JOURNAL OF BACTERIOLOGY, Oct. 1998, p. 5010–5019 Vol. 180, No. 19
0021-9193/98/$04.00+0

A New Class of *Caulobacter crescentus* Flagellar Genes

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Received 27 March 1998/Accepted 24 July 1998

Eight *Caulobacter crescentus* flagellar genes, *flmA*, *flmB*, *flmC*, *flmD*, *flmE*, *flmF*, *flmG*, and *flmH*, have been cloned and characterized. These eight genes are clustered in pairs (*flmAB*, *flmCD*, *flmEF*, and *flmGH*) that appear to be structurally organized as operons. Homology comparisons suggest that the proteins encoded by the *flm* genes may be involved in posttranslational modification of flagellins or proteins that interact with flagellin monomers prior to their assembly into a flagellar filament. Expression of the *flmAB*, *flmEF*, and *flmGH* operons was shown to occur primarily in predivisional cells. In contrast, the *flmCD* operon was expressed throughout the cell cycle, with only a twofold increase in predivisional cells. The expression of the three temporally regulated operons was subject to positive regulation by the CtrA response regulator protein. Mutations in class II and III flagellar genes had no significant effect on the expression of the *flm* genes. Furthermore, the *flm* genes did not affect the expression of class II or class III flagellar genes. However, mutations in the *flm* genes did result in reduced synthesis of the class IV flagellin proteins. Taken together, these data indicate that the *flm* operons belong to a new class of flagellar genes.

The life cycle of the aquatic bacterium *Caulobacter crescentus* provides a model system for the analysis of programmed developmental events. Each cell division produces two morphologically dissimilar progeny cells, a motile swarmer cell and a sessile stalked cell. Flagellum biogenesis is initiated in the predivisional cell and results in the synthesis of a basal body, hook, and flagellar filament at one pole of the developing swarmer cell (reviewed in reference 24). This process involves at least 50 genes (19). Most of the flagellar genes have been organized into a regulatory hierarchy that includes four classes of genes (reviewed in reference 54). In this hierarchy, the expression of genes in an earlier class is required for expression of the genes in subsequent classes. Furthermore, the genes that encode the structural components of the flagellum are transcribed in the order that their gene products are assembled into the structure. For instance, expression of the class II genes is required for expression of the class III hook and basal body genes. Similarly, expression of the class II and III genes and assembly of their products are required for expression of the class IV flagellin genes. One class II gene, *rpoN*, encodes sigma 54, the sigma factor that binds to class III and class IV promoters (11). In addition, integration host factor and transcriptional activators are required for expression of class III and class IV genes (7, 25, 26, 73). Synthesis of the flagellin subunits encoded by the class IV genes is also subject to posttranscriptional control mechanisms (3, 41, 42). Recently, Quon et al. (53) have shown that mutations in the class I *ctrA* gene result in altered expression of class II flagellar genes.

The flagellar filament is composed of three distinct flagellin monomers. A 29-kDa flagellin is initially assembled at the hook-proximal portion of the filament (18). Subsequently, the 27- and 25-kDa flagellins are synthesized and assembled consecutively (18, 71). The 25-kDa flagellin constitutes the distal two-thirds of the filament (18). In addition to these structural genes, the expression of the flagellar genes *flmA* (formerly *flaA*), *flmD* (*flaR*), *flmE* (*flaZ*), *flmG* (*flbA*), and *flmH* (*flaG*) is required for the synthesis of normal flagellin proteins (30). Strains containing mutations in any of these genes have a normal basal body and hook structure but fail to assemble a flagellar filament (30). Mutations in the *flmA*, *flmD*, *flmG*, and *flmH* genes result in the production of a novel 22-kDa flagellin protein. In addition, the production of the other flagellins is severely decreased (30, 57, 61). Mutations in the *flmE* gene resulted in the production of flagellins that migrate slightly faster than the wild-type flagellins on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Thus, flagellins from the *flmE* mutant have apparent molecular masses of 26 and 24 kDa instead of 27 and 25 kDa (30). The 22- and 24-kDa proteins are also produced in *flbT* mutants when the flagellins are overproduced (57). Recently, we have shown that degradation of the *fljK* mRNA is regulated by the *flbT* gene product (42).

To analyze the role of the *flm* genes in the production of these flagellin proteins, we have cloned and determined the nucleotide sequences of the *flmAB*, *flmCD*, *flmEF*, and *flmGH* operons. Homology searches revealed that the deduced amino acid sequences of the *flmA*, *flmB*, *flmC*, and *flmD* genes are similar to sequences of proteins involved in capsular and spore coat polysaccharide biosynthesis. Thus, the *flm* genes may be involved in glycosylation. To study the regulation of the *flm* operons, we have constructed fusions of the *flmA*, *flmC*, *flmE*, and *flmG* promoters to the *cat* or *lacZ* reporter gene. We demonstrate that expression of the *flmA*, *flmE*, and *flmG* genes is altered by a mutation in the *ctrA* gene, a class I flagellar gene. In addition, we show that the *flmA*, *flmE*, and *flmG* genes are expressed primarily in predivisional cells, indicating that these putative operons are temporally regulated. In contrast, the *flmC* gene is expressed throughout the cell cycle, with a twofold increase in the predivisional cell. These results, along with studies of the flagellar regulatory hierarchy, suggest that the *flm* genes belong to a new class of flagellar genes.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. All *C. crescentus* strains used are derivatives of the wild-type strain CB15. *C. crescentus* strains were grown at 33°C

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference or source
E. coli		
S17-1	*proA recA hsdR hsdM zzz*:RP4 (Tc::Mu) (Km::Tn*7*)	62
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* F^+ (*proAB lacI*^{q} M15 Tn*10*)	Stratagene
C. crescentus		
CB15	Wild type	
LS107	*syn-1000 bla-6*	Dickon Alley
LS2195	*syn-1000 ctrA401*	53
PC7070	*recA526 zzz*::Tn*5 str-30*	50
SC175	*flmE102* (formerly *flaZ102*)	29
SC229	*flmA104* (formerly *flaA104*)	29
SC278	*flmH131* (formerly *flaG131*)	29
SC305	*flmD148* (formerly *flaR148*)	29
SC1029	*flhB195*::Tn*5 str-152*	70
SC1030	*flmE618*::Tn*5* (formerly *flaZ618*::Tn*5*) *str-152*	19
SC1032	*flbD198*::Tn*5 str-152*	51
SC1055	*rpoN610*::Tn*5 proA 103 str-140*	19
SC1066	*fliL179 proA103 str-140*	19
SC1117	*flbN194*::Tn*5 str-152*	19
SC1127	*flmD614*::Tn*5* (formerly *flaR614*::Tn*5*) *str-142*	19
SC1128	*flmA613*::Tn*5* (formerly *flaA613*::Tn*5*) *str-142*	19
SC1132	*flhA608*::Tn*5 str-152*	51
SC1134	*flgK603*::Tn*5 str-152*	51
SC1135	*flbG602*::Tn*5 str-152*	51
SC2663	*fliM667*::Tn*5-lacI rif-175*	19
SC3090	*flmD651*::Tn*5* (formerly *flaR651*::Tn*5*) *str-152*	19
SC3809	*fliQR153 zzz*::Tn*5 recA526*	19
SC3898	*flmD148 recA526 zzz*::Tn*5*	φPC7070 × SC305
SC3899	*flmE102 recA526 zzz*::Tn*5*	φPC7070 × SC175
SC3971	*syn-1000 flmC*::pGL20	pGL20 integrated into LS107
SC3973	*syn-1000 flmE*::pGL21	pGL21 integrated into LS107
SC3975	*syn-1000 flmA*::pGL22	pGL22 integrated into LS107
SC4016	*syn-1000 flmG*::pGL41	pGL41 integrated into LS107
SC4250	*syn-1000* (*flmA*::*lacZ* on pRKlac290)	LS107 harboring pSPW1967

in peptone-yeast extract (PYE) medium or in defined minimal medium M2 (28). *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) or defined E medium supplemented with 1.0 mM proline (44). Complementation of motility defects was demonstrated in semisolid medium as previously described (59). Plasmids were transferred to the recipient *C. crescentus flm* mutants by conjugation from their *E. coli* host S17-1. Antibiotics were added, when appropriate, at the following concentrations: ampicillin, 50 μg/ml; sulfanilamide, 350 μg/ml in E medium; tetracycline, 15 μg/ml in LB and 1 μg/ml in PYE medium; and kanamycin, 50 μg/ml.

Molecular techniques. General cloning procedures were carried out as described by Sambrook et al. (55). *C. crescentus* chromosomal DNA was isolated as previously described by Malakooti and Ely (40). All enzymes used in the manipulations of DNA were used according to the specifications of the manufacturer. Transformation of *E. coli* was carried out as described by Sambrook et al. (55). Transformation of *C. crescentus* was carried out by electroporation according to the procedure of Gilchrist and Smit (23). The nucleotide sequence of both strands of DNA containing the gene of interest was determined from either double-stranded templates or single-stranded templates by the dideoxy-chain termination method (56) using a Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio). Nucleotide and amino acid sequence analyses were performed with the Wisconsin Package of the Genetics Computer Group (Madison, Wis.) (16).

Construction of *flmA*::*cat*, *flmC*::*cat*, *flmE*::*cat*, and *flmG*::*cat* gene fusions. For the *flmCD*, *flmEF*, and *flmGH* operons, a promoterless chloramphenicol acetyltransferase (CAT) cartridge contained in a 0.8-kb *Hin*dIII fragment was inserted into the unique *Hin*dIII sites of pGL39, pGL11, and pGL24, respectively. In the case of the *flmAB* operon, a 1.7-kb *Bam*HI-*Sal*I fragment containing the *flmA* promoter region was cloned into pGL30 that contained the *cat* gene inserted in the orientation opposite that of the *lacZ* promoter. The resulting plasmids, pGL20, pGL21, pGL22, and pGL41, were introduced in *C. crescentus* LS107 by electroporation. Transformants were selected for ampicillin resistance, causing the integration of the nonreplicating plasmid into the chromosome. Single-crossover recombinants were identified by Southern blot analysis (data not shown), resulting in strains SC3971, SC3973, SC3975, and SC4016, containing the chromosomal fusions *flmA*::*cat*, *flmC*::*cat*, *flmE*::*cat*, and *flmG*::*cat*, respectively.

CAT and β-galactosidase assays. Strains carrying a transcriptional fusion (plasmid borne or integrated into the chromosome by homologous recombination) were grown to the exponential growth phase (125 Klett units or A_{600} of 0.5) in PYE medium supplemented with appropriate antibiotics. Cell extracts were prepared by sonic disruption of the cells in 0.1× TE (10 mM Tris–0.1 mM EDTA [pH 8.0]). The cell debris was removed by centrifugation, and the supernatant was assayed to determine the protein concentration (9). CAT activity was assayed by using [^{3}H]acetyl coenzyme A according to the directions of the manufacturer (NEN, Boston, Mass.). Assays of β-galactosidase activity were performed as previously described (44). Totals of 5 and 12 μg of protein were assayed for β-galactosidase and CAT activities, respectively.

Cell synchronization and immunoprecipitation. Strains containing *flmC*::*cat*, *flmE*::*cat*, and *flmG*::*cat* chromosomal integrated fusions or *flmA*::*lacZ* plasmid (pSCW1967)-borne fusion were grown in M2 medium and synchronized by differential centrifugation (6). Swarmer cells were allowed to proceed synchronously through the cell cycle at 30°C. Samples were removed at specific times and pulse-labeled for 10 min with 10 μCi of Tran^{35}S-label (ICN, Costa Mesa, Calif.). Cell extracts were prepared and immunoprecipitated as described by Gomes and Shapiro (27), using antibody to CAT or β-galactosidase protein. Flagellin immunoprecipitation was used as a positive control for cell cycle-dependent expression. The cell cycle was also monitored by light microscopy. The immunoprecipitated proteins were resolved by SDS–10% polyacrylamide gel electrophoresis and visualized by autoradiography.

Nucleotide sequence accession numbers. The DNA sequences of *flmAB*, *flmCD-flmEF*, and *flmGH* operons described in this report have been assigned GenBank accession no. U27301, U27302, and U28867, respectively.

RESULTS

Isolation and characterization of *flmC*, *flmD*, *flmE*, and *flmF* genes. A cosmid, pLSG1, containing about 25 kb of *C. crescentus* chromosomal DNA was identified by complementation of the motility defect of strains SC1030 (*flmE*::Tn*5*), SC1127

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics or construction
pBEE302	Cloning vector (Sul^r SM^r (derivative of R300B) (58)
pBKSII(+/−)	pBluescript II KS +/− phagemid [Amp^r ColE1 replicon ori f1 (+/−)] (Stratagene)
pBSKII(+/−)	pBluescript II SK +/− phagemid [Amp^r ColE1 replicon ori f1 (+/−)] (Stratagene)
pKT230	Cloning vector (Kan Str IncQ replicon) (4)
pRKlac290	Promoterless *lacZ* gene cloned into pRK290 for transcriptional fusion (26)
pRK2L1	Cloning vector (Tet^r IncP replicon, Mob$^+$) (47)
pUC19	Cloning vector (Amp^r ColE1 replicon) (76)
R300B	Cloning vector (Sul^r Sm^r IncQ replicon, Mob$^+$) (5)
pCS91	*rsaA*::*lacZ* in pRKlac290 (65)
pGL2	pSCC33 with 1.6-kb *Hpa*I fragment deleted
pGL11	0.8-kb *Sac*I fragment from pGL14, containing the *flmC* promoter cloned into pBKSII(−)
pGL14	8.6-kb *Bam*HI-*Eco*RI fragment from pSCC2 into pBKSII(−)
pGL14-106	1.85-kb deletion from *Eco*RI site of pGL14 (exonuclease III deletion)
pGL14-312	4.35-kb deletion from *Eco*RI site of pGL14 (exonuclease III deletion)
pGL15	pGL14 with 3.2-kb *Hpa*I fragment deleted
pGL17	pGL14-106 cloned into pRK2L1 digested with *Bam*HI
pGL18	pGL14-312 cloned into pRK2L1 digested with *Bam*HI
pGL19	pGL15 cloned into pRK2L1 digested with *Bam*HI
pGL20	Promoterless CAT cartridge inserted into *Hin*dIII site of pGL11 (*flmC*::*cat*)
pGL21	Promoterless CAT cartridge inserted into *Hin*dIII site of pGL24 (*flmE*::*cat*)
pGL22	1.7-kb *Bam*HI-*Sal*I fragment containing *flmA* promoter cloned into pGL30 (*flmA*::*cat*)
pGL24	0.8-kb *Sal*I fragment from pSCC33 containing *flmE* promoter cloned into pBSKII(−) in opposite orientation of *lacZ* promoter
pGL30	Promoterless CAT cartridge inserted into *Hin*dIII site of pUC19 in opposite orientation of *lacZ* promoter
pGL36	3.16-kb *Sac*I-*Nco*I fragment from pLSG1 cloned in pBKSII(−)
pGL38	pGL36 cloned into pRK2L1 digested with *Kpn*I
pGL39	1.5-kb *Eco*RI-*Bam*HI fragment from pPVS149 containing *flmG* promoter cloned into pUC19
pGL41	Promoterless CAT cartridge inserted into *Hin*dIII site of pGL39 in opposite orientation of *lacZ* promoter
pGL48	pGL20 cloned into pRK2L1 digested with *Kpn*I (*flmC*::*cat*)
pGL49	pGL21 cloned into pRK2L1 digested with *Kpn*I (*flmE*::*cat*)
pGL50	pGL22 cloned into pRK2L1 digested with *Kpn*I (*flmA*::*cat*)
pGL53	pGL41 cloned into pRKlac290 digested with *Bam*HI (*flmG*::*lacZ*)
pLSG1	pLAFR1-7-derived cosmid containing 25 kb of *C. crescentus* DNA (*flmCD-flmEF* region) (58)
pLSG6	pLAFR1-7-derived cosmid containing 20 kb of *C. crescentus* DNA (*flmAB* region) (58)
pNC1341	3.5-kb *Bam*HI-*Eco*RI from pSCC7 cloned into pKT230
pNC1355	1.8-kb *Sal*I-*Eco*RI subcloned from pNC1341
pNC1356	1.7-kb *Bam*HI-*Sal*I subcloned from pNC1341
pPVS149	2.4-kb *Bam*HI-*Sac*I fragment cloned into pKT230 (59)
pPVS154	3.2-kb *Eco*RI fragment (*flmGH* region) in R300B (59)
pSCC2	14-kb *Eco*RI fragment subcloned from pLSG1
pSCC7	5-kb *Eco*RI fragment from pLSG6 cloned into pBEE302
pSCC33	3.7-kb *Sac*I from pLSG1 (*flmCD-flmEF* region) cloned in R300B
pSCW1355	1.8-kb *Sal*I-*Eco*RI fragment from pNC1355 cloned into pBKSII(−)
pSCW1356	1.7-kb *Bam*HI-*Sal*I fragment from pNC1356 cloned into pBKSII(−)
pSCW1967	1.7-kb *Bam*HI-*Sal*I fragment from pNC1356 cloned into pRKlac290 (*flmA*::*lacZ*)
pWZ162	*fliQ*::*lacZ* in pRKlac290 (79)

(*flmD*::Tn*5*), and SC3090 (*flmD*::Tn*5*) (58). Plasmid pLSG1 could also complement the SC175 (*flmE*) and SC305 (*flmD*) mutants. Deletion analysis of pLSG1 revealed that a 14-kb *Eco*RI fragment retained in pSCC2 was able to restore motility to the *flmD* and *flmE* Tn*5* insertion mutants. To define the locations of the *flmD* and *flmE* genes in pSCC2, DNA fragments were deleted or subcloned into plasmids that can replicate in *C. crescentus* (Fig. 1). Plasmids pSCC33, pGL2, and pGL38 could restore motility to the *rec*$^+$ strains SC305, SC1127, and SC3090, but they were unable to complement the motility defect of the recombination-deficient strain SC3898 (*recA526 flmD*). These results indicate that the restored motility in the *rec*$^+$ strains resulted from a recombinational event. Since pGL17 and pGL19 both complemented strain SC3898, and pGL18 and pSCC33 both failed to complement SC3898, we deduced that the *flmD* gene is contained within a fragment beginning 460 bp to the right of the *Sac*Ib site and ending at the *Hpa*I site. By contrast, pSCC33 was able to complement both SC175 (*flmE*) and SC3899 (*flmE recA*), indicating that the *flmE* gene is present entirely within the 3.7-kb *Sac*I fragment. Since both pGL18 and pGL38 were able to complement the *recA flmE* double-mutant strain SC3899, and since pGL19 failed to complement SC3899, we concluded that the *flmE* gene is located in a 0.8-kb fragment beginning 410 bp to the right of the *Hpa*I site and ending at the *Nco*I site. The locations of the *flmD* and *flmE* genes were confirmed by Southern analysis of chromosomal DNA from Tn*5* insertion mutants. Strains SC1127 and SC3090 contained a *flmD*::Tn*5* insertion located in the 1.2-kb *Sal*I fragment, and strain SC1030 had a *flmE*::Tn*5* insertion located in the 0.8-kb *Sal*I fragment (data not shown).

The nucleotide sequence of approximately 5 kb of DNA from the region containing the *flmD* and *flmE* genes was determined from both strands (GenBank accession no. U27302). Four open reading frames were identified as potential coding sequences by using the bias for high GC at the third position of each codon and the frequency of rare codon usage in *C. crescentus* coding regions (60). The *flmD* and *flmE* genes are each structurally organized as an operon with a previously unidentified gene (*flmC* and *flmF*, respectively). In both operons, the termination codon of the first gene overlaps the initiation codon of the second gene. Evidence that *flmCD* is transcribed as an operon is as follows: (i) plasmid pGL2 can correct the motility defect of strain SC305 (*flmD*) by recombination, and (ii) true complementation of a *recA flmD* double mutant can be

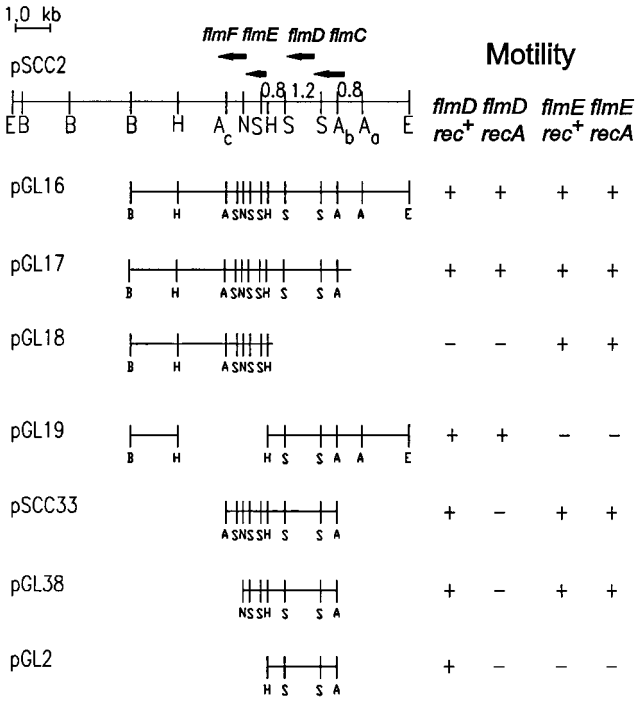

FIG. 1. Analysis of the *flmCD* and *flmEF* regions. Shown is a restriction map of plasmid pSCC2, which contains a 14-kb *Eco*RI DNA fragment of the *C. crescentus* chromosome. Subclones from this region in plasmid pRK2L1 or R300B were tested for the ability to complement the motility defect of strains SC305 (*flmD148*), SC175 (*flmE102*), SC3898 (*flmC148 recA526 zzz*::Tn*5*), and SC3899 (*flmE102 recA526 zzz*:Tn*5*). +, complementation of the motility defect; −, failure to complement. Solid arrows represent predicted open reading frames and direction of transcription. Abbreviations: A, *Sac*I; B, *Bam*HI; E, *Eco*RI; N, *Nco*I; P, *Hpa*I; S, *Sal*I.

obtained only with pGL17 and pGL19, which both contain the upstream *flmC* gene in addition to *flmD*.

Isolation and characterization of the *flmA*, *flmB*, *flmG*, and *flmH* genes. The *flmA* and *flmB* genes (formerly designated *flaA*) were identified by complementation of strains SC229 (*flmA104*) and SC1128 (*flmA*::Tn*5*) by the cosmid clone pLSG6 that contained about 20 kb of *C. crescentus* chromosomal DNA (58). Pulsed-field gel electrophoresis and Southern analysis of chromosomal DNA from SC1128 revealed that the *flmA* and *flmB* genes were present on an 18-kb *Eco*RI fragment of chromosomal DNA (data not shown). Cosmid pLSG6 contained two *Eco*RI sites, one in the vector and a second in the cloned *C. crescentus* DNA. A subclone of the 5-kb *Eco*RI fragment of pLSG6, pSCC7, could complement the motility defect of both strains SC229 and SC1128. Deletion analysis of pSCC7 revealed that a 3.5-kb *Bam*HI-*Eco*RI fragment (pNC1341) could fully complement strain SC229 (Fig. 2A). Analysis of additional subclones (pNC1355 and pNC1356) demonstrated that the DNA on both sides of the central *Sal*I site was required for *flmA* complementation (Fig. 2A). Nucleotide sequence analysis of the entire 3.5-kb *Bam*HI-*Eco*RI fragment (GenBank accession no. U27301) revealed two potential open reading frames with overlapping termination and initiation codons similar to those found in the *flmCD* and *flmEF* operons. The first open reading frame was designated *flmA* since it spanned the *Sal*I site required for complementation.

Previous studies (59) resulted in the isolation and characterization of the *flmG* (formerly *flbA*) and *flmH* (formerly *flaG*) genes. Using complementation analysis, Schoenlein et al. (59) demonstrated by that *flmG* and *flmH* were organized as an operon. Both genes were present on a 3.2-kb *Eco*RI fragment borne by pPVS154 (Fig. 2B) (59). The nucleotide sequence of the entire 3.2-kb *Eco*RI fragment was determined on both strands (GenBank accession no. U28867). Examination of the DNA sequence confirmed that *flmG* and *flmH* genes are organized as an operon (Fig. 2B). However, in this case, the two coding regions were separated by 152 bp.

Database comparisons. The deduced amino acid sequences of the eight Flm proteins were compared to entries in the GenBank database. As shown in Table 3, FlmA, FlmB, FlmC, and FlmD show significant levels of identity (23 to 41% identity) with proteins involved in capsular, lipopolysaccharide (LPS), and spore coat polysaccharide biosynthesis from *Bacillus subtilis*, *Methanococcus jannaschii*, and other bacteria. Furthermore, FlmC also shows homology with the CMP-KDO synthetase (3-deoxy-manno-octulosonate cytidylyltransferase) involved in LPS biosynthesis in *E. coli* (8), *Chlamydia trachomatis* (67), and *Haemophilus influenzae* (21). In *Helicobacter pylori*, a FlmA homolog, FlaA1, has been sequenced and found to show 61% identity over a 321-amino-acid overlap (GenBank accession no. AE00595) (68). Since *flm* mutants produce flagellins with altered migration in SDS-polyacrylamide gels (30), these results suggest that FlmA, FlmB, FlmC, and FlmD could be involved in the glycosylation of flagellin monomers or other proteins involved in flagellin biogenesis.

The predicted FlmH protein shows significant levels of homology with acetyltransferases from several bacteria (Table 3) (8, 22, 49, 69, 78). Similarly, the FlmE gene product shows a low level of homology with several methyltransferases (Table

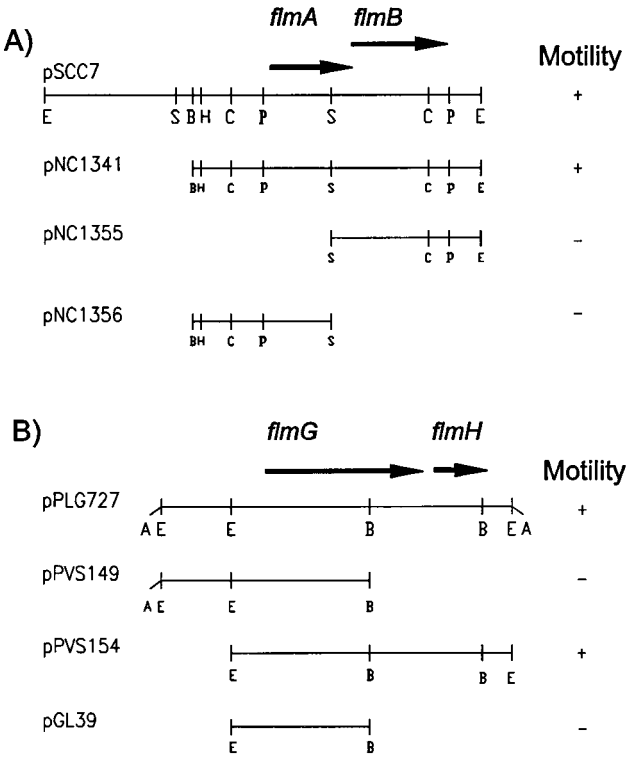

FIG. 2. (A) Analysis of the *flmAB* region. Genetic organization of plasmid pSCC7 harboring the 5.0-kb *Eco*RI fragment is represented. Solid arrows represent open reading frames and direction of transcription. The ability to complement strain SC229 (*flmA104*) is shown. (B) Organization of the *flmGH* region. Solid arrows represent open reading frames and direction of transcription. + and − denote the ability and inability, respectively to swarm in a semisolid medium. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Hpa*I; S, *Sal*I.

TABLE 3. Homology comparisons of the FlmA, FlmB, FlmC, FlmD, FlmE, FlmF, FlmG, and FlmH proteins

Protein (amino acids)	Homolog/organism	% Identity/length of amino acid overlap[a]	Function (accession no.)
FlmA (332)	D protein/*Methanococcus jannaschii*	41/330	Capsular polysaccharide biosynthesis (U67549)
	Cap8E/*Staphylococcus aureus*	38/327	Capsular polysaccharide biosynthesis (U73374)
	Cap5E/*Staphylococcus aureus*	38/314	Capsular polysaccharide biosynthesis (U81973)
	BpIL/*Bordetella pertussis*	34/331	LPS biosynthesis (X90711)
	CapD/*Staphylococcus aureus*	34/327	Capsular polysaccharide biosynthesis (P39853)
	TrsG/*Yersinia enterocolitica*	31/330	LPS biosynthesis (S51266)
	LpsB/*Rhizobium etli*	34/227	Putative dTDP-glucose 4,6-dehydratase (U56723)
	YveM/*Bacillus subtilis*	35/270	Hypothetical protein (Z71928)
	SpsJ/*Bacillus subtilis*	23/313	Spore coat polysaccharide biosynthesis (P39630)
FlmB (386)	SpsC/*Bacillus subtilis*	41/383	Spore coat polysaccharide biosynthesis (P39623)
	BplF/*Bordetella pertussis*	39/383	LPS biosynthesis (X90711)
	SpsC/*Escherichia coli*	37/382	Similar to *B. subtilis* SpsC protein (D90856)
	C protein/*Methanococcus jannaschii*	35/386	Spore coat polysaccharide biosynthesis (U67549)
	LmbS/*Streptomyces lincolnensis*	35/384	Lincomycin production (X79146)
	BplC/*Bordetella pertussis*	36/353	LPS biosynthesis (X90711)
	DegT/*Bacillus stearothermophilus*	34/372	Regulator of protease (M29002)
	SpsC/*Synechocystis* sp.	35/345	Spore coat polysaccharide biosynthesis (D90911)
	RfbE/*Escherichia coli*	31/342	Perosamine synthetase homolog (S83460)
FlmC (238)	SpsF/*Bacillus subtilis*	35/237	Spore coat polysaccharide (P39626)
	F protein/*Methanococcus jannaschii*	31/238	Spore coat polysaccharide biosynthesis (U67549)
	KdsB/*Escherichia coli*	45/59	CMP-KDO synthetase (P04951)
	KDO/*Chlamydia trachomatis*	33/110	CMP-KDO synthetase (U15192)
	KDO/*Haemophilus influenzae*	24/109	CMP-KDO synthetase (U32691)
FlmD (330)	MurG/*Mycobacterium tuberculosis*	29/170	UDP-*N*-acetylglucosamine transferase (Z95388)
	SpsH/*Bacillus subtilis*	25/112	Spore coat polysaccharide (P39628)
	G protein/*Methanococcus jannaschii*	23/124	Spore coat polysaccharide biosynthesis (U67549)
FlmE (216)	TCMO/*Streptomyces glaucescens*	39/51	Tetracenomycin methyltransferase (M80674)
	CobL/*Rhodococcus* sp.	28/113	Methyltransferase/Decarboxylase (L21196)
	ORF[b]/*Mycobacterium tuberculosis*	22/206	Unknown (Z80226)
	ORF/*Erwinia herbicola*	32/56	Similar to methyltransferase (AF006625)
FlmF (421)	IaaM/*Erwinia herbicola*	51/45	Tryptophan monooxygenase (L33867)
	Aux1/*Agrobacterium rhizogenes*	41/42	Tryptophan monooxygenase (Q09109)
	IaaM/*Pseudomonas syringae* pv. savastanoi	41/37	Tryptophan monooxygenase (P06617)
	IaaM/*Pseudomonas syringae* pv. syringae	39/38	Tryptophan monooxygenase (U04538)
FlmG (597)	ORF MJ1345/*Methanococcus jannaschii*	26/182	Predicted coding sequence (U67574)
	OGT/*Homo sapiens*	25/165	O-linked acetylglucosamine transferase (U77413)
	OGT/*Rattus norvegicus*	25/165	O-linked acetylglucosamine transferase (U76557)
	OGT/*Caenorhabditis elegans*	24/165	O-linked acetylglucosamine transferase (U77412)
	ORF/*Synechocystis* sp.	26/185	Hypothetical protein (D64003)
FlmH (197)	SpeG/*Escherichia coli*	25/180	Diamine acetyltransferase (spermidine) (P37354)
	YP20/*Bacillus licheniformis*	27/172	Unknown (PO5332)
	YdaF/*Bacillus subtilis*	27/110	Probable acetyltransferase (AB001488)
	AacA4/*Serratia* sp.	21/171	Aminoglycoside transferase (JC1322)
	RimJ/*Escherichia coli*	20/174	Ribosomal alanine acetyltransferase (P09454)
	AacA4/*Klebsiella pneumoniae*	20/173	Aminoglycoside acetyltransferase (P19650)
	AacA4/*Serratia marcescens*	19/168	Aminoglycoside acetyltransferase (P20092)
	ORF/*Mycobacterium tuberculosis*	25/101	Similar to *E. coli* RimJ (Z94752)
	AacA4/*Pseudomonas aeruginosa*	18/173	Acetyltransferase (X60321)

[a] The percentage of gap is less than 3% of the length of the amino acids sequences compared.
[b] ORF, open reading frame.

3) (15, 66). Also, the deduced amino acid sequence of the *flmF* gene shows a high level of homology (39 to 51% over a 38- to 45-amino-acid overlap) with tryptophan monooxygenases from *Erwinia herbicola*, *Agrobacterium rhizogenes* (13), and *Pseudomonas syringae* (43, 75). This homology extends from positions 4 to 48. More interestingly, a motif search revealed that FlmF contains a sugar transport signature—(LIVMSTAG) (LIVMF SAG) ×2 (LIVMSA) (DE) × (LIVMFYWA) G R (RK) ×6 (GSTA)—at residues 92 to 109 (Wisconsin Package version 9.0; Genetics Computer Group). Finally, the deduced FlmG product shows 24 to 25% identity over 165 amino acids to O-linked *N*-acetylglucosaminyltransferases from *Homo sapiens*, *Rattus norvegicus*, and *Caernorhabditis elegans* (Table 3) (33, 39). It is believed that this enzyme adds O-linked *N*-acetylglucosamine to transcription factors and nuclear pore proteins (39). A FlmG homolog has also been identified in *H. pylori* (GenBank accession no. AE000550) (68). Taken together, these results suggest that FlmA, FlmB, FlmC, FlmD, FlmE, FlmF, FlmG, and FlmH could be involved in glycosylation, acetylation, and/or methylation of flagellin subunits or proteins that interact with flagellins monomers prior to their assembly into a flagellar filament.

Effect of flagellar mutations on the expression of *flmA*, *flmC*, *flmE*, and *flmG* fused to *cat*. Quon et al. (53) have proposed that CtrA binds directly to promoters containing the (TTAA-N_7-TTAAC) consensus site to activate the flagellar regulatory hierarchy, to prevent replication of DNA, and to control DNA methylation and cell division. To test whether CtrA regulates

TABLE 4. Effects of *ctrA401* on *flmA*, *flmC*, *flmE*, and *flmG* transcription

Plasmid	Promoter fusion	Activity[a]	
		28°C	37°C
pGL50	*flmA*::*cat*	102 ± 11	28 ± 13
pGL48	*flmC*::*cat*	95 ± 15	81 ± 33
pGL49	*flmE*::*cat*	78 ± 12	30 ± 9
pGL53	*flmG*::*lacZ*	117 ± 3	41 ± 2
pWZ162	*fliQ*::*lacZ*	218 ± 2	133 ± 0.4
pCS91	*rsaA*::*lacZ*	85 ± 5	119 ± 2

[a] Strain LS2195 cells were grown to mid-log phase (120 to 150 Klett units) in PYE medium supplemented with tetracycline (1 μg/ml) under permissive (28°C) and restrictive (37°C) conditions. CAT and β-galactosidase specific activities were assayed and normalized to the level of activity found for control strain LS107. Data represent the mean ± standard deviation of duplicate samples from two independent experiments.

the expression of the *flmAB*, *flmCD*, *flmEF*, and *flmGH* operons, plasmids pGL48 (*flmC*::*cat*), pGL49 (*flmE*::*cat*), pGL50 (*flmA*::*cat*), pGL53 (*flmG*::*lacZ*), pCS91 (*rsaA*::*lacZ*), and pWZ162 (*fliQ*::*lacZ*) were mated into strain LS2195, which contains a temperature-sensitive *ctrA401* mutation. The resulting constructs were grown in PYE medium under the permissive condition (28°C) and then shifted to the restrictive condition (37°C) for 6 h. Cell extracts were prepared, and CAT and β-galactosidase activities were assayed (Table 4). As previously reported (53), expression of the *fliQ*::*lacZ* decreased about twofold in the *ctrA401* background at the restrictive temperature, and the crystalline surface array protein promoter, *rsaA*::*lacZ*, was relatively unaffected by the *ctrA401* mutation. However, the level of transcription of the *rsaA*::*lacZ* gene fusion showed a 2- to 2.5-fold increase at 37°C in both LS107 and LS2195 backgrounds (data not shown), suggesting that its expression is heat induced. More importantly, expression of the *flmA*::*cat*, *flmE*::*cat*, and *flmG*::*lacZ* gene fusion products was significantly (2.4- to 3.6-fold) reduced in the strain LS2195 (*ctrA401*) at the restrictive temperature. In contrast, *flmC* expression was relatively unaffected by the *ctrA401* mutation at either temperature. These results suggest that CtrA positively regulates the *flmA*, *flmE*, and *flmG* promoters either directly or indirectly.

To determine the effect of class II and class III flagellar mutations on transcription of the *flmAB*, *flmCD*, *flmEF*, and *flmGH* promoters, various Tn*5* insertion mutations in flagellar genes were introduced by transduction into strains SC3971, SC3973, SC3975, and SC4016, containing the integrated chromosomal fusions *flmC*::*cat*, *flmE*::*cat*, *flmA*::*cat*, and *flmG*::*cat*, respectively (see Materials and Methods). The expression of *flmA*, *flmC*, *flmE*, and *flmG* fused to *cat* was not altered more than twofold by a mutation in the *rpoN* gene (Table 5). Since the *rpoN* gene codes for the RNA polymerase sigma 54 subunit, these results indicate that the *flm* promoters are not transcribed by the sigma 54 holoenzyme. Therefore, they are not regulated like class III or IV flagellar genes. This conclusion is supported by the fact that mutations in other class II genes that greatly reduce class III and IV flagellar gene expression (3, 48, 74) cause only minor changes in the level of expression of the four genes (Table 5).

It has been reported that the transcriptional activity of class II gene promoters increased about twofold in the presence of other class II mutations (64). In our study, the only significant increases in *flm* promoter expression were the *flmA* and *flmG* promoters in a *fliM* mutant background. Furthermore, in contrast to class II genes, mutations in the *flmAB*, *flmCD*, *flmEF*, and *flmGH* operons do not show defects in cell division. Previous studies have shown that mutations in the *flmA*, *flmD*, *flmE*, and *flmH* genes do not regulate class II (*fliF* and *flhA*), class III (*flgE*, *flgK*, and *flbG*), or class IV (*fljK* and *fljL*) flagellar genes (3, 48, 74). Thus, the four *flm* operons do not have the properties of the previously studied class II genes even though the expression of three of these flagellar operons is affected by a *ctrA* mutation. Taken together, these results indicate that the four flagellar operons represent a new class or classes of flagellar genes.

TABLE 5. CAT activities of chromosomal *flm-cat* fusions in different *fla* mutants

Genetic background[a]	Flagellar mutation (class)	Relative sp act[b]			
		flmA::*cat*	*flmC*::*cat*	*flmE*::*cat*	*flmG*::*cat*
SC1029	*flhB* (II)	64 ± 27	46 ± 6	34 ± 3	121 ± 2
SC1032	*flbD* (II)	77 ± 2	57 ± 4	46 ± 9	116 ± 2
SC1055	*rpoN* (II)	142 ± 34	52 ± 5	75 ± 1	118 ± 8
SC1066	*fliL* (II)	47 ± 8	52 ± 16	36 ± 3	100 ± 11
SC1132	*flhA* (II)	107 ± 42	45 ± 22	44 ± 17	134 ± 24
SC2663	*fliM* (II)	188 ± 25	89 ± 26	ND	207 ± 14
SC3809	*flaS* (II)	89 ± 26	55 ± 12	42 ± 11	136 ± 6
SC1117	*flgH* (III)	77 ± 32	120 ± 20	55 ± 7	85 ± 2
SC1134	*flgK* (III)	80 ± 29	90 ± 15	60 ± 9	93 ± 25
SC1135	*flbG* (III)	88 ± 20	53 ± 8	71 ± 12	125 ± 18
SC1128	*flmA*	ND	84 ± 24	82 ± 5	92 ± 3

[a] Strains were grown in PYE medium supplemented with kanamycin (50 μg/ml) at 30°C.

[b] Normalized to a value of 100 for wild-type strain LS107. CAT activities were 498 ± 227, 197 ± 92, 500 ± 201, and 171 ± 58 cpm/μg of protein for *flmA*::*cat*, *flmC*::*cat*, *flmE*::*cat*, and *flmG*::*cat* gene fusions, respectively. CAT background activity for wild-type strain SC3844 was 7 ± 4 cpm/μg of protein. Values represent the mean ± standard deviation of duplicate samples from two or more independent experiments. ND, not determined.

To test whether *flmA*, *flmC*, *flmE*, and *flmG* genes are autoregulated or involved in the same regulatory pathway, we measured their transcription in each of the *flm* mutant backgrounds (Table 6). Plasmids carrying transcriptional fusions of the *flmA*, *flmC*, *flmE*, and *flmG* promoters to *cat* or *lacZ* were introduced into *flmA*, *flmD*, *flmE*, and *flmH* mutant strains. Cell extracts of mid-logarithmic-phase cultures were prepared and assayed for *cat* or *lacZ* activity. Mutations in *flmA*, *flmD*, *flmE*, and *flmH* have no significant effect on *flmA*, *flmC*, *flmE*, and *flmG* gene expression. Identical results were observed when the expression of chromosomal *flmC*::*cat*, *flmE*::*cat*, and *flmG*::*cat* gene fusions was measured in the presence of the *flmA* (*flaA104*) mutation (Table 5). These results indicate that

TABLE 6. CAT activities of *flm-cat* fusions in various *flm* mutant backgrounds

Genetic background[a]	Relative sp act[b]			
	pGL50 (*flmA*::*cat*)	pGL48 (*flmC*::*cat*)	pGL49 (*flmE*::*cat*)	pGL53 (*flmG*::*lacZ*)
flmA	108 ± 2	97 ± 5	97 ± 1	125 ± 4
flmD	112 ± 3	93 ± 8	88 ± 24	145 ± 1
flmE	95 ± 15	100 ± 11	67 ± 25	124 ± 17
flmH	111 ± 14	92 ± 12	92 ± 16	115 ± 1

[a] Strains were grown in PYE with tetracycline (1 μg/ml) at 30°C.

[b] Normalized to a value of 100 for wild-type strain LS107. CAT activities were 945, 382, 686, and 14 cpm/μg of protein for strain LS107 harboring plasmids pGL48, pGL49, pGL50, and pGL53, respectively. β-Galactosidase activities were 795 and 45 Miller units for LS107(pGL53) and LS107(pRKlac290), respectively. Data represent the mean ± standard deviation of duplicate samples from two or more independent experiments.

there is no autoregulation or regulatory interactions among the *flmAB*, *flmCD*, *flmEF*, and *flmGH* operons.

Temporal regulation of the *flmA*, *flmC*, *flmE*, and *flmG* genes. To determine whether expression of the *flmA*, *flmC*, *flmE*, and *flmG* genes is temporally regulated, strains containing a chromosomally inserted transcriptional *cat* fusion or a plasmid-borne *lacZ* fusion were synchronized and analyzed throughout the cell cycle. Expression of the *flmA*, *flmE*, and *flmG* operons occurred primarily in predivisional cells (Fig. 3). Transcription of both the *flmA* and *flmG* genes is very low or absent in swarmer cells and during the stalk-to-predivisional cell transition (0 to 0.5 cell division unit). It reaches a peak of expression in late predivisional cells (0.8 cell division unit) that coincides with the completion of filament assembly and the appearance of motility. The pattern of *flmE* expression is similar, but it shows a periodicity analogous to that observed for the 25-kDa flagellin where transcription continues in swarmer cells. In contrast, transcription of the *flmC* gene occurred throughout the cell cycle, with only twofold increase in predivisional cells (Fig. 3).

DISCUSSION

The regulation of flagellum biogenesis is complex, and many details remain to be elucidated. The results presented above demonstrate that the *flm* genes represent a new class of flagellar genes. DNA sequence analysis revealed that the *flmAB*, *flmCD*, and *flmEF* genes are structurally organized as operons. In each operon, the termination codon of the first gene overlaps the initiation codon of the second gene. DNA sequence analysis of the *flmG* and *flmH* genes confirmed that they also are organized in an operon as reported by Schoenlein et al. (59). However, in this case, the two coding regions are separated by 152 bp. The close spacing of the genes in the *flmAB*, *flmCD*, and *flmEF* operons suggests that translation of these operons may be governed by a translational coupling mechanism. In *E. coli*, there are many examples of translational coupling where the interruption of translation of the first gene causes a severe decrease in the expression of the translationally coupled distal gene (1, 52, 77). For translational coupling to occur, the efficient expression of the distal genes would be dependent on both translation of the first gene and termination of this translation in close proximity to the start codon for the second gene. Thus, translational coupling could be a mechanism to ensure equimolar synthesis of both proteins.

Homology searches of the deduced amino acid sequences revealed that FlmA, FlmB, FlmC, and FlmD have significant levels of identity with proteins involved in capsular, LPS, and spore coat polysaccharide biosynthesis from *B. subtilis*, *M. jannaschii*, and other bacteria. However, since FlmC also shared homology to the CMP-KDO synthetase from *E. coli*, *C. trachomatis*, and *H. influenzae*, these results suggested that these proteins could be involved in LPS biosynthesis. To test this hypothesis, we measured the KDO synthetase enzyme activity in *flmA*, *flmD*, *flmE*, *flmG*, and *flmH* mutants. Each mutant had wild-type levels of KDO synthetase activity and appeared to have wild-type LPS profiles (37). Thus, it does not appear that mutations in the *flm* genes affect LPS biosynthesis. The other Flm (FlmEFGH) proteins show homology to proteins involved in glycosylation, methylation, and/or acetylation in several bacteria (Table 3). Mutations in *flmA*, *flmD*, *flmE*, *flmG*, and *flmH* genes result in the production of a 22-kDa flagellin. Furthermore, we have shown that the 22-kDa protein results from a modification or a breakdown product of the 25-kDa flagellin proteins (20). Glycosylation of flagellin proteins has been reported for *Campylobacter* (17), *Spirochaeta aurantia* (10), some archaea (35, 63, 72), and *Azospirillum brasilense* (46). *Azospirillum* contains *flmAB* homologs, and a mutation in one of these genes prevents assembly of the flagellar filament (45). In addition, Wieland et al. (72) have suggested that in halobacteria, glycosylation of the flagellins was necessary for proper incorporation of the flagella into the cell envelope and that overproduction of flagellins resulted in subunits with lower molecular weights. In *Caulobacter*, the 22-kDa flagellin is present in a *flbT* mutant that overproduces flagellins (57). Flagellins also can be modified by methylation (2, 12, 36, 38), phosphorylation (31), and sulfation (36, 72). Strains containing mutations in *flmA*, *flmD*, *flmE*, *flmG*, and *flmH* genes have a normal basal body and hook structure but fail to assemble a flagellar filament (30). Therefore, our current hypothesis is that modification of the flagellin subunits or some other flagellar proteins by glycosylation, acetylation, and methylation is required for proper assembly of flagellin subunits into the filament. Clearly, this hypothesis has important implications for the structure and mechanism of assembly of the flagellar filament. Recently, we have determined the nucleotide sequences of five of the six flagellin genes in *C. crescentus* (20). Analysis of deduced amino acids indicated that there is a discrepancy between the calculated molecular weight and the actual mass determined by mass spectroscopy (37).

It has been demonstrated in *E. coli* (32), *Salmonella typhimurium* (34), and *C. crescentus* (14, 48, 74) that a cascade of positive and negative transcriptional control regulates the temporal expression of flagellar genes. Previously, the *flmAB*, *flmCD*, *flmEF*, and *flmGH* operons had been placed in class III in the flagellar gene regulatory hierarchy (48). However, the experiments presented in this report demonstrate that the *flm* operons represent a new class of flagellar genes. First, we have shown that none of the *flm* operons require the RNA polymerase sigma factor 54 for transcription, indicating that they are not class III or IV genes (Table 5). Second, we have shown that *flmAB*, *flmEF*, and *flmGH* are positively regulated by CtrA (Table 4), a transcriptional response regulator that controls class II flagellar genes (53). However, in contrast to class II flagellar genes, mutations in *flmAB*, *flmCD*, *flmEF*, and *flmGH* operons do not cause defects in cell division. In addition, previous studies (3, 48, 74) have shown that the *flmA*, *flmD*, *flmE*, and *flmG* genes do not regulate transcription of genes from class II (*fliF* and *flhA*), class III (*flgE*, *flgK*, and *flbG*), or class IV (*fljK* and *fljL*). Taken together, we conclude that the *flmAB*, *flmCD*, *flmEF*, and *flmGH* operons belong to a new class of flagellar genes.

It has been shown that synthesis of the flagellin subunits encoded by the class IV genes is subject to posttranscriptional control mechanisms (3, 41, 42). Anderson and Newton (3) showed that both *fljK*::*lacZ* transcriptional and translational fusions were expressed at nearly wild-type levels in strains carrying mutations in *flmA*, *flmD*, or *flmH*. Nevertheless, immunoprecipitation experiments measuring short (30-s or 1-min) pulses of flagellin protein synthesis demonstrated that mutations in these genes do result in reduced levels of flagellin synthesis (30). Our current hypothesis is that this reduced level of flagellin synthesis may be due to a feedback mechanism involving unassembled flagellin subunits rather than any direct action involving the *flm* gene products. Furthermore, since we have shown that the FlbT product regulates flagellin synthesis by altering mRNA stability (42), it is likely that the effects of *flm* mutations on flagellin gene expression involve mRNA stability as well.

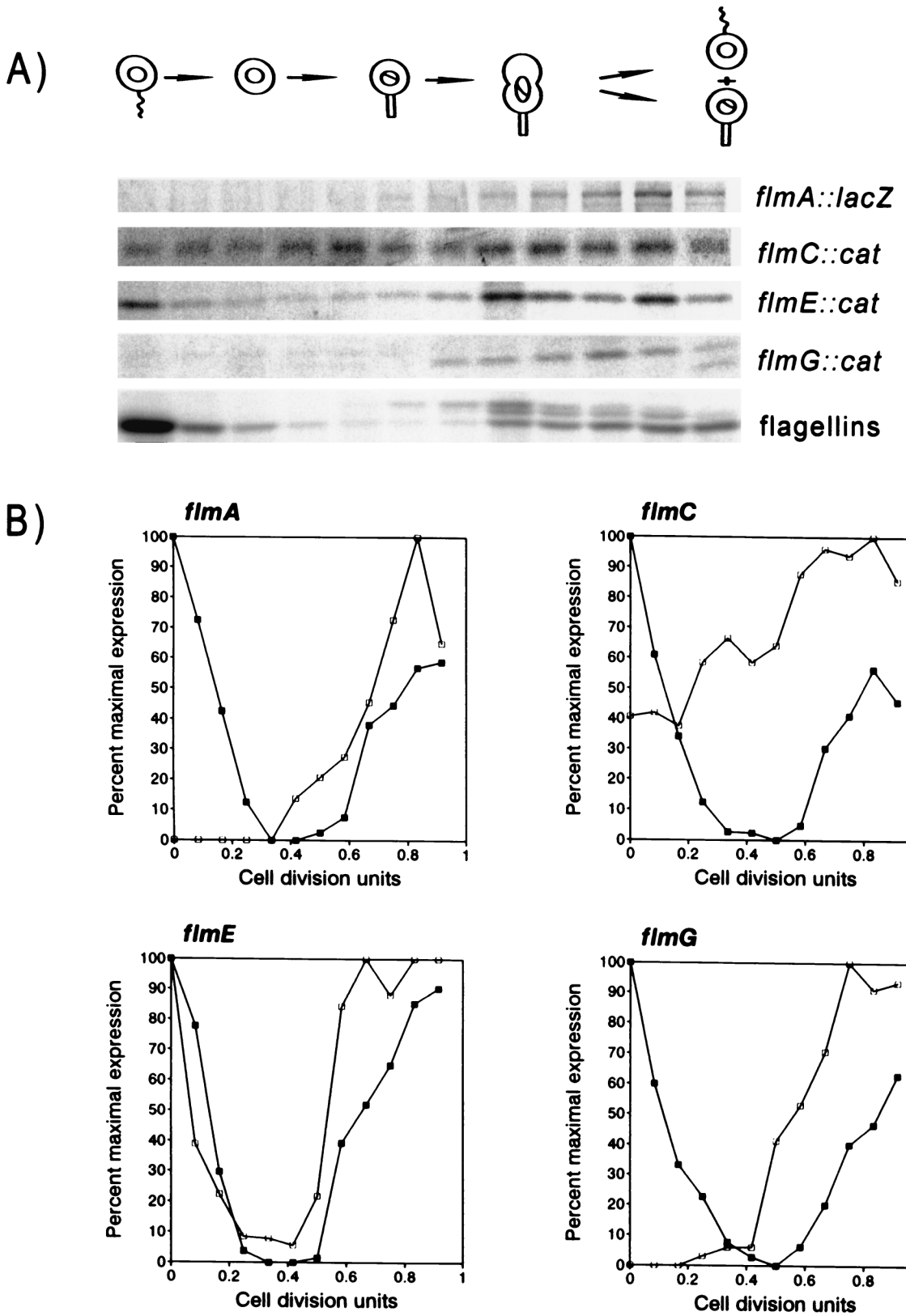

FIG. 3. Cell cycle expression of the *flmA*, *flmC*, *flmE*, and *flmG* operons. Synchronized populations of *Caulobacter* strains SC3971, SC3973, SC4016, and SC4250 were pulse-labeled with [^{35}S]methionine at 15-min intervals during the cell cycle. (A) Immunoprecipitation of labeled proteins with CAT, β-galactosidase, or flagellin antibodies. A cartoon showing progress through the cell cycle is shown at the top. The cell cycle-dependent expression of the flagellin genes is shown as a control. (B) Quantification of these data by using a Alpha Innotech photodocumentation system. Percentage of maximal expression of each sample is shown as a function of cell division units. One cell division unit is equivalent to a generation time of 180 min. Closed squares represent 25-kDa flagellin expression (recovered from SC3973 cells carrying the *flmE*::*cat* fusion); open squares represent expression of the *flmA*::*lacZ*, *flmC*::*cat*, *flmE*::*cat*, or *flmG*::*cat* fusion.

ACKNOWLEDGMENTS

We thank Gilles Leclerc for stimulating and helpful discussion. We also thank William B. Crymes for critical reading of the manuscript and Nelida Caballero and Bonsung Koo for expert technical assistance.

This work was supported by NIH grants GM50547 and GM34765 to B. Ely.

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