholerae expressing *flaA* from a class IV promoter (*flaDp-flaA*) was motile, whereas *V. cholerae* expressing *flaD* from a class III promoter (*flaAp-flaD*) was nonmotile (Fig. 1). This result indicates that class III expression within the flagellar hierarchy is not responsible for the essential role that FlaA plays in filament formation, since *flaA* can be expressed as a class IV gene and still mediate motility. The other flagellin, FlaD, cannot substitute for FlaA by simply being expressed as the product of a class III gene; KKV2431, expressing *flaD* from either *flaAp* or *flaDp*, remained nonmotile. These results demonstrate that the FlaA amino acid sequence, rather than temporal expression, plays an essential role in filament formation. Notably, *flaA* expressed from *flaDp* led to greater motility than *flaA* expressed from its native promoter, *flaAp*. Measurement of *flaAp-lacZ* and *flaDp-lacZ* transcription in wild-type, Δ *flaAC*, Δ *flaEDB*, and Δ *flaABCDE* *V. cholerae* strains confirmed that *flaDp* is transcribed at higher levels than *flaAp* in all strain backgrounds, which has been demonstrated previously (11), and also showed that presence or absence of the various flagellin genes does not dramatically alter transcription from these promoters (see Fig. S1 in the supplemental material).

High levels of FlaA required for monoflagellin filament formation, very little FlaA required for multiflagellin filament formation. As shown above, in a *V. cholerae* strain lacking any flagellins, *flaA* expressed from the more highly transcribed *flaDp* allows for better motility than *flaA* expressed from the less transcribed *flaAp*, suggesting that the amount of FlaA flagellin is critical for motility when cells are expressing a monoflagellin composed only of FlaA. To verify this, *flaA* was expressed within *V. cholerae* at increasing levels in the absence of the other flagellins (Fig. 2A) by (i) expression from its native promoter from the chromosome (in a Δ *flaBCDE* strain), (ii) expression from its native promoter from a relatively low-copy-number plasmid (pACYC *ori*; ~20 copies per cell [26]) in the Δ *flaA-E* strain, (iii) expression from its native promoter from a high-copy-number plasmid (pGEM *ori*; >300 copies per cell; Promega) in the Δ *flaA-E* strain, and (iv) expression from the arabinose-inducible pBAD promoter

FIG 2 Large amount of FlaA required for monoflagellin-based motility and small amount of FlaA required for multiflagellin-based motility. *V. cholerae* strains were inoculated into motility agar at 30°C; motility is visualized by swarm diameter. (A) Strains shown (see Table S2 in the supplemental material) are KKV2431 ($\Delta flaA-E$), KKV2530 ($\Delta flaBCDE$), and KKV2431 carrying pKEK89 (*flaAp-flaA* low copy), pKEK1340 (*flaAp-flaA* high copy), and pKEK1948 (*pBAD-flaA*). The plate contained 0.2% arabinose. (B) Strains shown are KKV2431 ($\Delta flaA-E$), KKV2431 carrying pKEK1948 (*pBAD-flaA*), KKV2506 ($\Delta flaA$), and KKV2506 carrying pKEK1948. The plate contained no arabinose; motility in the presence of arabinose is shown in Fig. S2.

(pBR322 *ori*; ~20 copies per cell [27]) in the $\Delta flaA-E$ strain in the presence of 0.2% arabinose. Motility increased with increasing copy number of *flaA* expressed from *flaAp*, demonstrating that relatively large amounts of FlaA are necessary for monoflagellin filament formation. Induction of FlaA expression from pBAD in the presence of 0.2% arabinose resulted in the most motility, so we utilized this expression system for the detailed analyses of FlaA described below.

To determine if similarly large amounts of FlaA are required for filament formation in the presence of the other four flagellins, *flaA* was expressed from the pBAD promoter within *V. cholerae* either lacking all five flagellins (KKV2431; $\Delta flaA-E$ strain) or only lacking *flaA* (KKV2506; $\Delta flaA$ strain) (i.e., in the presence of FlaBCDE). Strains were analyzed for motility either in the absence or presence of 0.2% arabinose. In the absence of arabinose, very little transcription occurs from this promoter (28), yet the $\Delta flaA$ strain carrying the pBAD-*flaA* plasmid was motile; the $\Delta flaA-E$ strain carrying the same plasmid was nonmotile (Fig. 2B). This indicates that the small amount of FlaA in the absence of arabinose induction is sufficient for motility when the other flagellins are present but not when the other flagellins are absent. When these strains were grown in the presence of arabinose, both $\Delta flaA-E$ and $\Delta flaA$ strains carrying pBAD-*flaA* were motile (Fig. S2). These results demonstrate that very little FlaA is required for multiflagellin filament formation, but a large amount of FlaA is required for monoflagellin filament formation.

FlaA-FlaD chimeric flagellins reveal residue in D_{1N} required for FlaA function.

The FlaA flagellin alone facilitated monoflagellin motility, whereas the other four flagellins, including FlaD, did not (Fig. S3). Bacterial flagellins have a structure composed of conserved D₀ and D₁ domains at the N and C termini and variable D₂ (and sometimes D₃) domains in the middle of the protein, depicted linearly as D_{0N}-D_{1N}-D₂-D_{1C}-D_{0C}. Alignment of the *V. cholerae* FlaA, FlaB, FlaC, FlaD, and FlaE proteins with the FliC protein of *S. Typhimurium* allowed for approximate localization of D₀, D₁, and D₂ domains in the *V. cholerae* flagellins (Fig. S4; due to lack of homology within the central region, we have indicated a single D₂ domain within the *V. cholerae* flagellins rather than the D₂ and D₃ domains present in *S. Typhimurium*). Chimeric flagellin proteins were constructed between FlaA and FlaD to determine which domain(s) of FlaA allowed it to form filaments. Schematically representing the flagellin as composed of five linear domains with either FlaA (A) or FlaD (D) sequence, the following six chimeric proteins were constructed and expressed in KKV2431: DAAAD, ADDDA, AADAA, DDADD, DADAD, and ADADA, along with the wild-type FlaA (AAAAA; Fig. 3). The chimeric proteins that promoted motility in the $\Delta flaA-E$ strain (Fig. 3) were DAAAD, AADAA, and DADAD, while ADDDA (not shown), DDADD, and ADADA did not stimulate motility. These results demonstrate that the D₁ domain(s) of FlaA contains some critical

FIG 3 The D_{1N} domain contains a region critical for the function of *V. cholerae* FlaA. *V. cholerae* strains were inoculated into motility agar containing 0.2% arabinose at 30°C; motility is visualized by swarm diameter. Chimeric protein construction between the various domains of FlaA and FlaD, indicated schematically to the left. Strains shown (see Table S2 in the supplemental material) are KKV2431 (ΔflaA-E) and KKV2431 carrying pKEK1948 (AAAAA), pKEK1968 (DAAAD), pKEK1970 (AADAA), pKEK1973 (ADADA), pKEK1976 (DADAD), and pKEK1977 (DDADD).

element required for its essential role in filament formation, whereas the D₀ and D₂ domains do not. Results were identical when these plasmids were expressed in the *V. cholerae* ΔflaA strain (which expresses the other four flagellins; not shown).

To further narrow down the inhibitory residue(s) within FlaD_{D1} that prevents FlaA function, a chimeric FlaA protein, ADAAA, that only contained the D_{1N} domain from FlaD was constructed, and this also did not stimulate motility (Fig. 4A) of the ΔflaA-E strain, implicating the D_{1N} domain of FlaD as containing sequences that inhibit FlaA function. Further chimeric proteins were constructed that contained even smaller regions of FlaD (aa 131 to 153): FlaA¹⁻¹³⁰-FlaD¹³¹⁻¹⁵³-FlaA¹⁵⁴⁻³⁷⁹ (FlaA::D¹³¹⁻¹⁵³), FlaA^{G143S, E144T, A145K} (FlaA STK), and finally the single-amino-acid substitution, FlaA^{A145K} (FlaA A145K). All of these chimeric proteins failed to stimulate motility of the *V. cholerae* ΔflaA-E strain (Fig. 4A). Additional mutant FlaA proteins with substitutions from

FIG 4 Substitution of lysine at position 145 (A145K) in FlaA prevents motility of *V. cholerae*. *V. cholerae* strains were inoculated into motility agar containing 0.2% arabinose at 30°C; motility is visualized by swarm diameter. (A) Strains shown are KKV2431 (ΔflaA-E), KKV2431 carrying pKEK1948 (AAAAA), pKEK1990 (ADAAA), pKEK1991 (FlaA¹⁻¹³⁰-FlaD¹³¹⁻¹⁵³-FlaA¹⁵⁴⁻³⁷⁹; A::D¹³¹⁻¹⁵³), pKEK2034 (FlaA^{G143SE144TA145K}; FlaA STK), and pKEK2035 (FlaA^{A145K}; FlaA A145K). Below is shown an alignment of the putative beta hairpin region of the five *V. cholerae* flagellins with the two beta sheets indicated, and the lysine residues present in βD1a of FlaB, FlaC, FlaD, and FlaE are shown in red. (B) Transmission electron microscopy of *V. cholerae* strain KKV2431 (ΔflaA-E) carrying pKEK1948 (FlaA) and pKEK2035 (FlaA^{A145K}; FlaA A145K) grown in 0.2% arabinose. Arrows indicate suspected hook protrusions.

FIG 5 A region of the *V. vulnificus* D_{1N} domain of FlaA prevents the function of *V. cholerae* FlaA. *V. cholerae* strains were inoculated into motility agar containing 0.2% arabinose at 30°C; motility is visualized by swarm diameter. (A) Strains shown (see Table S2 in the supplemental material) are KKV2431 (Δ *flaA-E*) and KKV2431 carrying pKEK1948 (FlaA^{VC}), pKEK2022 (FlaA^{VP}), and pKEK2021 (FlaA^{VV}). (B) Strains shown are KKV2431 (Δ *flaA-E*) and KKV2431 carrying pKEK1948 (FlaA^{VC}), pKEK2021 (FlaA^{VV}), pKEK1974 (FlaA^{VV1-180-VC181-379}; FlaA^{VV-VC}), and pKEK1979 (FlaA^{VC1-86-VV87-153-VC154-379}; FlaA^{VV87-153}).

the FlaD region of aa 131 to 153 were constructed that maintained the motility of the Δ *flaA-E* strain, including FlaA^{R135N}, FlaA^{S141T}, FlaA^{E144T}, FlaA^{S152D}, and FlaA^{S151A, S152D, S153N} (not shown). These results clearly demonstrate that the presence of a lysine at position 145 inhibits the function of FlaA in motility. Interestingly, FlaB and FlaC also contain a lysine at position 145, and FlaE contains a lysine at position 144 (Fig. 4A).

Transmission electron micrographs (TEM) of the Δ *flaA-E* strain expressing FlaA^{A145K} revealed that these cells are nonmotile due to the lack of filament formation (Fig. 4B); only a small protrusion can be seen at the pole of the cell that resembles the protrusions seen in cells that lack FlaA (11), which likely represents the flagellar hook. In contrast, the Δ *flaA-E* strain expressing FlaA forms a flagellar filament (Fig. 4B), demonstrating that the introduction of a lysine residue at position 145 prevents filament formation of FlaA.

A region in the D_{1N} domain of the FlaA flagellin from *V. vulnificus* (FlaA^{VV}) prevents motility in *V. cholerae*. Other *Vibrio* spp. also swim via a polar flagellum that is composed of multiple flagellins. *V. parahaemolyticus* (FlaA^{VP}; 77% identity; also referred to as FlaC [29]) and *V. vulnificus* (FlaA^{VV}; 75% identity; also referred to as FlaC [30]) both encode FlaA flagellins that share homology with *V. cholerae* FlaA (FlaA^{VC}) (Fig. S5). To determine if either of these flagellins could substitute for FlaA^{VC}, FlaA^{VP} and FlaA^{VV} were expressed from the pBAD promoter in KKV2431 (Δ *flaA-E*), and the swimming behavior of these strains in motility agar were compared to that of KKV2431 expressing FlaA^{VC} (Fig. 5A). FlaA^{VP} facilitated motility of KKV2431 whereas FlaA^{VV} did not, indicating that amino acid differences between these proteins were responsible for their differences in motility. To further narrow down the region of FlaA^{VV} that was preventing motility, chimeric proteins were expressed that contained aa 1 to 180 of FlaA^{VC} fused to aa 181 to 385 of FlaA^{VV} (FlaA^{VC-VV}) and aa 1 to 180 of FlaA^{VV} fused to aa 181 to 379 of FlaA^{VC} (FlaA^{VV-VC}). FlaA^{VV-VC} failed to provide motility to KKV2431 (Fig. 5B), whereas FlaA^{VC-VV} did (not shown), indicating that the inhibitory residue(s) was within the first 180 aa of FlaA^{VV}.

To further narrow down the inhibitory residue(s), a chimeric FlaA protein containing the same inhibitory region (aa 87 to 153) of FlaA^{VV} as FlaD (described above) was constructed. This chimeric protein (FlaA^{VC1-86-FlaA^{VV}87-153-FlaA^{VC}154-379}, termed FlaA^{VV87-153}) (Fig. 5B) also failed to provide motility to KKV2432, indicating that the inhibitory region lies between aa 87 and 153 of FlaA^{VV}. This region contains differences in 10 amino acids between FlaA^{VV} (which fails to stimulate motility of the Δ *flaA-E* strain) and FlaA^{VC}/FlaA^{VP} (which both stimulate motility of the Δ *flaA-E* strain) (Fig. S5). However, attempts to narrow down the inhibitory region/residue further by introducing either aa 87 to 108, aa 109 to 130, or aa 131 to 153 from FlaA^{VV} into FlaA^{VC} resulted in

FIG 6 Transmission electron microscopy of *V. cholerae* strain KKV2431 ($\Delta flaA$ -E) carrying pKEK1948 (FlaA) and pKEK1979 (FlaA^{VC1-86-VV87-153-VC154-379}; FlaA^{VV87-153}), grown in 0.2% arabinose.

chimeric proteins that all facilitated motility of KKV2431 (FlaA^{VV87-108}, FlaA^{VV109-130}, and FlaA^{VV131-153}) (Fig. S6). These results indicate that, minimally, two amino acid changes within aa 87 to 153 of FlaA^{VV} are required to prevent FlaA^{VC} from providing motility to KKV2431.

Transmission electron micrographs of the $\Delta flaA$ -E strain expressing FlaA^{VV87-153} revealed that cells are flagellated and thus are not defective for filament formation (Fig. 6); however, the filament appeared straight rather than the curved filament typical for the cells expressing the wild-type FlaA^{VC}. This suggests that the altered amino acids in D_{1N} lead to the formation of a straight filament that is locked into one of the L or R forms (31).

DISCUSSION

The polar flagellum of *V. cholerae* facilitates motility, which allows the bacteria to colonize preferential sites within the human small intestine and cause disease, as well as to navigate the aquatic environment and form biofilms during interepidemic periods. Given the integral role the flagellum plays in the life cycle of *V. cholerae*, understanding its biogenesis will give insights that may lead to novel cholera intervention strategies. One of the mysteries of *V. cholerae* flagellar synthesis has been the preferential role of the flagellin FlaA in filament formation. All five flagellins are found within the filament, but only FlaA is absolutely required and sufficient for filament formation (11). When FlaA is not present, the other flagellins are still expressed but are secreted rather than assembled into the filament (12, 13). This suggests the other four flagellins fail to provide some crucial attribute for assembly of the filament, which FlaA alone possesses.

The flagellar genes are expressed in a four-tiered hierarchy, with class III gene transcription preceding class IV gene transcription (4, 14). Because *flaA* is expressed from a class III promoter while the other flagellins are expressed from class IV promoters, the temporal position within the hierarchy could have been an explanation for the crucial role of FlaA in filament formation. However, *flaA* transcribed from a class IV promoter still promoted filament formation, whereas transcription of one of the other flagellin genes (*flaD*) from the *flaA* promoter still failed to stimulate filament formation (Fig. 1). This clearly demonstrates that the critical attribute of FlaA for filament formation resides within the FlaA amino acid sequence.

FIG 7 Model of FlaA lacking the D_0 domains (D_{1N} - D_2 - D_{1C}), with the critical alanine at position 145 labeled in boldface.

By testing the ability of chimeric proteins constructed between FlaA and FlaD to stimulate motility, we identified the D_{1N} domain of FlaA as containing the critical residues for filament formation, since FlaA with the D_{1N} domain from FlaD did not provide motility to *V. cholerae*. Considering that the overall structure of the D_1 domain is well conserved among bacterial flagellins whose structures have been solved (19–21), we predict a similar structure in the FlaA^{VC} flagellin and have modeled the protein (D_{1N} - D_2 - D_{1C}) based upon the crystal structure of the FliC flagellin from *P. aeruginosa* (4NX9_A; lacking the D_0 domains at the N and C termini) (19), because this gave the highest-confidence threading model in HHPred ($4.9e-25$) (32) (Fig. 7). The inhibitory region of FlaD was narrowed down further to residues contained within aa 131 to 153 and finally to the lysine residue present at position 145. This residue lies within the first of two predicted antiparallel beta sheets ($\beta D1a$ and $\beta D1b$) that form a beta hairpin, and there is an alanine at the corresponding position in FlaA^{VC}. This region containing the beta hairpin forms a concave interaction surface in the *S. Typhimurium* flagellin (aa 132 to 151 [see yellow highlights in Fig. S5 in the supplemental material]) that interacts with the convex interaction surface formed at the turn of the alpha helices of D_{1N} (aa 89 to 107 [Fig. S5, yellow highlights]) of the adjacent flagellin in the protofilament (20).

The first beta sheet in the predicted beta hairpin of FlaA^{VC} ($\beta D1a$ aa 141 to 147; SFG^{EASF}) contains the critical A145 residue (boldface); when changed to the lysine found at this position in FlaD, it prevented the motility of *V. cholerae*. Interestingly, there is a lysine residue present at aa 145 in FlaB, FlaC, and FlaD and at aa 144 in FlaE (Fig. 4A and Fig. S4). None of the other four flagellins is capable of monoflagellin filament formation in *V. cholerae* (Fig. S3), so we conclude that the lysine present within this beta sheet prevents the correct incorporation of the alternate flagellins into a monofilament structure, possibly by destabilizing the contact between the interacting surfaces of adjacent flagellins with a large positively charged side chain (i.e., flagellin-flagellin interactions). This predicts that the alternate flagellins only interact correctly with FlaA^{VC} within the filament, perhaps because the interaction surface on FlaA^{VC} has evolved to productively interact with the lysine residue on the adjacent flagellin.

Alternatively, the lysine side chain may prevent correct folding of the alternate flagellins, which can only occur in the presence of FlaA^{VC}, because it acts like a chaperone by providing a template that neutralizes the lysine and facilitates correct

folding. This chaperone function of FlaA^{VC} only being required at the initiation of filament formation would be consistent with the extremely small amount of FlaA^{VC} that is necessary for multiflagellin filament formation (Fig. 2B). It is also clear that the presence of the lysine within the putative β D1a region of FlaD is not the only aspect preventing FlaD from forming a monoflagellin filament, because substitution of the D_{1N} region from FlaA into FlaD (i.e., DADDD) did not result in a chimeric protein that was able to promote motility in the flagellin-less *V. cholerae* strain (Fig. S7). The fact that FlaD protein with both D1 domains from FlaA was motile (i.e., DADAD) indicates that there is an additional residue(s) within the D_{1C} region of FlaA necessary for its ability to form a monoflagellin filament.

The beta hairpin region of the *S. Typhimurium* flagellin is also within the switch region, which permits the filament to switch between different supercoiled forms that allow the bacteria to run and tumble (20). These interactions lead to a slight pitch to the 11 individual protofilaments that make up the filament, resulting in the curved, corkscrew appearance of the flagellum rather than a straight filament. In our studies, we discovered that FlaA^{VV} had a region in D_{1N} that, when inserted into FlaA^{VC}, prevented motility. Cells of *V. cholerae* expressing FlaA^{VC} with aa 87 to 153 from FlaA^{VV} synthesize a flagellar filament, but the filament appears straight with no curvature. The flagellar filaments of other bacteria have been locked by genetic mutation into two different L and R straight forms, which represent the two different short and long states that the protofilaments switch between to facilitate the supercoiling that allows motility (20, 31, 33). The insertion of aa 87 to 153 from FlaA^{VV} into FlaA^{VC} appears to lock the *V. cholerae* filament into one of these straight forms. There are differences in 15 aa between the two proteins in this region, with only 10 aa that are different between FlaA^{VV} and FlaA^{VC}/FlaA^{VP} (Fig. S5, residues in red;) (we assume that since FlaA^{VP} is functional in *V. cholerae*, any amino acid differences with FlaA^{VC} they share are likely irrelevant). Our attempts to narrow down this region have indicated that differences in at least 2 aa are required (Fig. S6). The corresponding aa 89 to 107 and aa 132 to 151 regions are known to interact in the protofilament of *S. Typhimurium* (Fig. S5, highlighted yellow), so perhaps the straight filament formed by FlaA^{VC} with aa 87 to 152 from FlaA^{VV} reflects interactions between these regions in two adjacent flagellins that lock the flagellum into a straight form.

Other *Vibrio* spp. also have flagella composed of multiple flagellins, but thus far only *V. cholerae* appears to have a dominant flagellin subunit absolutely required for filament formation. *V. vulnificus*, *V. parahaemolyticus*, *V. fischeri*, and *V. anguillarum* mutant strains with the gene corresponding to *flaA* inactivated are still motile (29, 30, 34, 35), although all show some level of decreased motility. A *V. anguillarum flaA* mutant is defective for virulence in fish, and a *V. fischeri flaA* mutant is defective for symbiotic colonization of the squid (34, 35). Interestingly, these other *Vibrio* spp. have at least one alternate flagellin protein besides FlaA without a lysine at position 145 in the beta hairpin and thus may be able to substitute another flagellin for the essential role only FlaA^{VC} can perform in *V. cholerae* filament formation. Given that *V. cholerae* can synthesize a monoflagellin filament with FlaA, the question remains as to what functions the alternate flagellins play in *V. cholerae* motility and virulence.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains DH5 α , DH5 α pir, TOP10, XL1-Blue, and EC100D pir⁺ were used for cloning, and SM10 λ pir was used for conjugation. *V. cholerae* O1 strain O395, which is streptomycin resistant, and its derivatives were used for all experiments. Strains were grown in Luria broth (LB) at 37°C overnight. Antibiotics were used in the following concentrations: ampicillin (Ap), 100 μ g/ml; streptomycin (Sm), 100 μ g/ml. When required, arabinose was added to the medium at a concentration of 0.2%.

A complete list of primers and plasmids used is provided in Tables S1 and S2 in the supplemental material. Details on plasmid construction are included in the supplemental material. All plasmid constructs were verified by sequencing. *V. cholerae* strains with chromosomal deletions were constructed by sucrose counterselection utilizing pKEK229 (16), a derivative of pCVD442 (36), as described previously. *V. cholerae* chromosomal deletions removed all coding sequence from the corresponding gene by fusing the start codon to the stop codon. The entire *flaAC* (Δ *flaAC*) and *flaEDB* (Δ *flaEDB*) loci were removed by fusing the start codon of the first gene to the stop codon of the last gene.

β -Galactosidase assays. *V. cholerae* strains carrying the promoter-*lacZ* plasmids listed in Table S2 were assayed for β -galactosidase activity by the method described by Miller (37).

Motility assays. Strains were grown at 37°C overnight on an appropriate agar plate and then inoculated into motility agar (0.3% LB agar), supplemented with 0.2% arabinose as necessary, and incubated at 30°C.

Electron microscopy. Strains were inoculated into LB medium containing appropriate antibiotics, supplemented with 0.2% arabinose as necessary, and incubated at 37°C overnight. Imaged strains were washed using 2 mM CaCl₂, inoculated on a carbon type B, 300-mesh copper grid (Ted Pella, Inc.), and stained with 1% uranyl acetate. Cells were imaged using a JEOL 1230 TEM and Hitachi STEM S5500.

Modeling of FlaA. Molecular graphics and analyses were performed with the UCSF Chimera package (38). FlaA (NCBI protein accession number [ACP10294](#); VC395_2304) was modeled on *Pseudomonas aeruginosa* FlhC (PDB entry [4NX9](#)).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00029-18>.

SUPPLEMENTAL FILE 1, PDF file, 4.0 MB.

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