

Characterization of Strains Containing Mutations in the Contiguous *flaF*, *flbT*, or *flbA-flaG* Transcription Unit and Identification of a Novel Fla Phenotype in *Caulobacter crescentus*

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During the *Caulobacter crescentus* cell cycle, flagellin synthesis and filament assembly are temporally controlled events which require the products encoded by the contiguous *flaF*, *flbT*, and *flbA-flaG* transcription units (P. V. Schoenlein, L. S. Gallman, and B. Ely, J. Bacteriol. 171:000-000, 1989). To better define the functions of these genes, immunoprecipitation studies, Western blot (immunoblot) analyses, and electron microscopic analyses characterized flagellin synthesis and assembly in mutant and merodiploid strains. Mutations in the *flaF* or *flbA-flaG* transcription unit resulted in reduced synthesis of the 25- and 27-kilodalton (kDa) flagellins. In contrast, mutations in *flbT* resulted in overproduction of these flagellins. The F1bT phenotype is unique, since all other identified *C. crescentus* *fla* mutations cause a reduction in the levels of the 25- and 27-kDa flagellins. Furthermore, the *flbT* mutant showed a chemotaxis deficiency even though it was motile. Thus, the *flbT* gene product appears to be involved in the regulation of both flagellin synthesis and chemotactic function. Mutations in the *flbT* and *flbA-flaG* transcription units also resulted in the production of a 22-kDa flagellin species that is not normally detected in wild-type cells. This flagellin species was not detected in the *flbT* filaments. Furthermore, the 22-kDa flagellin was no longer detected in *flbA* pseudorevertants that assembled functional filaments. Thus, the 22-kDa flagellin does not appear to be assembled into filaments. Since many of the *flbT* filaments are shorter than wild-type filaments, we discuss the possibility that the 22-kDa flagellin species may adversely affect flagellin assembly in this mutant.

Flagellum biogenesis in *Caulobacter crescentus* occurs during a specific time interval of the cell cycle and results in the synthesis of a basal body, hook, and flagellar filament at one pole of the developing swarmer cell. The flagellar filament is composed of at least two homologous protein monomers, a 27-kilodalton (kDa) flagellin that is located proximal to the hook structure and a 25-kDa flagellin that makes up most of the distal portion of the filament (21, 38). Recently, it has been shown that small amounts of a third homologous flagellin (29 kDa) occur in a small segment at the beginning of the flagellar filament (A. Driks, R. Bryan, L. Shapiro, and D. J. DeRosier, J. Mol. Biol., in press). Furthermore, preliminary studies have correlated the absence or reduction of the 27-kDa flagellin with shorter flagellar filaments (13, 20, 38), and more recent studies have demonstrated that both the 27- and 29-kDa flagellins are required for normal filament assembly (27).

Mutations in at least 30 genes (*fla* or *flb*) (9, 16, 30) result in decreased levels of the 25- and 27-kDa structural flagellin proteins (17), which suggests that wild-type synthesis of these flagellins is one of the last steps in a cascade of gene expression. This hierarchical regulatory cascade has been further characterized (4, 5, 26-29) and appears to be similar to the regulation of flagellin gene (*hag*) expression in *Escherichia coli* (18, 19). Additional studies have indicated that DNA replication may play a role in regulating the periodic expression of the flagellar cascade during the *C. crescentus* cell cycle (29, 31, 32, 37).

One of the major clusters of flagellar genes contains three

homologous flagellin genes, *flgJ*, *flgK*, and *flgL*, in addition to the *flaY*, *flaE*, *flaF*, and *flaG* genes (Fig. 1). Mutations in *flaE*, *flaF*, or *flaG* result in the production of a structure which contains the basal body and hook but lacks the flagellar filament. Mutations in *flaY* result in the production of an extremely short flagellar filament (stub) in addition to the basal body and hook (17). When the production of flagellin proteins was examined by immunoprecipitation, strains containing *flaY* or *flaE* mutations produced the same flagellin patterns as did the wild-type strain, but the proteins were present at reduced levels (17, 34). Strains containing *flaF* mutations showed an unusual flagellin pattern in that they overproduced the 29-kDa flagellin but synthesized only trace amounts of the 25- and 27-kDa flagellins. In addition, strains containing the *flaG* mutations were unique since they produced a novel 22-kDa flagellin and only trace amounts of the 25-kDa flagellin (17). The *flaY* and *flaE* genes have been isolated (34) and characterized (3, 24), and the roles of these genes in flagellum biogenesis are currently being studied by Shapiro and co-workers.

More recently, the *flaF* and *flaG* genes and two newly identified *fla* genes, *flbT* and *flbA*, were isolated on a 4.0-kilobase (kb) *Sst*I fragment (Fig. 1; 36). To begin to define the roles of these genes in flagellum biogenesis, we used Western blot (immunoblot) analyses and immunoprecipitation studies to characterize flagellin synthesis in *flaF*, *flbT*, *flbA*, and *flaG* mutants. All of these mutants except *flbT* produced significantly reduced levels of the 25- and 27-kDa structural flagellins. In contrast, the *flbT* mutant showed increased amounts of these flagellins but normal amounts of the 29-kDa flagellin. Therefore, these data suggested that the F1bT protein negatively regulates the synthesis of the 25- and 27-kDa flagellins. Further evidence for the regulatory role of *flbT* was obtained when flagellin synthesis

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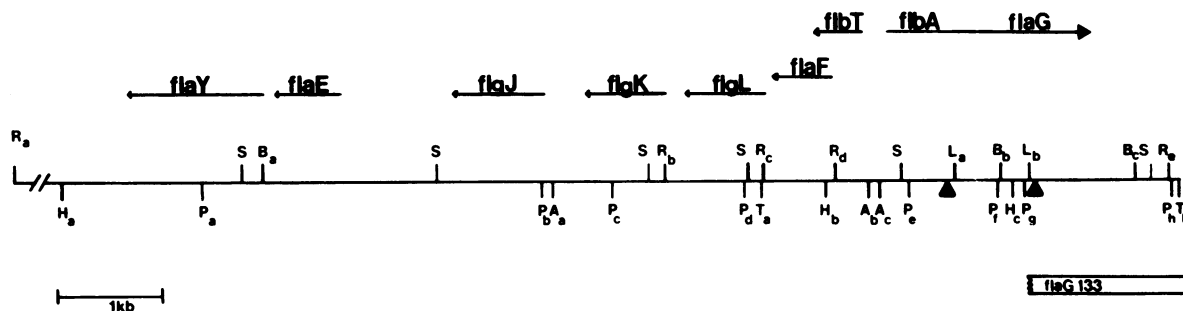


FIG. 1. Composite representation of the *flaYG* gene cluster resulted from studies by Purucker et al. (34), Bryan et al. (3), and Schoenlein et al. (36). Locations of *flgJ* (29-kDa flagellin) by Gill and Agabian (14) and Minnich and Newton (27) and *flgL* (27-kDa flagellin) and *flgK* (25-kDa flagellin) and their directions of transcription by C. Lagenaur and N. Agabian (personal communication) and Minnich and Newton (27) are shown. Locations of the *flaG133* deletion mutation (open bar) and of the *flbA604::Tn5* and *flaG617::Tn5* insertions (triangles) were determined by Schoenlein et al. (36). Abbreviations: T, *SstI*; R, *EcoRI*; H, *HindIII*; A, *HpaI*; S, *Sall*; L, *BglII*; B, *BamHI*; P, *PstI*.

was examined in merodiploid strains. When *flbT* was provided in *trans*, reduced synthesis of the 25- and 27-kDa flagellins was observed. In contrast, the 29-kDa flagellin appears to be regulated by an independent mechanism that may involve the FlaF protein. The *flbA*, *flaG*, and *flbT* mutants also produced a 22-kDa flagellin species that is not normally detected in wild-type cells. The function of this flagellin was addressed by analyzing filament assembly in the *flbT* mutant and in motile *flbA* pseudorevertants.

MATERIALS AND METHODS

Strains, plasmids, and media. The *C. crescentus* and *E. coli* strains and the plasmids used in this study are presented in Tables 1 and 2, respectively. *C. crescentus* strains were grown as described in the accompanying paper (36). Solid, semisolid (motility), and liquid complex growth media (PYE) have been described by Johnson and Ely (15) and Schoenlein et al. (36). Defined liquid medium (M2) was described by Johnson and Ely (15), and defined solid medium (PIG) was described by Schoenlein et al. (36). When appropriate, solid and liquid media were supplemented with 50 and 10 µg of

kanamycin per ml, respectively. *E. coli* was grown in Luria-Bertani (LB) broth supplemented with kanamycin (50 µg/ml) for plasmid selection.

Detection and characterization of assembled flagellins. Assembled flagellar structures were characterized by electron microscopy as described by Johnson and Ely (16). Phase-contrast microscopy was used to determine whether mutant cultures were motile. Cultures stained for flagella (M. E. Heimbrook, W. L. L. Wang, and G. Campbell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, R-22, p. 240) provided additional information on the length and quantity of assembled flagella. Released flagellar filaments also were purified from culture fluids as described by Johnson et al. (17a) with the following modifications. Cultures were grown overnight to stationary phase in 350 ml of PYE broth and centrifuged at 5,000 × *g* for 10 min. The supernatant was spun a second time to remove residual cells or cell debris. After centrifugation, Triton X-100 was added to the supernatant to a final concentration of 2%, and the mixture was spun at 50,000 × *g* for 2 h. Resulting pellets from the same culture fluid were combined, suspended in 4.0 ml of TE buffer (10 mM Tris, 1.0

TABLE 1. Strains used

Organism and strain	Genotype	Reference or source
<i>C. crescentus</i>		
CB15	Wild type	33
CB15N	Synchronizable variant of CB15	N. Agabian
SC276	<i>flbT650</i>	10
SC278	<i>flaG131</i>	16
SC279	<i>flaF132</i>	16
SC280	<i>flaG133</i>	16
SC282	<i>flaF134</i>	16
SC517	<i>flaG161</i>	16
SC519	<i>flaG163</i>	16
SC1047	<i>flaG617::Tn5 proA103 str-140</i>	Tn5 mutagenesis of SC859 (8)
SC1065	<i>flaA604::Tn5 proA103 str-140</i>	8
SC2006	<i>flbA604::Tn5</i>	SC1065 phage × CB15N
SC2007	<i>flaG617::Tn5</i>	SC1047 phage × CB15N
SC2176	<i>flbA604::Tn5-Tet</i>	Contact of NC9762 with SC2006
<i>E. coli</i>		
C600	<i>r⁻ m⁻ thr leuB6 lacY⁺ tonA thi supE44</i>	L. Shapiro
C600SF8	<i>r⁻ m⁻ pro leu thi thr lop lig⁺ str</i>	W. Reznikoff
HB101(pRK2013)	HB101 (pRK2013)	12
NC9762	C600 (pBEE132)	7
SK1592(pKT230)	F ⁻ <i>gal thi Ti^r endA r⁻ m⁺ sbcB15</i> (pKT230)	1

TABLE 2. Recombinant plasmids used

Plasmid	Source
pPLG727	The 4.0-kb <i>flaFG</i> region cloned into the <i>SstI</i> site of R300B. All derivatives described below were cloned from this region and are shown in Fig. 5.
pPVS150	The 3.2-kb <i>EcoRI flbA-flaG</i> region in the <i>EcoRI</i> site of pKT230; <i>flbA</i> is proximal to the <i>amp</i> promoter of pKT230.
pPVS151	The 3.2-kb <i>EcoRI flbA-flaG</i> region in the <i>EcoRI</i> site of pKT230; <i>flaG</i> is proximal to the <i>amp</i> promoter of pKT230.
pPVS152	The 0.8-kb <i>EcoRI flaF</i> region in the <i>EcoRI</i> site of pKT230; the <i>HindIII</i> site is proximal to the <i>amp</i> promoter of pKT230.
pPVS153	The 0.8-kb <i>EcoRI flaF</i> region in the <i>EcoRI</i> site of pKT230; the <i>HindIII</i> site is distal to the <i>amp</i> promoter of pKT230.
pPVS162	The 3.0-kb <i>HpaI-EcoRI flbA-flaG</i> region in the <i>HpaI-EcoRI</i> region of pKT230; <i>flbA</i> is proximal to the <i>amp</i> promoter of pKT230.
pPVS149	The 2.4-kb <i>BamHI-SstI</i> fragment containing the <i>flbT</i> and <i>flaF</i> transcription units in the <i>BamHI-SstI</i> region of pKT230; <i>flbA</i> is proximal to the <i>amp</i> promoter of pKT230.
pPVS163	The 4.0-kb <i>SstI flaFG</i> region in the <i>SstI</i> site of pPKT230; <i>flaG</i> is proximal to the <i>amp</i> promoter of pKT230.
pPVS164	The 4.0-kb <i>SstI flaFG</i> region in the <i>SstI</i> site of pKT230; <i>flaF</i> is proximal to the <i>amp</i> promoter of pKT230.
pPVS190	The 4.0-kb <i>SstI flaFG</i> region containing a minimum deletion of 0.7 kb in the <i>flbT</i> region between the <i>EcoRI</i> _b and <i>BglII</i> _a sites (Fig. 5); <i>flaF</i> is proximal to the <i>amp</i> promoter of pKT230.

mM EDTA) containing 2% Triton X-100, and spun at 150,000 × *g* for 2 h. The resulting pellet was resuspended in 0.1 ml of TE and transferred to a microfuge tube; then 40 μl of this flagellum suspension was mixed with a denaturing buffer (22) and electrophoresed through a 15% polyacrylamide-sodium dodecyl sulfate gel.

Detection of intracellular flagellins. Total intracellular flagellins of *C. crescentus* strains were assayed in radioimmunoassays by the procedure of Lagenaur and Agabian (23) as modified and described by Johnson et al. (17), using ¹⁴C-labeled amino acids (1.78 mCi/mg; yeast profile reconstituted protein hydrolysates; Amersham Corp., Arlington Heights, Ill.). Electrophoresis was performed on 7.5 to 20% gradient sodium dodecyl sulfate-polyacrylamide slab gels and examined by autoradiography. Total intracellular flagellins also were assayed by Western analyses as described in the Immun-Blot (GAR-horseradish peroxidase) assay kit (Bio-Rad Laboratories, Inc., Gaithersburg, Md.). For these analyses, *C. crescentus* cells were grown in PYE medium, spun, washed, and suspended in 0.5 ml of 10 mM Tris hydrochloride–0.1 mM EDTA (pH 7.9). The cells were sonically disrupted, and protein concentration was determined by the method of Bradford (2). Electrophoresis was performed in 15% polyacrylamide-sodium dodecyl sulfate gels for maximum separation of flagellins for the Western transfer.

Recombinant DNA methods, enzymes, and biochemicals. Chromosomal DNA and plasmid DNA were isolated as described elsewhere (35). Restriction endonucleases and T4 DNA ligase were obtained from New England BioLabs, Inc. (Beverly, Mass.), Bethesda Research Laboratories (Gaithersburg, Md.), or International Biotechnologies, Inc. (New Haven, Conn.). Restriction endonuclease digestions were

performed as suggested by the manufacturer. Other recombinant DNA techniques were as described by either Davis et al. (6) or Maniatis et al. (25). [α -³²P]dCTP was obtained from Dupont, NEN Research Products (Boston, Mass.).

Genetic techniques and selections for pseudorevertants. Genetic techniques used in this study are described in the accompanying paper (36) and included complementation analysis and segregation analysis of the plasmid provided in *trans*. To obtain motile revertants, mutant cells were stabbed into semisolid agar and incubated at 4, 23, and/or 30°C for at least 14 days. Mutants at 4°C were incubated for 30 days because of the slower generation time. Growth that flared away from the point of inoculation in only one direction was streaked for single colonies. These colonies were tested for motility and the ability to swarm in motility medium.

Transduction analyses. Preparation of bacteriophage lysates and the transduction procedure were previously described by Ely and Johnson (11). Transductions with the SC2006 R1 to R6 pseudorevertants as the recipients, using bacteriophage grown on SC2176, used Tn5-Tet-encoded tetracycline resistance (Tc^r) as the selected marker and were performed directly on complex medium supplemented with tetracycline. To allow time for the recipients to express Tc^r, the PYE-tetracycline plates contained a 10.0-ml PYE agar overlay that had solidified for several hours before use. Transductions with CB15N as the recipient using bacteriophage grown on SC2006 R1 to R6 used Tn5-encoded kanamycin resistance (Km^r) as the selected marker and were performed directly on complex medium supplemented with kanamycin. Ten Tc^r and ten Km^r colonies from each transduction were purified and stabbed into semisolid motility medium.

RESULTS

Flagellar filament formation in *flbA* and *flbT* mutants. Previous genetic studies determined that the *flaF*, *flbT*, and *flbA-flaG* transcription units encode *trans*-acting factors that are required for flagellum biogenesis (Fig. 1) (36). To analyze the possible functions of these gene products, we examined flagellin synthesis (see below), filament assembly, and flagellar function (motility and chemotaxis) in *flaF*, *flbT*, *flbA*, and *flaG* mutants. The *flbA* mutant SC1065 (*flbA604::Tn5*) showed a temperature-sensitive phenotype. At 30°C, SC1065 was unable to swarm in motility medium, and no motile cells were observed. Electron microscopic examination of SC1065 grown at 30°C determined that the swarmer cells assembled an extremely short, straight flagellar filament (stub). In contrast, at 23°C SC1065 formed small concentric swarms in motility medium, and a small number of motile cells were observed in broth cultures. These cells formed shorter filaments, ranging from two to five sine waves in length, compared with wild-type filaments (seven to eight sine waves). Once the filaments were assembled at 23°C, they appeared to be stable, since motile cells were still observed after the *flbA* culture was shifted from 23 to 33 or 37°C.

The *flbT* mutant, SC276, was previously characterized as a *che* mutant (*cheP115*) because SC276 was motile but unable to swarm in motility medium (10). However, we observed that broth cultures of SC276 in late logarithmic growth appeared to contain significantly fewer motile cells than did similar cultures of wild-type cells. Furthermore, the filaments assembled by the *flbT* mutant were more variable in length (five to eight sine waves) than were filaments

TABLE 3. Flagellin assembly and function in *C. crescentus* *fla* mutants

Mutant gene	Assembled flagellar filaments	Motility ^a	Chemotaxis ^a
Wild type	7-8 sine waves	+	+
<i>flaF</i>	None	-	-
<i>flaG</i>	None	-	-
<i>flbA</i>	Stub at 30°C; 2-5 sine waves at 23°C	-	-
<i>flbT</i>	5-8 sine waves	+	-

^a +, Wild-type function; -, complete absence of function; ±, reduced function (as described in text).

assembled by wild-type CB15 cells (seven to eight sine waves). Released filaments containing as few as only one sine wave were also present in the culture fluid. These abbreviated filaments and the inability of SC276 to swarm in motility medium demonstrated that the *flbT* mutant SC276 has both a Fla and a Che phenotype.

Microscopic examination of the *flaF* mutants (SC282 and SC279) and the *flaG* mutants (SC278, SC280, SC519, SC517, and SC1047) confirmed previous studies by Johnson and Ely (16) and showed that these mutants lack any detectable portion of a flagellar filament (Table 3).

Effect of *flbT*, *flbA*, and *flaG* mutations on flagellin synthesis. Three homologous flagellins (29, 27, and 25 kDa) are periodically synthesized and assembled into the filament during

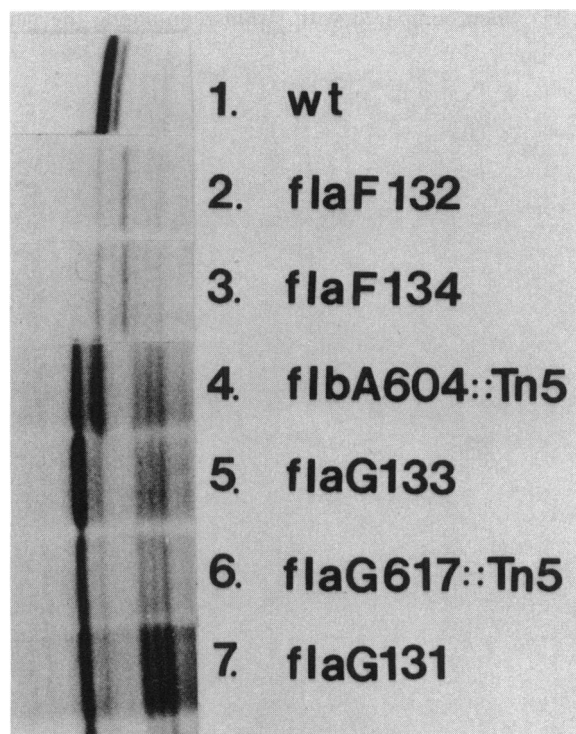


FIG. 2. Immunoprecipitations with anti-flagellum antibody of pulse-labeled proteins from cell extracts of strains containing mutations in the *flaFG* region. Cultures grown at 30°C to 125 Klett units (green filter no. 54) were pulse-labeled with a mixture of ¹⁴C-amino acids for 10 min, and equivalent amounts of labeled proteins as determined by trichloroacetic acid-precipitable counts were subjected to immunoprecipitation with anti-flagellum antibody. Positions of the flagellin proteins are indicated.

TABLE 4. Flagellin species characterized from *C. crescentus* *fla* mutants

Strain	Relevant genotype ^a	Flagellin ^b				
		29	27	25	24	22
CB15 ^c	Wild type	+	+	+	±	-
SC279	<i>flaF132</i>	++	±	±	-	-
SC282	<i>flaF134</i>	++	±	±	-	-
SC276	<i>flbT650</i>	+	++	++	++	++
SC1065	<i>flbA604::Tn5</i>	-	-	R	-	+
SC278	<i>flaG131</i>	-	-	±	-	+
SC517	<i>flaG161</i>	-	-	±	-	+
SC519	<i>flaG163</i>	-	-	±	-	+
SC1047	<i>flaG617::Tn5</i>	-	-	±	-	+
SC280	<i>flaG133 (ΔflaG)</i>	-	-	±	-	+

^a Mutations were characterized by Southern analysis and complementation studies (36).

^b Immunoprecipitation and/or Western analyses of mutant cell extracts, using anti-flagellum antibody identified the relative amounts and species of flagellins (indicated in kilodaltons) produced by these strains at 120 Klett units (green filter). +, Amount of flagellin species present in wild-type cells; -, nondetectable amount of flagellin species; ++, increased amount of flagellin species; ±, trace levels of flagellin species; R, reduced levels of flagellin species. For the 22-kDa flagellin which was not detected in wild-type cells, the level of 22-kDa flagellin produced by the *flaG* mutants is defined as the norm (+).

^c Parent in which all mutations used in this study were isolated.

the cell cycle. To characterize flagellin synthesis in the *flaF* mutants SC279 and SC282 and the *flaG* mutants SC278, SC519, SC517, SC1047, and SC280, immunoprecipitations with anti-flagellum antibody were performed (Fig. 2, Table 4). In accordance with previous results (17), *flaF* mutants synthesized increased amounts of the 29-kDa flagellin but only trace amounts of the 25- and 27-kDa flagellins. The *flaG* mutants did not produce detectable levels of the 27- or 29-kDa flagellin and produced only trace levels of the 25-kDa flagellin (see below). In addition, a 22-kDa flagellin species that was not detected in wild-type cells was precipitated from all *flaG* mutants with the anti-flagellum antibody. Western analyses of *flaF* and *flaG* mutants (Fig. 3) showed flagellin patterns similar to those identified by immunoprecipitation studies except that the trace level of the 25-kDa flagellin was not detected in any of the *flaG* mutants (Fig. 3). These results suggest that the 25-kDa flagellin is not stable in the *flaG* mutants.

Since the *flbA* mutant (SC1065 or SC2006) has a temperature-sensitive phenotype (Table 3), immunoprecipitation studies (Fig. 2) and Western analyses (Fig. 3) using anti-flagellum antibody were performed at both 30 and 23°C. The *flbA* mutant grown at 30°C produced a 22-kDa flagellin and reduced levels of the 25-kDa flagellin. Neither the 27- nor the 29-kDa flagellin was observed. In contrast, the *FlbA* mutant grown at 23°C produced an increased amount of 25-kDa flagellin and trace levels of the 27- and 29-kDa flagellins. No 22-kDa flagellin was observed at this temperature. Thus, the species of flagellin produced by the *flbA* mutant was dependent on the growth temperature. However, the level of flagellin synthesis appeared to be unaffected by changes in growth temperature and continued to be significantly less than that of the wild type (Table 4).

When the *flbT* mutant SC276 was examined, wild-type amounts of the 29-kDa flagellin and increased levels of the 27- and 25-kDa flagellins were observed (Table 4, Fig. 4A). This increased level of flagellin synthesis in the *flbT* mutant is a unique phenotype among a wide spectrum of *fla* mutations in *C. crescentus*. All other *fla* mutations have been shown to decrease the intracellular level of the 25- and

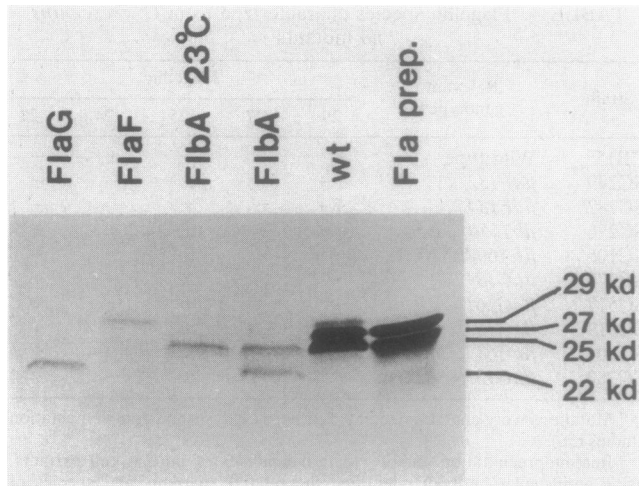


FIG. 3. Western analyses of the flagellin profiles of a representative FlaG mutant (SC280) and FlaF mutant (SC282) grown at 30°C (lanes 1 and 2, respectively), F1bA grown at 23°C (lane 3) and 30°C (lane 4), and wild-type CB15 (lane 5). The flagellin species of wild-type cells represented in denatured flagellar filaments isolated from wild-type cells grown to stationary phase is shown in lane 6. Equivalent amounts of protein from cellular extracts were loaded onto the gel. Trace amounts of the 25- and 27-kDa flagellins could be observed in the *flaF* and *flbA* (23°C) lanes, respectively.

27-kDa flagellin proteins (17). Therefore, synthesis of these structural flagellins appears to be negatively regulated by the *flbT* gene product. In addition, SC276 produced large quantities of 24- and 22-kDa flagellins whose function and origin are unknown. Only trace amounts of the 24-kDa flagellin species were detected in wild-type cells (Fig. 4 and 5).

Flagellin composition of the *flbA* and *flbT* flagellar filaments. Released filaments from the *flbT* mutant SC276 were isolated and characterized to determine whether the 22- and 24-kDa flagellin species produced by this mutant (Fig. 4A) were assembled into filaments. Neither the 22- nor the 24-kDa flagellin was detected in the isolated filaments, whereas both the 25- and 27-kDa flagellins were assembled (Fig. 4B). However, the ratio of 25-kDa to 27 kDa flagellin was approximately 1:1, not the 4:1 ratio present in wild-type CB15 (38). In these filaments, the amount of assembled 27-kDa flagellin was approximately equivalent to the amount of assembled 27-kDa flagellin in the wild-type filament. In contrast, the amount of assembled 25-kDa flagellin was reduced approximately three- to fourfold. The reduction in the amount of the assembled 25-kDa flagellin is consistent with the observation that many *flbT* filaments are shorter than wild-type filaments and therefore would be expected to contain smaller amounts of the 25-kDa flagellin. Furthermore, these results clearly demonstrate that the large intracellular levels of the 25-kDa flagellin (Fig. 4A, Table 4) are not efficiently or stably assembled into a filament.

Repeated attempts to isolate intact stub structures from *flbA* mutants (SC1065 and SC2006) grown at 30°C were unsuccessful, which indicated that these structures were fragile. Therefore, we could not determine whether the 22-kDa flagellin was assembled into these structures. However, analysis of filaments assembled by the *flbA* mutant grown at 23°C detected small levels of assembled 25-kDa flagellin. The 29- and 27-kDa flagellins were not detected in these structures. These results are consistent with reduced levels of the 25-, 27-, and 29-kDa flagellins being synthesized

by a small number of motile *flbA* cells (Fig. 2, 3, and Table 4).

trans-Acting effect of the *flbT* gene product. Complementation studies demonstrated that all of the mutant defects in the *flaFG* region could be corrected by the introduction of the corresponding cloned gene (36). However, during the course of these studies, we observed that swarm sizes of the complemented mutants were often smaller than those observed for the wild type. Therefore, to determine whether this variability resulted from an effect mediated by a gene product(s) encoded by the *flaFG* region, merodiploid strains that contained different fragments from this region in the same parent vector, pKT230, were constructed (Table 2). Motility, the ability to swarm in motility medium, and flagellin synthesis patterns of these strains were characterized. The *flaF*, *flbT*, *flbA*, *flaG*, and wild-type CB15 merodiploid strains containing the 4.0-kb *SstI* *flaFG* region (pPVS164) formed smaller swarms and appeared to have fewer motile cells in broth culture than did wild-type cells containing the pKT230 parent vector. Somewhat unexpectedly, all of these strains, including the wild type, did not form swarms when they contained pPVS163 (the 4.0-kb *SstI* region cloned into pKT230 such that *flaG* is proximal to the *amp* promoter). Furthermore, Western analysis demonstrated that when the entire 4.0-kb *SstI* *flaFG* region was provided in *trans* to *flaF*, *flbA*, *flbT*, *flaG*, and wild-type strains, flagellin synthesis was altered. Strains containing pPVS164 showed reduced amounts of the 27-kDa flagellin, and strains containing pPVS163 showed even greater reductions in the level of the 27-kDa flagellin as well as a reduction in the level of the 25-kDa flagellin (e.g., Fig. 5, lanes 4, 10, and 11) when compared with strains containing the parent

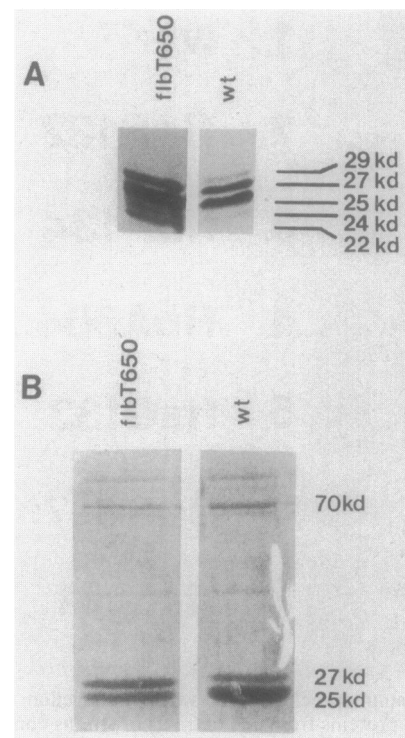


FIG. 4. (A) Western analysis of the flagellin profiles of the *flbT* mutant (SC276) (lane 1) compared with equivalent amounts of protein from wild-type CB15 cellular extract (lane 2). (B) Flagellin species isolated from released flagellar filaments of the *flbT* mutant (SC276) and wild-type CB15.

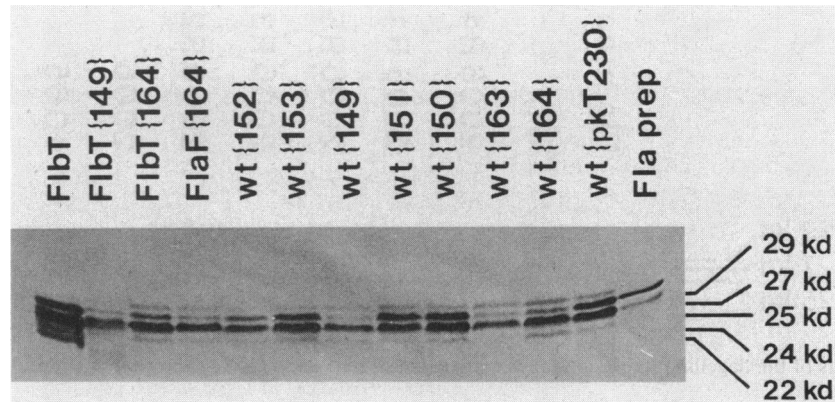


FIG. 5. Western analysis of the flagellin profiles of wild-type CB15 and SC276 (FibT) containing specific regions of the *flaFG* region cloned into pKT230 (see Table 2 for descriptions of plasmids): pPVS149, the 2.2-kb *Bam*HI₆-*Sst*I_a fragment containing the *flaF* and *flbT* transcription units; pPVS164 and pPVS163, the 4.0-kb *Sst*I *flaFG* fragment in opposite orientations; pPVS152 and pPVS153, the 0.8-kb *Eco*RI *flaF* fragment in opposite orientations; pPVS151 and pPVS150, the 3.2-kb *Eco*RI *flbAflaG* fragment in both orientations.

plasmid pKT230 (Fig. 5, lane 12). However, production of the 29-kDa flagellin was consistently unaltered. The smaller swarms formed by these merodiploid strains are consistent with the observed reduction in the amount of the 27- or 25-kDa flagellin.

Further studies using specific cloned genes from the *flaFG* region (Table 2, Fig. 1) demonstrated that *flaF*, *flbT*, and wild-type strains containing pPVS149 (the 2.2-kb *Bam*HI₆-*Sst*I_a *flaF-flbT* fragment) also produced small swarms in motility medium and showed significant reductions in levels of the 25- and 27-kDa flagellins (e.g., Fig. 5, lanes 1 and 2). Similarly, *flaF* and wild-type strains containing pPVS152 formed smaller swarms and showed reductions in the amounts of the 25- and 27-kDa flagellins (e.g., Fig. 5, lane 5). Comparison of the recombinant plasmids that mediated the repression of flagellin synthesis indicated that the one common feature was the presence of the *flbT* region. Furthermore, in pPVS163, the *flbT* gene was cloned such that readthrough from the *amp* promoter could have resulted in even higher levels of expression of *flbT*. Thus, these data are consistent with a putative negative regulatory role for the *flbT* gene product (see above).

In contrast to the results presented above, providing the *flaF* gene in pPVS153 (the 0.8-kb *Eco*RI *flaF* fragment) to the *flaF* mutants or the *flbA-flaG* transcription unit in pPVS150 and pPVS151 (the 3.0-kb *Eco*RI fragment in opposite orientations) or pPVS162 (the 2.8-kb *Hpa*I_c-*Eco*RI_c fragment) to the *flaG* mutants resulted in normal-size swarms. Flagellin synthesis in all of these strains was similar to that in CB15(pKT230), and these transcription units did not alter the motility or flagellin synthesis patterns of the wild-type strain (Fig. 5, lanes 6, 8, 9, and 12). These data demonstrate that the *flaF* or *flbA-flaG* transcription unit functions efficiently in *trans* to restore normal flagellin synthesis patterns. Furthermore, since the 22-kDa flagellin was no longer detected in any of the *flbA* or *flaG* merodiploid strains, these data clearly demonstrate that restoration of the *flbA* or *flaG* gene product, or both, prevented the production of detectable levels of this flagellin species.

Absence of the 22-kDa flagellin in assembled flagellar filaments of *flbA* pseudorevertants. By analyzing pseudorevertants, previous studies have demonstrated a correlation between the disappearance of the 22-kDa flagellin species and the ability of the mutant cell to assemble a functional filament (13, 20). Therefore, we isolated second-site muta-

tions that compensated for the *flbA604::Tn5* insertion in SC2006 (Fig. 1). SC2006 formed one to three motile flares from ca. 10⁸ cells. Analyses of six flares from independent colonies of SC2006 showed that the cells forming these flares were kanamycin resistant. Southern analyses using Tn5 as a probe confirmed that SC2006 and these resulting pseudorevertants (SC2006 R1 to R6) contained only one Tn5 element, which appeared to be inserted at the original position in the *flbA* gene. However, a chromosomal deletion (approximately 2.0 kb) that deleted a portion of the Tn5 element was observed in SC2006 R5. Deletion of this portion of the Tn5 element probably allowed transcription of the *flaG* gene, the distal gene of the *flbAflaG* operon (Fig. 1), resulting in partial filament assembly (see below). Further studies using transductional analyses indicated that the second-site mutations in the remaining strains were not linked to the *flbA-flaG* region. No second-site mutations were obtained from any of the *flaG* mutants or from the *flbT650* insertion mutation (36).

The swarms formed by SC2006 R1 to R6 were variable in size but consistently smaller than swarms formed by wild-type CB15. Microscopic examination showed that pseudorevertants (SC2006 R1 to R6) assembled variable-length flagellar filaments (two to five sine waves) and that many swarmer cells were observed at both 23 and 30°C. Western analysis of these strains determined that at both 23 and 30°C, the 22-kDa flagellin was no longer produced at detectable levels. However, trace amounts of the 27- and 29-kDa flagellins were present, and a four- to fivefold increase in the level of 25-kDa flagellin was observed (Fig. 6). Since the ability of the SC2006 R1 to R6 pseudorevertants to assemble flagellar filaments was correlated with the absence of the 22-kDa flagellin and a concomitant increase in the amounts of the 25-, 27-, and 29-kDa flagellins. These studies demonstrated that both the low level of flagellin synthesis and the altered flagellin species resulting from the *flbA604::Tn5* mutation could be compensated for by mutations in other genes. Studies are in progress to determine the map locations of these unlinked second-site mutations.

DISCUSSION

In this study, we demonstrated that mutations in the contiguous *flaF*, *flbT*, and *flbA-flaG* transcription units (Fig. 1) affect the synthesis of the 29-, 27-, and 25-kDa structural

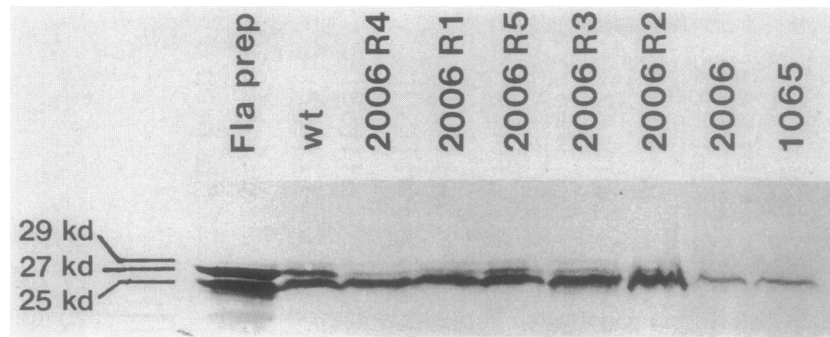


FIG. 6. Western analysis of the flagellin profiles in the *flbA* mutants (SC2006 and SC1065) and *flbA* pseudorevertants (SC2006 R1 to R5) grown at 23°C.

flagellins (Table 4) and their assembly into filaments. As observed previously (17), immunoprecipitations and Western analyses with anti-flagellum antibody of *flaF* mutant cell extracts showed only trace amounts of the 27- and 25-kDa flagellins but increased levels of the 29-kDa flagellin (Fig. 2 and 3). Similar analyses of *flaG* mutants demonstrated that only trace amounts of the 25-kDa flagellin, along with a 22-kDa flagellin species, were produced (Fig. 2 and 3). The severe reduction in synthesis of the 25- and 27-kDa flagellins in *flaF* and *flaG* mutants would explain the absence of a filament (Table 3).

The *flbA* mutant (SC1065) showed a temperature-sensitive phenotype: at 30°C, SC1065 assembled only a very short, straight filament (stub) (Table 3) and synthesized small amounts of only the 25-kDa structural flagellin in addition to a 22-kDa flagellin species (Fig. 2 and 3). At 23°C, a small number of swarmer cells assembled longer filaments. The 22-kDa flagellin was no longer detected, but larger amounts of the 25-kDa flagellin and trace amounts of the 27- and 29-kDa flagellins were present. Although the flagellin species were different at 23 and 30°C, the level of flagellin synthesis did not appear to be altered.

Of particular interest was the flagellin profile of strain SC276 (*flbT650*). Although wild-type amounts of the 29-kDa flagellin were produced, increased levels of the 25- and 27-kDa flagellins, along with large amounts of a 24-kDa and a 22-kDa flagellin species, were detected (Fig. 4A). This is a unique phenotype, since all other characterized *C. crescentus* flagellar mutants produce reduced levels of the 25- and 27-kDa flagellins, and indicates that these flagellins are negatively regulated through some mechanism that involves the *flbT* gene product. SC276 was characterized previously as a chemotactic mutant (*cheP115*) because the strain was motile but unable to swarm in motility medium (10, 16). However, both the frequency at which swimming cells reversed direction and the methylation of methyl-accepting chemotaxis proteins were normal in this strain (10; B. Ely, unpublished data). This study demonstrated that the *flbT* mutant assembles filaments that are more variable in length than are the filaments assembled by wild-type cells. These filaments show a two- to threefold reduction in assembled 25-kDa flagellin protein (Fig. 4B), demonstrating a defect in the assembly of this flagellin. However, the altered filament length of the *flbT* mutant cannot account for the Che phenotype, since the *flbA* mutant grown at 23°C assembles even shorter filaments (see above; Table 3) but still forms small swarms in motility medium. Since the *flbT650* (36) mutation in SC276 results in both a Che and a Fla phenotype, the *flbT* gene product appear to regulate both flagellin

synthesis and chemotactic function(s). Similarly, some genes in *Salmonella typhimurium* are involved in both flagellum biogenesis and chemotaxis (39).

Further analyses of merodiploid strains demonstrated that the *flaF* or *flbA-flaG* transcription unit restored normal amounts of the 25-, 27-, and 29-kDa flagellins to *flaF* or *flbA* and *flaG* mutants. However, during these studies we observed that all merodiploid strains, including the wild type, which harbored the *flbT* region on a plasmid, produced the 27- and/or the 25-kDa flagellin at reduced levels (Fig. 5). Since *flbT* was present on a plasmid, the extra copies of the *flbT* gene product may be preventing synthesis of these flagellins. These observations, combined with the unique flagellin synthesis pattern of the *flbT* mutant in which both the 25- and 27-kDa flagellins are overproduced, indicate that *flbT* may encode a repressor protein.

The function and significance of the 24- and 22-kDa flagellins that are produced as a result of *flbT*, *flbA*, and *flaG* mutations are unknown. In our study, neither the 22- nor the 24-kDa flagellin protein was detected in the isolated F1bT filaments (Fig. 4B). These results indicated that these flagellins are not assembled into any portion of a filament when the 25-, 27-, or 29-kDa structural flagellin is also present. Further analyses of the *flbA* mutant and pseudorevertants provided additional evidence that the 22-kDa flagellin species is not assembled into functional filaments. Previous studies have shown that even when very large amounts of this flagellin are produced, the 22-kDa protein is assembled only into stub structures, not into functional filaments (13, 17, 20). Furthermore, Koyasu (20) showed that large amounts of the 22-kDa flagellin were localized in the membrane fraction of mutant cells. Thus, incorporation of the 22-kDa, and possibly the 24-kDa, flagellin species into the *flbT* membrane may disrupt the integrity of the membrane at this pole and interfere with assembly of the 25-kDa flagellin (see above; Fig. 4). Ongoing studies of the *flbT* mutant have demonstrated other phenotypic abnormalities that would lend support to the existence of a membrane defect. One example is that this mutant shows abnormal stalk development (A. Driks, P. V. Schoenlein, and B. Ely, unpublished data).

Although previous studies have proposed that the 22- and 24-kDa flagellins may be breakdown products of the structural flagellins (17), additional studies, using pulse-chase experiments and anti-flagellum antibody, indicated that the 22-kDa flagellin may result from expression of a novel flagellin gene (20). Therefore, further experiments are necessary to determine the relationship of the 22- and 24-kDa flagellins to the other structural flagellins. Even if these

flagellins are breakdown products of any one of the structural flagellins, the processing event may be genetically programmed. Elucidation of the specific role(s) of *flbT* and *flbA* and *flaG* in preventing 22- and 24-kDa flagellin synthesis should help define the origin and function of these flagellin species. In addition, studies of the putative repressor function of *flbT* will determine whether the 25- and 27-kDa flagellins are negatively regulated by *flbT* only during flagellum biogenesis or throughout the cell cycle.

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