Flagellin Redundancy in *Caulobacter crescentus* and Its Implications for Flagellar Filament Assembly^{▽†}

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Bacterial flagella play key roles in surface attachment and host-bacterial interactions as well as driving motility. Here, we have investigated the ability of *Caulobacter crescentus* to assemble its flagellar filament from six flagellins: FljJ, FljK, FljL, FljM, FljN, and FljO. Flagellin gene deletion combinations exhibited a range of phenotypes from no motility or impaired motility to full motility. Characterization of the mutant collection showed the following: (i) that there is no strict requirement for any one of the six flagellins to assemble a filament; (ii) that there is a correlation between slower swimming speeds and shorter filament lengths in $\Delta fljK$ $\Delta fljM$ mutants; (iii) that the flagellins FljM to FljO are less stable than FljJ to FljL; and (iv) that the flagellins FljK, FljL, FljM, FljN, and FljO alone are able to assemble a filament.

Movement allows organisms to localize to nutrient-rich habitats and move away from unfavorable environments (26). Flagellum-mediated motility facilitates movement in 45% of bacterial species, based on annotated genomes (Fig. 1) (18). The bacterial flagellum is a macromolecular machine that generates thrust via rotation of the long whip-like extension known as the filament (30). Flagellar filaments also play a key role in surface attachment and host-bacterial interactions.

Flagellin is the major flagellar structural protein that makes up the flagellar filament. It is estimated that a flagellar filament of *Salmonella enterica* serovar Typhimurium utilizes up to 30,000 flagellin monomers (30). This estimate is based on the average length of an *S. Typhimurium* flagellar filament (approximately 7 μm), the helical symmetry, and the repeat distance of the flagellar filaments (45). Due to the number of flagellin monomers per filament, flagellins comprise a major pathogen-associated molecular pattern that is recognized by both innate and adaptive immune responses of eukaryotic hosts (3).

The flagellum is generally comprised of approximately 12 structural subunits plus a number of auxiliary proteins that

form the flagellar-specific type III secretion apparatus. In most bacterial species the majority of the structural subunits are encoded by one gene per genome. When divergence from this rule is observed, it is usually consistent for all structural components (Fig. 1) (18). In such cases the bacterium in question encodes two independent flagellar systems. The flagellin genes depart from this rule of one gene, one subunit. Approximately 45% of annotated flagellar systems possess more than one flagellin gene (Fig. 1). The number of flagellin genes in such systems generally ranges between two (*S. Typhimurium*) and seven (*Vibrio parahaemolyticus*). The annotated genome of *Magnetococcus* sp. MC-1 states that it possesses 15 flagellin genes (18).

The flagellar systems of *S. Typhimurium* and *Escherichia coli* have been well characterized and are regarded as paradigm systems. *S. Typhimurium* belongs to the multiple-flagellin systems, producing two flagellins, FljB and FljC (23, 33). However, *S. Typhimurium* exhibits phase variation between these flagellins and consequently utilizes only one flagellin at any given time to assemble its filaments. In other bacteria encoding multiple flagellins such as the *Vibrio* spp., *Helicobacter pylori*, *Sinorhizobium meliloti*, *Bdellovibrio bacteriovorus*, and *Caulobacter crescentus*, evidence suggests that all encoded flagellins are utilized (8, 19, 20, 28, 29, 37). Some of these flagellar systems have even been shown to exhibit a degree of ordered flagellin incorporation (7, 19, 20). The majority of flagellar systems that encode multiple flagellins share several common features. First, the flagellin genes are encoded within monocistronic operons often located at the same locus. Second, the flagellin genes always encode flagellins that possess molecular masses within approximately 2 to 5 kDa of each other; FljB and FljC are no exception (6). For example, *Vibrio fischeri* encodes

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FIG. 1. Bioinformatic analysis of flagellar gene dosage of annotated bacterial genomes in the KEGG database. The genes are organized by their approximate location in the flagellum structure starting with *fliF* on the left and finishing with the filament cap, *fliD*, on the right. To be included in the analysis, the genome was required to contain the inner membrane anchor FliF, on the assumption that a foundation is required to build a flagellum. The gene dosage was calculated using the IF and COUNT functions of Microsoft Excel 2004 generating a gene dosage matrix. The gene dosage matrix was then converted to color using the image function of the statistical software package R, using a defined set of colors for 0 to 7 genes plus purple for the 15 flagellins of *Magnetococcus*. The key feature with respect to this study is the variation in gene dosage seen for the flagellin genes. From 607 *fliF*-containing genomes, 45% possess multiple flagellins compared to approximately 10% that encode two complete flagellar systems. The KEGG database as of January 2011 containing 1,144 annotated bacterial genomes was used for this analysis. The mixed group (*) includes the genomes of species belonging to the families of *Acidobacteria*, *Verrucomicrobia*, *Gemmatimonadetes*, *Planctomycetes*, *Synergistetes*, and the green nonsulfur bacteria. All other families are named, and the Gram-negative families are given as Greek symbols.

six flagellins, all approximately of 40 kDa (29), while *C. crescentus* encodes a 29-kDa, a 27-kDa, and four 25-kDa flagellins (Fig. 2) (8). Third, a level of redundancy is evident. In some systems, however, one (or more) flagellin is essential for filament assembly and has thus been defined as the major flagellin.

Our current understanding of filament assembly, filament structure, and the diversity of habitats colonized by bacteria encoding multiple flagellins does not satisfactorily explain why their utilization is an advantage. It has been suggested that flagellin utilization may be environment specific (28). This is

consistent with the observation that locked *fliB* “on” and *fliC* “on” phase variants in *S. Typhimurium* exhibit altered phenotypes with respect to systemic infections of infected mice (11).

C. crescentus is a nonpathogenic, vibroid alphaproteobacterium that colonizes freshwater habitats (34). *C. crescentus* divides asymmetrically to produce two morphogenetically distinct daughter cells: the sessile stalked (ST) cell and the motile swarmer (SW) cell (14). The motile SW cell utilizes a single, unsheathed polar flagellum to generate motility. The SW cell retains the flagellum for approximately a third of the *C. crescentus* cell cycle, after which it is ejected when the SW cell

FIG. 2. Genetic organization of the α - and β -flagellin loci of *C. crescentus*. Flagellar genes are shown as arrows while predicted hypothetical open reading frames are boxes. A deletion of the ORF *cc1462* had no observable phenotype with respect to motility (data not shown). Upstream of *fljL* are located the flagellin translation regulators *flaF* and *flbT* (see discussion). The α -locus is found among a cluster of nine known flagellar genes while the β -locus is found at an unlinked location on the *C. crescentus* chromosome. The theoretical masses of the six flagellins are shown in parentheses under each gene.

enters the differential transition to a ST cell. There are six flagellin genes annotated in the *C. crescentus* genome: *fljJ*, *fljK*, *fljL*, *fljM*, *fljN*, and *fljO* (Fig. 2), plus a putative flagellin predicted to encode a protein of 43 kDa (32). Previous work has shown that $\Delta fljJ$ and $\Delta fljL$ mutants alter motility on swarmer plates and that a $\Delta fljM$ mutant has no effect, while mutants with other deletions spanning *fljJ*, *fljK*, and/or *fljL* were still motile (8, 31). This led us to investigate why *C. crescentus* maintains the use of multiple flagellins.

In this study we examined the role of the six *C. crescentus* flagellins, FljJ to FljO, during filament assembly. Our hypothesis argues that an intrinsic feature of filament assembly rather than environmental pressure drives *C. crescentus* to utilize multiple flagellins. By generating a collection of flagellin gene deletions, we show that there is a significant degree of structural redundancy in the system. The flagellins FljK, FljL, FljM, FljN, and FljO were sufficient alone to assemble a functional flagellar filament, but FljJ was not. We characterized the effect of the mutations on total flagellin concentration, flagellin stability, filament composition, the physical characteristics of the filaments produced, and the swimming speed of the mutants compared to the wild type. We discuss the implications of our data for filament assembly and the utilization of multiple flagellins in flagellar systems.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *C. crescentus* strains were grown in peptone-yeast extract (PYE) complex medium at 30°C (1). Medium was supplemented with antibiotics and growth supplements (kanamycin, 20 $\mu\text{g ml}^{-1}$; naladixic acid, 20 $\mu\text{g ml}^{-1}$; sucrose, 0.2%). *Escherichia coli* strains were grown at 37°C in Luria Bertani (LB) broth and supplemented with antibiotics and growth supplements (kanamycin, 50 $\mu\text{g ml}^{-1}$; ampicillin, 100 $\mu\text{g ml}^{-1}$; sucrose, 5%). For solid medium, irrespective of bacterial species, 1.5% agar was used. In motility assays of *C. crescentus* strains PYE containing 0.3% agar was used where a single colony of *C. crescentus* strains was stabbed into the agar using a toothpick and left to incubate at 30°C for 3 to 5 days.

Cloning strategies. In-frame deletions were constructed as previously described using pNPTS128 (35). Specific primers were designed for the amplification of approximately 300 to 500 bp of the upstream and downstream regions of each gene (see Table S1 in the supplemental material). The resulting PCR products amplified from *C. crescentus* NA1000 genomic DNA were cloned into

pGEM-T Easy (Promega). The upstream and downstream regions were then subcloned together with appropriate enzymes. These regions were subsequently subcloned into the suicide plasmid pNPTS128 and conjugated into NA1000. Double-crossover mutants were selected and screened as described previously (1).

For plasmid-based flagellin expression experiments, full-length open reading frames (ORFs) of *fljJ*, *fljK*, *fljL*, *fljM*, *fljN*, and *fljO* were cloned into either pRXMCS-2 or pBXMCS-2 from PCR products (41). An NdeI restriction site at the start of a polylinker in both plasmids was utilized to provide the flagellin start codon (see Table S1 in the supplemental material). All constructs were confirmed by sequencing (GATC, Germany). To induce flagellin expression, 0.3% xylose was added to the growth medium.

Analysis of swimming speeds. Overnight cultures were diluted 1:10 with fresh PYE broth and grown to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8. Free swimming was observed with a phase-contrast microscope (CH-40; Olympus) and recorded on VHS videotape at 1/30-s intervals through a charge-coupled-device (CCD) camera (C5405-50; Hamamatsu Photonics). The necessary parts of the movies were captured on a computer as BMP images with video capture software (CosmoCapture, Library, Japan). The swimming speed, determined by the software, was calculated for a minimum of 25 swimming cells by tracing the centers of the cells.

Alternatively, the motion of swarmer cells more than 30 μm away from the glass surfaces in the slide sample was recorded with a CCD camera under a Nikon Eclipse E800 microscope in the phase-contrast mode with an oil immersion 100 $\times$ objective lens (Plan Apo) at room temperature. Video segments containing 100 frames were taken at 10 frames per second with version 6 of the MetaMorph software package (Universal Imaging, Downingtown, PA). The swimming speed of a cell was calculated by measuring its travel distance over more than 1 s. Statistical analysis of data sets was performed using Microsoft Excel 2004 or the statistical software package R (www.r-project.org) using both traditional pairwise *t* tests and analysis of variance (ANOVA).

Western blot analysis of whole-cell lysates. Proteins were separated using Tricine-SDS-PAGE as previously described (4). Western blot analysis was performed as described previously for between 100 and 200 OD units using the following formula: OD units = (culture volume/resuspension volume) $\times$ OD_{600} , where volumes are measured in milliliters (4). Detection was achieved using ECL-Plus and exposing the membranes to Hyperfilm (GE-Healthcare). Quantification of immunoblots was performed using ImageQuant software as previously described after scanning 5- to 15-s exposures using a standard Epson scanner (4). Statistical analysis of data sets was performed using the ANOVA analysis tool in Microsoft Excel 2004.

***C. crescentus* filament preparations.** *C. crescentus* strains were cultured overnight in 250 ml to 1 liter of PYE medium and centrifuged at 10,000 $\times g$ for 15 min to remove cells. The supernatant was ultracentrifuged at 27,000 rpm for 30 min using an SW-32 Ti rotor; the supernatant was removed, and the pellet was resuspended in 20 ml of phosphate-buffered saline (PBS). This mix was centrifuged again at 17,500 $\times g$ to remove any remaining cells, and the supernatant was ultracentrifuged at 27,000 rpm for 30 min using an SW-32 Ti rotor. The pellet was then resuspended in a small volume of PBS (e.g., 50 μl per 250 ml of culture), and the mixture was used immediately if needed for the preparation of grids for transmission electron microscopy (TEM) analysis or matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis after Tricine-SDS-PAGE.

Transmission electron microscopy analysis. Copper Grids (200 mesh) were prepared by glow discharging and used within 1 h. Five microliters of filament preparations was placed onto the carbon-coated surface of the grid, and the excess was removed with blotting paper. The grids were then stained with 10 μl of either 3% uranyl acetate (UA) or 3% phosphotungstic acid (PTA), and the excess liquid was removed with blotting paper. Grids were allowed to dry and were then observed with the Philips CM100 TEM.

Quantitative filament parameters were determined using ImageJ software (rsb.info.nih.gov/ij/download.html) for between 10 and 30 filaments per mutant. The lengths of each filament, pitch length, and pitch height were measured from CCD-captured TEM images. Data and statistical analysis were performed using Microsoft Excel 2004 or R. The statistical significance of parameters compared to the wild type was calculated using a standard *t* test in R.

Mass spectrum analysis. The mass spectrum analysis in this project was performed using in-house proteomics facilities at Newcastle University. Once samples were produced consisting of a suitable concentration of protein, they were transferred to the mass spectrum unit for trypsin digestion, zip-tip purification, MALDI-TOF mass spectrum analysis, or liquid chromatography–tandem mass spectrometry (LC-MS/MS) and peptide mass finger printing (PMF).

TABLE 1. Strains and plasmids used in this study

Organism and mutation or plasmid	Strain	Genotype or description	Reference or source
Strains			
<i>E. coli</i>	DH5 α	λ^- ϕ 80 Δ lacZ Δ M15 Δ (lacZYA-argF)U169 <i>recA1</i>	New England Biolabs
	S17-1	<i>endA1 hsdR17</i> (r $_{K^-}$ m $_{K^-}$) <i>supE44 thi-1 gyrA relA1 thi pro hsdR</i> mutant <i>hsdM⁺ recA</i> RP4-2 (Tc::Mu-Tn7)	39
<i>C. crescentus</i>			
Wild type	NA1000	syn-1000, synchronizable derivative of a <i>C. crescentus</i> wild-type strain	9
Δ <i>fliF</i>	LS1218	Δ <i>fliF</i>	13
Δ <i>fljJ</i>	TPA663	Δ <i>fljJ</i>	This study
Δ <i>fljK</i>	TPA2234	Δ <i>fljK</i>	This study
Δ <i>fljL</i>	TPA916	Δ <i>fljL</i>	This study
Δ <i>fljM</i>	TPA541	Δ <i>fljM</i>	This study
Δ <i>fljJL</i>	TPA934	Δ <i>fljJ</i> Δ <i>fljL</i>	This study
Δ <i>fljJK</i>	TPA933	Δ <i>fljJ</i> Δ <i>fljK</i>	This study
Δ <i>fljKL</i>	TPA971	Δ <i>fljK</i> Δ <i>fljL</i>	This study
Δ <i>fljJM</i>	TPA2099	Δ <i>fljJ</i> Δ <i>fljM</i>	This study
Δ <i>fljKM</i>	TPA2341	Δ <i>fljK</i> Δ <i>fljM</i>	This study
Δ <i>fljLM</i>	TPA1300	Δ <i>fljL</i> Δ <i>fljM</i>	This study
Δ <i>fljJKL</i>	TPA970	Δ <i>fljJ</i> Δ <i>fljK</i> Δ <i>fljL</i>	This study
Δ <i>fljJKM</i>	TPA1298	Δ <i>fljJ</i> Δ <i>fljK</i> Δ <i>fljM</i>	This study
Δ <i>fljJLM</i>	TPA1138	Δ <i>fljJ</i> Δ <i>fljL</i> Δ <i>fljM</i>	This study
Δ <i>fljKLM</i>	TPA1139	Δ <i>fljK</i> Δ <i>fljL</i> Δ <i>fljM</i>	This study
Δ <i>fljMNO</i>	TPA1299	Δ <i>fljM</i> - <i>fljO</i>	This study
Δ <i>fljJKLM</i>	TPA1140	Δ <i>fljJ</i> Δ <i>fljK</i> Δ <i>fljL</i> Δ <i>fljM</i>	This study
Δ <i>fljJMNO</i>	TPA2344	Δ <i>fljJ</i> Δ <i>fljM</i> - Δ <i>fljO</i>	This study
Δ <i>fljKMNO</i>	TPA2352	Δ <i>fljK</i> Δ <i>fljM</i> - Δ <i>fljO</i>	This study
Δ <i>fljLMNO</i>	TPA2356	Δ <i>fljL</i> Δ <i>fljM</i> - Δ <i>fljO</i>	This study
Δ <i>fljJKMNO</i>	TPA2346	Δ <i>fljJ</i> Δ <i>fljK</i> Δ <i>fljM</i> - Δ <i>fljO</i>	This study
Δ <i>fljJLMNO</i>	TPA2353	Δ <i>fljJ</i> Δ <i>fljL</i> Δ <i>fljM</i> - Δ <i>fljO</i>	This study
Δ <i>fljKLMNO</i>	TPA2354	Δ <i>fljK</i> Δ <i>fljL</i> Δ <i>fljM</i> - Δ <i>fljO</i>	This study
Δ <i>fljJKLMNO</i>	TPA2357	Δ <i>fljJ</i> Δ <i>fljK</i> Δ <i>fljL</i> Δ <i>fljM</i> - Δ <i>fljO</i>	This study
Δ <i>flmD</i>	TPA1642	Δ <i>flmD</i>	This study
Δ <i>flmD</i> Δ <i>fljJKL</i>	TPA1679	Δ <i>flmD</i> Δ <i>fljJ</i> Δ <i>fljK</i> Δ <i>fljL</i>	This study
Δ <i>flmD</i> Δ <i>fljMNO</i>	TPA1878	Δ <i>flmD</i> Δ <i>fljM</i> - Δ <i>fljO</i>	This study
Plasmid vectors			
pGEM-T		Amp r cloning vector	Promega
pBXMCS-2			41
pRXMCS-2			41
pNPTS128		<i>sacB⁺ mob⁺ Kan^r</i> vector	35

RESULTS

Δ *flj* strains are less motile due to changes in swimming speed. A bioinformatics analysis for gene dosage of flagellar systems from annotated bacterial genomes deposited in the KEGG database shows that 45% of flagellated bacteria encode multiple flagellins (Fig. 1). The genomes encoding multiple flagellins were not restricted to a specific group of bacteria but were spread across the bacterial kingdom. Furthermore, both nonpathogenic and pathogenic species possessed this trait. This suggests that there is selective pressure across the bacterial kingdom that drives such a significant number of flagellar systems to utilize multiple flagellins.

This bioinformatics analysis identified a further noteworthy feature of flagellar systems. A significant number of Gram-negative bacteria potentially encode two flagellar systems per genome, some of which are experimentally defined as doing so (17, 28, 40). Interestingly, this trait was not observed among annotated Gram-positive bacterial genomes. Several other fea-

tures are apparent from the overview this analysis gives to flagellar systems of bacteria. However, a number of these features require more in-depth analysis and experimental investigation for confirmation of their importance and are out of the scope of this current study.

A plausible explanation for the wide use of multiple flagellins is that a structural constraint exists with respect to flagellar filament assembly. An alternative hypothesis is that the flagellins are utilized in different environments or at different time points during the bacterial life cycle. In order to investigate the use of multiple flagellins, we focused on the flagellins encoded in the *C. crescentus* genome.

The flagellins of *C. crescentus* are located in two clusters known as the α - and β -clusters (8, 32). The α -cluster flagellin genes are *fljJ*, *fljK*, and *fljL* while the genes *fljM*, *fljN*, and *fljO* are located within the β -cluster (Fig. 2). It has previously been shown that in *C. crescentus* some of the flagellins encoded in the α - and β -clusters are not essential to motility; however, the

extent of redundancy for the system has not been fully investigated (8, 31).

We addressed the question of flagellin redundancy by making combinations of flagellin mutants and determining their effect on motility. A series of in-frame deletion constructs of the α -flagellins and the β -flagellins was generated. The deletion constructs were then used in two-step recombination mutagenesis to produce a collection of 23 strains (12). The collection of mutants included combinations of gene deletions of *fljJ*, *fljK*, *fljL*, and *fljM*; a complete deletion of the β -cluster (using a DNA fragment upstream of *fljM* and downstream of *fljO* during the deletion method [Table 1, $\Delta fljM\text{-}\Delta fljO$]); and a strain with all of the α - and β -cluster flagellins deleted. Attempts to isolate single deletions of *fljN* and *fljO* were not successful. The β -cluster exhibits significant similarity between *fljM*, *fljN*, and *fljO* at the DNA level, suggesting that it originates from gene duplications. We have assumed that the lack of success in obtaining single and double deletions of *fljN* and *fljO* is due to some aspect of this locus that prevents recombination in this region of the chromosome.

The effect of flagellin gene deletions was measured in motility agar (data not shown) and quantified by microscopic evaluation of the swimming speeds of the mutants in comparison to the speed of the wild-type strain NA1000 (Fig. 3). Strikingly, out of the entire flagellin deletion collection, a non-motile phenotype was observed for only two mutants: the $\Delta fljKLMNO$ (*fljJ*⁺) and $\Delta fljJKLMNO$ strains. Single gene deletions had little or no effect on motility. The swimming speeds of eight of the flagellin mutants were significantly different ($P < 0.001$, calculated by ANOVA) from the swimming speed of the wild type (Fig. 3). This supports the previous observations that FljJ, FljK, FljL, and FljM are not essential for motility (8, 31).

The increase in the number of flagellin gene deletions led to a greater reduction in swimming speed. The most severe motility defects were observed for the $\Delta fljKMX$ combinations (where X stands for any other α - or β -flagellin) but not in the $\Delta fljKM$ strain (Fig. 3). The data suggest that the flagellins FljK and FljM play a major structural role in filament assembly. This conclusion is supported by the $\Delta fljJLMNO$ (*fljK*⁺) strain, which retains the ability to swim at a speed comparable to that of the wild type when the filament is made up of FljK alone (Fig. 3). Even though the most dramatic drop in speed correlated to the loss of *fljK* and *fljM*, the $\Delta fljJKMNO$ (*fljL*⁺) mutant remains motile, with an average speed of 19.58 $\mu\text{m/s}$. This suggests that FljL plays an important role in filament assembly.

Δflj deletion mutant combinations possess flagellar filament populations comprised of all remaining flagellins. The motility analysis of the deletion strains has provided evidence that the flagellins of *C. crescentus* exhibit a higher degree of structural redundancy than expected. The extent of this redundancy suggests that no single flagellin is required for motility, but it does not inform us whether all of the flagellins or only a subset of flagellins are used to assemble the filament. To investigate the composition of wild-type and mutant filaments, we performed an extensive MALDI-TOF analysis of tryptic peptides from isolated flagellar filament populations. MALDI-TOF was chosen as the observed similarity between five of the six flagellins renders immunoblot analysis extremely difficult (8).

FIG. 3. Comparison of the swimming speeds and physical parameters of the flagellar filaments produced by the flagellin gene mutants. Average values for each strain are shown, with the standard deviations plotted as error bars. The mutants with asterisks return a P value of less than 0.001 compared to the wild type in a pairwise t test. The presence and absence of specific flagellin genes are highlighted at the bottom. Black boxes indicate intact genes; blank boxes indicate deletions; gray bars highlight the $\Delta fljKMX$ mutants.

MALDI-TOF analysis can identify a series of signature tryptic peptides that define each flagellin (Fig. 4A). For example, FljK, FljL, and FljM/FljN can be differentiated by the N-terminal peptides (3,257 m/z for FljK, 3,240 m/z for FljL, and 3,296 m/z for FljM/FljN) (Fig. 4A and B). As the $\Delta fljJLMNO$ (*fljK*⁺) and $\Delta fljJKMNO$ (*fljL*⁺) strains were motile, our initial analysis of the mass spectra obtained confirmed that these two strains produced filaments comprised of only FljK and FljL, respectively (Fig. 4C and Table 2).

FljM and FljN exhibit the highest level of similarity (95%) differing by only two detected peptides (1,878 and 1,934 m/z and the C-terminal FljN/FljO 2,390 m/z peptide) (8). A comparison of the 3,296 m/z FljM/FljN common peptide throughout the data set identified a consistent drop in the intensity of

FIG. 5. A selection of electron micrographs of flagellar filaments from NA1000 flagellin gene deletion mutants. These images show some of the mutants that exhibit a short filament distribution but still have the occasional filament of wild-type length, for example, the $\Delta fljKL$ mutant. The $\Delta fljJ$ mutant is shown for comparison. White arrowheads identify hooks on ejected filaments. Bar, 0.5 μm .

came less noisy when fewer flagellins were utilized. For example, the $\Delta fljJLMNO$ and $\Delta fljJKMNO$ filaments showed a lot less variation in filament length than the wild-type or the $\Delta fljK$ or $\Delta fljL$ strain (see Fig. S1). The majority of short filament mutants also exhibited tight distributions around their average length (see Fig. S1). One mutant to show a clear change in its distribution profile was the $\Delta fljKL$ double mutant (see Fig. S1). The majority of observed filaments grouped in a distribution between 0.75 and 2.0 μm . However, a number of filaments (3 out of 15) had lengths of approximately 5 μm . $\Delta fljKL$ motile cells were recorded to have typical swimming speeds of approximately 47 $\mu\text{m/s}$. One explanation for this distribution is that these filaments are fragile and are prone to breaking during the method used to isolate them. A similar phenomenon could explain other mutants that also possessed a low number of outlying filaments of normal length, for example, the $\Delta fljLM$ and $\Delta fljJLM$ strains. The same assumption also holds for the

observed changes in length distributions between wild-type filaments and the $\Delta fljJLMNO$ and $\Delta fljJKMNO$ filaments. It is plausible that a filament made from one or two flagellins will be stronger due to the packing nature of the subunits.

In contrast to filament length, the pitch length was found to lie within a typical distribution range of 0.5 to 1.5 μm for all mutants (Fig. 3; see also Fig. S1 in the supplemental material). Even though the distributions appear similar, ANOVA defined the data set as significant ($P = 0.006$). The average pitch length of all the mutants was $0.96 \pm 0.23 \mu\text{m}$, compared to $0.99 \pm 0.16 \mu\text{m}$ for the wild type. Comparing each mutant to the wild type using the t test found that only four mutants showed a significant ($P < 0.001$) change in their pitch lengths (Fig. 3). In contrast, the pitch height fluctuated more significantly, ranging between 1.3- and 1.9-fold higher than the wild type ($P < 0.001$) (Fig. 3). t test results of the pitch height data compared to the wild type showed that 14 mutants returned a P value of less than 0.001.

FIG. 7. Ability of plasmid-based expression of the flagellins to restore motility in the $\Delta fljJLMNO$ (Δfla) mutant. (A) Motility swarms after 5 days at 30°C on a single motility agar plate containing kanamycin and xylose (see Materials and Methods) of pBX-*fljJ*, -*fljK*, -*fljL*, -*fljM*, -*fljN*, or -*fljO*, the vector control pBXMCS-2, and the wild type. (B) Motility swarms on a single motility agar plate of the vector control pRXMCS-2, pRX-*fljK*, and pRX-*fljM* compared to the wild type. (C) Immunoblot analysis of flagellin levels in the strains shown in panels A and B. Below each lane are shown the swimming speeds of selected motile mutants. For comparison, the $\Delta fljJLMNO$ and $\Delta fljJLMNO$ mutants are also included on these gels.

type but comparable to the speeds of both strains with plasmid-based expression of *fljK* and *fljL* (Fig. 7C). Plasmid-based expression of the flagellins *fljN* and *fljO* showed that these two flagellins were also able to support filament assembly in the $\Delta fljJLMNO$ mutant (Fig. 7A). This analysis would therefore suggest that, out of the six flagellins, all but FljJ are able to form a filament alone. However, a plausible reason for a non-motile phenotype for FljJ was the low expression levels obtained. Importantly, the levels obtained were higher than those of some of the motile deletion mutants, and no FljJ was detected in spent supernatants (data not shown).

Does stability of the flagellins play a role in filament assembly in *C. crescentus*? The strongest correlation observed throughout this analysis has been between the swimming speeds and flagellin concentration as determined by immunoblot analysis. We were interested in determining whether flagellin stability also played a role in the motility phenotypes observed. We tested the stability of the flagellins in the $\Delta fljJKL$ and $\Delta fljMNO$ mutants in comparison to the wild type by the addition of spectinomycin (which inhibits protein synthesis) to mid-log-phase cultures (Fig. 8) (2, 4). This allowed us to differentiate between the stability of the α - and β -flagellins. In the wild type the detectable pool of flagellins is stable over 120 min (Fig. 8). Similar stabilities were also observed for the $\Delta fljMNO$ mutant, suggesting that the α -flagellins remain stable. In contrast, a 20% decrease of the β -flagellins levels was observed in the $\Delta fljJKL$ mutant at 120 min (Fig. 8) ($P < 0.02$, by ANOVA). This finding is consistent with the motility phenotypes of these

two triple mutants: the $\Delta fljMNO$ strain possesses a wild-type swimming speed, whereas the $\Delta fljJKL$ mutant swims at a speed of 46 $\mu\text{m/s}$ (Fig. 3).

Protein stability can be affected by the protein's spatial location, as well as other factors. To compare the stability of unsecreted flagellins, we deleted the flagellin modification gene *flmD* in NA1000 and the $\Delta fljJKL$ and $\Delta fljMNO$ mutants (Fig. 8). The *flmD* gene product is predicted to be involved in the terminal transferase reaction that couples the glycosylation of the *C. crescentus* flagellins (16, 22). In many systems studied, nonglycosylated flagellins are not secreted (25).

The $\Delta flmD$ mutant generated in this study produced no detectable flagellins in spent culture supernatants, and the mutant was nonmotile (data not shown). Steady-state flagellin levels are reduced in a $\Delta flmD$ mutant, and the flagellins produced have altered migration patterns in SDS-Tricine gels (Fig. 8B). The stability phenotypes of the flagellins in the $\Delta flmD$ strain compared to those of the $\Delta fljMNO$ $\Delta flmD$ and $\Delta fljJKL$ $\Delta flmD$ strains reflect the stability observed in the *flmD*⁺ parental strains when each individual zero time point was taken as 100% (Fig. 8A). The single $\Delta flmD$ mutant showed an intermediate phenotype, while in the $\Delta fljMNO$ strain the remaining flagellins (FljJ, FljK, and FljL) showed the highest level of stability. In contrast, the $\Delta fljJKL$ mutant showed complete degradation of the flagellins FljM, FljN, and FljO over the 120 min assayed. Therefore, the data suggest that the stability of the α - and β -flagellins plays, if any, a very minor role in the system. The data also suggest that glycosylation

FIG. 8. (A) Comparison of the stability of the flagellins in the wild type (squares) and the $\Delta fljJ$ (circles) and $\Delta fljMNO$ (triangles) mutants in $flmD^+$ (solid lines) and $\Delta flmD$ (dashed lines) strains. Stability of the flagellins was assayed by inhibiting protein synthesis with the addition of spectinomycin (see Materials and Methods) over 120 min. A slight change in flagellin stability is observed in the $\Delta fljJ$ $flmD^+$ mutant, suggesting that the β -flagellins are not as stable as the α -flagellins. The analysis of the corresponding $\Delta flmD$ strains confirms the ability to differentiate the α - and β -flagellins by their stability. These data were statistically significant ($P < 0.02$). (B) Selected immunoblots of flagellins in the strains used in the assays shown in panel A. Flagellin levels in general were lower in the $\Delta flmD$ mutants. The mobilities of the flagellins through SDS-PAGE were also changed. Blots are aligned using protein markers and the nonspecific band seen above the flagellin band.

plays a role in protecting flagellins from degradation prior to secretion in *C. crescentus*.

DISCUSSION

Approximately 45% of flagellated bacteria encode multiple flagellins in their genomes, suggesting that there is a selective advantage to having multiple copies of these genes (Fig. 1). Previous studies of a small fraction of bacterial species belonging to this group of flagellar systems suggest that a level of redundancy is always evident with respect to flagellin utilization (8, 19, 20, 28, 29, 37). In this study we have investigated the working hypothesis that there is a structural aspect to filament assembly that drives the maintenance of multiple flagellin genes. We chose the model system of *C. crescentus* to follow this hypothesis. We have shown that all six flagellins utilized by *C. crescentus* exhibit significant structural redundancy in a collection of 23 mutants. In fact, the observed redundancy in

C. crescentus is unlike all other systems studied, where there is strong evidence to suggest that at least one flagellin is essential for motility (20, 29, 37). The level of observed redundancy is so great that a functional filament can be formed from FljK, FljL, FljM, FljN, or FljO alone. In contrast, the flagellin FljJ was unable to form a functional filament, by itself, even though MALDI-TOF analysis showed that it was being utilized in other mutants and the wild type.

Our data suggest that the β -flagellins do not contribute to the ability of *C. crescentus* to achieve wild-type swimming speeds. The majority of motile $\Delta fljMNO$ strain derivatives exhibited swimming speeds comparable to the swimming speed of the wild type (Fig. 3). The MALDI-TOF analysis, albeit not strictly quantitative, showed that FljM/FljN is a major component of the wild-type filament. Integration of peak volumes from LC-MS/MS analysis agreed with this observation and suggests that FljK and FljM are the most abundant flagellins (data not shown). Consistently, all $\Delta fljKMX$ mutant combinations exhibited a significant drop in the swimming speeds as a result of shorter filaments (Fig. 3).

Flagellin gene expression is regulated at both the transcriptional and translational levels (10). Previous work has shown that the transcription of the β -flagellins, i.e., *fljMNO*, is directly regulated by CtrA rather than by the flagellar-specific transcriptional activator FlbD (15, 21). FlbD is responsible for coupling flagellar gene transcription to the assembly pathway in *C. crescentus* (43, 44). Interestingly, we also observed that a basal level of translation of the β -flagellins is seen in the $\Delta fliF$ mutant. The flagellar-independent timing of β -flagellin production suggests that a plausible role of the β -flagellins is to maintain filament length or integrity during the SW cell stage of the cell cycle.

This analysis of flagellin utilization in *C. crescentus* has shown that there is no strict requirement for ordered assembly of the flagellar filament (7). This would imply that after secretion, the incorporation of individual flagellins is stochastic, meaning that any of the six flagellins can be incorporated into the growing structure. We suggest that when order is observed in *C. crescentus* filaments, it is a reflection of a regulatory input prior to or during secretion (7). This model also explains the apparent unbalanced utilization of the flagellins in the population to generate filaments. Our data, however, are unable to pinpoint what type of regulation this could be. It is plausible that the observed order of flagellins in filaments from other bacterial species could also be a reflection of a regulatory input during flagellin secretion or production rather than a structural requirement. Utilizing an internal checkpoint to dictate flagellin secretion rather than regulating flagellin incorporation into the filament outside the cell is consistent with the proposed environmental use of alternative flagellins. However, it is important to note that in other systems there may be alternative selective pressures driving ordered filament assembly over what we observe in *C. crescentus*.

The presence of a regulatory checkpoint prior to or during secretion of flagellins is consistent with expressing the flagellins in a flagellar-independent manner. A maximum speed of only 36.9 $\mu\text{m/s}$ was achieved on expressing *fljK* from a plasmid. In comparison, the wild type and a number of our mutants reach speeds between 55 and 48 $\mu\text{m/s}$ (Fig. 3). Two posttranscriptional regulators control flagellin translation in response to the

flagellar assembly pathway in *C. crescentus*, FlaF and FlbT, and are likely candidates for the source of the observed regulation (5, 24, 27).

Other than a regulatory mechanism to control secretion, the data suggest that generating a flagellar filament from a subset of flagellins in response to environmental changes would not promote a significant selective advantage. We argue that, to benefit in a given environment, *C. crescentus* cells would require a specific combination of flagellins that would promote a significant increase in speed compared to that of the wild type. However, the data suggest otherwise as all but one mutant showed a reduced average speed compared to that of the wild type, with eight mutants exhibiting a significant drop in speed (Fig. 3). Schneider and Doetsch (38) showed that the pitch height and pitch length (also known as wavelength) are critical components of the filaments' physical properties that aid the efficiency of swimming in viscous environments (38). They argued that efficiency was maintained when the height of the wave and its length were modified. From our analysis, we have shown that all but four mutants had similar pitch lengths. In contrast, for 14 of the flagellin mutants the pitch height significantly changed (Fig. 3). As no consistent change in both pitch height and length was observed, the data suggest that no specific flagellin combination would present a selective advantage for *C. crescentus* to swim away or toward a given stimulus at a faster speed by changing the composition of filaments made in the population.

In conclusion, we have observed a striking and unexpected level of structural redundancy among the flagellins of *C. crescentus*. We show that flagellin protein levels and filament length correlate and are key to the production of a flagellar filament of optimal length for optimal swimming behavior.

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