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Identification of the *fliI* and *fliJ* Components of the *Caulobacter* Flagellar Type III Protein Secretion System

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***Caulobacter crescentus* is motile by virtue of a polar flagellum assembled during the predivisional stage of the cell cycle. Three mutant strains in which flagellar assembly was blocked at an early stage were isolated. The mutations in these strains mapped to an operon of two genes, *fliI* and *fliJ*, both of which are necessary for motility. *fliI* encodes a 50-kDa polypeptide whose sequence is closely related to that of the *Salmonella typhimurium* FliI protein, an ATPase thought to energize the export of flagellar subunits across the cytoplasmic membrane through a type III protein secretion system. *fliJ* encodes a 16-kDa hydrophilic protein of unknown function. Epistasis experiments demonstrated that the *fliIJ* operon is located in class II of the *C. crescentus* flagellar regulatory hierarchy, suggesting that the gene products act at an early stage in flagellar assembly. The expression of *fliIJ* is induced midway through the cell cycle, coincident with other class II operons, but the FliI protein remains present throughout the cell cycle. Subcellular fractionation showed that FliI is present both in the cytoplasm and in association with the membrane. Mutational analysis of FliI showed that two highly conserved amino acid residues in a bipartite ATP binding motif are necessary for flagellar assembly.**

The bacterium *Caulobacter crescentus* constructs a single polar flagellum during each cell cycle. The synthesis of the flagellum in *C. crescentus* has been studied extensively as a model for cell cycle regulation of gene expression (5, 14). A transcriptional hierarchy activated by cues from the cell cycle controls expression of a network of 50 or more genes that are necessary to assemble an active flagellum. Considerably less is known about how the products of this array of genes assemble the flagellar structure at the cell pole. In particular, the manner in which flagellar-subunit proteins are exported across the cytoplasmic membrane to their site of assembly in *C. crescentus* and other bacteria is poorly understood. As part of our efforts to understand flagellar biogenesis, we describe here the characterization of an operon encoding two components necessary for flagellar assembly, at least one of which is thought to be involved in flagellar protein secretion.

Substructures of the flagellum are assembled sequentially from the cytoplasmic membrane outward (21, 40). The most complex of these substructures, the basal body, is anchored in the cell envelope. The basal body contains an axial rod and three rings, with each residing in one of the layers of the envelope of the gram-negative cell. The rod terminates in a curved hook just beyond the cell surface, which connects the rod to the helical filament that extends out from the cell. The ordered assembly process arises at least in part from the hierarchical nature of flagellar gene expression, in which the filament subunits (flagellins) are the last gene products expressed. The *C. crescentus* flagellar hierarchy has four classes of genes which are sequentially activated during the cell cycle (5, 14). The function of each gene product in a class is necessary for activation of expression of genes in lower classes; in addition, there tends to be negative autoregulation of gene expression within each class. The CtrA transcription factor resides at the pinnacle of the known hierarchy and activates class II operons (32). Class II gene products include the MS ring subunit (FliF), proteins known to be associated with the MS ring (e.g., FliG and FliM), several proteins thought to contribute to the flagellar export apparatus (FlbF, FliQ, and FliR), and the transcription factors (σ^{54} and FlbD) necessary for expression of class III and IV genes. Class III genes encode various axial components, out to and including the hook subunit, whereas class IV genes encode the major flagellin subunits.

Though the vast majority of the protein mass of the flagellum resides outside the cytoplasmic membrane, none of the axial rod, hook, and filament subunit proteins possess signal peptide sequences at their amino termini. These subunits are apparently not exported by the bacterial general secretory pathway. Rather, they are thought to exit the cytoplasm via a specialized pore located in the center of the MS ring, thereby entering an axial channel which carries them to the ultimate site of polymerization (25, 28). This localized flagellum-specific protein export apparatus has been proposed to be made up of eight or more proteins which have homology with components of the type III virulence secretion pathway found in several gram-negative pathogens (4, 16, 25, 36, 41, 45). Most of these proteins are predicted from sequence analysis to reside in the cytoplasmic membrane, perhaps forming a gated channel in the core of the MS ring.

How the flagellar export apparatus accomplishes the selection of flagellar proteins and their subsequent translocation across the membrane is not understood, but *C. crescentus* is a useful experimental organism with which to address such questions. The majority of the genes necessary to construct a flagellum in *C. crescentus*, including those encoding most of the membrane proteins thought to make up the export channel, have previously been identified (13, 33, 38, 51). Flagellar assembly in *C. crescentus* is subject to profound temporal and spatial constraints, which leads to interesting questions vis-à-vis the flagellar export apparatus. Is the export channel localized specifically to the pole of the swarmer cell, and if so,

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant genotype or description
Strains	
NA1000	Synchronizable derivative of *C. crescentus* CB15
LS107	NA1000 Δ*bla* (ampicillin sensitive)
JM63	NA1000 *fliI*
LS2481	NA1000 *fliJ*::Tn*5*
LS2482	NA1000 *fliI*::Tn*5*
Plasmids	
pCS267	pLAFR5 cosmid containing 20-kb *C. crescentus* genomic DNA insert, including *fliIJ* operon
pMR20	10.7-kb Tet^r broad-host-range vector with polylinker for cloning
pRKlac290	20-kb Tet^r broad-host-range low-copy-number vector for making *lacZ* transcriptional fusions
pCS271	pMR20 with 3.2-kb *Bam*HI-*Sac*I fragment containing *fliI* and *fliJ* truncated at 3′ end at codon 129
pCS274	pMR20 with 3-kb *Bam*HI-*Pst*I fragment containing *fliI* truncated at 3′ end at codon 440
pCS277	pMR20 containing 1.6-kb *Nco*I codon with *fliI* truncated at 3′ end at codon 261
pCS292	pMR20 with 0.9-kb *Nco*I-*Sac*I fragment containing 3′ portion of *fliI* (starting at codon 261) and *fliJ* truncated at codon 129
pCS321	pRKlac290 with 2.2-kb *Pvu*II fragment containing *fliIJ*
pCS322	pRKlac290 with 1.7-kb *Pvu*II-*Pst*I fragment containing *fliI*, truncated at 3′ end at codon 440
pET21c	Amp^r vector with T7 promoter for protein overexpression and generation of carboxyl-terminal polyhistidine tag (Novagen)
pCS312	*fliI* cloned as 1.4-kb *Bam*HI-*Xho*I fragment (generated by PCR) into pET21c
pCS321	*fliIJ* locus cloned as 2.2-kb *Pvu*II fragment into pRKlac290, generating a transcriptional fusion to *lacZ*
pCS322	*fliI* locus cloned as 1.7-kb *Pvu*II-*Pst*I fragment into pRKlac290, generating a transcriptional fusion to *lacZ*

how? During the *Caulobacter* cell cycle, the flagellated swarmer cell differentiates into a nonmotile stalked cell by shedding its flagellum and growing a stalk at the same pole. Is the flagellar export apparatus degraded when the swarmer cell differentiates into a stalked cell, as is the case for the FliF MS ring protein and chemoreceptors (3, 18)?

We describe here the identification of an operon encoding a critical component of the export pathway, the FliI protein. FliI is a highly conserved ATP-binding protein that has been proposed to couple energy from ATP hydrolysis to translocation of substrates through the type III membrane channel (9, 12, 25, 50). In *C. crescentus*, *fliI* is closely followed by a second open reading frame (ORF) encoding a small hydrophilic protein that is also necessary for flagellar assembly and that we postulate functions as a chaperone in flagellar-subunit secretion.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth supplemented with ampicillin (50 μg/ml), tetracycline (10 μg/ml), or kanamycin (50 μg/ml) as necessary (37). *C. crescentus* strains were grown at 30°C in either peptone-yeast extract (PYE) or M2 minimal salts-glucose (M2G) medium (10), supplemented with tetracycline (2 μg/ml) or kanamycin (20 μg/ml), as necessary.

Mutant screens, mapping, and DNA sequencing. Two of the nonmotile *C. crescentus* mutant strains used were generated by Tn*5* transposon insertion mutagenesis. Suicide plasmid pSUP202 carrying Tn*5* (Km^r) was introduced into *C. crescentus* LS107 (NA1000 Δ*bla*) by conjugation as previously described (10). Cultures of mutagenized cells were plated in semisolid swarm agar containing kanamycin. Colonies which failed to form swarms were screened for mutations in class II flagellar genes. Since strains with mutations in class II genes tend to show cell division defects, cultures of nonswarming strains were examined for filamentous cells. Nonmotile filamentous strains were tested for the restoration of motility upon introduction of plasmids containing known class II operons. Mutations in two strains were not complemented by any known flagellar genes and were further characterized. The Tn*5* insertions in these strains were transduced into wild-type strain NA1000 (selecting for Km^r) to generate strains LS2481 and LS2482, respectively.

Strain JM63 was isolated as a nonswarming mutant generated by mutagenesis of NA1000 with UV light. Cells were irradiated with a germicidal lamp in shallow dishes at 14 ergs/mm^2 for 75 s. Under these conditions, a 4% survival rate was obtained. After a 2-h postmutagenesis incubation to account for segregation lag, independent pools were plated for single colonies at room temperature. JM63 was identified as a strain that failed to migrate through semisolid agar. Microscopic examination of JM63 revealed the lack of a flagellar filament.

Cosmid pCS267 was obtained by mating a cosmid library of *C. crescentus* genomic DNA (2) from *E. coli* S17-1 into JM63 and screening for the restoration of motility in semisolid agar. Restriction fragments of pCS267 were subcloned into plasmid pMR20, a 10.7-kb tetracycline-resistant broad-host-range plasmid. pMR20 constructs were mated from *E. coli* S17-1 into *C. crescentus* mutant strains with selection for Tet^r. Tetracycline-resistant isolates were examined for motility by stabbing on semisolid agar and by microscopic analysis. Although the strains used were recombination proficient, it is unlikely that motility arose from rare recombination events between plasmids and chromosomal loci. Microscopic analyses of samples taken directly from selection plates and after growth in PYE broth showed little difference in the numbers of motile swarmer cells between complemented strains and wild-type controls.

DNA sequencing of relevant subclones was carried out by the dideoxynucleotide chain termination method (39), with single- or double-stranded DNA as the template. Large portions of the sequence were determined by automated sequencing at the Beckman Center PAN Facility at Stanford University. Sequence compilation was carried out by using the Genetics Computer Group package of the University of Wisconsin (6); database searching employed the BLAST algorithm.

The sites of Tn*5* insertions in LS2481 and LS2482 were determined by amplifying fragments of the *fliIJ* operon flanking Tn*5* by PCR. In each case, one of the PCR primers was located in the Tn*5* sequence (primer R3, ACCATGTTAGGA GGTCACAT) and the other was located either 5′ to *fliI* or 3′ to *fliJ*. The resulting PCR products were subcloned into the pCRII cloning vector (Invitrogen Corp.) for sequencing to identify insertion sites.

Electron microscopy. Bacterial cultures were grown in PYE medium at 30°C to an optical density at 600 nm (OD_{600}) of 0.5, transferred to a sterile 1.5-ml microcentrifuge tube, and concentrated by gentle centrifugation. The supernatant was removed, and pellets were resuspended by gentle pipetting into the residual fluid. The concentrated cell suspension was transferred to a Formvar-coated grid and treated with uranyl acetate. Grids were examined with a Phillips electron microscope.

Production of His-tagged FliI and generation of antiserum. The *fliI* coding region was amplified by PCR with the following primers: FliI5′Bam, CGAACG TATGGATCCCGCTGACCATC, and FliI3′Xho, ACTCACTCTCGAGGATC TGG. Primers were synthesized by Operon Technologies (Alameda, Calif.). The resulting 1.4-kb PCR product was digested with *Bam*HI and *Xho*I and ligated into the same sites in the pET21c plasmid expression vector (Novagen). The resulting 6.7-kb plasmid, pCS312, expresses an N-terminal fusion of the T7 leader to residue 14 of FliI. This FliI construct is fused to a polyhistidine tag at residue 440. The expression of FliI-His from the T7 promoter was induced in *E. coli* BL21(λDE3)/pCS312 by the addition of isopropyl-β-D-thiogalactoside (IPTG). IPTG was added to cultures grown at 30°C in Luria-Bertani broth to an OD_{600} of 0.5. Cells were harvested 2 h after the addition of IPTG. Under these conditions, the 50-kDa FliI-His protein comprised roughly 30% of total cell protein. Cells were lysed by sonication in ice-cold buffer (20 mM Tris [pH 7.9], 5 mM imidazole, 0.5 M NaCl) and centrifuged at 20,000 × *g* for 20 min. A significant fraction of FliI-His (30 to 50%) pelleted with the insoluble material at this stage, with the fraction of insoluble FliI-His increasing significantly with growth temperature (~75% when grown at 37°C) and when cell pellets were frozen and thawed prior to lysing. Only the soluble portion was used for further

purification. FliI-His was purified from the soluble fraction by chromatography on His Bind resin (Novagen) according to the manufacturer's instructions. The purified FliI-His protein showed a strong tendency to precipitate when it was frozen and thawed. Rabbits were injected with a slurry of soluble and precipitated proteins at Berkeley Antibody Company (Berkeley, Calif.) by conventional protocols. Booster injections with the same purified FliI-His were given at 3-week intervals. Because the initial immune response was relatively weak, after 3 months, rabbits were given booster injections twice with purified FliI-His excised from a 10% polyacrylamide gel.

Western blots. To examine flagellar-protein levels by Western blotting, total cellular protein from *C. crescentus* strains was prepared by lysing cells directly in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (37). Proteins were resolved by SDS-PAGE on 10% acrylamide gels and transferred to Immobilon P membranes by standard Western blotting techniques. Blots were probed with primary antiserum at a dilution of 1:5,000, followed by secondary antibody (goat anti-rabbit immunoglobulin G; Boehringer Mannheim; 1:20,000 dilution). Bound antigen was visualized by chemiluminescence. Antisera to various other *C. crescentus* flagellar proteins have been described previously (17, 18).

Cytoplasmic and membrane protein fractions were prepared from a log-phase NA1000 culture as previously described (17). Briefly, cells were harvested by centrifugation and resuspended in 10 mM Tris (pH 7.5)–1 mM EDTA. Cells were lysed by treatment with lysozyme and sonication. Unbroken cells were removed by low-speed centrifugation. The membrane fraction was isolated by ultracentrifugation at 150,000 × *g* for 2 h. The remaining supernatant proteins constituted the cytoplasmic fraction. To prepare extracellular proteins, a separate 100-ml sample of the initial culture was centrifuged at 8,000 × *g* for 20 min. Then the culture supernatant was passed through a 0.45-μm-pore-size filter. Proteins were precipitated from the supernatant by the addition of ammonium sulfate to 50% saturation at 4°C. Precipitated materials were collected by centrifugation at 9,000 rpm for 20 min. The pellet was washed with 70% ethanol, dried under vacuum, and resuspended in 1 ml of 10 mM Tris (pH 7.5)–1 mM EDTA.

Assay of transcriptional activity. DNA fragments were inserted into the multiple cloning site of the pRKlac290 vector to generate transcriptional fusions to *lacZ* (44). The resulting clones were mated from the *E. coli* host strain S17-1 into NA1000 (10). β-Galactosidase activity (27) was measured at 30°C with log-phase cultures grown in PYE-tetracycline. Assays were done at least in duplicate with a minimum of two independent cultures for each promoter construct. The standard error was ≤10% in each case.

Cell regulation of *fliJ* expression was assayed by immunoprecipitation of newly synthesized β-galactosidase from synchronous cell cultures. *C. crescentus* cultures for synchronization were grown in M2G medium, and swarmer cells were isolated by Ludox density gradient centrifugation (11). Swarmer cells were released into fresh M2G medium at 30°C at an OD_{600} of 0.3 to 0.4. Progression through the cell cycle was monitored microscopically. The initial swarmer cell populations contained <2% predivisional cells. Samples for immunoprecipitation were labelled and prepared as described previously (45).

Construction and analysis of site-directed mutants. Site-directed mutagenesis of the *fliI* K170 and D254 codons was done as described previously (45) with an Altered Sites kit (Promega, Madison, Wis.). The mutagenic oligonucleotides were FliMutKA, GGGCGTCGGCGCGTCGGTGCTG, and FliMutDA, GCCT GATGGCCTCGGTCACC. Mutant *fliI* genes were cloned into pMR20 and the high-copy-number vector pJS14 and introduced by conjugation into *C. crescentus* strains for complementation and dominance analyses.

Nucleotide sequence accession number. The sequence of the 2,252-bp *Pvu*II fragment has been deposited in the GenBank database under accession no. U93180.

RESULTS

Identification of new genes involved in flagellar assembly. To identify new *C. crescentus* genes involved in flagellar assembly, nonmotile mutant strains were generated by UV and Tn*5* mutagenesis. Strains JM63 (UV), LS2481 (Tn*5*), and LS2482 (Tn*5*) form tight nonspreading colonies on semisolid swarm agar, compared to the diffuse swarm of the wild-type parent (NA1000) (Fig. 1A). Microscopic observations of these three strains grown in liquid PYE medium indicated a complete lack of motility, and further analysis by electron microscopy showed no apparent external flagellar structure (i.e., hook or filament). In addition to being nonmotile, the average cell length in cultures of each strain grown in PYE medium was longer than that of the wild type (Fig. 1C and D). Abnormalities in cell division are often associated with mutations in class II flagellar genes (for examples, see references 13 and 51), perhaps due to an as-yet-undefined coupling between early stages of flagellar assembly and the cell cycle.

A cosmid library containing *C. crescentus* genomic DNA was mated into JM63, and a single cosmid (pCS267) that restored motility to this strain was isolated (Fig. 1B). pCS267 was subsequently found to restore motility to strains LS2481 and LS2482 as well. Fragments of the pCS267 genomic DNA insert were subcloned to identify the smallest region which could complement the motility defects of these strains. A restriction map of the relevant region is shown in Fig. 2. The locations of the Tn*5* insertions in LS2481 and LS2482 were determined by Southern blotting (not shown). The minimal restriction fragment that restored motility to all three mutants was a 2.2-kb *Pvu*II fragment (Fig. 2). Only JM63 and LS2482 were complemented by a 2.1-kb *Bam*HI-*Pst*I fragment overlapping the left side of the *Pvu*II fragment, whereas only LS2481 was complemented by a 0.9-kb *Nco*I-*Sac*I fragment contained within the right side of the *Pvu*II fragment. Thus, the JM63 and LS2482 mutations were likely in a separate gene(s) from the LS2481 mutation.

The sequence of the 2,252-bp *Pvu*II fragment is shown in Fig. 3. It contains three extended ORFs with patterns of codon usage and third-position G+C bias that are typical of *C. crescentus* translated sequences, suggesting that these are legitimate genes which encode proteins. The first gene has been designated *fliI*, as the predicted product is a 50-kDa polypeptide whose amino acid sequence is very similar to those of the *S. typhimurium* FliI protein (46) (Fig. 4) and FliI proteins from several other bacteria. FliI is an ATPase which is necessary for flagellar assembly; it is thought to be involved directly in the secretion of flagellar subunit proteins across the cytoplasmic membrane (7, 12, 50). Other FliI homologs, such as the *Yersinia enterocolitica* YscN protein, are involved in the secretion of nonflagellar virulence factors (50). Despite careful and repeated sequencing of the proximal end of *C. crescentus fliI*, no ATG or GTG codons were found. The TTG sequence at nucleotide 345 is tentatively assigned here to encode the start codon, as UUG is occasionally used as a start codon in bacteria, a reasonable Shine-Dalgarno sequence is located 6 to 10 bp upstream, and the length of the protein generated from this start site is consistent with those of other FliI proteins and their homologs. The Tn*5* insertion in LS2482 is located in codon 82 of the *fliI* gene (Fig. 3). The mutation causing the loss of motility in JM63 is most likely in the *fliI* gene as well, as motility was restored to both JM63 and LS2482 by the presence in *trans* of only the *fliI* gene, truncated at a *Pst*I site located at the extreme 3′ end of the coding region.

The gene immediately 3′ to *fliI*, which we have designated *fliJ*, is predicted to encode an 18-kDa polypeptide. The Tn*5* insertion in LS2481 is between codons 69 and 70 of this gene (Fig. 3), and motility was restored to LS2481 by fragments extending to the *Sac*I site near the 3′ end of the gene. Thus, this gene product is necessary for flagellar assembly. The polypeptide encoded has an abundance of charged residues, slightly more acidic than basic, and its structure is predicted by a variety of algorithms to be almost exclusively alpha-helical. BLAST searches with this sequence showed weak similarities to helical coiled-coil domains of myosins and tropomyosins and to the *fliJ* gene products of *E. coli* and *Bacillus subtilis* (not shown). These genes are located immediately 3′ to the *fliI* genes of *B. subtilis* and *E. coli*; indeed, virtually all *fliI* homologs, including those involved in virulence factor secretion, are followed by genes encoding small (14- to 18-kDa), acidic, hydrophilic polypeptides that typically show little sequence similarity. How these proteins function in flagellar assembly or virulence factor secretion is unknown.

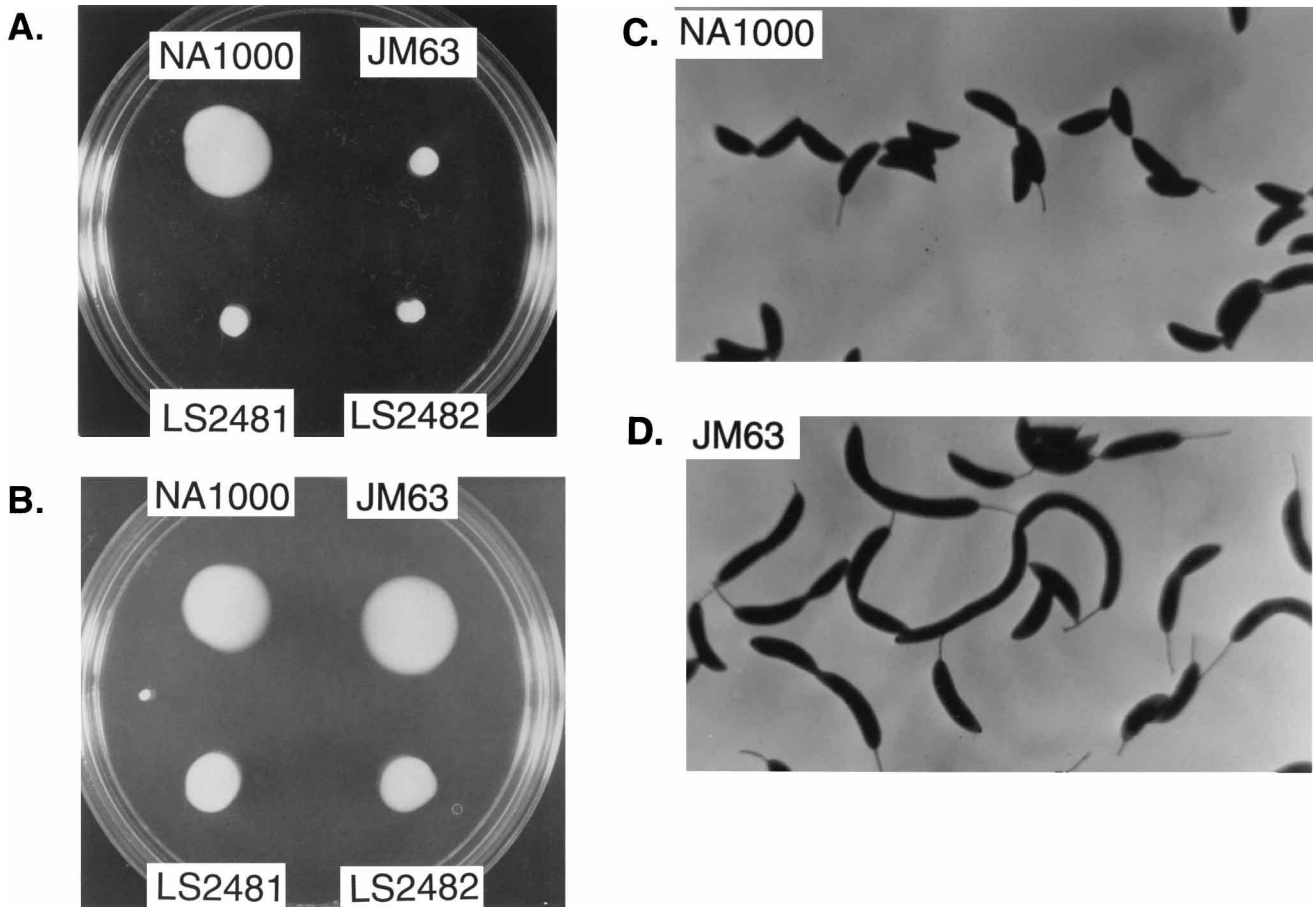

FIG. 1. Phenotype of three new nonmotile *C. crescentus* mutants. (A) Wild-type (NA1000) and mutant strains were stabbed onto semisolid PYE plates (0.25% agar) and incubated at 30°C for 4 days. (B) Wild-type and mutant strains transformed with cosmid pCS267, containing the intact *fliI* and *fliJ* genes, were stabbed onto semisolid PYE agar as described for panel A. (C) Electron micrograph of uranyl acetate-stained NA1000 cells, showing normal swarmer, stalk, and predivisional cells. (D) Electron micrograph of JM63 culture, showing significant numbers of elongated predivisional cells. Micrographs of strain LS2481 and LS2482 (not depicted) showed similar cell distribution and morphology.

An ORF begins immediately downstream of *fliJ*. The predicted product of this coding region has a signal sequence that is characteristic of secreted proteins which are acylated at a conserved cysteine residue (31), but otherwise this partial ORF has no significant resemblance to any protein in available databases. Because motility was restored to Tn*5* insertion strains with mutations in the *fliI* and *fliJ* genes by DNA fragments terminating at the *Sac*I site upstream of this ORF, there is no evidence at this time that the product of this ORF has a role in motility.

The location of the *fliIJ* locus on the physical map of the *C. crescentus* chromosome was determined by hybridization to Southern blots of genomic DNA. On the map, the *fliIJ* operon is located in the interval from 2500 to 2600 (data not shown), the region to which the *podU* locus has previously been mapped (48). The *podU* mutant strain SC2185 is nonmotile and mildly filamentous but is unusual in that it is resistant to the pole-specific *Caulobacter* phage φCbK. During the course of this work, it was discovered by one of our colleagues that the nonmotile phenotype of SC2185 is in fact due to a Tn*5* insertion in *fliI* (34). The phage resistance of SC2185 is due to a second unlinked mutation, as transduction of the *fliI*::Tn*5* locus from SC2185 into NA1000 resulted in a nonmotile φCbK-sensitive strain. (LS2482 is φCbK-sensitive as well.) We also found that the *fliIJ* operon is separated by only 2.7 kb from *ctrA*, with the two loci oriented in opposite directions (43) (Fig. 2B). CtrA is the only known class I master regulator of the *C. crescentus* flagellar regulatory hierarchy (32). *ctrA* is not present in cosmid pCS267, as the boundary of the fragment inserted in this cosmid is located between the *Bam*HI and *Sac*I sites in the region between *fliIJ* and *ctrA*. Preliminary sequencing and analysis of the region between *ctrA* and *fliIJ* revealed no notable similarities to genes with established functions.

Location of the *fliIJ* operon in the flagellar hierarchy. The *C. crescentus* flagellar hierarchy has four classes of genes which are sequentially activated during the cell cycle (see the introduction). To assess the position of the *fliI* and *fliJ* genes in the flagellar regulatory hierarchy, low-copy-number plasmids carrying transcriptional fusions of flagellar-gene promoters to *lacZ* were introduced into *fliI* and *fliJ* mutant strains and β-galactosidase activities were measured and compared to that of wild-type NA1000 (Table 2). Class II promoters showed elevated expression, whereas the expression of class III promoters was greatly reduced. These results were confirmed by Western blot analysis of the amounts of class II, III, and IV flagellar-gene products in the *fliI* and *fliJ* mutants (not shown). Higher levels of class II FliM and FliF proteins were present in the mutant strains compared to those in the wild type, but the class III FlgH protein (L ring) and class IV 25- and 27-kDa flagellins were undetectable in the mutants. Thus, *fliI* and *fliJ* are class II

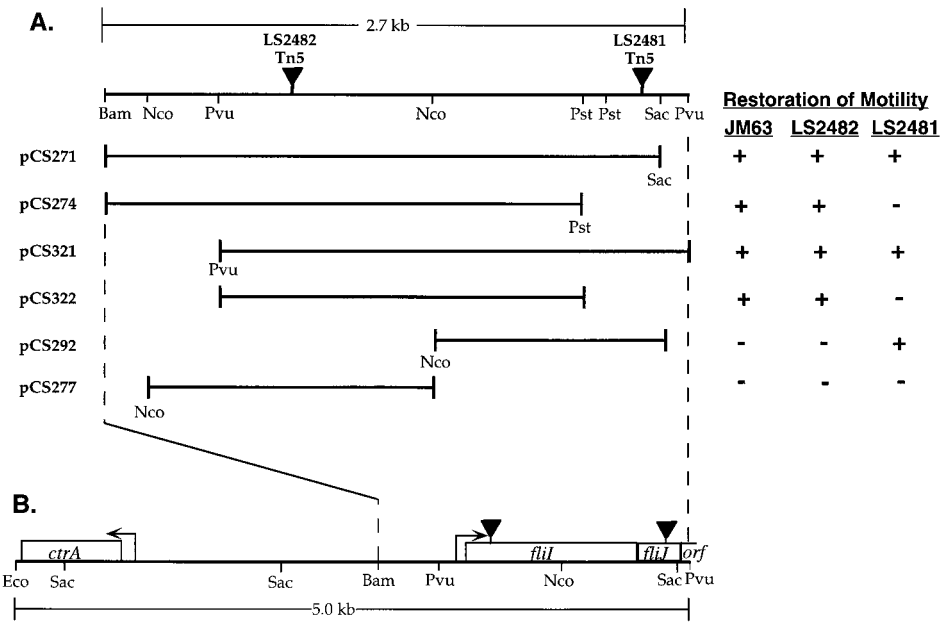

FIG. 2. Complementation of the nonmotile phenotype of mutant strains. (A) Schematic of the locations of relevant restriction sites and Tn*5* insertions in the LS2481 and LS2482 chromosomes. Restriction fragments from cosmid pCS267 were cloned into either pMR20 or pRKlac290 and introduced by conjugation into mutant strains, with selection for Tet^r. Motility was assayed by using swarm plates and microscopy. (B) Map of the chromosomal region containing the *fliIJ* operon and *ctrA*. Dashed lines extending from panel A show the locations of *Bam*HI and *Pvu*II sites flanking the region shown in panel A. Tn*5* insertions are indicated by inverted triangles as they are in panel A. Arrows indicate the orientations of the *fliIJ* and *ctrA* loci. The boundaries of the *fliI*, *fliJ*, and *orf* coding regions are based on the sequence data in Fig. 3. More detailed characterization of the *ctrA* locus can be found elsewhere (32).

genes whose products act at an early stage of flagellar assembly. As noted above, the aberrant cell division phenotypes of JM63, LS2481, and LS2482 (Fig. 1D) are consistent with mutations in class II genes, as such mutants typically show defects in cell division when they are grown on PYE medium, whereas class III and IV mutants are nonmotile but normal in length.

Expression of the *fliIJ* operon was examined by using transcriptional fusions to *lacZ* in a low-copy-number plasmid (pRKlac290). A DNA fragment extending from 0.3 kb upstream of the *fliI* coding region to the *Pst*I site near the 3′ end of *fliI* was inserted into pRKlac290 to yield plasmid pCS322. The introduction of pCS322 into wild-type NA1000 produced roughly 1,000 units of β-galactosidase activity (Table 3). As expected for expression of a class II operon, transcriptional activity increased roughly twofold in other class II mutants but was not affected by mutations in class III genes (Table 3). Most class II promoters are dependent on the CtrA transcription factor for activity (32). The activity of the *fliI*::*lacZ* transcriptional fusion was examined in a strain (LS2195) that contains a temperature-sensitive mutation in *ctrA*. β-Galactosidase activity was reduced by 48% at 2 h after a shift to the nonpermissive temperature (37°C). The expression of several other class II promoters was similarly reduced after LS2195 was shifted to 37°C (32).

An examination of *fliIJ* promoter activity over the course of the *C. crescentus* cell cycle showed that expression peaked at 0.5 to 0.6 division units (Fig. 5), as with other class II promoters (29, 33, 38, 45, 51). As expected, this preceded flagellin expression and the appearance of motility indicative of filament assembly at 0.8 to 0.9 division units. A transcriptional fusion in which the 3′ end of the fused fragment was located at the *Pvu*II site distal to *fliJ* (pCS321), rather than in *fliI* (pCS322), resulted in a slight increase in *lacZ* activity, with an identical cell cycle expression pattern (not shown), suggesting that transcription from the *fliIJ* promoter continues into the downstream ORF.

The expression of motility-related genes in *C. crescentus* is generally limited to the predivisional-cell stage, but the persistence of motility-related proteins during the cell cycle can vary. By definition, flagellar structural proteins remain through the flagellated swarmer cell stage. The FliF protein, which composes the MS ring in the cytoplasmic membrane, is rapidly degraded when the swarmer is converted to a stalked cell (18). Likewise, the McpA chemoreceptor protein is proteolytically removed at the swarmer-to-stalk transition (3). In contrast, the FliL protein, which is necessary for flagellar motor function in *C. crescentus*, remains throughout the cell cycle (17). To examine FliI protein levels, it was necessary to generate antibodies to the protein. A polyhistidine-tagged version of the *C. crescentus* FliI protein was purified and used to generate rabbit polyclonal antibodies. The resulting antiserum recognized the 50-kDa FliI protein on Western blots of whole-cell lysates of NA1000 (Fig. 6A). This band was absent in LS2482, the *fliI* null strain. FliI levels were greatly increased in a strain expressing *fliI* from a multicopy plasmid and increased slightly in LS2481, as expected from our analysis of *fliI* promoter activity. The JM63 strain clearly did not produce a stable 50-kDa FliI. A weak band at ca. 48 kDa was present, but whether this represented a truncated or unstable mutant FliI was unclear. We do not yet know the nature of the FliI mutation in JM63.

FliI levels during the *C. crescentus* cell cycle were examined in protein samples taken from a synchronous culture, with FliF and McpA protein levels tested as controls (Fig. 6B). The amount of FliI protein was nearly constant throughout the cell cycle, including the swarmer-to-stalked-cell transition period during which FliF and McpA were degraded. Induction of the *fliI* promoter in the predivisional stage probably accounted for the slight rise in FliI levels late in the cell cycle; induction of

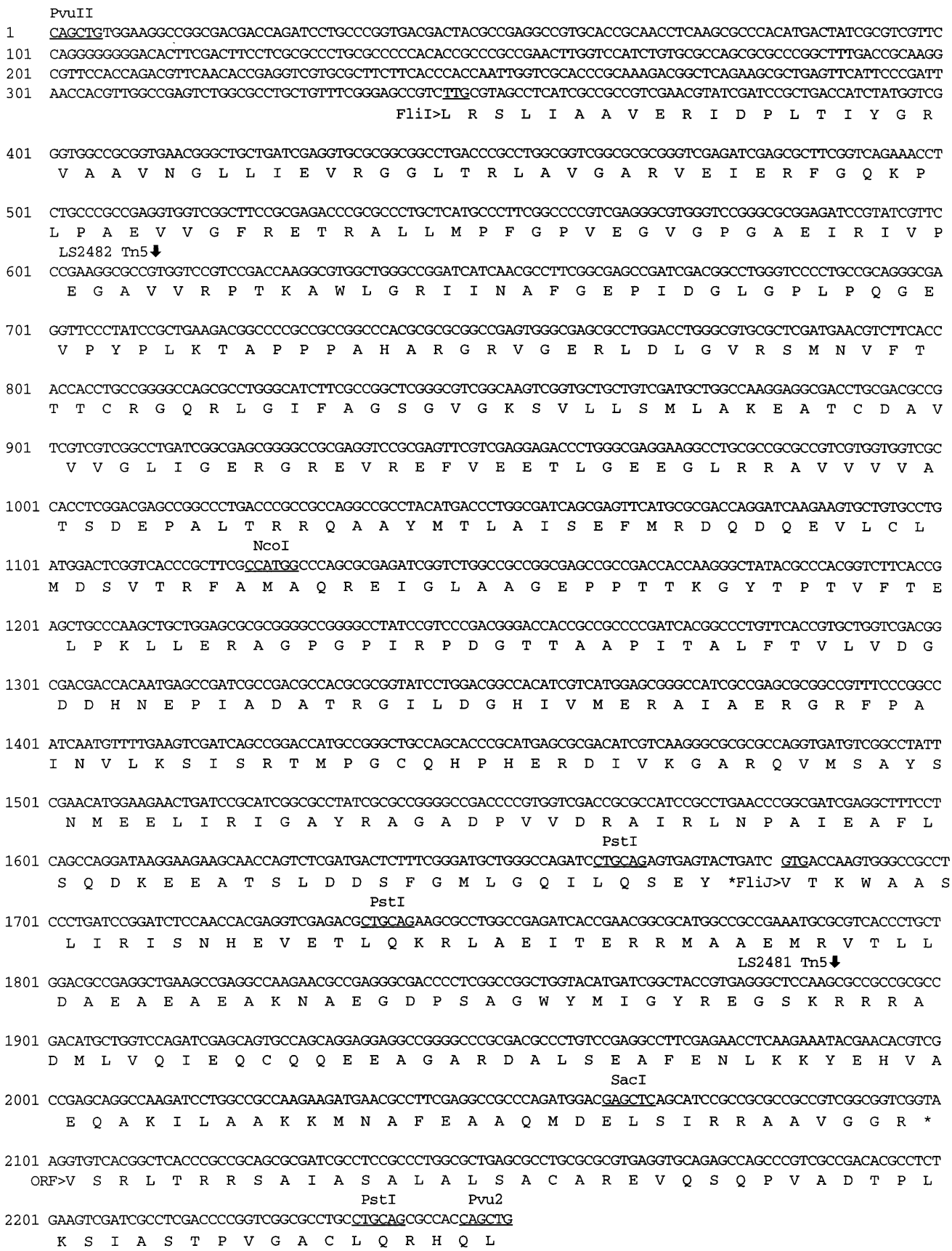

FIG. 3. Sequence of the 2.2-kb *Pvu*II fragment encompassing the *fliIJ* locus. The predicted polypeptide sequences of FliI, FliJ, and the 3′ ORF frame are indicated beneath the nucleotide sequence. The locations of Tn*5* insertions in *fliI* and *fliJ* are shown, and the important restriction sites noted in Fig. 2 are underlined.

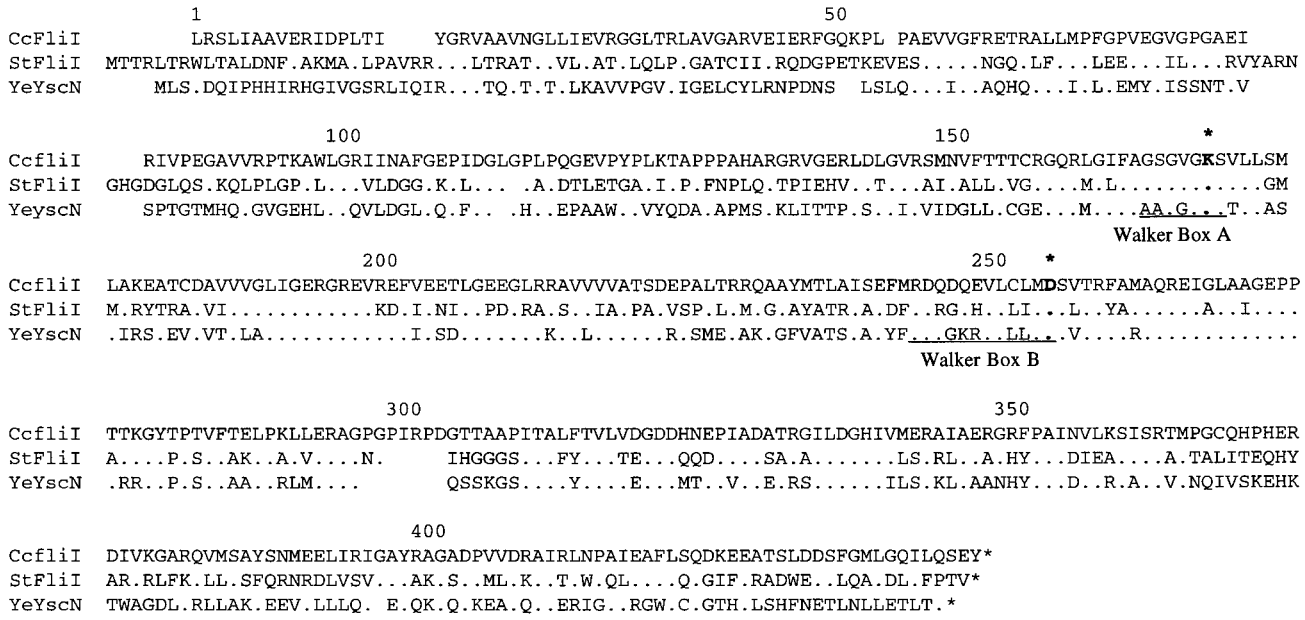

FIG. 4. Comparison of *C. crescentus* FliI and FliJ primary sequences with those of other homologs. The alignment of *C. crescentus* FliI (CcFliI) with *S. typhimurium* FliI (StFliI) (46) and *Y. enterocolitica* YscN (YeYscN) (50) was generated by using the Pileup program (6). Dots in the *S. typhimurium* FliI and *Y. enterocolitica* YscN sequences indicate residues that are identical to those in *C. crescentus* FliI. Blank spaces indicate gaps introduced into the alignment by the Pileup algorithm to maximize similarity. Walker box residues mutated in subsequent experiments are indicated by asterisks. In pairwise comparisons, the *C. crescentus* FliI sequence has 45% identity (54% similarity) with *S. typhimurium* FliI and 43% identity (63% similarity) with *Y. enterocolitica* YscN.

the *fliF* and *mcpA* promoters in predivisional cells generated a dramatic increase in the levels of these proteins. Despite the constitutive presence of FliI, Kornacker and Newton (20) have shown that the flagellar hook subunit can be exported only during the predivisional and swarmer stages; perhaps the availability of export factors other than FliI or the activities of FliI and other components of the export system are cell cycle regulated.

Subcellular location of FliI. Although FliI and its homologs are clearly necessary for type III protein secretion pathway function, neither *C. crescentus* FliI nor any of its homologs has any significantly hydrophobic regions that would be expected to span a membrane. This suggests that FliI is likely to be located in the cytoplasm, but one model for FliI function predicts that like the SecA component of the general secretory pathway, it can reversibly associate with the membrane secretory pore (see Discussion for more details). To analyze the subcellular location of FliI, *C. crescentus* cells were fractionated into cytoplasmic, membrane, and extracellular components. The presence of FliI in each fraction was assayed by Western blotting (Fig. 7). FliI protein was found in both cytoplasmic and membrane fractions. To verify that this behavior was not an artifact of inefficient separation of cytoplasmic and membrane fractions, each sample was also probed with antisera to the CcrM DNA methyltransferase (44), FlgH flagellar ring protein (17), and flagellins (Fig. 7). CcrM was observed only in the cytoplasmic fraction, and FlgH was observed only in the membrane fraction. Flagellins, which are extremely abundant proteins, appeared primarily in the extracellular fraction due to cell-cycle-dependent ejection of the flagellum and shearing of the filament during sample preparation. The faint

TABLE 2. Flagellar-gene expression in LS2481 and LS2482

Promoter(s)	β-Galactosidase activity[a]		
	NA1000 (wild type)	LS2481 (*fliI*::Tn*5*)	LS2482 (*fliI*::Tn*5*)
Class II			
fliF	1,170 (1.00)	2,440 (2.09)	2,420 (2.07)
fliLM	970 (1.00)	1,990 (2.05)	1,890 (1.95)
fliQ	1,190 (1.00)	2,240 (1.88)	2,080 (1.75)
Class III			
flgE	1,290 (1.00)	111 (0.08)	117 (0.09)
flgH	675 (1.00)	115 (0.17)	210 (0.31)
flgI	243 (1.00)	50 (0.21)	59 (0.24)
Class IV, *fljL*	1,940 (1.00)	139 (0.07)	166 (0.09)

[a] Transcriptional fusions of class II and III flagellar promoters to *lacZ* on the pRKlac290 plasmid vector were mated into NA1000 and each of the mutant strains. β-Galactosidase activities were measured in cultures grown in PYE broth at 30°C to an OD_{600} of 0.3 to 0.6 and were calculated in Miller units (27). Data in parentheses are the relative β-galactosidase activities compared to the activity of the same promoter construct in NA1000.

TABLE 3. Effects of flagellar-gene mutations on *fliI* transcription

Strain	Mutant gene	Mutant class	*fliI* promoter activity[a]
NA1000	Wild type	II	990 (1.00)
LS1218	*fliF*	II	1,680 (1.70)
LS391	*fliQ*	II	1,640 (1.66)
LS2482	*fliI*	II	2,370 (2.39)
LS2481	*fliJ*	II	1,630 (1.64)
SC1117	*flgH*	III	1,080 (1.09)
SC518	*flgF*	III	1,130 (1.14)

[a] Data are for strains containing plasmid pCS322, bearing a *fliI*::*lacZ* transcriptional fusion. β-Galactosidase activities were measured in cultures grown in PYE broth at 30°C to an OD_{600} of 0.3 to 0.6 and were calculated in Miller units (27). Data in parentheses are the relative β-galactosidase activities compared to the activity of the same promoter construct in NA1000.

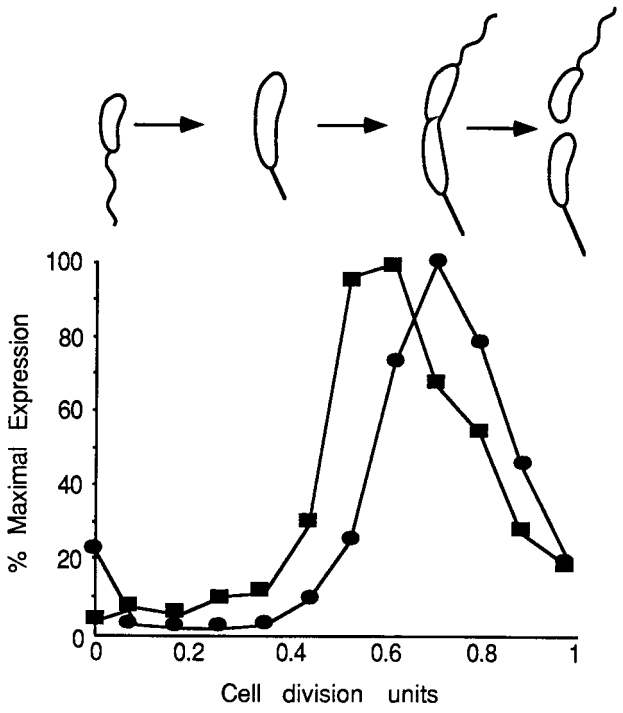

FIG. 5. Activity of the *fliIJ* promoter during the cell cycle. *fliIJ* expression (squares) and the expression of flagellins (circles) are shown. Expression of the *fliIJ* promoter was examined in a synchronous culture of strain NA1000/pCS322, which contains a transcriptional fusion of *fliI* to *lacZ*. At 15-min intervals, culture samples were exposed to [^{35}S]methionine to label newly synthesized proteins. β-Galactosidase was immunoprecipitated with anti-β-galactosidase antibody and analyzed by SDS-PAGE and autoradiography. As a control, flagellins were immunoprecipitated with antiflagellin antibodies. Signals were quantitated with a PhosphorImager (Molecular Dynamics).

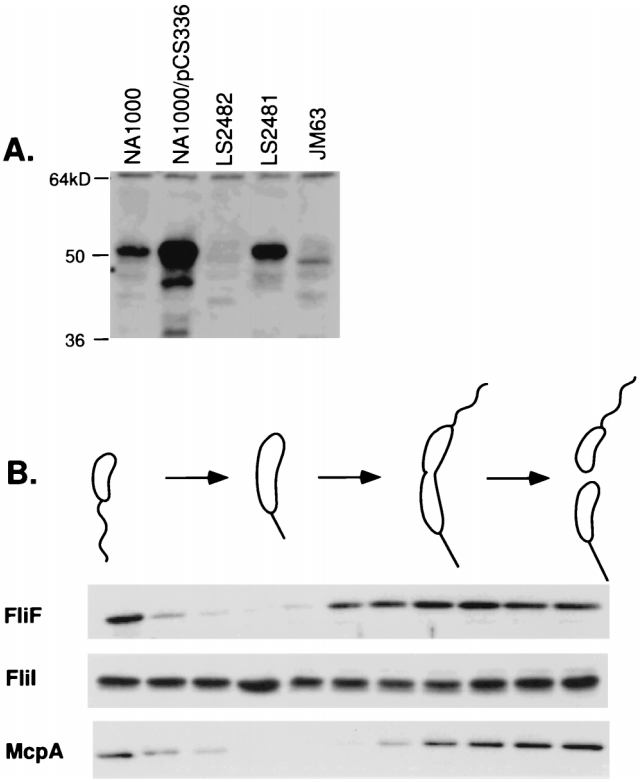

FIG. 6. Western blot analysis of FliI. (A) Testing of antiserum specificity. Equivalent amounts of protein from all the strains indicated were resolved, blotted, and probed with polyclonal antiserum generated to His-tagged FliI. The 50-kDa protein detected in NA1000 was absent in LS2482. The levels of this protein were slightly enhanced in a *fliF* mutant strain and in LS2482 complemented with cosmid pCS267. (B) Analysis of FliI protein levels through the cell cycle. Samples representing equivalent culture volumes were taken at 15-min intervals from a synchronous culture and examined by Western blotting with antisera to the FliI, FliF, and McpA proteins.

band of cytoplasmic flagellin likely reflected newly synthesized but as-yet-unsecreted protein.

Mutational analysis of FliI. FliI and its homologs that act in virulence factor export in gram-negative pathogens (e.g., *Y. enterocolitica* YscN and *S. typhimurium* InvC) show significant relatedness to the β-subunit of the F_0F_1 ATPase and have common Walker box motifs (Fig. 4A), which are involved in ATP binding (12, 47). To determine whether this ATP binding motif is important for the function of the *C. crescentus* FliI protein, two highly conserved amino acid residues in the Walker box motifs were altered. Changing aspartate 254 to alanine (D254A mutant) destroyed the activity of FliI in vivo, based on the inability of this mutant to restore motility to strains JM63 and LS2482 when it was expressed from a low- or high-copy-number plasmid (Table 4). Likewise, a mutation of lysine 170 to alanine (K170A) or glycine (K170G) resulted in the loss of complementation ability. Interestingly, the K170A mutant also greatly reduced the motility of wild-type *C. crescentus* when it was expressed from either a low- or high-copy-number plasmid vector. This dominant negative effect, which was not observed with the K170G and D254A alleles, was evident as both a reduction in swarm size on soft agar and a severe reduction in the number of motile swarmers observed microscopically. This observation suggests that the K170A mutant protein can interfere with the function of wild-type FliI, at least when it is more abundant than wild-type FliI.

DISCUSSION

Here we have described the isolation of an operon of at least two genes, *fliI* and *fliJ*, whose functions are necessary for motility in *C. crescentus*. These genes reside in class II of the flagellar genetic regulatory hierarchy, indicating that the products of these genes function early in flagellar assembly. This is consistent with the involvement of the FliI protein in secretion of flagellar subunits across the cytoplasmic membrane, as previously suggested by the following observations. Vogler et al. (46) found that an *S. typhimurium* temperature-sensitive *fliI* mutant was unable to regrow sheared flagellar filaments from intact basal bodies at the nonpermissive temperature. It was subsequently recognized that FliI homologs are also present in type III systems used for secretion of nonflagellar virulence factors in various gram-negative pathogens (4, 9, 16, 50), implying that FliI homologs have an integral role in the secretory process.

The structural and functional properties of the type III secretory system are poorly understood. FliI may have a role similar to that of SecA of the general secretory pathway, since both of them bind and hydrolyze ATP (9, 12, 23). SecA recognizes the N-terminal signal peptide of nascent polypeptides destined for secretion while the proteins are still being translated. The secretion signal for type III pathway substrates also appears to be at or near the N termini of the substrates that have been investigated (22, 42), including the *C. crescentus*

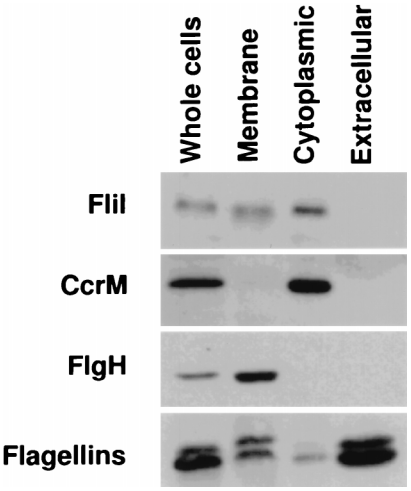

FIG. 7. Subcellular localization of FliI. Cytoplasmic, membrane, and extracellular protein fractions were prepared from a mid-log-phase PYE culture of *C. crescentus* NA1000, as described in Materials and Methods. Equivalent amounts of protein from all fractions, including a whole-cell control, were resolved and blotted onto a polyvinylidene difluoride membrane. These were probed separately with polyclonal antisera recognizing the FliI protein, CcrM DNA methyltransferase, FlgH flagellar ring protein, and *C. crescentus* flagellins. There were two major *C. crescentus* flagellins, of 27 and 25 kDa. The apparent difference in migration between flagellins in the lanes labelled "Whole cells" and "membrane" was probably due to a gel anomaly.

flagellar hook protein (20), but this signal is not obvious as a conserved sequence motif. In the general secretory pathway, the SecA-substrate complex, often in conjunction with the SecB chaperone, interacts with the SecE/SecY secretory pore, at which point ATP binding by SecA drives partial insertion of the SecA-substrate complex into the membrane (8, 19, 24). Hydrolysis of ATP allows SecA to retract from the membrane, but the substrate protein continues through. Whether FliI can bind substrates directly, what effect it has on substrates, and how it interacts with components of the membrane pore remain to be determined. Our observation that FliI is found both in the cytoplasm and in association with the membrane is consistent with a SecA-like transient association with the membrane pore of the type III system.

TABLE 4. Effects of mutant *fliI* alleles on motility

Strain background	Plasmid-borne *fliI* allele	Motility with *fliI* allele expressed from[a]:	
		pMR20 (low copy number)[b]	pJS14 (high copy number)[c]
LS2482 (*fliI*::Tn*5*)	Wild type	+	+
	K171A	−	−
	K171G	−	−
	D255A	−	−
NA1000 (wild type)	Wild type	+	+
	K171A	±	−
	K171G	+	+
	D255A	+	+

[a] Motility was assayed by microscopic examination of logarithmically growing PYE cultures. +, motile swarmer cells at levels similar to the wild type level; −, motile swarmer cells present at <1% of the wild-type level; ±, motile swarmer cells present at ~5 to 10% of the wild-type level.

[b] The pMR20 vector is based on the RK2 replicon and is estimated to be present at two to five copies per cell.

[c] The pJS14 vector is present in *C. crescentus* at a minimum of 10 to 20 copies per cell (41).

Mutations in the ATP binding domains (Walker boxes A and B) of FliI homologs (*S. typhimurium* FliI [7], *S. typhimurium* InvC [9], and *Y. enterocolitica* YscN [50]) block flagellar assembly or virulence factor secretion, suggesting that ATP binding and possibly ATP hydrolysis are essential for secretion via the type III pathway. We found that mutational alterations of lysine 170 in box A and Asp254 in box B of the *C. crescentus* FliI protein blocked flagellar assembly. In work published during the preparation of this paper, Fan and Macnab (12) showed that mutations at K188 of *S. typhimurium* FliI (homologous to *C. crescentus* FliI K170) resulted in a 100-fold reduction in ATPase activity and a 10-fold increase in K_m for ATP. *S. typhimurium* FliI K188 mutations showed a dominant negative effect similar to that observed with *C. crescentus* FliI K170 mutations. If FliI functions as part of a multiprotein complex, as discussed above, one possible explanation for the dominant effect is that the mutant competes with wild-type FliI for complex formation; a mutant protein defective in ATP binding and/or hydrolysis may enter the complex but subsequently fail to support protein secretion (12). Interestingly, no dominant negative effect on virulence factor secretion was seen with the InvC K165E mutant (9) or the YscN Δ169-177 mutant (50). The *C. crescentus* FliI D254A mutant generated here did not have a dominant effect, but the mutant protein appeared to be completely insoluble when it was expressed in *E. coli*, suggesting that the structure of the mutant protein is abnormal. Whether this abnormality is due directly to the substituted amino acid or indirectly to defective interaction with ATP is unknown.

The function of FliJ in flagellar assembly remains obscure. Virtually all of the known *fliI* homologs are followed on the chromosome by a gene encoding a small protein that is similar in size and hydrophilicity to FliJ, suggesting a functional linkage between FliI and these gene products. We are exploring the possibility that these small proteins are functionally analogous to the *Yersinia* SycE (14-kDa) and SycH (15-kDa) proteins, which interact with the YopE and YopH substrates of the type III secretory system to prevent premature association of these proteins in the cytoplasm (49). The action of these proteins also resembles somewhat that of the 17-kDa SecB chaperone of the general secretory pathway, which associates with proteins destined for export to prevent their folding in the cytoplasm (35).

The flagellar regulatory hierarchy has been assumed to operate by the recognition of completed structural elements of the flagellum and the activation of expression of the next class of genes. How structures are recognized and how this information is transduced to the transcriptional apparatus are unknown. All class II structural components, with the exception of the transcription factors σ^{54} and FlbD, appear to be associated with the inner membrane directly or indirectly via the MS ring (FliF protein). It could be rationalized that this entire inner membrane structure, presumably containing the type III secretory pore, must be complete and active to allow the transition to class III gene transcription. The observation reported here that the cytoplasmic components of class II which are necessary for secretion are also necessary for the transition to class III expression suggests not only that a structure is necessary but also that secretion itself is required. In *S. typhimurium*, the FlgM anti-sigma factor is exported from the cell via the flagellum-specific secretion system to release inhibition of class III flagellar gene expression (15). A negative regulator of tran-

scription has also been found to be exported through the *Yersinia* type III secretory system, resulting in the induction of expression of *yop* genes (30). No flagellar regulatory factor(s) exported through the *C. crescentus* flagellar channel is known yet, but a candidate locus has been identified genetically. This locus has been designated *bfa* (for bypass of flagellar assembly) because mutants express class III genes in the presence of class II mutations, including mutations in components of the secretory system (26). Understanding the connections among targeted protein secretion, flagellar assembly, and flagellar-gene regulation in *C. crescentus* and other bacteria clearly requires more understanding of the structure and activity of the type III secretory system.

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