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Multiple Structural Proteins Are Required for Both Transcriptional Activation and Negative Autoregulation of *Caulobacter crescentus* Flagellar Genes

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The periodic and sequential expression of flagellar (*fla*) genes in the *Caulobacter crescentus* cell cycle depends on their organization into levels I to IV of a regulatory hierarchy in which genes at the top of the hierarchy are expressed early in the cell cycle and are required for the later expression of genes below them. In these studies, we have examined the regulatory role of level II *fliF* operon, which is located near the top of the hierarchy. The last gene in the *fliF* operon, *flbD*, encodes a transcriptional factor required for activation of σ^{54}-dependent promoters at levels III and IV and negative autoregulation of the level II *fliF* promoter. We have physically mapped the *fliF* operon, identified four new genes in the transcription unit, and determined that the organization of these genes is 5′-*fliF-fliG-flbE-fliN-flbD*-3′. Three of the genes encode homologs of the MS ring protein (FliF) and two switch proteins (FliG and FliN) of enteric bacteria, and the fourth encodes a predicted protein (FlbE) without obvious similarities to known bacterial proteins. We have introduced nonpolar mutations in each of the open reading frames and shown that all of the newly identified genes (*fliF*, *fliG*, *flbE*, and *fliN*) are required in addition to *flbD* for activation of the σ^{54}-dependent *flgK* and *flbG* promoters at level III. In contrast, *fliF*, *fliG*, and *flbE*, but not *fliN*, are required in addition to *flbD* for negative autoregulation of the level II *fliF* promoter. The simplest interpretation of these results is that the requirements of FlbD in transcriptional activation and repression are not identical, and we speculate that FlbD function is subject to dual or overlapping controls. We also discuss the requirement of multiple structural genes for regulation of levels II and III genes and suggest that *fla* gene expression in *C. crescentus* may be coupled to two checkpoints in flagellum assembly.

The procaryotic flagellum is a complex organelle composed of three structural elements: the basal body or motor, which is embedded in the membrane and peptidoglycan layers of the cell envelope; the external hook, which attaches the basal body to the flagellar filament; and the filament itself, which rotates to move the cell (35, 62). In the dimorphic bacterium *Caulobacter crescentus*, the flagellum is assembled at one pole of the dividing cell late in the cell cycle and then segregates with the motile swarmer cell at division. Its biosynthesis requires the activity of 40 to 50 flagellar (*fla*) genes (15), and, as in the enteric bacteria, expression of these genes is regulated by their organization in a regulatory hierarchy in which expression of genes at the top of the hierarchy is required for expression of genes lower in the hierarchy (reviewed in references 7 and 44). In the *C. crescentus* hierarchy, there is also evidence that *fla* gene interactions mediate the negative, as well as the positive, transcriptional control of gene expression (45, 49, 70).

A unique feature of flagellum biosynthesis in *C. crescentus* is the cell cycle regulation of *fla* gene expression; the genes are expressed periodically in a sequence that corresponds to the order in which the gene products are assembled. The time of expression depends in part on the relative position of these genes in the hierarchy (48), with genes at the top of the hierarchy expressed earliest in the cell cycle and those at the bottom expressed late. These results and those from epistasis experiments (45, 70) suggest that the genes required for flagellum biosynthesis are organized into four levels, I to IV (see Fig. 1 below and references 45 and 66). Many *C. crescentus* *fla* genes have been recently renamed to correspond with the names of their homologs in *Escherichia coli* and *Salmonella typhimurium* (see references 23 and 25), as summarized in Table 1.

The level IV genes at the bottom of the hierarchy are the 25- and 27-kDa flagellin genes, and just above them are the level III genes that encode the hook, ring, and axial proteins. Both level III and level IV genes are transcribed from specialized σ^{54} promoters (40, 42, 46). The level II genes are near the top of the hierarchy. They include the level IIB genes of the *fliL* operon (63, 73) and *fliQ* operon (13) and genes at the *flaO* locus, which we show in this paper are contained within the *fliF* operon. These transcription units have a common consensus promoter, and we have grouped them together at level IIB. The level IIB promoters are unlike others described in bacteria and are recognized by a specialized sigma factor designated as σ^{Y} (see Table 1 and reference 5) or σ^{R} (8). *rpoN*, which encodes σ^{54} (3, 8), appears to have a similar promoter, and we have placed it at the same level (3). The *flhA* gene has been assigned to level IIA because its 5′ sequence is not similar to the *fliF*, *fliL*, and *fliQ* promoter consensus (54, 56). Level I genes at the top of the hierarchy have not been identified, and this level has been reserved for genes that couple expression of level II genes to cell cycle events (Fig. 1).

Several findings indicate that genes in the *fliF* operon play a pivotal role in regulating the transcriptional cascade. The last gene of this operon encodes the FlbD protein (53), which belongs to a family of specific activators of σ^{54} promoters that includes the *E. coli* NtrC protein (33). Genetic analysis has shown that activation by the *flbD* gene product requires *ftr*

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TABLE 1. Nomenclature of *C. crescentus fla* genes

C. crescentus gene		*E. coli* or *S. typhimurium* gene	Function or structure	Promoter[a]	Reference(s)
Old	Revised				
Level IIA[b]					
flbF	*flhA*	*flhA*	Export (?)	σ^X	54, 56
Level IIB					
flaO	*fliF*	*fliF*	MS ring	σ^Y	66 and this report
	fliG	*fliGm*	Switch		This report
	flbE	None	Unknown		This report
	fliN	*fliN*	Switch		This report
flbD	*flbD*	None	Transcription Regulation		53
flbO	*fliL*	*fliL*	Unknown	σ^Y	73
	fliM	*fliM*	Switch		
flaS	*fliQ*	*fliQ*	Unknown	σ^Y	13
	fliR	*fliR*	Unknown		
Level III					
flbG[c]			Hook initiation (?)	σ^{54}	41
(flaJ)[c]		*(fliK)*	Hook length		59, 60
flbH	*flgD*	*flgD*	Hook initiation (?)		41
flaK	*flgE*	*flgE*	Hook		49
flaN	*flgK*	*flgK*	HAP1	σ^{54}	41
flaQ			?		
flaC	*flgF*	*flgF*	Proximal rod	σ^{54}	11
flaB	*flgG*	*flgG*	Distal rod		
flaD		None	E ring (?)		21
flbN	*flgH*	*flgH*	L ring		12
flaP	*flgI*	*flgI*	P ring	σ^{54}	29
Level IV					
flgJ	*fljJ*	*hag*	29-kDa flagellin	σ?	18
flaL	*fljL*		27-kDa flagellin	σ^{54}	38
flgK	*fljK*		25-kDa flagellin	σ^{54}	38

[a] The promoter type is listed for the first gene in an operon. Level IIA and Level IIB promoters are presumed to be recognized by different sigma factors, provisionally designated σ^X and σ^Y, on the basis of sequence comparisons.

[b] Level designations apply only to *C. crescentus* genes.

[c] *flaJ*, which is homologous with *fliK*, may be part of the *flbG* ORF (41).

(flagellar transcription regulation) sequence elements located approximately 100 bp from the level III *flbG* (4, 42, 43) and *flgK* (20, 42, 43) promoters. Pairs of *ftr* sequence elements have now been identified in the promoters of all published σ^{54} *fla* genes, and FlbD has been proposed as a global regulator of these level III and IV genes (69). An *ftr* sequence element, *ftr4*, also overlaps the *fliF* promoter and has been implicated in the negative autoregulation and the correct cell cycle timing of this level II transcription unit (45, 66). Results of in vitro assays with highly purified components have now demonstrated that FlbD activates the σ^{54} *flbG* promoter by its interaction with the upstream *ftr* sequences and represses the σ^Y-dependent *fliF* promoter by binding to *ftr4* (5, 6). Genetic results suggest, however, that the *flbD* gene alone is not sufficient for activation of transcription from level III promoters in vivo (9, 45, 70). The experiments described here were undertaken to determine the role of other genes in the *fliF* operon in the regulation of FlbD activity.

We report below an analysis of the *fliF* transcription unit that has identified four new open reading frames (ORFs) upstream from *flbD* and shown that the organization of the genes in the operon is 5′-*fliF-fliG-flbE-fliN-flbD*-3′. The genes identified encode predicted homologs of the MS ring protein FliF and the two switch proteins FliG and FliN of enteric bacteria, plus a protein, FlbE, without obvious similarity to known bacterial proteins. To examine the role of these proteins in *fla* regulation, we have introduced nonpolar mutations in each of the newly identified ORFs and tested their effects on transcriptional regulation in vivo. Our results show that all of these genes are required in addition to *flbD* for activation of the σ^{54} *flbG* and *flgK* promoters at level III of the hierarchy but that only *fliF-fliG-flbE* are required in addition to *flbD* for the negative autoregulation of *fliF*. These results indicate that multiple structural genes in the *fliF* operon are required for transcriptional regulation by FlbD. We discuss the possibility that the two functions of FlbD as a transcription activator and repressor may be subject to dual or overlapping controls.

MATERIALS AND METHODS

Strains and media. *C. crescentus* strains were all derived from strain CB15 (ATCC 19089), and they are listed in Table 2. Plasmids were introduced into *C. crescentus* by triparental matings or electroporation and selected on minimal M2 medium supplemented with 0.2% glucose or on peptone-yeast extract (PYE) supplemented with spectinomycin as described previously (51). Following electroporation, the integration of pUC-based plasmids in *C. crescentus* was selected for on PYE medium containing spectinomycin and streptomycin. Motility was assayed in swarm plates containing 0.5× PYE plus 0.35% agar. *E. coli* cells were routinely grown in ML (51). Drugs were included at the following concentrations (micrograms per

TABLE 2. *C. crescentus* strains and genotypes

Strain	Genotype	Source or reference
CB15	Wild type	ATCC 19089
SC290	*fliG138*	25
PC7070	*rec-526* Sm^r *zbe*::Tn*5*	50
PC7812	II.1::Ω	48
PC5509	*flbE188*::Tn*5*	51
SC1967	*flbD620*::Tn*5*	15
PC8638	*flbD358*	NTG mutagenesis
PC8652	*fliG372*	NTG mutagenesis
PC8660	*fliF380*	NTG mutagenesis
PC8661	*fliF381*	NTG mutagenesis
PC8683	*fliF403*	NTG mutagenesis
PC8742	*fliF462*	NTG mutagenesis
PC8750	*fliG470*	NTG mutagenesis
PC8760	*flbD480*(Ts)	NTG mutagenesis
PC8795	*flbD215*	NTG mutagenesis
PC9263	Δ*flbE* II.1::Ω	Materials and Methods
PC9264	Δ*fliN* II.1::Ω	Materials and Methods
PC9249	Δ(*fliF-flbD*) *fliF*::Ω	Materials and Methods
PC9255	Δ(*fliF-flbD*)*rec-526 zbe*::Tn*5*	φ(PC7070) × PC9249
PC9256	*fliF462 rec-526 zbe*::Tn*5*	φ(PC7070) × PC8742
PC9257	*fliG470 rec-526 zbe*::Tn*5*	φ(PC7070) × PC8750
PC9258	*flibD480*(Ts) rec-526 zbe::Tn*5*	φ(PC7070) × PC8760
PC9259	*flbD215 rec-526 zbe*::Tn*5*	φ(PC7070) × PC8795
PC9260	*fliF403 rec-526 zbe*::Tn*5*	φ(PC7070) × PC8683

milliliter): ampicillin, 50; tetracycline, 15 for *E. coli* and 2 for *C. crescentus*; kanamycin, 50; spectinomycin and streptomycin, 50 for *E. coli* and 200 for *C. crescentus*.

Plasmid construction. Plasmids used in this study include pUC-based plasmids (72) that do not replicate in *C. crescentus* and pRK290-based plasmids that are maintained in *C. crescentus*. Plasmids containing *fliF* DNA were derived from the original pRK290 clone pS1505 (reference 51 and Fig. 2B). DNA fragments from these plasmids were subcloned in pUC18 and in pBluescript KS + (Stratagene Corp.) and then in the pRK290 derivative pRK2L1 (42) for use in complementation. The *flap-lacZ* fusion plasmids have been described previously (45).

Chemical mutagenesis. *C. crescentus* CB15 was subjected to mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), essentially as detailed by Miller (37). Cultures grown in PYE medium to saturation were subjected to NTG treatment at 125 μg/ml for 15 min at 30°C. The cells were spun down and washed repeatedly in PYE medium before being plated in motility agar. Colonies defective in swarm formation after growth at 30°C for 2 to 3 days were purified and rechecked for the nonswarming phenotype. Temperature-sensitive nonmotile mutants were identified by testing for swarming at 24°C as the permissive temperature and at 37°C as the restrictive temperature. Mutants that could be complemented by *fla*$^+$ genes were presumed to be *fla* mutants.

Mapping of *fliF* mutations. NTG-derived *fla* mutants were tested for gain of motility in a *rec* background following introduction of the *fliF* plasmid clone pS1505 or pGIR342 (see Fig. 2B and the next section). Mutations complemented by these plasmids were mapped more accurately and tested for polarity in complementation and recombination tests with plasmids deleted for various fragments of *fliF* (see Fig. 2B). Another test of polarity was the expression of *flbD* at the 3′ end of the operon as assayed in Western blots (immunoblots) (53).

Construction of deletions in the *fliF* operon. We attempted to make deletions in each of the first four ORFs in the *fliF* operon first by constructing in vitro, in-frame internal deletions in pUC18 clone pGIR195 (Fig. 2B and 3). These deletions were designed to allow any translational coupling between adjacent ORFs to occur normally.

Suitable restriction sites at the 5′ and 3′ ends of each ORF (Fig. 3 and 4) were targeted for linker mutagenesis by use of *Xho*I 8-mer (CCTCGAGG) or 10-mer (CTCTCGAGAG) linkers as required to maintain the translational reading frame. The intervening sequence was deleted to generate the deletion derivative with a unique *Xho*I site within the mutated gene. In the case of the *fliF* ORF, the 5′ and 3′ sites used 10-mer and 8-mer linkers, respectively, with the resulting deletion derivative having a 9-mer *Xho*I linker. The resulting deletion derivatives in pUC18 in ORFs 1, 2, 3, and 4 were designated pGIR199, pGIR230, pGIR224, and pGIR225, respectively (Fig. 3).

The DNA fragments with the deletions were then subcloned as *Kpn*I-*Xba*I inserts in pRK2L1 to test for complementation of the *C. crescentus fla* mutations. The corresponding pRK2L1 clones are pGIR346, pGIR347, pGIR348, and pGIR349, respectively (Fig. 2B and 3). The plasmids pGIR347 to pGIR349 were also deleted in vitro for sequences between their unique *Xho*I and *Xba*I sites to generate a series of 3′ deletions of ORF2 to ORF5 (*fliG-flbE-fliN-flbD*), ORF3 to ORF5 (*flbE-fliN-flbD*), and ORF4 to ORF5 (*fliN-flbD*). These clones, carrying either *fliF* alone, *fliF-flbG*, or *fliF-flbG-flbN*, correspond to plasmids pGIR350, pGIR351, and pGIR352, respectively (Fig. 2B). These plasmids were used to complement mutations in the *fliF* operon to test for polarity of deletion and NTG-induced mutations in the first four ORFs. Mutations that were not complemented to give a full wild-type Mot$^+$ phenotype by stabbing in soft agar or by direct microscopic observation were considered to be polar for expression of downstream genes.

The pUC18 ΔORF plasmid clones described above (pGIR199, pGIR230, pGIR224, and pGIR225) contain two *Hin*dIII sites, one in the 5′ region of the *fliF* operon and the other in the 3′ polylinker region of the vector (Fig. 3). The 3′ *Hin*dIII sites were selectively destroyed by partial digestion and filling in of the overhanging end. The *Kpn*I-*Hin*dIII promoter

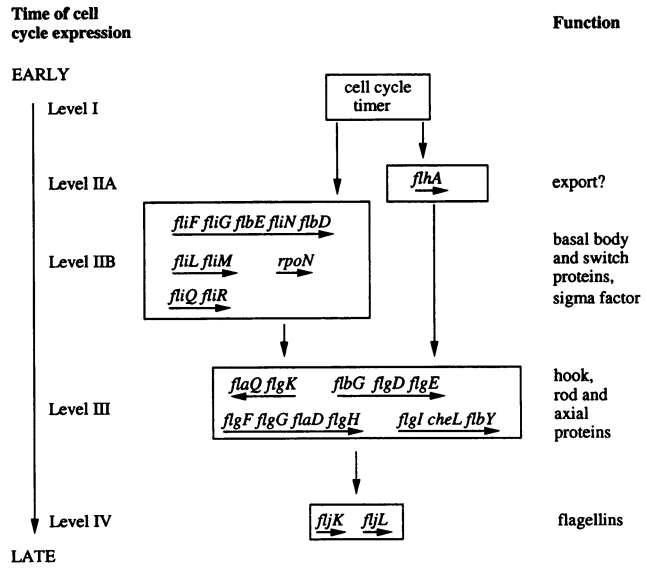

FIG. 1. Organization of *C. crescentus fla* genes in a regulatory hierarchy. The *fla* genes are described in Table 1, and the organization of hierarchy is discussed in the text.

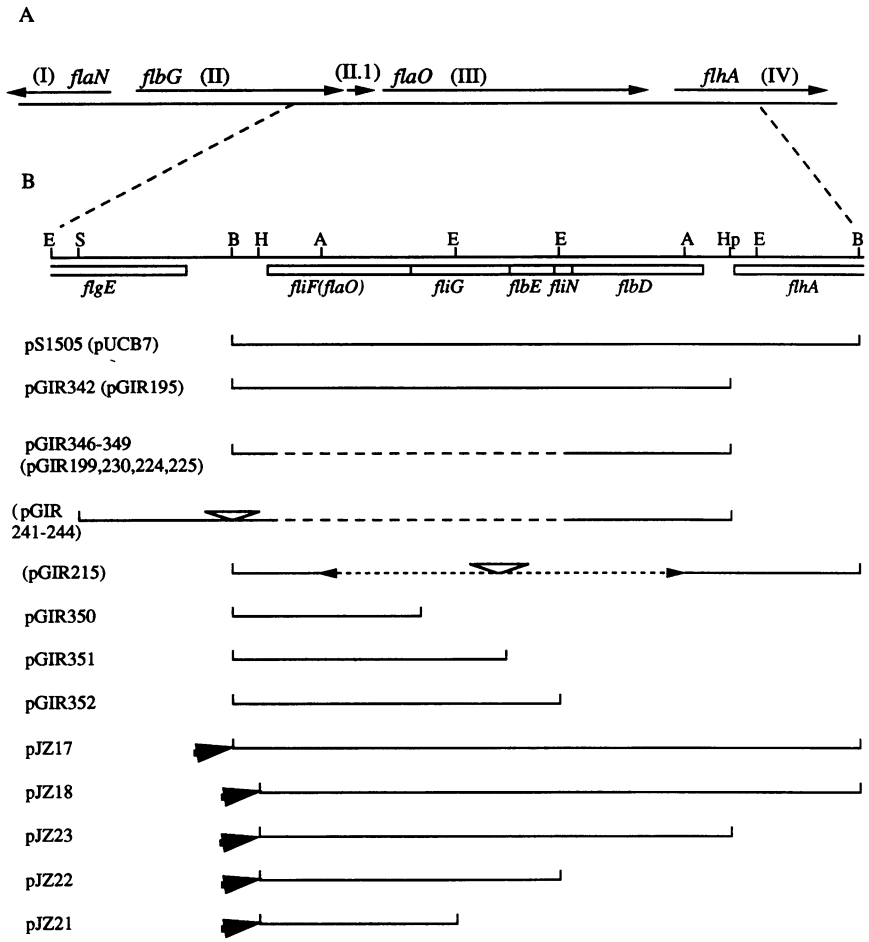

FIG. 2. Map of the *fliF* (*flaO*) region of the hook gene cluster and related plasmids. (A) The top line shows the organization of the hook gene cluster, with arrows showing the directions and extents of the transcription units. The first genes in the different transcription units (I to IV) are shown above the arrows. (B) The *fliF* operon region and the various genes are shown in more detail, and plasmids used in this study are delineated below. Plasmids, which are derivatives of either pRK2L1 or pUC (indicated in parentheses), are described in the text. Dashed lines represent the various in-frame deletions in the *fliF* operon genes (presented in more detail in Fig. 3). The inverted triangles in plasmids pGIR241 to -244 and pGIR215 represent the sites of insertion of the Ω cassette. The filled arrowheads in the pJZ plasmids represent the *lppp lacZp* promoters driving expression of the *fliF* genes in the pINIII*lpp5* vector. Relevant restriction sites are designated as follows: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; S, *Sma*I.

regions of these deletion plasmids were then replaced with *Kpn*I-*Hin*dIII fragments carrying an additional 2 kb of upstream DNA along with the Spc^R Str^R Ω cassette (52) inserted in the *Bam*HI site within transcription unit II.1. These Ω-tagged ΔORF clones in pUC18 were designated plasmids pGIR241 to -244, respectively (Fig. 2B).

A pUC18 derivative carrying a deletion of all five ORFs (*fliF-fliG-flbE-fliN-flbD*) was also generated by insertion of the Ω cassette at the deletion site. The Ω insertion spanned *Apa*I sites in *fliF* and *flbD* to generate plasmid pGIR215 (Fig. 2B). The parent plasmid in this construction was pUCB7, which carried ca. 1.6 kb of the *flhA* sequence downstream of *flbD*, the last gene of the *fliF* operon (Fig. 2B).

Cells of *C. crescentus* CB15 were electroporated with the deletion derivatives pGIR243 (Δ*flbE*) and pGIR244 (Δ*fliN*), and Spc^r Str^r transformants were selected. Stable Spc^r Str^r recombinants depend upon integration of the plasmid by homologous recombination into the chromosome, because ColE1 replicons are not maintained in *C. crescentus*. Bacteriophage φCr30 lysates made on motile, Spc^r Str^r transformants were transduced into strain CB15, and the deletion strains were screened for Spc^R Str^R transductants that were nonmotile. Of the Spc^R Str^R transductants from the screen, 4 to 5% were nonmotile deletion mutants in *flbE* (PC9263) and *fliN* (PC9264). The general strategy for the isolation of gene replacements used here has been described previously (39). Plasmid pGIR215, which carries the Ω insertion in place of the major part of the *fliF* operon, was similarly used to obtain strain PC9249 lacking the entire operon. For reasons that are not clear, plasmids pG1R241 (Δ*fliF*) and pG1R242 (Δ*fliG*) did not yield Mot^- recombinants at the transduction step, and, consequently, it was not possible to generate strains carrying in-frame deletions in *fliF* and *fliG*.

Assays of *fla* transcription. Promoter activity was assayed by measurement of levels of β-galactosidase in *fla* mutant strains carrying the various *flap-lacZ* fusion plasmids. The cells were grown at 30°C overnight in PYE medium containing tetracycline and diluted into the same medium. Early-log-phase cultures were assayed for β-galactosidase activity as described by Miller (37).

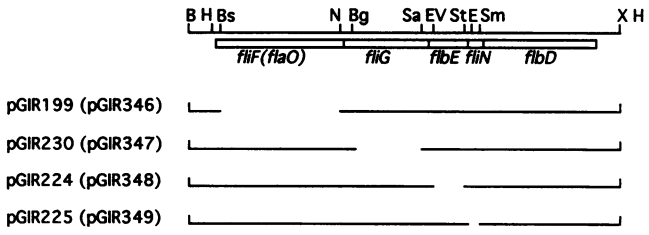

FIG. 3. Construction of in-frame deletion clones. The DNA fragment carried in parental plasmid pG1R195 is shown on the top line with relevant restriction sites. The deletions are indicated as gaps in the lines representing the regions contained in each of the deletion derivatives. The plasmid names in parentheses represent pRK2L1 derivatives of the pUC deletion clones used in complementation studies. Restriction sites are indicated as follows: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*NI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; N, *Nru*I; Sa, *Sal*I; Sm, *Sma*I; St, *Stu*I; X, *Xba*I.

Nucleotide sequencing and analysis. Sequencing was carried out by the method of dideoxy chain termination (57) with ^{35}S-dATP. The DNA was sequenced on both strands by using nested-deletion clones made on fragments subcloned in pBluescript SK −. Oligonucleotides were synthesized for sequencing through gaps and regions where reading the sequence was otherwise difficult. The work was done in part by Lark Sequencing Technologies, Inc.

The sequence information was processed and analyzed by using the University of Wisconsin Genetics Computer Group package (10). After identification, the newly identified ORFs in the *fliF* operon were compared with the sequences in the database (release number 84) by TFASTA search.

Expression of gene products in minicells. Plasmid pJZ17 (Fig. 2B) was constructed by insertion of the 7-kb *Bam*HI fragment shown in Fig. 2B that contains the intact *fliF* operon into the *Bam*HI site of expression vector pINIII*lpp*5 (24). This plasmid was then digested with *Xba*I and *Hin*dIII, cleaving upstream within the vector and at nucleotide 290 of the insert, respectively. This removed the II.1 terminator and *fliF* promoter contained between the 5′ *Bam*HI site and the first 290-bp DNA segment cloned in pJZ17. In the resulting plasmid, pJZ18, the *fliF* transcription unit is under the direct control of the vector-borne *E. coli lpp* promoter and *lacUV5* promoter-operator (Fig. 2B). Plasmid pJZ18 was later used to generate 3′ deletions by digestion of the distal *Bam*HI site and partial digestion of the internal *Eco*RI sites, releasing up to three fragments. The ends of the digested DNA were filled in with Klenow fragment and religated. The resulting plasmids, pJZ21, pJZ22, and pJZ23, containing 0, 1, or 2 internal *Eco*RI sites, respectively, were used in experiments to identify proteins encoded by the *fliF* operon.

Continuous sucrose gradient centrifugation (55) was used to isolate minicells from strain P678-54 (1), carrying the pINIII-*lpp*5 derivatives described above. The cells, with and without added isopropyl-β-D-thiogalactopyranoside (IPTG) were labeled with [^{35}S]methionine as described previously (53). The protein products were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and subjected to autoradiography.

Nucleotide sequence accession number. The nucleotide sequence reported in Fig. 4 has been deposited in the GenBank data base under accession number M98855.

RESULTS

Organization of the *fliF* operon. The *fliF* transcription unit was originally located in the hook gene cluster by mapping of a series of Tn*5* insertions and the spontaneous mutation *flaO138* that conferred a nonmotile phenotype. The mutations could be complemented by a 7-kb *Bam*HI fragment in plasmid pS1505 (Fig. 2 and reference 51) that extends from within the upstream transcription unit II.1 (9, 40, 48) to the *Bam*HI site within the downstream *flhA* transcription unit (54). The 5′ end of the *fliF* transcript has been mapped approximately 80 bp upstream of the *Hin*dIII site (Fig. 2B and references 40 and 66), and the 3′ end of the transcription unit is bounded by *flhA* (formerly designated *flbF*; Fig. 2A and references 51 and 53), whose transcription is initiated ca. 200 bp 3′ of the unique *Hpa*I site (Fig. 2B and references 54 and 56). We conclude that the complete *fliF* transcription unit is contained within the 5.6-kb *Bam*HI-*Hpa*I fragments on the basis of the ability of plasmid pGIR342 to complement all Tn*5* insertion mutations at the *flaO* locus (Fig. 2B).

Nucleotide and translated amino acid sequence of the *fliF* operon. To identify the *fliF* operon genes required for the regulatory experiments described below, we determined the nucleotide sequence of 4003 bp that extends from the *Bam*HI site in transcription unit II.1 to the first codon of the *flbD* ORF (53). On the basis of GCG TERMINATOR analysis, the sequence between nucleotides 55 to 105 can form a short stem-loop structure and contains the probable terminator of transcription unit II.1 (Fig. 4).

Translation of the nucleotide sequence (Fig. 4) identified the four ORFs, as summarized in Fig. 2B. Analysis of the sequence by the criteria of codon usage, homology, and the effects of mutations (see below) indicated that the ORFs correspond to new genes. As discussed in the next section, the first ORF appears to be a homolog of the *S. typhimurium fliF* gene (26, 64), the second and the fourth ORFs appear to be homologs of the *S. typhimurium* switch protein genes *fliG* and *fliN* (30), respectively, and the third ORF, designated *flbE*, shows no homology with any known genes.

We have designated the ATG at base 338 as the *fliF* start codon, although a good consensus Shine-Dalgarno sequence (14) could not be identified upstream (Fig. 4). We think it less likely that the in-frame GTG at base 284 is the translation start, because it extends the N terminus of FliF 18 residues and includes codons that are infrequently used in *C. crescentus* (34, 58), and as shown below, deletion of all sequences upstream of the *Hin*dIII site at base 289 in plasmid pJZ18 allowed expression of the *fliF* polypeptide. Possible ribosome binding sites were identified upstream of *flbE* (GAGG, 8 bp from the ATG start) and of *fliN* (AAGG, 10 bp from the ATG start). The termination codon TGA of the *fliF* ORF overlaps the putative start codon of the *fliG* ORF, which suggests that translation of the two genes is coupled. The *fliG* and *flbE* genes are separated by 10 bp, the *flbE* and *fliN* genes are separated by 16 bp, and the *fliN* and *flbD* genes are separated by 33 bp. The GC content of *C. crescentus* is about 67%, and codon usage analysis showed that all these ORFs have a similar third-base GC bias, as observed in other known *C. crescentus* genes described previously (34, 58).

Comparisons of *fla* genes from *C. crescentus* and enterobacteria. The *fliF* genes of *S. typhimurium* encode the MS ring protein (26, 64), which may be the first protein assembled in the inner membrane during flagellar biogenesis (27, 32). The *fliF* gene of *C. crescentus* should encode a protein of 56.8 kDa ($FliF_C$), and its alignment with the *S. typhimurium* homolog $FliF_S$ is shown in Fig. 5. $FliF_C$ may have undergone a large

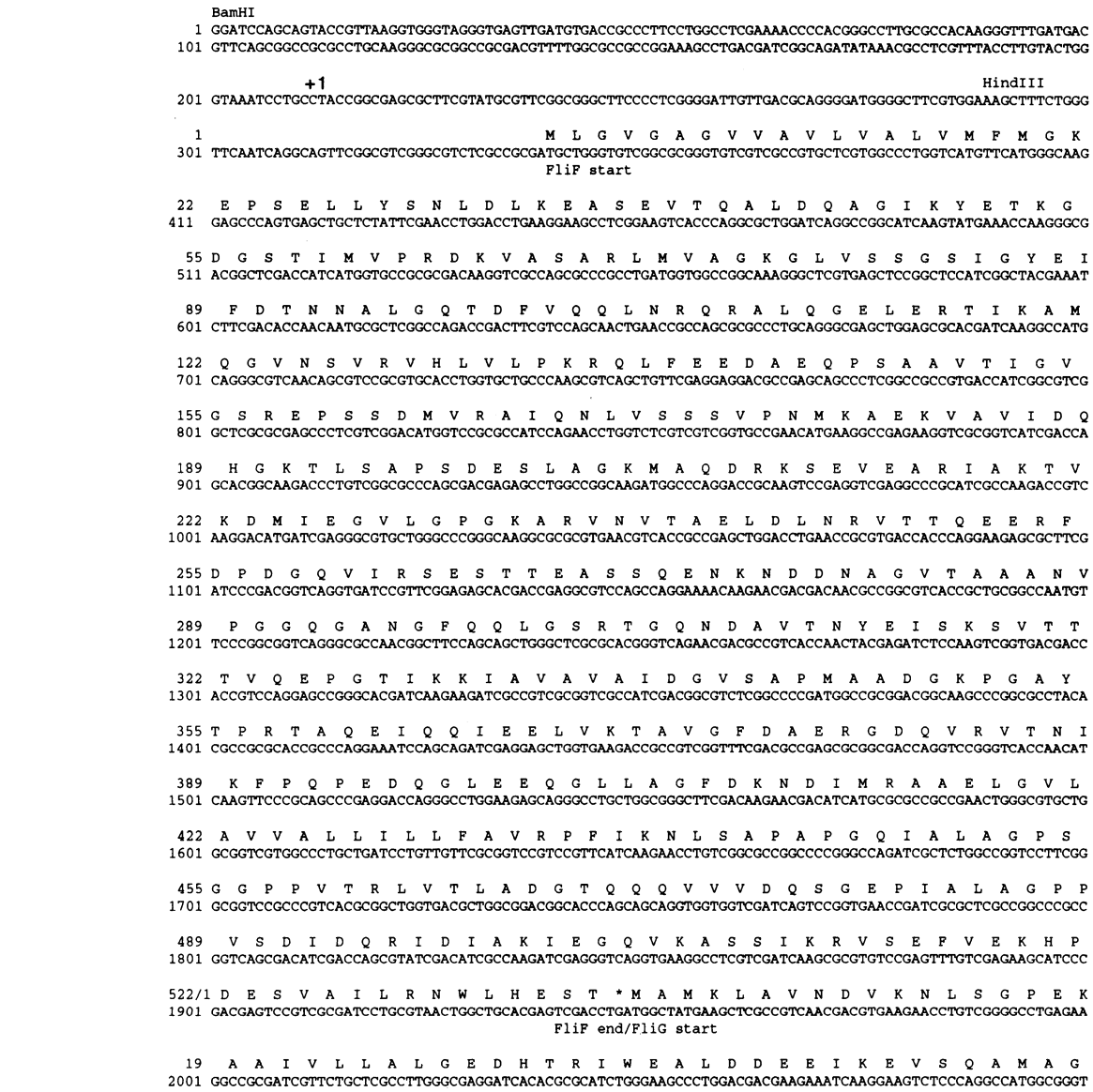

FIG. 4. Nucleotide sequence of the *fliF* operon. The sequence shown contains the first four ORFs of the operon up to the start codon of *flbD*. The translated sequences of FliF, FliG, FlbE, and FliN are shown in the one-letter amino acid code above the corresponding ORFs. Relevant restriction sites are indicated. Transcription initiates at nucleotide 208, indicated by +1.

number of changes in its primary sequence, because when aligned with $FliF_S$ (26) and the FliF sequence from *B. subtilis* ($FliF_B$ [74]), a number of deletions or insertions could be identified. The degrees of overall identity of $FliF_C$ with $FliF_B$ and $FliF_S$ are 24 and 30%, respectively (Table 3). The degree of identity between $FliF_B$ and $FliF_S$ has been reported to be 22% (74). The low degree of sequence identity among the FliF proteins is mainly the result of nonconserved substitutions over the entire length of the protein (Fig. 5).

The deduced molecular mass of the *C. crescentus* switch protein $FliG_C$ is 37.7 kDa, and comparison of $FliG_C$ with $FliG_S$ (Fig. 6 and reference 30) and with $FliG_B$ (2) shows degrees of sequence identity of 32 and 30%, respectively (Table 3). The sequence identity is distributed over the entire length of the protein, and large deletions or insertions were not necessary to align the proteins (Fig. 6). The *fliF* and *fliG* genes of *S. typhimurium* map adjacent to one another (35), as observed here in *C. crescentus*, and the start codon of *fliG* overlaps the end of *fliF* (26, 30), which suggests that translation of the two genes is coupled in both organisms.

The fourth ORF of the *C. crescentus fliF* operon, *fliN*, encodes a protein ($FliN_C$) with a deduced size of 11.6 kDa. $FliN_C$ has degrees of sequence identity with the switch proteins of *E. coli* ($FliN_E$) (36) and *S. typhimurium* ($FliN_S$) (30) of 43.6 and 37.3%, respectively (Table 3). The degree of identity is higher in the carboxyl terminus, and the amino-terminal portion of $FliN_C$ is considerably shorter than that of $FliN_E$ or $FliN_S$ (Fig. 7; see Discussion).

The FlbE protein, which encodes a 213-amino-acid protein with a predicted mass of 22.5 kDa, contains a hydrophilic N terminus and no hydrophobic regions. The protein is acidic, with a predicted pI value of 4.88. Although sequences similar

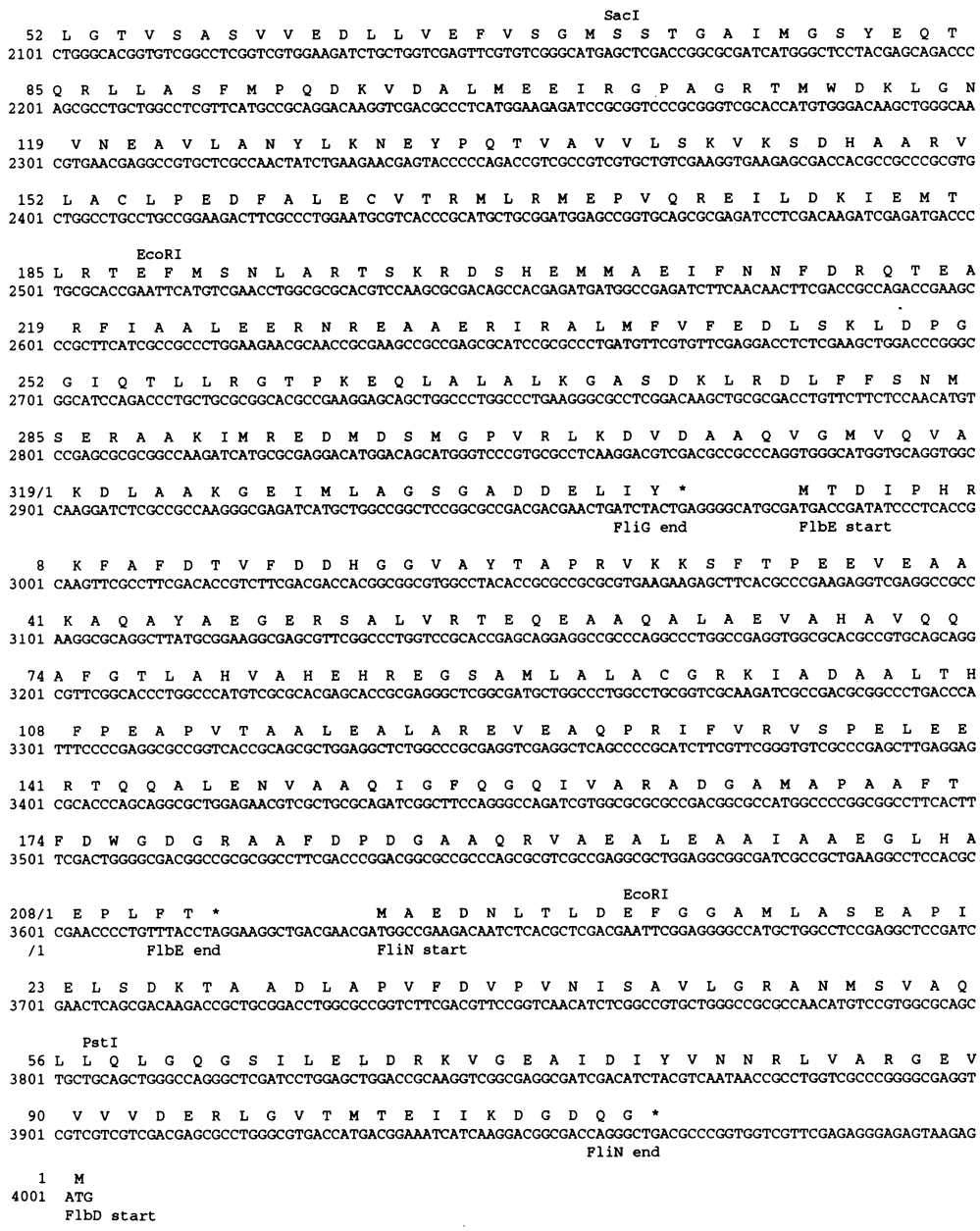

FIG. 4—*Continued.*

to the *C. crescentus flbE* gene were not identified in the database and its function remains to be determined, experiments described below show that the gene is essential for motility and *fla* gene regulation.

Identification of proteins encoded by the *fliF* operon. High-level expression vector pINIII*lpp*5 (24) was used to examine the gene products encoded by the *fliF* operon after induction with IPTG in *E. coli* minicells (Materials and Methods). The appearance of the 52-kDa FlbD protein (53) was monitored as an internal control for expression of the *fliF* operon.

The expression of proteins in minicells with the vector plasmid is shown in Fig. 8A, lanes 1 and 2. In the absence of induction (Fig. 8A, lane 3), no new protein bands were detected in minicells carrying plasmid pJZ17, which contains the *fliF* operon preceded by the terminator of the upstream transcription unit II.1 (Fig. 2B; Materials and Methods). Upon induction with IPTG, a band at about 52 kDa corresponding to FlbD was observed (Fig. 8A, lane 4). Removal of the II.1 terminator (plasmid pJZ18 [Fig. 2B]) resulted in increased expression of the operon, as judged from the levels of the FlbD protein both with (Fig. 8A, lane 6) and without (Fig. 8A, lane 5) addition of IPTG. Furthermore, after induction, bands corresponding in size to the predicted FliF (56.8-kDa) and FliG (37.7-kDa) proteins were expressed from plasmid pJZ18 (Fig. 8A, lanes 5 and 6). A 17-kDa polypeptide was also expressed at an intensity similar to that of FlbD.

To confirm the origins of the 56.8-, 37.7-, and 17-kDa proteins, the expression patterns were examined with the plasmids pJZ23, pJZ22, and pJZ21, in which progressively larger fragments at the 3′ end of the insert had been deleted

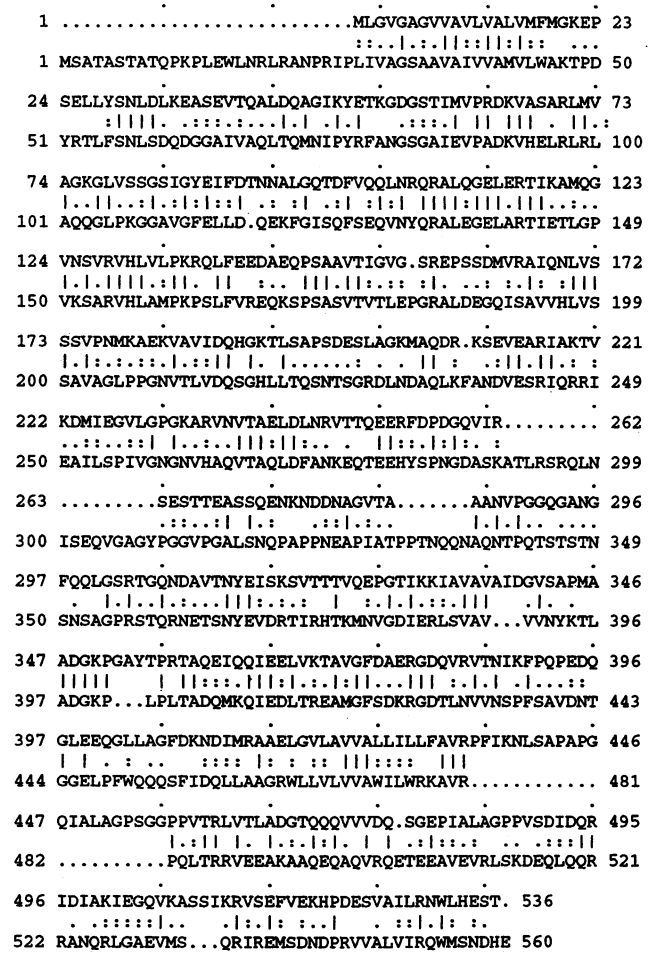

FIG. 5. Alignment of FliF sequences from *C. crescentus* and *S. typhimurium*. The *C. crescentus* sequence is shown on the top lines. Periods in the sequences are introduced to maximize alignment. Identical residues are indicated by vertical bars. Conservative replacements are indicated by colons.

(Fig. 2B and 8B). All three gene products were expressed from plasmid pJZ23 at a level similar to that from parent plasmid pJZ18 (Fig. 8A, lanes 5 and 6). Plasmid pJZ22, which lacks *flbD*, expressed both FliF and FliG, but both the 52-kDa FlbD band and the 17-kDa product were absent (Fig. 8B, lanes 3 and 4), suggesting that the 17-kDa polypeptide may be a degradation product of the FlbD protein. Plasmid pJZ21, which should contain only *fliF* and the 5′ portion of *fliG* (Fig. 2B), expressed the 56.8-kDa band identified above as FliF and a ca. 28-kDa band corresponding to the predicted size of the amino-terminal portion of FliG (FliG′) encoded by the truncated gene (Fig. 8B, lanes 1 and 2). These results are consistent with *fliF* mapping at the proximal end of the transcription unit, with *fliG* immediately 3′ to it, as diagrammed in Fig. 2B.

We were unable to identify induced gene products in the 11- to 25-kDa range that would correspond to FlbE (22.5 kDa) or FliN (11.6 kDa). Failure to detect these polypeptides could be due to the inefficient recognition of the ribosome binding sites in *E. coli*, the lower abundance of methionine residues in these proteins (3, 4, and 9 residues in FlbE, FliN, and FlbD, respectively), or the instability of the expressed proteins in minicells.

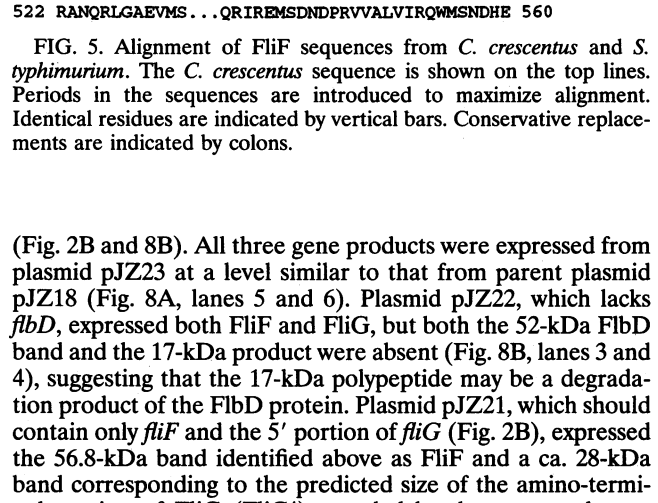

FIG. 6. Alignment of FliG sequences for *C. crescentus* and *S. typhimurium*. The *C. crescentus* sequence is shown on the top lines. Periods in the sequences are introduced to maximize alignment. Identical residues are indicated by vertical bars. Conservative replacements are indicated by colons.

Construction of chromosomal deletions by gene replacement. Previous conclusions about the role of *fliF* genes in the positive and negative regulation of *fla* gene expression were based on an analysis of Tn*5* insertions in the operon and one spontaneous mutant that had not been precisely mapped (45). To determine the involvement of individual genes in transcriptional regulation, we constructed strains carrying in-frame, chromosomal deletions in *flbE* and *fliN*, as well as an essentially complete deletion of the *fliF* operon (Materials and Methods).

Strains PC9263(Δ*flbE*), PC9264(Δ*fliN*), and PC9249(Δ*fliF-flbD*) were tested by complementation and by recombination with the set of plasmids pGIR350, pGIR351, and pGIR352, which carry inserts deleted for various extents of the 3′ end of the *fliF* operon (Fig. 2B). The results are consistent with the predicted locations of the deletions and showed that PC9263 and PC9264 are not polar for expression of the downstream gene(s) (Materials and Methods); genomic Southern blot analysis verified the expected location of deletions and the Ω cassette (data not shown).

Identification, mapping, and characterization of NTG-in-

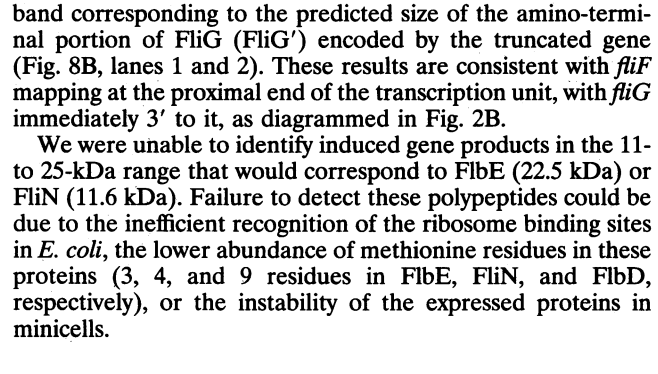

FIG. 7. Alignment of FliN sequences from *C. crescentus* and *S. typhimurium*. The *C. crescentus* sequence is shown on the top lines. Periods in the sequences are introduced to maximize alignment. Identical residues are indicated by vertical bars. Conservative replacements are indicated by colons.

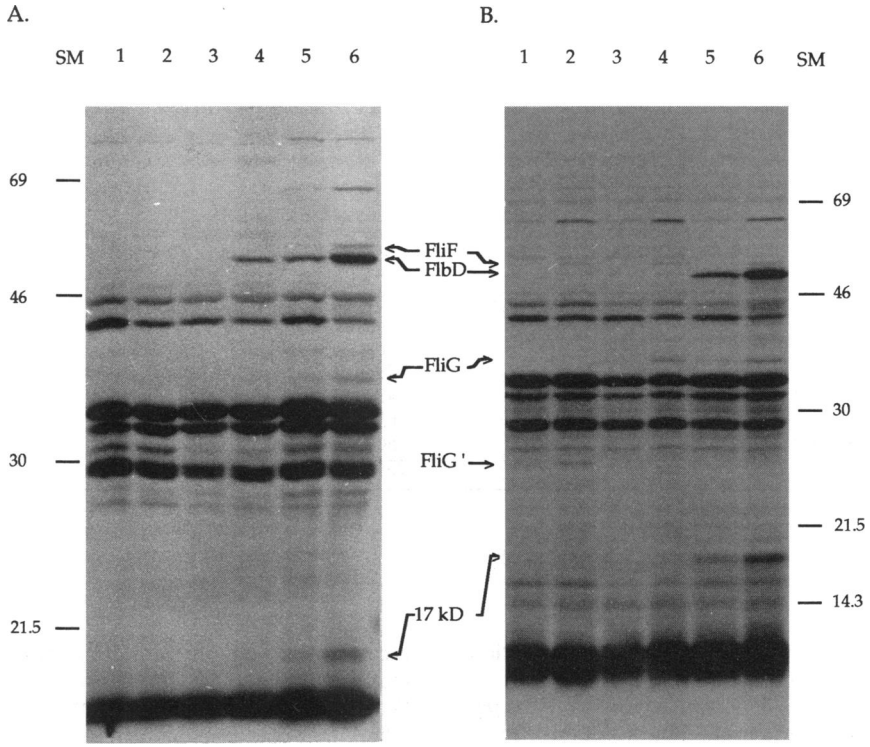

FIG. 8. Identification of *fliF* and *fliG* products in minicells. Minicells of *E. coli* P678-54 carrying different inducible *flaO* region clones were labeled with [^{35}S]methionine. Proteins were analyzed by electrophoresis in SDS-polyacrylamide gels along with ^{14}C-labeled markers followed by autoradiography. (A) 10% gel; (B) 10 to 15% gradient gel. Minicells harbored plasmids as follows. (A) Lanes 1 and 2, pINIII*lpp* expression vector; lanes 3 and 4, pJZ17; lanes 5 and 6, pJZ18. (B) Lanes 1 and 2, pJZ21; lanes 3 and 4, pJZ22; lanes 5 and 6, pJZ23. Lanes 2, 4, and 6 in both panels correspond to induced expression with IPTG. Size markers (SM) and sizes (in kilodaltons) are shown at the left and right margins of the figure.

duced mutations in the *fliF* operon. Nine *fla* strains that could be complemented with a plasmid containing the intact *fliF* operon (pG1R342; Fig. 2) were identified after NTG mutagenesis. Mutations in these strains (PC8638, PC8652, PC8660, PC8661, PC8683, PC8742, PC8750, PC8795, and PC8760) and in SC290 (51) (Table 2) were further mapped by recombination with plasmids pGIR346 to pGIR349, containing a series of overlapping 5′ fragments of the *fliF* operon (Fig. 2B), and plasmid clone pGIR315, containing the *flbD* gene (53). The polarity of these mutations on expression of downstream genes was tested by genetic complementation in a *rec* background and Western blot assays as described in Materials and Methods.

TABLE 3. Sequence comparison of *fliF*, *fliG*, and *fliN* genes and products in *C. crescentus*, *B. subtilis*, *E. coli*, and *S. typhimurium*.

Gene	Organism (reference)	Gene product		Identity (%)[a]
		No. of residues	Deduced mass (Da)	
fliF	*C. crescentus*	536	56,856	100
	B. subtilis (74)	536	59,300	24
	S. typhimurium (26)	560	61,230	31
fliG	*C. crescentus*	340	37,684	100
	B. subtilis (2)	338	38,191	30
	S. typhimurium (30)	331	36,851	32
fliN	*C. crescentus*	110	11,668	100
	E. coli (36)	137	14,855	44
	S. typhimurium (30)	137	14,789	37

[a] The translated gene sequences from different organisms were compared with their respective counterparts in *C. crescentus*.

NTG-induced mutations were mapped in *fliF*, *fliG*, and *flbD*, and the mutations in strains PC8742 (*fliF462*) and PC8750 (*fliG470*) were shown to be nonpolar when tested by complementation; strain SC290 was also shown to contain a nonpolar mutation in *fliG* (here renamed *fliG138* [Table 2]). Mutations in strains PC8683 (*fliF403*) and PC8652 (*fliG372*) were polar, however. Three NTG-generated mutations were identified in *flbD* and one of them [*flbD*480(Ts)] was shown to confer a temperature-sensitive motility phenotype in strain PC8760. Strains PC8660 (*fliFp380*) and PC8661 (*fliFp381*) were shown to carry polar mutations that map to the *Bam*HI-*Hin*dIII fragment that contains the *fliF* promoter (data not shown).

MS-ring and switch protein genes are required for activation of σ^{54}-dependent promoters and for negative autoregulation of *fliF* transcription. The nonmotile phenotype of the in-frame deletions and nonpolar NTG-induced mutations in the five ORFs of the *fliF* operon indicates that all of the genes are required for a functional flagellum. This includes the *flbE* gene, which has no obvious sequence similarity to identified *fla* genes in other bacteria. Previous results had shown that *flbD*, the last gene in the transcription unit, activates transcription from the σ^{54}-dependent *flbG* promoter in *E. coli* and negatively autoregulates the *fliF* promoter (45). The roles of the four newly identified ORFs upstream of *flbD* in regulating expression from σ^{54} *flgK* and *flbG* promoters and the *fliF* promoter were examined in constructs with each promoter fused to the *lacZ* reporter gene in plasmid vector pANZ5 (45).

TABLE 4. Effects of mutations in the *fliF* operon on *fla* gene transcription

Strain	Mutation[a]	Level of expression of β-galactisodase (Miller units) by reporter plasmid:			
		pANZ5 (vector)	pANZ404 (*flgKp-lac*)	pANZ405 (*flbGp-lac*)	pANZ406 (*fliFp-lac*)
CB15	wt[b]	20	122	209	154
PC9266	II.1::Ω	23	116	268	115
PC8742	*fliF462*	22	30	21	415
PC8750	*fliG470*	26	29	5	588
PC9263	Δ*flbE*	21	39	10	1,800[c]
PC9264	Δ*fliN*	18	26	6	176
PC8283	*flbE188*::Tn*5*	ND[d]	29	11.6	754[c]
PC8795	*flbD215*	ND	28	4.9	479
PC8638	*flbD358*	19.6	34	58	981
SC1967	*flbD*::Tn*5*	ND	26.4	3.5	975
PC9249	Δ(*fliF-flbD*)	25	29	6	1,018[c]
PC8760	*flbD*(Ts) (24°C)	13	42	43	100
CB15	wt (24°C)	12.5	105	180	126
PC8760	*flbD*(Ts) (37°C)	36	50	16.5	290
CB15	wt (37°C)	39	234	400	327

[a] Cells were grown at 30°C unless otherwise indicated.
[b] wt, wild type.
[c] The level of expression was variable and dependent on the isolate; see Discussion.
[d] ND, not determined.

The plasmids were introduced into strains with the mutations described above and assayed for *lacZ* expression.

The levels of β-galactosidase activity measured from the *flgK* and *flbG* promoter fusions in the mutant backgrounds were in most cases no higher than those with the plasmid vector pANZ5 alone (Table 4). Thus, transcription from the σ^{54} *flgK* and *flbG* promoters at level III of the *fla* gene hierarchy requires products encoded by all five ORFs of the *fliF* operon, including the MS ring protein, the switch proteins FliG and FliN, FlbE, and the transcriptional activator FlbD.

Assays of *lacZ* expression driven by the *fliF* promoter in the same mutant backgrounds showed that the negative autoregulation of the level II *fliF* promoter requires *fliF*, *fliG*, *flbE*, and the activator gene *flbD*, but not the switch protein gene *fliN* (Table 4). NTG-induced mutations in MS ring gene *fliF* and switch protein gene *fliG* resulted in a three- to fourfold increase in the level of expression from the *fliF* promoter, while mutations in *flbD* result in a 5- to 10-fold increase (Table 4). The β-galactosidase activities in strains with *flb*E mutations were also extremely high, in some cases 10- to 15-fold higher than that observed in the wild-type background. The variability in levels of *fliF* expression in this genetic background (Table 4) could be explained by instability of the reporter plasmid due to deleteriously high levels of β-galactosidase expression or, alternatively, secondary mutations which we have observed to accumulate in these strains (4). We propose in the Discussion that the *flbE* gene product, along with the activator FlbD, may play a direct role in transcriptional regulation of *fliF*.

DISCUSSION

Genes in the *fliF* operon occupy a key position near the top of the *C. crescentus* flagellar gene hierarchy, where they function to control the cell cycle timing both of their own expression and that of late genes during flagellum biosynthesis. Our analysis of this level II transcription unit has identified four new ORFs that are located upstream from the σ^{54} transcription activator gene *flbD* (53). Three of the ORFs encode homologs of enterobacterial structural proteins required for flagellum biosynthesis, and all four of the newly identified genes are required in addition to *flbD* for transcription of the σ^{54} *flgK* and *flbG* promoters at level III. With the exception of the switch protein gene *fliN*, these genes are also required for the negative autoregulation of the *fliF* promoter (Table 4). We suggest below that these results may reflect different mechanisms controlling the activity of FlbD as an activator of late *fla* σ^{54} promoters and repressor of *fliF* transcription.

Characterization of genes in the *fliF* operon. Nucleotide sequence analysis of the *fliF* operon showed that the organization of genes in this transcription unit is 5′-*fliF-fliG-flbE-fliN-flbD*-3′. The FliF protein is responsible for the MS ring structure of the flagellar basal body in the cytoplasmic membrane of the bacterium (16, 64, 65). Although the $FliF_C$ sequence is not highly homologous to $FliF_B$ (74), the degree of sequence identity between $FliF_C$ and $FliF_S$ is higher, and the hydropathy profiles of $FliF_C$ and $FliF_S$ are almost identical (data not shown). The MS ring contains about 26 subunits of FliF (28, 61, 64), and this multimeric structure may allow for some variation of sequence within the protein. The carboxy terminus of $FliF_C$ is less conserved, consistent with observations that the C terminus is not required for either subunit assembly (64) or for functional assembly and interaction with switch proteins (16).

The switch protein FliG in enteric bacteria interacts directly with the cytoplasmic face of FliF (16, 17) and with the other switch proteins FliM and FliN in the C-ring complex, which in turn may directly interact with the motor complex MotA/MotB (17). It is therefore not surprising that with the constraint of multiple interactions, the $FliG_C$ sequence is quite well conserved over its entire length with $FliG_S$ (Fig. 6).

The sequence of $FliN_C$ is well conserved at the carboxy-terminal region, but it is much shorter at the amino terminus than the enterobacterial protein $FliN_S$ (Fig. 7). FliN in enteric bacteria interacts not only with the other switch proteins but also may be involved in interaction with *mot* products (71) and with the flagellum-specific export apparatus (67). The more conserved C terminus of $FliN_C$ may be responsible for these functions.

The *C. crescentus* FlbE protein has as yet no known homolog in other bacteria. Any structural role for this protein remains to be determined, but our results do show it to have strong regulatory effects on *fla* gene transcription. These effects are

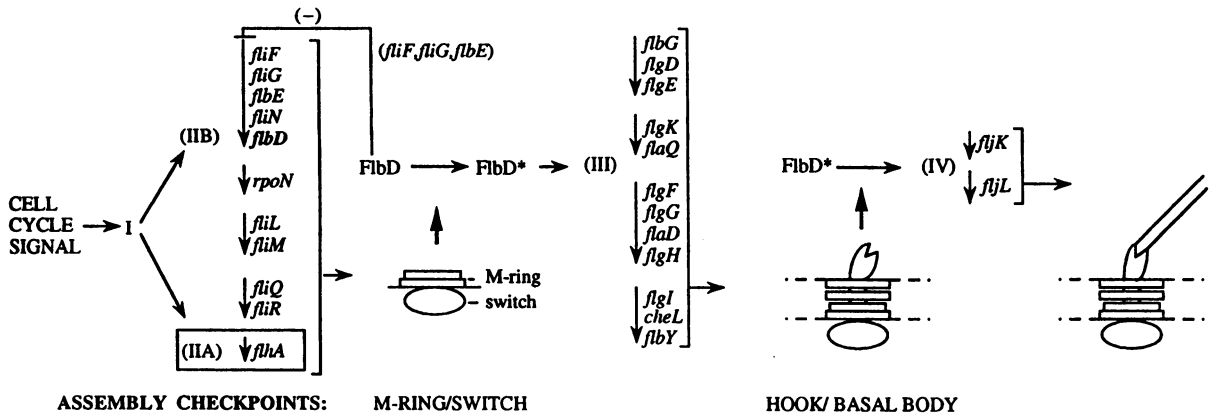

FIG. 9. Regulation of FlbD in activation of level III genes and repression of the *fliF* promoter. This model proposes that, in addition to transcription factors encoded by *flbD* and *rpoN*, expression of σ^{54} genes at level III requires assembly of the MS ring-switch complex and that expression of genes at level IV requires assembly of the completed hook-basal body complex. Possible mechanisms for modulating FlbD activity as a positive or negative transcription factor are considered in Discussion. There is no direct evidence to indicate whether the same form of FlbD (FlbD or FlbD*) acts as an activator and repressor.

particularly dramatic in the negative regulation of the *fliF* promoter (Table 4), as discussed below.

Positive regulation of *fla* gene expression by genes in the *fliF* operon. From work on enterobacteria, FliF is known to be the earliest-assembled flagellar component, followed by the switch proteins and the putative flagellar protein export apparatus (32). Like the enterobacterial flagella, the *C. crescentus* flagellum is also built from the inside of the cell out (21). Consequently, it is not surprising that *fliF* is among the earliest operons transcribed in the *C. crescentus* cell cycle (48), encoding as it does components that are assembled first into the flagellum, as well as the FlbD protein, which is called into play at this early stage of flagellar assembly to regulate expression of the next level of *fla* products and thereby the next level of flagellar assembly (5, 53).

The results presented here demonstrate, however, that additional genes within the *fliF* operon are also required for *flbD* function in *C. crescentus*. Our analysis of in-frame deletions and NTG-induced mutations in each of the five ORFs of *fliF* show that all genes in this transcription unit are essential not only for flagellum biosynthesis and motility but also for activation of the level III *flgK* and *flbG* operons (Table 4). Mutations in *rpoN*, which encodes the σ^{54} protein (3, 8), *fliM*, which encodes the third switch protein (73), the *fliQ* operon (13), and *flhA* (54) also prevent expression from these same promoters. Thus, all level II genes, including *flbD*, are required for expression of level III genes, as well as level IV genes, lower in the hierarchy (Fig. 1).

Negative regulation of *fla* gene expression by genes in the *fliF* operon. The presence of the FlbD protein alone is sufficient to repress transcription from the *fliF* promoter in a reconstituted in vitro system (5). Thus, the requirement of multiple genes for the negative autoregulation of the *fliF* promoter was unexpected and suggests an unusual aspect of *fla* gene regulation by FlbD in vivo. The function of *flbE* in flagellum biosynthesis is not known, but it is striking that mutations in this gene exert negative regulatory effects at least as strong as those displayed by *flbD* itself. The severe effects of *flbE* mutations on *fliF* transcription suggest that FlbE protein, along with FlbD, may be directly involved in negative regulation. Mutations in *fliF* and *fliG* resulted in only a four- to fivefold derepression, although the few mutations examined may not be null alleles (Table 4). Mutations in level IIB genes outside of the *fliF* operon have been shown to derepress *fliF* transcription only slightly (45), which suggests that the effect of these genes on *fliF* regulation may be indirect.

The finding (Table 4) that the switch protein gene *fliN* is needed for activation of level III and IV genes but not for repression of *fliF* indicates that the requirements for FlbD in transcription activation and repression are not identical. The simplest explanation of these observations is that the function of FlbD as a transcription activator and repressor in vivo requires different gene products. Possible mechanisms for FlbD regulation in vivo include covalent modification and direct interaction with another protein or proteins (see below). Whatever the mechanism(s), we suggest that FlbD function may be subject to dual or overlapping controls.

Is *fla* gene expression in *C. crescentus* coupled to flagellum assembly? As discussed above, all level II genes, which encode the protein subunits of the MS ring-switch structure, are required for transcription of level III genes. Previous results have shown that all genes in the level III transcription units, including *flbG* and *flgK* (51), *flgF* (11), and *flgI* (29), which encode the outer portion of the basal body and the hook, are required in turn for expression of the level IV flagellin genes (25, 45, 51, 70). How can multiple structural gene products regulate flagellar gene expression? Although it is formally possible that each of the proteins acts independently at the *fla* gene promoters, it is more likely that the proper assembly of these proteins into a completed structure directs the next stage of *fla* gene expression, as proposed originally as a mechanism for regulation of *fla* gene expression in *E. coli* (31). This is assumed to be the operative mechanism in the model diagrammed in Fig. 9, in which FlbD is shown as the transcriptional activator of level III and IV genes (5, 6).

The results considered above suggest that the ordered expression of *C. crescentus fla* genes is coupled to flagellum assembly at two morphological checkpoints: the first corresponds to the assembly of the inner basal body (level II MS ring and associated switch complex), which would be required for expression of genes at level III, and the second corresponds to completion of the hook-basal body, which would be required for expression of the 25- and 27-kDa flagellin genes at level IV. This is a more complex regulatory arrangement than the one described for *E. coli* and *S. typhimurium*, in which there is apparently a single morphological checkpoint. In these bacte-

ria, a complete hook-basal body encoded by the middle genes must be assembled before the late *fla* genes can be expressed (summarized in reference 35).

We can imagine two general models by which *fla* gene expression at level III could be coupled to assembly of the MS ring-switch protein complex in *C. crescentus*. In the first, a protein needed for transcription of level III genes, FlbD, would be synthesized along with other level II gene products, but the transcription factor would be sequestered and its activity somehow masked by an inhibitor. Completion of the basal body substructure would act to remove or otherwise inactivate the inhibitor, thereby permitting expression of the level III genes. This is similar to the model proposed by Komeda for coupling flagellin synthesis in *E. coli* to the completion of the hook-basal body structure (31). In *S. typhimurium*, the *flgM* gene encodes such a negative regulator (19), which blocks transcription of the flagellin genes by complexing with the flagellin-specific σ^{28} factor and prevents its association with the core RNA polymerase (47). Activation of flagellin gene expression results from the export of the FlgM protein outside of the cell, presumably through the export channel formed by the completed hook-basal body structure (22, 33a). It seems unlikely that a homolog of FlgM is involved in *C. crescentus fla* gene regulation, because level III and IV genes are not transcribed from σ^{28} promoters, but a negative regulatory protein in *C. crescentus* analogous to FlgM could inhibit either FlbD function or σ^{54} activity.

In the second general model, FlbD would be synthesized as an inactive protein and its activation would depend on a positive signal generated by completion of the MS ring-switch complex, perhaps by modification of the FlbD protein. Although modification could occur by a variety of mechanisms, phosphorylation may be the most likely given the homology of FlbD with NtrC (53), the recent observations that the FlbD protein can be phosphorylated in a cell cycle-specific fashion (68), and the finding by Benson et al. that a small high-energy phosphate donor stimulates FlbD activity as a transcriptional activator of σ^{54} promoters (6).

It should be noted that these two mechanisms are not mutually exclusive and both protein inhibition and modification could be used to regulate FlbD activity. As considered above, the requirement of *fliN* for activation of the σ^{54} promoters, but not for repression of *fliF* (Table 4), is consistent with the control of FlbD function at two different levels. In Fig. 9, we speculate that modification of FlbD to FlbD* for activation of the σ^{54} promoters is triggered by completion of the MS ring-switch complex, which is the first morphological checkpoint in flagellum assembly and requires all level II genes. We have no evidence for the mechanism by which the *flbE*, *fliG*, and *fliF* gene products function in repression of *fliF* by the FlbD protein (Fig. 9), but we speculate that they could act indirectly, perhaps as antagonists of an unidentified negative regulator of FlbD. The availability of purified FlbD protein and in vitro assays for its function as a transcriptional activator and repressor of level III and level II promoters (5) should allow the direct investigation of the regulatory interactions proposed here.

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