Early Caulobacter crescentus Genes fliL and fliM Are Required for Flagellar Gene Expression and Normal Cell Division

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The biogenesis of the Caulobacter crescentus polar flagellum requires the expression of more than 48 genes, which are organized in a regulatory hierarchy. The fliO locus is near the top of the hierarchy, and consequently strains with mutations in this locus are nonmotile and lack the flagellar basal body complex. In addition to the motility phenotype, mutations in this locus also cause abnormal cell division. Complementing clones restore both motility and normal cell division. Sequence analysis of a complementing subclone revealed that this locus encodes at least two proteins that are homologs of the Salmonella typhimurium and Escherichia coli flagellar proteins FlIL and FlIM. FlIM is thought to be a switch protein and to interface with the flagellum motor. The C. crescentus fliL and fliM genes form an operon that is expressed early in the cell cycle. Tn5 insertions in the fliM gene prevent the transcription of class II and class III flagellar genes, which are lower in the regulatory hierarchy. The start site of the fliLM operon lies 166 bp from the divergently transcribed flaCBD operon that encodes several basal body genes. Sequence comparison of the fliL transcription start site with those of other class I genes, flaS and flaO, revealed a highly conserved 29-bp sequence in a potential promoter region that differs from the FlA promoter, the 3'. flbO locus.

The developmental program of Caulobacter crescentus directs its differentiation into two dissimilar progeny at each cell division (Fig. 1). The stalked-cell and flagellated swarmer-cell progeny differ not only in morphology but in their ability to initiate DNA replication. The stalked-cell progeny initiates DNA replication, but the swarmer cell must first shed its flagellum and differentiate into a stalked cell before initiating chromosomal replication. Prior to division into progeny with different developmental programs, cellular asymmetry is expressed in the predivisional cell. At least two different mechanisms are responsible for this asymmetry: the differential transcription of specific genes from the newly replicated chromosomes in the predivisional cell (18, 20, 51) and the targeting of proteins, by virtue of information contained within their amino acid sequences, to either the stalked or incipient swarmer pole (2, 33, 47).

An important aspect of C. crescentus cell differentiation is the polar assembly of a single flagellum. Cell cycle cues appear to regulate the timed expression of flagellar genes in a temporal sequence that reflects the ordered assembly of their gene products (3–5, 21, 41). The C. crescentus flagellar genes can be grouped into three classes on the basis of the time of gene expression and the position of the gene in the regulatory hierarchy, as shown diagrammatically in Fig. 1. Epistasis experiments have placed the fliO locus in class I (22, 42). Two independent Tn5 insertions in the fliO locus result in a nonmotile cell that fails to assemble a basal body, hook, and filament and is unable to express the class II and class III flagellar genes. This mutant phenotype suggests that the fliO locus is involved in the early stages of flagellar biogenesis. In addition to the motility phenotype, mutations in the fliO locus also cause abnormal cell division. To define the initial steps in the flagellar regulatory hierarchy and to identify early components needed for the recognition of the swarmer-cell pole, we have characterized the fliO locus.

We report here that the fliO locus comprises of an operon that contains at least two genes. These genes are homologs of the fliL and fliM flagellar genes that have been identified in Escherichia coli (31), Salmonella typhimurium (27), and Bacillus subtilis (1, 57). As with other class I genes, expression of the fliLM operon occurs early in the cell cycle. Furthermore, the promoter regions of three class I genes, fliLM, flaS, and flaO, have a conserved 29-bp sequence that differs from those of the fla genes, the 3'. C. crescentus heat shock promoter (21), and the promoters used by some C. crescentus flagellar genes (9, 39, 40, 43), as well as the fla promoters that appear to be used by the chemotaxis genes (12).

MATERIALS AND METHODS

Bacterial strains and growth media. A derivative of the C. crescentus wild-type strain CB15N (renamed NA1000) (11) and mutant strains were grown in PYE medium (45) at 30°C. M2 minimal glucose medium (24) was used when cells were synchronized by Ludox density gradient centrifugation (11). E. coli TG-1, XL-1 blue, and S17.1 (53) were grown in LB medium or Superbroth (48) at 37°C.

Measurement of the time of expression of the fliLM operon. C. crescentus NA1000 containing a fliL transcriptional fusion was grown in minimal M2 glucose medium to an A600 of 0.8 to 1.0 and swarmer cells were collected in Ludox density gradients, as described previously (11). Swarmer cells were resuspended in M2 medium supplemented with glucose and allowed to proceed synchronously through the cell cycle at 30°C. At the division units shown in Fig. 10, 5-ml samples were removed and pulse-labeled for 10 min with 15 μCi of [35S]methionine (1,197 Ci/mmol). Cell extracts were prepared from the labeled cells and immunoprecipitated as previously described (21) with antibody to either β-galactosi-
The response defective in dependent on RNA to based transcription. separated on amide subcloned into DNA or vectors. Plasmid constructions. DNA was subcloned into either M13-based vectors or Bluescript vectors from Stratagene to generate single strands to be used for sequencing. Nested deletions were generated by the Nested deletion kit (Pharmacia) in the Bluescript vectors. The sequence of 2.8 kb of DNA was completed on both DNA strands by the method of Sanger et al. (49). DNA sequence analysis was done using the GCG package (7).

Plasmid constructions. DNA fragments were isolated and subcloned into pUC-based vectors (36) or Stratagene pBlue-scriptSK or pBluescript KS vectors. They were then cloned into a pRK290 derivative plasmid (pRK290-20R) that is able to replicate in C. crescentus (19). These plasmids were conjugated into SC1066 and SC1131 mutant strains and tested for complementation of motility.

Portions of the flbO locus were fused to a promoterless lacZ reporter gene in the plac290 vector (16). The plac290 vector contains the lacZ gene from pRSZ3 with the mp18 polynucleotide inserted into pRK290. Plasmid pJY400L was generated by a polymerase chain reaction by using genomic DNA. The DNA from positions 18 to 326 was amplified and then fused to lacZ in plac290. Plasmids containing these transcription fusions were transformed into E. coli S17.1 and then conjugated from E. coli into C. crescentus. β-Galactosidase activity was determined as described by Miller (38).

**Primer extension analysis.** The flbL transcription start site was mapped by primer extension analyses (21), using the four oligonucleotides C, D, E, and F, shown in Fig. 4. Total C. crescentus RNA (40 μg) was annealed to 5'-end-labeled γ-32P-oligonucleotide for 16 h at 45°C. The methods and solutions used were as previously described by Gomes and Shapiro (21). Products extended by avian myeloblastosis virus reverse transcriptase were analyzed next to a DNA sequencing ladder generated by the same primer on an 8% polyacrylamide gel.

**Nucleotide sequence accession number.** The nucleotide sequence reported in this paper was submitted to GenBank and assigned the accession number M85232.

**RESULTS**

**Identification and isolation of the flbO locus.** The flbO locus is defined by two independent Tn5 insertions (Ω1066, Ω1131) in the nonmotile strains SC1131 and SC1066 (23). These mutations map to a group of genes encoding structural components of the flagellar basal body (Fig. 2). Both SC1131 and SC1066 are nonmotile and have a cell division defect (Fig. 3A and B). Although both mutant strains form filaments, SC1131 (Fig. 3A) has a higher percentage of long, snakelike cells than SC1066 does (Fig. 3B).

Genetic complementation analysis was used to identify the functional gene boundaries within the flbO locus in strains carrying Ω1131 and Ω1066 insertions. Both Tn5 insertions lie adjacent to the 3' side of a SacI (A4) restriction site (Fig. 2), as determined by Southern blot analysis (22). A series of DNA fragments spanning this region was subcloned into the vector pRK290-20R. The plasmids, shown in Fig. 2, were then tested for their ability to complement the motility and cell division defects in SC1131 and SC1066. All subclones that complemented the motility phenotype also complemented the cell division phenotype. A 6.6-kb EcoRI-ClaI fragment (pKH6.6R) complemented mutations in the entire cluster, including flbN, flaD, flaB, flaC, and flbO (Fig. 2). The SC1131 strain containing the complementing plasmid pHX2.5R exhibited normal motility and the same morphology as that of the parental strain (Fig. 3C and D). Plasmids pHX2.5R and pHX1.9R were able to complement the flbO mutations but not mutations in either flaC or motC. DNA fragments contained in pKH1.3R, pHX5.3R, and pJY1.0R did not complement flbO mutations, thus defining pHX1.9R as the smallest complementary clone. The 2.5-kb SalI fragment in pKH2.5R complemented a mutation in flaC which lies 5' to the flbO mutations. motC, which lies 3' to flbO, is complemented by pKH410R. Neither pKH2.5R nor pKH410R complemented the flbO mutations. Therefore, flbO is defined as a separate transcriptional locus from the flanking genes flaC and motC.

**Nucleotide sequence of the flbO locus.** The DNA sequence of the flbO locus was determined and is shown in Fig. 4. Analysis of predicted amino acid sequences allowed us to identify two potential open reading frames (ORFs), by using the bias for G or C at the third position of each codon (26) and the frequency of rare C. crescentus codon usage (26, 50). Both ORFs are contained within the complementing clone pHX1.9R. The first ORF is predicted to encode a 167-amino-acid protein of approximately 18.4 kDa; the second ORF is predicted to encode a 373-amino-acid protein of approximately 41 kDa. A search of GenBank data base v67.0 using the TFASTA (7, 54) program showed significant similarities between ORF1 and flbL and between ORF2 and flbM from E. coli (30), B. subtilis (1, 57), and S. typhimurium (27) (Fig. 5).
and 6). In each of these organisms, both \( \text{flIL} \) and \( \text{flIM} \) are required for flagellar biogenesis and are adjacent genes within an operon. The \( \text{S. typhimurium} \) \( \text{flIM} \) gene encodes a flagellar switch protein that coordinates a chemotaxis signal with the direction of flagellar rotation (34, 55, 56). The function of the \( \text{flIL} \) gene is unknown.

The \( \text{FliL} \) proteins from different bacteria are of similar sizes: \( \text{S. typhimurium} \) \( \text{FliL} \) is 155 amino acids, \( \text{B. subtilis} \) \( \text{FliL} \) is 140 amino acids (1), and \( \text{C. crescentus} \) \( \text{FliL} \) is 167 amino acids. The putative initiation codon for \( \text{C. crescentus} \) \( \text{FliL} \) is a GTG, which encodes a valine. This codon can be used as an initiation codon in \( \text{C. crescentus} \), although no obvious ribosome-binding site precedes this sequence. \( \text{C. crescentus} \) \( \text{FliL} \) appears to contain an extra domain that is not found in other \( \text{FliL} \) proteins. Amino acid residues between positions 24 and 57 are not homologous to the predicted \( \text{E. coli} \) protein (Fig. 5) nor to the \( \text{B. subtilis} \) or \( \text{S. typhimurium} \) \( \text{FliL} \) proteins. Significant differences in amino acid sequence between \( \text{E. coli} \) and \( \text{S. typhimurium} \) were also found in the first third of the \( \text{FliL} \) proteins, suggesting that the function of this region is not highly conserved (27).

The predicted \( \text{C. crescentus} \) \( \text{FliL} \) protein contains a membrane-spanning domain at its amino terminus. In \( \text{E. coli} \), \( \text{FliL} \) fractionates exclusively to the membrane (35). A hydrophathy profile of the \( \text{C. crescentus} \) \( \text{FliL} \) protein shows a hydrophobic domain at the amino terminus, followed by a large hydrophilic domain (data not shown). The hydrophilic domain in the \( \text{C. crescentus} \) \( \text{FliL} \) protein is significantly larger than the domain found in the \( \text{E. coli} \), \( \text{S. typhimurium} \), and \( \text{B. subtilis} \) homologs. This domain contains a highly charged sequence consisting of lysine and glutamate residues. The \( \text{flIL} \) DNA sequence that encodes this region is underlined in Fig. 4. This is an unusual \( \text{C. crescentus} \) sequence for two reasons. First, it is a series of 47 purine residues. Second, such an AT-rich region (57%) is unusual for \( \text{C. crescentus} \) DNA, which is 33% AT. To ensure that this sequence was correct, multiple independent clones were sequenced by the single-stranded method (49) and a double-stranded genomic polymerase chain reaction product of this region was sequenced as well. Both methods verified the existence of this region.

An alignment of the predicted \( \text{FliM} \) amino acid sequences from \( \text{E. coli} \) (30), \( \text{C. crescentus} \), and \( \text{B. subtilis} \) (57) is shown in Fig. 6. The \( \text{C. crescentus} \) \( \text{FliM} \) protein shares 22% amino acid identity and 48% similarity with the \( \text{FliM} \) protein from \( \text{B. subtilis} \) and 25% amino acid identity and 51% similarity with the \( \text{FliM} \) protein from \( \text{E. coli} \). This level of amino acid identity is similar to that found between the \( \text{B. subtilis} \) \( \text{FliM} \) protein and the \( \text{E. coli} \) (29%) and \( \text{S. typhimurium} \) (28%) predicted amino acid sequences. Interestingly, there are few amino acid identities common to all three proteins, although there are many synonymous amino acid changes dispersed throughout the protein. The most highly conserved region in the \( \text{FliM} \) protein from \( \text{B. subtilis} \), \( \text{E. coli} \), and \( \text{S. typhimurium} \) is a 10-amino-acid sequence, LSOQ-EIDALL, found near the amino terminus (57). A similar sequence of LNOQ-EIDSSL was found in the \( \text{C. crescentus} \) \( \text{FliM} \) homolog near the amino terminus (Fig. 4, boxed).

Within the first 36 codons of \( \text{FliM} \) (Fig. 6), there are four possible initiation codons at amino acids 1, 11, 25, and 35 (Fig. 6, arrows). The first two potential initiation codons are not preceded by a consensus for the \( \text{C. crescentus} \) ribosomal
binding site. However, the GTG at position 26 is preceded by a good ribosomal binding site. There is as yet no experimental evidence to determine which initiation codon is used in vivo. The switch protein homology begins after the initiation codon at amino acid 47. Initiation at either amino acid position 26 or 36 would yield a protein comparable in size to that predicted for the FliM protein (37,000 Da) from B. subtilis (57), E. coli, or S. typhimurium (27).

**Transcription of the fliLM genes.** S1 nuclease protection assays were done to determine the direction of transcription. A DNA fragment of 1.2 kb extending from the SalI (S3) site to the XhoI site (see restriction map at the top of Fig. 7) was used as a probe in these assays. The 1.2-kb probe end-labeled at the XhoI restriction site resulted in a fully protected fragment, whereas the same fragment end-labeled at the SalI (S3) site did not protect an RNA fragment (data not shown). Thus, the direction of transcription is from SalI (S3) to XhoI.

Deletions in the fliL 5′ region were constructed to identify DNA sequences necessary for transcription (Fig. 7). A series of DNA fragments was fused to a promoterless lacZ reporter gene on plasmid plac290. These plasmids were conjugated into wild-type C. crescentus, and β-galactosidase activity was determined. A 2.5-kb SalI fragment, containing 1,735 bp upstream of the fliL coding region (pJYSSL), has 2,000 U of β-galactosidase activity, as compared to 70 U obtained with the plac290 vector that does not contain a DNA insert and to 20 U obtained with pJYSXL that deletes all of fliL and the first third of the fliM coding region. A deletion that retains 29 bp 5′ to the fliL transcription initiation site (see Fig. 9) in pJYHSL and pJYSAL results in an approximately fourfold decrease in promoter activity to 680 and 500 U, respectively. The plasmid pJY400L that retains 263 bp 5′ to the fliL transcription start site but lacks the fliL coding sequence and 52 bp of untranslated leader mRNA has 3,000 U of β-galactosidase activity. The additional 234 bp 5′ to the HpaI (H3) site in pJY400L, compared to pJYHSL and pJYSAL, appears to be responsible for increasing the promoter activity.

FIG. 3. Electron micrographs of C. crescentus mutant strains SC1131 (A), SC1066 (B), and SC1066 containing the complementary plasmid pHX2.5R (C), and of parent strain NA1000 (D). Cells on carbon-coated grids were stained with 1% uranyl acetate and examined in a Philips 300 electron microscope.
Fig. 4. DNA sequence of the flbO locus shown schematically by the bar in Fig. 2. This figure shows the nucleotide sequences of two ORFs and their deduced amino acid sequences. The flbO locus was found to contain two genes that are similar to the E. coli, S. typhimurium, and B. subtilis flL and flM genes. ORF1 (flL) begins at position 425 and ends at position 928. ORF2, corresponding to flM, begins at position 956 and ends at position 2077. An unusual sequence of 47 alternating purines, within the putative flL coding sequence, is underlined (positions 509 to 555). Arrows indicate the possible start sites for the FlL and FlM proteins. The arrow below the nucleotide sequence shows the transcriptional start site. The underlined and boldfaced amino acid sequence within the flM coding region is similar to the highly conserved amino terminus of flM in S. typhimurium, E. coli, and B. subtilis. Boxes labeled A, B, C, D, E, and F above the DNA sequence depict the primers used in primer extension analysis.
FIG. 5. Amino acid sequence comparison of the predicted FliL proteins from *C. crescentus* (C.c.) and *E. coli* (E.c.) (30). Sequences were aligned by using the TAP program from the GCG package (7), beginning with the first initiation codon of both proteins. Identities between residues are boxed with solid lines, and conserved amino acid changes are boxed with dashed lines. The two proteins have 26.2% identity and 50% similarity. There is a charged domain containing predominantly Lys and Gln in the *C. crescentus* FliL protein that is not present in the *E. coli* FliL protein. The *C. crescentus* *fli* gene encodes 167 amino acids, corresponding to an approximate size of 18.4 kDa.

by five- or sixfold. The plasmid pJP300L, containing sequences 3' to the *fli* coding region (27 bp) and the beginning of the *fliM* coding region, does not have significant transcriptional activity, indicating that there is no promoter activity between the two genes. The results obtained with these reporter gene transcription fusions suggest that the promoter for *fli* resides within a 300-bp fragment and that this promoter directs the transcription of both the *fli* and *fliM* genes.

**Identification of the transcription initiation site.** Primers A and B, which are complementary to sequences found within the *fliM* coding region (Fig. 4), were used in primer extension assays to determine the transcription start site. Reverse transcriptase was used to extend the sequence from the annealed primer-RNA hybrid. Primer extension with either primer A or B resulted in an extended product that mapped well beyond the predicted FliM initiation codon (data not shown). This result, in conjunction with the transcriptional fusions shown in Fig. 7, argues that the *fliM* gene does not have its own promoter; rather, it most likely resides within an operon initiating with the *fliL* gene. An RNA transcript begins 5' to the *SalI* (S) site and terminates beyond the *XhoI* site.

To map the transcription start site more precisely, four oligonucleotide primers were used (labeled C, D, E, and F in Fig. 4) for primer extension analysis. Primers C, D, and E are complementary to sequences upstream of the *fliL* gene. All four primers extended to the same 5' end of the RNA transcript. Primers C, D, E, and F are 22, 45, 61, and 204 bp from the start of transcription, respectively. Primer extension analysis with primer D is shown in Fig. 8. The tran-
transcription start site of *flil* lies within the pJY400L transcriptional fusion which gave strong promoter activity (Fig. 7). This single transcription start site is located 30 bp 3’ to the *HpaI* (H3) site, and the transcript extends past the *flil* gene. Thus, pJYHSL and pJYHAL, shown in Fig. 7, would have a deletion extending into a possible promoter region. The residual β-galactosidase activity observed with both deletions could be due to vector sequences in conjunction with the 5’ *flil* sequences generating weak promoter activity.

The DNA sequence surrounding the start of transcription is shown in Fig. 9. The *flil* operon is divergently transcribed from the *flac* operon (8). The start site of the *flac* transcript, mapped by primer extension analysis (8), is 166 bp 5’ to the *flil* transcription start site. The DNA sequence within 50 bp of the region 5’ to the *flil* start site does not contain sequences that conform to the promoters known to be recognized by σ70, σ24, σ28, or the heat shock sigma factor σ32 (6). However, the sequence beginning 11 bp from the start of *flil* transcription (Fig. 9, boxed) has a 29-bp conserved region (between -11 and -39) that is also found at -7 and -10 from the *flaO* (40) and *flaS* (10) start sites, respectively. All three genes, *flil*, *flaS*, and *flaO*, are class I genes that reside at or near the top of the flagellar regulatory hierarchy. Mutations in these genes yield a nonmotile phenotype that also exhibits cell division defects. It seems likely that the consensus promoter motif for these genes reflects their parallel patterns of regulation.

**Temporal expression of the *flilM* operon.** Transcription of both the *flaO* (39) and the *flaS* (10) genes is initiated during a defined time in the cell cycle, prior to the initiation of transcription of the hook and flagellin structural genes. To test whether expression of the *flilM* operon is temporally regulated, a *Sall* (S3)-XhoI (Fig. 7) transcription fusion to a promoterless *lacZ* reporter gene was integrated into the *C. crescentus* chromosome at the *flbo* locus. The resulting integrant truncates the chromosomal copy of the *flil* gene, and the expression of the *lacZ* reporter is driven by the *flil* promoter (Fig. 10B). Cultures containing this *flil*:lacZ fusion were synchronized. Samples were taken at the times indicated in Fig. 10A and incubated with [35S]methionine for 10 min. Cell extracts were prepared, and protein was immunoprecipitated with antibody to β-galactosidase as described in Materials and Methods. In this assay, the synthesis of β-galactosidase is a reflection of *flil* promoter activity. Transcription initiates at the swarmer-to-stalk transition, between 0.2 and 0.5 division units. Maximum expression occurred in predivisional cells at 0.8 division units. The gene was not transcribed in the progeny cells. Thus, transcription of *flil* initiates early in the cell cycle, coincident with the initiation of transcription of *flaS* (10) and *flaO* (39). Moreover, since the junction between *flilM* and the *lacZ* reporter gene is 404 bp downstream of the *flil* coding region, transcription of the operon continues beyond *flilM*, which is consistent with the SI analysis. (We show in Fig. 7, by using
stalled cells contained β-galactosidase synthesized from the fliL::lacZ transcription fusion, suggesting that the fusion transcript was not specifically segregated to the swarmer cell.

DISCUSSION

Two Tn5 insertions within the fliB locus result in nonmotile strains (SC1131, SC1066) that lack a basal body complex (23). We have shown here that the fliB locus is an operon that contains at least two genes that are homologous to the E. coli and S. typhimurium fliL and fliM genes. Both Tn5 insertions map within the fliM coding region, implying that either fliM alone or fliM in conjunction with fliL is required for normal flagellar biogenesis.

Transcriptional regulation of the fliLM operon. It was found that class II genes (Fig. 1), such as the hook operon (fliG) (39-41), and class III flagellin gene products, the 25- and 27.5-kDa flagellins encoded by figK and figL, respectively, are not expressed in a fliM mutant background. In an attempt to identify genes that are involved in the regulation of the fliL operon, fliL::lacZ transcription fusions were tested in a variety of flagellar mutants from all three classes (data not shown). None of the 25 flagellar mutant strains tested decreased fliL transcription. However, mutations in class I genes such as flaS, flaO, and fliL increase fliL transcription by approximately twofold (data not shown). These results suggest a coordinate regulation of class I genes and possible autoregulation of fliL transcription. Another possible class I gene was revealed when fliLM expression was found to increase twofold in strains (10a) carrying a mutation in the fliJ gene. The genes that initiate the flagellar cascade, comparable to the fliC and flhD genes identified in E. coli (31) and S. typhimurium (28, 29, 52), have not yet been identified in C. crescentus. It may be that mutations in the regulators of the C. crescentus class I genes have not been identified in multiple flagellar mutant screens because they are involved in other essential cellular functions and would thus be lethal to the cell.

All of the known class II and class III genes such as fliG, fliK, fliN, and fliL (31) and fliB (9) require a σ^54 RNA polymerase for expression. The C. crescentus chemotaxis operon has a promoter sequence 5' to its transcription start site (12, 17) that is similar to that utilized by the E. coli, S. typhimurium, and B. subtilis σ^70 RNA polymerase that is used for a subset of flagellar and chemotaxis genes (31, 44, 57). Identification of the transcription start site and sequence analysis revealed that the fliL promoter does not conform to the σ^54, σ^28, σ^22, or σ^30 promoter consensus sequences. Furthermore, the expression of fliL was not affected in a σ^54 mutant strain. Other class I genes, such as flaS (10) and flaO (39), share this unique promoter sequence with fliL (Fig. 9). This potentially new promoter might account for the similar temporal expression and regulation of these class I genes, perhaps mediated by a previously unidentified sigma factor.

The fliL operon maps between the flaC (22) and motC operons, as determined by Southern blots and complementation analysis (Fig. 2). The flaC and fliL operons form divergent units, with 166 bp between the two transcription start sites. An 18-bp inverted repeat sequence (Fig. 9) that lies between the two transcription units (63 bp from the start of fliL transcription) may be a control element for either the fliLM or the flaC operon. It may be that the two operons share common motifs that regulate their expression. However, mutations in any of the class II genes, including the flaC operon, do not affect fliL expression. In contrast,
mutations in the fliL operon inactivated expression of the flaC operon. Although these divergent operons are differentially controlled, their expression might be temporally coordinated by a shared regulatory region.

**Analysis of the predicted FliL and FliM proteins.** Of the three genes in the *S. typhimurium* switch gene cluster, i.e., fliL, fliM, and fliN, the fliL gene and its product diverge the most from their *E. coli* homologs (27). The predicted amino acid sequence of the *S. typhimurium* FliL is 77% identical to its *E. coli* homolog, whereas FliM and FliN have 90 to 95% identity with their *E. coli* counterparts (27). Both the *C. crescentus* and *B. subtilis* FliL proteins appear to have diverged even further from the *E. coli* and *S. typhimurium* homologs. The *B. subtilis* FliL is 21% identical to the *E. coli* FliL (1), and the *C. crescentus* FliL shares 26% amino acid identity and 50% similarity with the *E. coli* FliL protein (Fig. 5).

The FliL protein from *E. coli* and *S. typhimurium* contains a membrane-spanning domain at its amino terminus that anchors it within the membrane (27, 35) and a hydrophilic domain that is thought to reside within the periplasm (35). The *C. crescentus* FliL homolog has a similar hydrophobic domain at its amino terminus that may function as a membrane-spanning domain. It is followed by a large hydrophilic domain composed of a cluster of lysines, glutamates, and aspartate residues. The specific function that FliL plays in cell motility has yet to be determined. However, another switch protein from *S. typhimurium*, FliG, also contains clusters of charged amino acids, namely, Asp, Glu, His, Lys, and Arg (27). Of these residues in FliG, 52% are adjacent to another charged residue and 26% of the residues are in clusters of three or four. Although the function of these charged clusters is not known, one hypothesis is that they play a role in the proton translocation process that is required for flagellar rotation (27).

FliM is one of three proteins (*fliG, fliN, fliM*) that form a switch complex that is proposed to be located at the base of the basal body (34, 55, 56). This complex interacts with the CheY and CheZ chemotaxis proteins, in addition to contacting components of the motor that determine the direction of flagellar rotation (55). In *C. crescentus*, the absence of FliM results in the loss of the earliest flagellar structure, the basal body complex (23). Similarly, the *S. typhimurium* flagellar assembly is arrested shortly after the insertion of the first basal body ring if the cell has a temperature-sensitive mutation in *fliG* or *fliN* (25). The *E. coli* and *S. typhimurium* FliM proteins are associated with the inner membrane and do not contain a cleavable signal sequence (27). *C. crescentus* FliM also does not appear to have a cleavable signal sequence. The PEPTIDESTRUCTURE program (7) showed that the organization of the hydrophobic and hydrophilic domains throughout the FliM protein are similar in *C. crescentus*, *E. coli*, and *S. typhimurium*. Both the amino and carboxy termini of these proteins are polar (27).

In this study, we have found that a homolog of an early structural component of the flagellar rotor is necessary for
expression of class II and class III flagellar genes. Likewise, mutations in the *B. subtilis* *fliM* gene affect the expression of chemotaxis and motility genes that are transcribed by σ^70 (57). How does a component of the flagellar motor, such as a switch protein, affect the transcription of other flagellar genes? It seems unlikely that the FliM protein directly regulates the transcription of other flagellar genes; rather, it is probably involved in the accurate assembly of the flagellum complex. It may be that if the flagellum is not properly assembled, a signal is generated that prevents the expression of structural flagellar components normally produced later in the assembly process. It has been demonstrated in *S. typhimurium* that the *flgM* gene product prevents transcription of late flagellar genes if the intermediate steps in flagellum assembly are aberrant (14, 15).

Flagellar biogenesis in *C. crescentus* is the result of a complex coordination of flagellum assembly, flagellar gene expression, DNA replication, and cell division (Fig. 1). It has recently been demonstrated that transcription of *fliL* (46) and other class I genes, including *flaS* and *flbO* (10), are affected if DNA replication is disrupted. Furthermore, mutations in the class I genes such as *flaS*, *flaO*, and *fliLM* affect normal cell division. Defects in cell division are not apparent in strains carrying class II or class III flagellar mutations, even though some of these mutants lack a basal body complex. These results imply that class I gene expression is coordinated with events associated with cell division (Fig. 1). The regulation of flagellar gene expression is also cued to the stages of flagellar assembly so that additional structural components are not made if an aberrant structure has been formed. Therefore, although FliM most likely does not directly affect flagellar gene expression or cell division, it lies within the pathway that coordinates and senses these different cellular processes.

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REFERENCES


7. Devereux, D., P. Haeberli, and O. Smithies. 1984. A comprehen-
EARLY C. CRESCENTUS GENES ftll AND ftIM

3337

46. Quon, K. Unpublished data.