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A Mutation That Uncouples Flagellum Assembly from Transcription Alters the Temporal Pattern of Flagellar Gene Expression in *Caulobacter crescentus*

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The transcription of flagellar genes in *Caulobacter crescentus* is regulated by cell cycle events that culminate in the synthesis of a new flagellum once every cell division. Early flagellar gene products regulate the expression of late flagellar genes at two distinct stages of the flagellar *trans*-acting hierarchy. Here we investigate the coupling of early flagellar biogenesis with middle and late flagellar gene expression. We have isolated mutants (*bfa*) that do not require early class II flagellar gene products for the transcription of middle or late flagellar genes. *bfa* mutant strains are apparently defective in a negative regulatory pathway that couples early flagellar biogenesis to late flagellar gene expression. The *bfa* regulatory pathway functions solely at the level of transcription. Although flagellin promoters are transcribed in class II/*bfa* double mutants, there is no detectable flagellin protein on immunoblots prepared from mutant cell extracts. This finding suggests that early flagellar biogenesis is coupled to gene expression by two distinct mechanisms: one that negatively regulates transcription, mediated by *bfa*, and another that functions posttranscriptionally. To determine whether *bfa* affects the temporal pattern of late flagellar gene expression, cell cycle experiments were performed in *bfa* mutant strains. In a *bfa* mutant strain, flagellin expression fails to shut off at its normal time in the cell division cycle. This experimental result indicates that *bfa* may function as a regulator of flagellar gene transcription late in the cell cycle, after early flagellar structures have been assembled.

Caulobacter crescentus divides asymmetrically, forming two distinct cell types: a motile swarmer cell, with a single polar flagellum and chemotaxis machinery, and a sessile stalked cell. Transcription of flagellar genes is coordinated with cell cycle events, so that a new flagellum is synthesized and assembled at the swarmer pole of the predivisional cell before cell division is completed (reviewed in references 5 and 21). The flagellum consists of three major subassemblies (Fig. 1). The basal body contains a series of membrane-bound ring proteins which serve to anchor the flagellum within the cell membrane (30, 58, 63). The rings are traversed by a rod-like structure composed of the products of the *flgF* and *flgG* genes (12, 24). Attached to the rod, outside of the cell is a flexible hook (61), encoded by the *flgE* gene (48). The most distal structure, composed of flagellin monomers, is the flagellar filament (13, 63). The assembly of the polar flagellar structure proceeds from the inside out; the assembly of basal body is completed before assembly of the external hook and filament structures (26).

Flagellar gene expression in *C. crescentus* is controlled by a *trans*-acting regulatory hierarchy (Fig. 1) (7–9, 26, 38, 47, 48, 54, 67). One possible consequence of this regulatory hierarchy is that the order of expression of flagellar genes within the cell cycle reflects the order of flagellum assembly (Fig. 1). An unknown cell cycle event, possibly DNA replication (11, 59), triggers the expression of early flagellar genes (class II). The expression of these genes is dependent on an unidentified *trans*-acting factor, tentatively called σ^R (for regulatory sigma factor) (11, 59, 68, 69). The gene encoding σ^R would occupy the hypothetical class I of the flagellar regulatory hierarchy. Following expression and assembly of early flagellar gene products, the class III and IV flagellar genes are expressed. The class III and IV flagellar genes have conserved promoter sequences (reviewed in references 5 and 21). They are transcribed by RNA polymerase containing the alternative sigma factor, σ^{54}, and contain binding sites for the DNA-bending protein, integration host factor. Transcription is activated by factors that bind to distant enhancer sequences often located 100 bp upstream or downstream of the transcription start site. For example, the transcriptional activator FlbD binds to a conserved enhancer located in the promoters of flagellar genes that code for external structures such as the hook complex and flagellins (3, 4, 46, 64). FlbD is thought to be active only at times during the cell cycle when its target promoters are expressed. The activity of FlbD is regulated by temporal phosphorylation, and this in turn results in cell cycle transcription of its target promoters (64).

In addition to the positive activation of transcription by cell cycle cues, the expression of class III and IV genes is also dependent on the expression and, presumably, assembly of class II gene products (47, 54, 67). For example, epistasis experiments have demonstrated that strains containing mutations in early flagellar structural genes (class II) do not express genes encoding later, more external structures (class III and IV genes) (47, 54, 67). Additionally, late class IV flagellin gene expression is dependent on the expression of middle genes (class III). Therefore in *C. crescentus*, flagellar biogenesis regulates gene expression at two distinct stages of the *trans*-acting hierarchy (Fig. 1).

In this paper, we report the investigation of the coupling of early flagellar biogenesis with middle and late flagellar gene expression. We report the isolation of mutants that do not require early class II flagellar gene products for the transcription of middle or late flagellar genes. These mutants, *bfa* (for bypass of flagella assembly) mutants, were isolated by selecting for cells that could transcribe the *fljL* flagellin gene promoter in a class II mutant background. This mutation can bypass the transcriptional requirement for all class II genes, with the ex-

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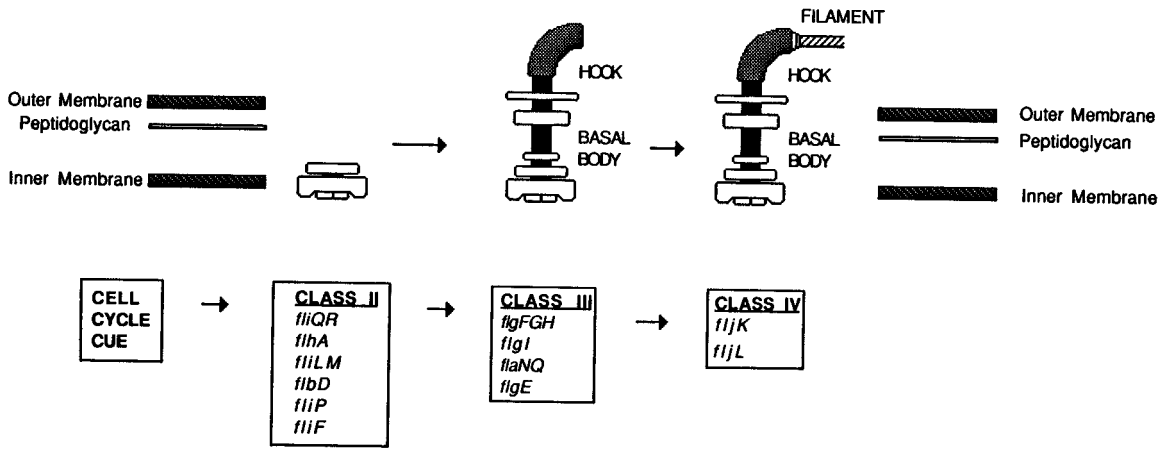


FIG. 1. The *Caulobacter* flagellar regulatory hierarchy. The genes at each level of the hierarchy are contained in boxes. The hypothetical structures encoded by each set of genes are depicted above each box. Flagellar assembly is coupled to gene expression at two distinct levels of the regulatory hierarchy. A cell cycle cue initiates the transcription of the class II subset of flagellar genes. These genes encode regulatory proteins as well as the components of the early flagellar structure. The expression of these early genes is required for the expression of class III genes. These genes encode components of the basal body and hook structures. The expression of these genes is, in turn, necessary for the expression of the class IV genes, which encode flagellins.

ception of those encoding essential transcription factors. In a *bfa* mutant strain, *fljL* expression fails to shut off before cell division, indicating that *bfa* may function as a negative regulator of flagellar gene transcription late in the cell cycle.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *C. crescentus* NA1000, a motile, synchronizable strain, was used as the wild-type strain. *Caulobacter* cells were grown at 31°C in either PYE medium (50) or minimal M2-glucose medium (28). Cells harboring p*lacZ*/290 or pAGM*fljL*/*neo* were maintained by supplementing medium with 1 μg of tetracycline or 5 μg of gentamicin, respectively, per ml. A 700-bp *Hin*dIII-*Eco*RI fragment from pKic7 (1) containing the *fljL* promoter was subcloned into pAGM, a pACYC184 derivative containing mobilization functions from RP4 (mob^+) and a gentamicin resistance gene (1), in front of the *neo* reporter gene. The *fljL*/*neo* fusion was integrated by homologous recombination into the *Caulobacter* chromosome by selecting for gentamicin resistance. p*fljL*/*lacZ*/290 was generated by subcloning the 700-bp *Hin*dIII-*Eco*RI fragment containing the *fljL* promoter into p*lacZ*/290 (22). Similarly, a 1.3-kb *Pst*I-*Xho*I fragment containing the *flbG* promoter was subcloned into pRK290 in front of the *lacZ* reporter gene (23). p*flgF*/*lacZ*/290 was generated by using a 1.9-kb *Sal*I-*Cla*I fragment subcloned into p*lacZ*/290.

Genetic manipulations. Ethyl methanesulfonate (EMS) mutagenesis was performed as described previously (55) on the class II mutant strain SC508, which contains a deletion in the *fliQR* operon. This strain also carried the integrated reporter plasmid pAGM*fljL*/*neo*. Following mutagenesis, cells expressing *fljL*/*neo* were isolated by selecting for resistance to 50 and 300 μg of kanamycin per ml. Transducing phage and all transductions were performed as previously described by Johnson and Ely (29).

Recombination-deficient (Rec^-) strains were created by using a SC508 strain containing a Tn*5* insertion in *cysB*, a gene required for cysteine biosynthesis which is located within transducing distance of *rec*. The five *Caulobacter* strains containing a *bfa* mutation were made Rec^- by transducing *cysB*::Tn*5tet* with ϕCR30 and selecting for tetracycline resistance at 1 μg/ml. Phage ϕCR30 was plated on a *Caulobacter* strain that was Rec^- but $cysB^+$. This phage was used to transduce SC508 containing *bfa* and the *cysB*::Tn*5tet*. The resulting colonies were selected for growth on minimal M2 plates and tested for UV sensitivity (28). Those strains that returned to cysteine prototrophy and demonstrated UV sensitivity were considered Rec^-.

Epistasis experiments. The effect of *bfa* on the expression of flagellar *lacZ* fusions was tested in class II mutant strains with Tn*5* insertions. To accomplish this, the kanamycin cassette was switched with a spectinomycin cassette, since the *bfa* mutants were initially kanamycin resistant. Class II mutant strains (Table 1) were mated with *Escherichia coli* S17-1 containing a ColE1-based plasmid with an *oriT* and spectinomycin-resistant Tn*5* (Tn*5spc*). Colonies were selected for resistance to 50 μg of spectinomycin per ml. To identify colonies that had undergone double-crossover events, 100 colonies were replica plated onto kanamycin and spectinomycin plates. Those that were sensitive to kanamycin but resistant to spectinomycin were selected. Class II strains that contain Tn*5spc* were used to generating transducing lysates.

To screen for β-galactosidase activity expressed by bacterial colonies, 3 ml of molten top agar containing 0.3 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml was used to overlay bacterial growth (32). Quantitative measurements of β-galactosidase activity were performed as previously described (22).

Pulse-field gel electrophoresis. *Caulobacter* chromosomal DNA samples were prepared as previously described by Ely and Gerardot (15). Strains UC1010, UC1020, UC1030, UC1040, and UC1050 were mapped by using a pulsed-field gel apparatus. The cells were embedded in agarose plugs, the cells were lysed, and a restriction digestion with *Ase*I, *Dra*I, and *Spe*I was performed. The agarose plugs were loaded in a 1% agarose gel, and the DNA was electrophoresed for 36 h at 14°C in 0.5× Tris-borate-EDTA with pulse times beginning at 10 s with a linear increase to 30 s over the course of the run. Gels were stained with ethidium bromide and transferred to nitrocellulose (2, 10). A 1,050-bp *Hin*dIII fragment from pKic7 containing the *neo* gene of Tn*5* was randomly labeled with [α-^{32}P]dGTP (Amersham, Arlington Heights, Ill.) for use as a hybridization probe (2, 9).

Cell cycle expression experiments. *Caulobacter* cultures were grown in M2-glucose to an optical density at 660 nm of 1.0 to 1.4. Swarmer cells were isolated by centrifugation through a Ludox gradient (16). Swarmer cell populations (greater than 97% pure) were suspended in fresh M2-glucose medium and allowed to progress through the cell cycle. At various times throughout the cell cycle, samples were removed and proteins were pulse-labeled with ^{35}S-Trans-labeled methionine (ICN, Irvine, Calif.) for 10 min. Labeled protein was immunoprecipitated with either a monoclonal anti-β-galactosidase antibody (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) or a polyclonal antiflagellin antibody (25).

Immunoblots. Whole-cell extracts of overnight cultures were obtained by sonication. Protein concentration of cellular extracts was determined by the Bradford assay (Bio-Rad, Hercules, Calif.). Equal amounts of protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose, and immunoblotting was performed as described previously (60). A polyclonal antiflagellin antibody was used as the primary antibody. Secondary goat anti-rabbit immunoglobulin G antibody conjugated to alkaline phosphatase (Bio-Rad) was used to visualize protein bands.

RESULTS

Isolation of *bfa* mutants. A positive selection scheme was used to isolate mutants (*bfa*) that could express late flagellar genes (class IV) in the absence of early flagellar gene products (Fig. 2). The late flagellin promoter *fljL* (encoding 27-kDa flagellin) was subcloned upstream of a promoterless *neo* reporter gene in plasmid pKic7. *neo* encodes neomycin phosphotransferase II and when expressed in *C. crescentus* confers resistance to kanamycin. When the *fljL*/*neo* transcription fusion was integrated by homologous recombination into wild-type *C. crescentus* NA1000, *fljL* promoter activity upstream of *neo* generated sufficient levels of neomycin phosphotransferase

TABLE 1. Bacterial strain and plasmids used in this study

Bacterial strain or plasmid	Genotype	Reference(s)
E. coli S17-1	Rp4-2, Tc::Mu, Km::Tn*7*	51
C. crescentus		
NA1000*bla*	*syn-1000 bla*	16
SC508	*flaS153* (Δ*fliQR*)	29
SC1029	*podW*::Tn*5*	62
SC1032	*flbD198*::Tn*5*	52
SC1042	*fliF106*::Tn*5 proA str-140*	47
SC1048	*fliP*::Tn*5*	19
SC1055	*rpoN*::Tn*5 proA str-140*	4, 14
SC1088	*cysB135*::Tn*5 str-152*	15
SC1131	*fliLM196*::Tn*5*	68
SC1132	*flhA608*::Tn*5 str-152*	14, 53, 56
UC900	*flaS153* pAGM*fljL*/*neo*	This paper
UC1010	*bfa-1201 flaS153*	This paper
UC1020	*bfa-1202 flaS153*	This paper
UC1030	*bfa-1203 flaS153*	This paper
UC1040	*bfa-1204 flaS153*	This paper
UC1041	*bla bfa-1204*	This paper
UC1042	*bla bfa-1204 rpoN*::Tn*5spc*	This paper
UC1043	*bla bfa-1204 flbD198*::Tn*5spc*	This paper
UC1044	*bla bfa-1204 fliF106*::Tn*5spc*	This paper
UC1045	*bla bfa-1204 fliLM196*::Tn*5spc*	This paper
UC1046	*bla bfa-1204 fliP*::Tn*5spc*	This paper
UC1047	*bla bfa-1204 podW*::Tn*5spc*	This paper
UC1048	*bla bfa-1204 flbF-608*::Tn*5spc*	This paper
UC1049	*bla bfa-1204 fla-1213*::Tn*5spc*	This paper
UC1050	*bla bfa-1205 flaS153*	This paper
UC2113	*fla-1213*::Tn*5*	This paper
Plasmids		
pSUP2021	pBR325-Mob::Tn*5*	57
pAGM	Deletion of Tet^r from pAGMT	This paper, 1
pKic7	Promoterless *neo* reporter	1
p*lacZ*/290	*lacZ* reporter vector	23
pJBZ282	*lacZ* protein fusion vector	1
pKic7 *fljL*/*neo*	700-bp *Hin*dIII-*Eco*RI *fljL* promoter fragment in pKic7	This paper
pAGM *fljL*/*neo*	*fljL*/*neo* in pAGM	This paper
p*fljL*/*lacZ*/290	700-bp *Hin*dIII-*Eco*RI *fljL* promoter fragment in p*lacZ*/290	This paper
p*flbG*/*lacZ*/290	1.3-kb *Pst*I-*Xho*I fragment containing the *flbG* promoter inserted into p*lacZ*/290	23
p*flgF*/*lacZ*/290	1.9-kb *Sal*I-*Cla*I fragment containing the *flgF* promoter inserted into p*lacZ*/290	This paper
p*fljK*/*lacZ*/290	580-bp *Pst*I-*Eco*RI fragment containing the *fljK* promoter inserted into p*lacZ*/290	66
p*fljK*::*lacZ*	580-bp *Pst*I-*Eco*RI fragment containing the *fljK* promoter and 29 codons inserted in frame to *lacZ* in pJBZ282	This paper

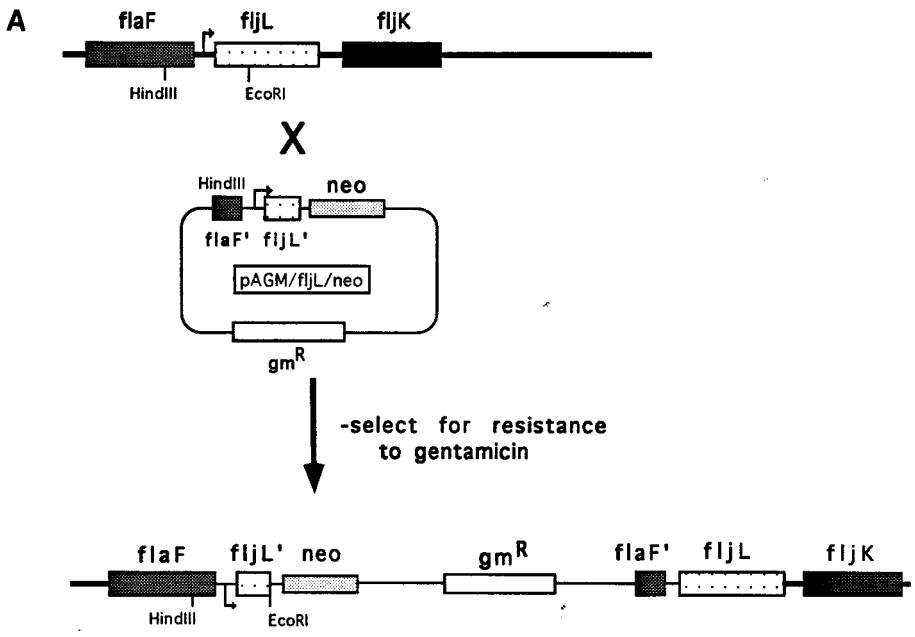
II to confer resistance to high levels of kanamycin (500 μg/ml) (Fig. 2). In contrast, relatively low levels of kanamycin resistance were generated when this fusion was introduced class II mutant strain SC508, which contains a deletion in the *fliQR* operon (Fig. 2).

To isolate mutants that expressed *fljL* in the absence of *fliQR*, EMS mutagenesis was performed on UC900, the *fliQR* deletion strain containing the *fljL*/*neo* transcription fusion. EMS mutagenesis was used because initial attempts at isolating this type of mutant by using Tn*5* mutagenesis failed. Following EMS mutagenesis, the cells (1.5×10^9 per plate) were selected for resistance to kanamycin at concentrations of 50 and 300 μg/ml. Approximately 500 kanamycin-resistant colonies grew on each plate; therefore, the frequency of kanamycin resistance was ca. 3.3×10^{-7}. When cells were not treated with EMS, the frequency of kanamycin resistance was less than 10^{-8}. To determine whether the *fljL* promoter was actually expressed in these kanamycin-resistant cells, a secondary screen for *fljL* promoter activity was used. A plasmid containing a *fljL*/*lacZ* transcription fusion was introduced into 300 kanamycin-resistant clones, and β-galactosidase assays were performed to provide a measure of *fljL* promoter activity. β-Galactosidase activity was detectable only in those cells selected for growth at the lower kanamycin concentration of 50 μg/ml, indicating that growth at higher concentrations was probably not due to expression of the *fljL*/*neo* fusion. Wild-type levels of β-galactosidase activity were expressed in approximately 18% of the original kanamycin-resistant colonies from the plates containing 50 μg of antibiotic per ml. Cells that both were resistant to kanamycin and expressed wild-type levels of β-galactosidase activity from a *fljL*/*lacZ* reporter fusion were considered to contain bypass mutations. Five different mutant strains were chosen for mapping and characterization of this bypass phenotype (Table 1).

Genetic mapping of the *bfa* mutations. To physically map the *bfa* mutation, as well as move it into different genetic backgrounds, *bfa* was genetically linked to the transposon Tn*5*. The five *Caulobacter* strains containing the *bfa* mutation were first made recombination deficient (Rec$^-$) as described in Materials and Methods. This step was required to ensure that Tn*5*, when introduced into these cells on a nonreplicating plasmid, transposed randomly and did not integrate into the chromosome by homologous recombination. Note that this would be a relatively frequent event since these *bfa* mutant cells contain an integrated copy of a ColE1-type plasmid.

Tn*5* was introduced into the five Rec$^-$ *bfa* mutant strains by mating with an *E. coli* strain containing pSUP2021 (57). Tn*5*-containing transconjugants were selected on PYE plates containing 1 mg of kanamycin per ml. Note that Tn*5* confers significantly greater resistance to kanamycin than the integrated *fljL*/*neo* fusion in these mutants. A φCR30 transducing lysate was prepared from a pool of the resistant colonies from each mating. This lysate was then used to transduce a Δ*fliQR* strain (SC508) containing a *fljL*/*lacZ* transcription fusion. Transductants were selected on medium containing 50 μg of kanamycin per ml to select for Tn*5* and then screened for β-galactosidase activity by agar overlay with X-Gal. The presence β-galactosidase activity indicated that the Tn*5* and the *bfa* mutation cotransduced and therefore were in relatively close physical proximity to each other. The cotransduction frequency was determined for each of the five *bfa* mutant strains in which Tn*5* was now physically linked, by transducing the Tn*5* from the linked strain into a new *fliQR* deletion strain carrying the reporter fusion. The cotransduction frequency ranged from approximately 20 to 86% (data not shown).

To map the position of the *bfa* mutations on the *Caulobacter* chromosome, the location of the Tn*5* transposon in each of the linked strains was physically mapped by pulse-field gel electrophoresis. Following electrophoresis, the DNA was transferred to nitrocellulose filter and Southern blotting was performed with the *neo* gene from Tn*5* as a probe. The *bfa* mutations apparently map to the same chromosomal location in all five of



B

[kanamycin] (μg/ml)	Relative Growth NA1000/ fljL-neo	SC508/ fljL-neo
25	+++	+
50	+++	-
100	+++	-
500	++	-

FIG. 2. (A) Construction of a *fljL/neo* reporter gene fusion. A *fljL/neo* reporter fusion was introduced into *C. crescentus* on plasmid pAGM, a pACYC184 derivative containing mobilization functions from RP4 and a gentamicin resistance (gm^R) gene (see Materials and Methods). This fusion plasmid integrates into the chromosome by homologous recombination when introduced in *C. crescentus* by conjugation. H, *Hin*dIII; R, *Eco*RI. (B) Relative levels of kanamycin resistance when strains carrying this fusion were plated on PYE plates containing different concentrations of kanamycin. Wild-type *Caulobacter* cells (strain NA1000) carrying this fusion are resistant to relatively high levels of kanamycin as a result of normal expression of *fljL* promoter activity. In contrast, strain SC508, which possesses a deletion in the class II genes, *fliQ* and *fliR*, is sensitive to moderate concentrations of kanamycin as a consequence of low expression of the *fljL* promoter. +++, growth unaffected by kanamycin; ++, moderate growth; +/−, little growth; −, no growth.

the chosen mutants (Fig. 3). The Southern blot of DNA isolated from strain UC1040 is shown in Fig. 3B. The *neo* probe hybridized to the 340-kb *Ase*I fragment, the 400-kb *Dra*I fragment, and the 330-kb *Spe*I fragment. The Southern blotting results for the other four *bfa* mutant strains were the same as those for UC1040 (data not shown), indicating the isolated *bfa* mutations map to one region of the *Caulobacter* chromosome. This region of the chromosome is located adjacent to the large hook cluster of flagellar genes (Fig. 3A). We then tested whether other *bfa* mutants mapped near this region by performing a cotransduction experiment using the Tn*5* linked to *bfa-1204* in strain UC1040. Of the 68 independent *bfa* mutant strains tested, 67 cotransduced with Tn*5* at a frequency similar to that for the *bfa-1204* allele (data not shown). This result suggests that these additional *bfa* mutations map relatively close to the *bfa-1204* mutation.

Epistasis experiments. The *bfa* mutation permits the expression of *fljL* in the absence of *fliQ* and *fliR*, two class II flagellar genes. Epistasis experiments were performed to determine whether this mutation could also bypass the requirement for other class II genes. The *bfa* mutant strain that contained the Tn*5* in closest proximity (UC1040) to *bfa* was used to construct *bfa*/class II double mutants. As a first step, ϕCR30 lysates of UC1040 were transduced into the wild-type *Caulobacter* strain and selected for Tn*5* by plating on medium containing kanamycin. Since the Tn*5* in UC1040 is 86% linked to *bfa*, approximately 8 of 10 colonies should also contain the *bfa* mutation. To determine which of the kanamycin-resistant colonies contained *bfa*, a Tn*5spc*, class II mutation (*fliP*), was then introduced by transduction. The resulting spectinomycin-resistant colonies were screened for lack of motility with light microscopy to ensure that the transductants had acquired the *fliP* mutation. The *fljL-lacZ* reporter fusion was then introduced into the *fliP*-containing strains, and β-galactosidase assays were performed to determine which of these isolates also contained a *bfa* mutation.

The parent strain which contained the *bfa* mutation as identified by the assay above was then used as a host to construct other *bfa*/class II double mutants by transduction. The *fljL/lacZ* transcriptional reporter fusion was introduced into each of these double mutant strains, and β-galactosidase activity was measured. The *bfa* mutation could relieve the transcriptional

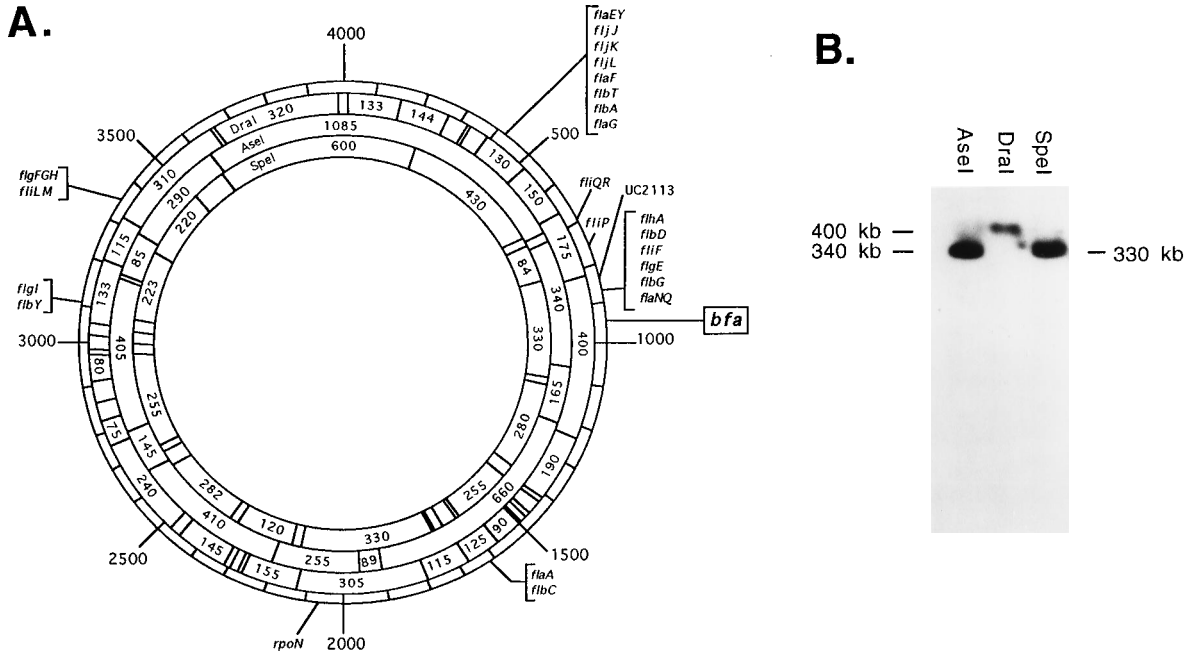


FIG. 3. Physical mapping of the *bfa* mutation. (A) Physical map of the *Caulobacter* chromosome generated by restriction digest with *Ase*I, *Dra*I, and *Spe*I showing the relative locations of Tn*5* insertions linked to *bfa* by transduction and flagellar genes (genetic map data from reference 14). (B) An autoradiogram of a Southern blot from strain UC1040 containing Tn*5*. To determine the locations of Tn*5* insertions that cotransduced with the *bfa* mutation, pulsed-field gel electrophoresis was performed on DNA samples that had been digested with *Ase*I, *Dra*I, and *Spe*I. Following electrophoresis, DNA samples were transferred to nitrocellulose, and a Southern hybridization was performed with a ^{32}P-labeled *neo*-containing fragment from pKic7 as a probe for Tn*5*. The sizes of the labeled fragments were determined by comparing migration with that of phage lambda DNA concatemers.

requirement for several other class II genes, including *fliLM*, *fliP*, and *flhA* (Table 2). In each of these mutant strains, *fljL*/*lacZ* expression is low, with β-galactosidase levels at or near background (90 U). When the *bfa* mutation from UC1040 was introduced into these strains, expression of *fljL*/*lacZ* returned to levels that were comparable to those in wild-type cells (Table 2). We also tested two other nonmotile Tn*5* mutants, SC1029, which has an insertion in a gene required for polar organelle development (*podW*) (62), and UC2113, a recently isolated flagellar mutation (51). Strains carrying these mutations are nonmotile and do not express class III and IV flagellar genes. Therefore, both of these mutants are likely to possess Tn*5* insertions in class II flagellar genes. In support of this idea, the *bfa* mutation could also bypass the transcriptional requirement for these two genes (Table 2). The *bfa* mutation cannot bypass the requirement for *rpoN* and *flbD*. Both of these genes encode essential transcription factors. *rpoN* encodes the σ^{54} subunit of RNA polymerase (6), and *flbD* encodes the transcriptional activator of *fljL* (3, 4, 46, 52, 66). These results suggest that *bfa* functions to regulate *fljL* transcription in the absence of class II flagellar gene products. We next tested whether *bfa* also functioned to couple the transcription of class III genes to class II gene expression. To accomplish this, we tested the expression of both a hook operon/*lacZ* reporter fusion (*flbG*/*lacZ*) and a basal body/*lacZ* reporter (*flgF*/*lacZ*) in class II mutant backgrounds, in the presence and absence of *bfa*. As was the case with the *fljL*/*lacZ* fusion, *bfa* effectively bypassed the transcriptional requirement for class II gene products for both transcriptional fusions (Table 2). This set of experiments indicates that *bfa* may be a general negative regulator of both class III and class IV flagellar genes. In support of this idea, the *bfa* mutation in strain UC1040 can be complemented in *trans* with DNA contained in a plasmid library prepared from wild-type cells. Clones containing inserts that mapped to the same region of the genome as *bfa* could reverse the *bfa* phenotype in a Δ*fliQR bfa* double mutant (data not shown). This result may indicate that the *bfa-1204* phenotype is attributable to a loss of function mutation in a negative regulator.

TABLE 2. Effects of *bfa* and class II mutations on flagellar promoter activity

Strain	β-Galactosidase activity (U)					
	fljL/*lacZ*		*flbG*/*lacZ*		*flgF*/*lacZ*	
	bfa$^+$	*bfa*	*bfa*$^+$	*bfa*	*bfa*$^+$	*bfa*
SC508 (*fliQR*)	87	8,912	336	7,148	70	5,061
SC1131 (*fliLM*)	181	2,499	33	2,938	75	4,494
SC1048 (*fliP*)	193	1,043	30	932	60	1,838
SC1029 (*podW*)	110	3,364	38	1,014	66	3,597
SC1132 (*flhA*)	90	3,042	33	1,599	125	1,054
UC2113	190	2,607	34	1,542	51	1,062
SC1055 (*rpoN*)	122	85	45	26	41	83
SC1032 (*flbD*)	343	102	263	43	64	41
NA1000 (wild type)	5,658	5,811	2,468	1,031	1,666	1,897

Effect of *bfa* on the temporal pattern of flagellar gene expression. One possible function of the *bfa* regulatory system may be to control or influence the cell cycle timing of late flagellar gene expression in response to early events in flagellar biogenesis. To test this idea, we assayed the temporal transcription of the *fljL* promoter in both wild-type and *bfa* mutant cells. Cell cycle expression of *fljL* was assayed by synchronizing a culture containing the *fljL*/*lacZ* transcriptional reporter plasmid. The isolated swarmer cells were suspended in fresh me-

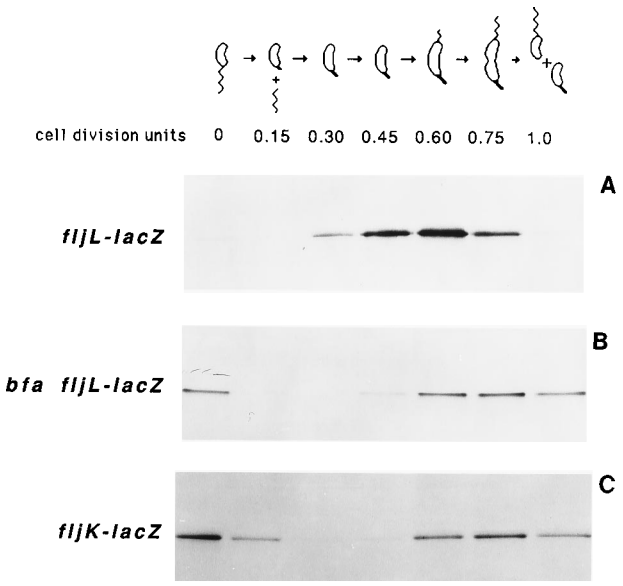


FIG. 4. Effect of *bfa* on the cell cycle expression of *fljL*. The temporal expression of β-galactosidase was assayed in *Caulobacter* cells carrying flagellin-*lacZ* transcription reporter fusions. Isolated swarmer cells were suspended in fresh M2 medium and were permitted to progress through the cell cycle. At various times during the cell cycle (0, 30, 60, 90, 120, 150, and 180 min), an aliquot was removed and proteins were labeled with ^{35}S-Trans label for 10 min. Labeled protein was immunoprecipitated with an anti-β-galactosidase antibody. (A) *fljL*/*lacZ* expression in wild-type strain NA1000. The drawing above the fluorogram shows the cell types present at each time point, as determined by light microscopy. Labeled β-galactosidase is indicated. (B) *fljL*/*lacZ* expression in *bfa* mutant strain UC1040. (C) Cell cycle expression of a non-*bfa*-regulated *fljK*/*lacZ* reporter fusion in wild-type strain NA1000.

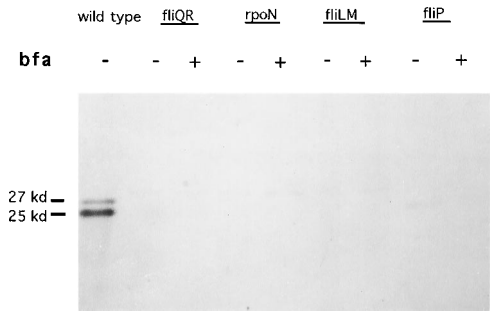


FIG. 5. Flagellin protein synthesis in a class II *bfa* double mutant. Flagellin protein expression in class II mutants in the presence (+) or absence (−) of a *bfa* (*bfa-1204*) mutation was assayed by immunoblotting. Cellular extracts of overnight cultures were obtained by sonication. Equal amounts of protein were electrophoresed on an SDS-polyacrylamide gel. Protein concentration was determined by the Bradford assay. Immunoblotting was performed with a polyclonal antiflagellin antibody. Secondary goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase was used to visualize flagellin proteins.

dium and allowed to progress through the cell division cycle. At various time points, proteins were pulse-labeled with ^{35}S-Trans-labeled methionine. Labeled β-galactosidase was immunoprecipitated from the cell extracts following incubation with anti-β-galactosidase antibody and then subjected to polyacrylamide gel electrophoresis. In wild-type cells, the *fljL*/*lacZ* fusion is expressed under temporal control. Expression begins at approximately 0.3 cell division unit (60 min) and peaks between 0.45 and 0.6 cell division unit (90 to 120 min) (Fig. 4A). Expression of this fusion ceases abruptly at the time of cell division. The *fljL*/*lacZ* fusion displays a different pattern of expression in *bfa* mutant cells (Fig. 4B). The peak in temporal expression is delayed by approximately 30 min. One remarkable difference in the cell cycle expression pattern of *fljL* expression in *bfa* mutant cells occurs at the time when expression normally ceases. *bfa* mutants continued to express *fljL*/*lacZ* up to the time of cell division, with expression continuing into the swarmer cell stage (Fig. 4B). This experimental result suggests that *bfa* may normally function to regulate transcription of *fljL* late in the cell division cycle. As a routine control, newly synthesized flagellin protein was also immunoprecipitated in each of these experiments (not shown). Interestingly, the temporal pattern of flagellin protein synthesis in *bfa* mutants decreased at the same time in the cell cycle as in wild-type cells, indicating that *bfa* functions solely at the level of transcription (see below).

Genes encoding flagellar distal structures, including the basal body rods, the outer rings, the hook, hook-associated proteins, and flagellins, occupy either class III or IV of the flagellar regulatory hierarchy when assayed in epistasis experiments using transcriptional fusions. One notable exception is the promoter for the major 25-kDa flagellin gene, *fljK*. Although it is well documented that flagellin protein is not synthesized in class II mutants, *fljK* promoter activity is not affected (64). Therefore, *fljK* transcription is not controlled by the *bfa* regulatory system. Comparison of the temporal pattern of *fljL*/*lacZ* expression with that of a *fljK*/*lacZ* transcription fusion shows that *fljK* expression in wild-type cells is strikingly similar to that of *fljL* in a *bfa* mutant (Fig. 4). Although *fljK*, *fljL*, the hook operon, and *flaNQ* promoters are activated by the same transcription factor (3, 4, 46, 64), *fljK* is the only one of these promoters that demonstrates this late pattern of expression. These experiments raise the possibility that the distinct temporal pattern of *fljK* expression may reflect a difference in regulation by *bfa*.

Posttranscriptional regulation of flagellin expression. The results presented above demonstrate that *fljL* transcription fusions are expressed in class II/*bfa* double mutants. We wished to determine if flagellin protein was also expressed in these mutant strains. Immunoblotting with an antiflagellin antibody was performed on wild-type cells as well as class II/*bfa* double mutants (Fig. 5). Although late flagellar genes are transcribed in *bfa* mutants, there is no detectable flagellin protein present in cell extracts, suggesting that flagellin mRNA may be subject to posttranscriptional regulation. To test this, we assayed the effect of a class II flagellar mutation on the expression of a *fljK*/*lacZ* transcription fusion and *fljK*::*lacZ* protein fusion (Fig. 6). The expression of a *fljK*/*lacZ* transcription fusion is unaffected in the class II mutant strain SC508 (Δ*fliQR*) (Fig. 6). In contrast, a fusion of 29 amino acids of FljK to β-galactosidase is not expressed in this same mutant strain (Fig. 6). These experiments suggest that the levels of flagellin expression are also regulated by a posttranscriptional mechanism.

DISCUSSION

One remarkable feature of flagellar biogenesis in *C. crescentus* is the cell cycle-controlled synthesis of a single polar flagellum. Flagellar biogenesis in *C. crescentus*, as in other bacteria, requires the expression of approximately 50 genes (14). The first genes expressed in the cell cycle (class II) encode early flagellum structures, such as the MS ring of the basal body and switch proteins (reviewed in references 5 and 21). Structural components of the flagellum comprise only a fraction of this early group of genes. Also in this class are tran-

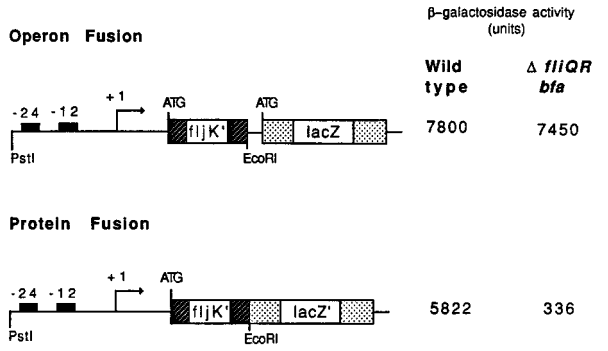


FIG. 6. Flagellin protein reporter fusions are not expressed in class II mutants. Depicted is a schematic diagram of a *fljK/lacZ* operon fusion and a *fljK*::*lacZ* protein fusion. The *fljK/lacZ* fusion represents a 580-bp *Pst*I-*Eco*RI fragment containing the *fljK* promoter subcloned 5′ to the *lacZ* gene in p*lacZ*/290. The protein fusion contains the same fragment of *fljK* DNA cloned in frame to the *lacZ* gene coding sequence in pJBZ282. The resulting fusion contains the first 29 codons of *fljK* fused to codon 8 of *lacZ*. These fusions were introduced into both the wild-type strain and strain UC1040 (Δ*fliQR bfa-1204*), and β-galactosidase levels were measured as an index of gene expression.

scription factors and components of a flagellum-specific protein export system. The expression and, presumably, successful assembly of this entire set of gene products are required for the subsequent expression of genes that encode later flagellum structures. Therefore, the expression of class III and IV genes is influenced by the cell cycle as well as earlier events in flagellar biogenesis.

Two mechanisms regulate flagellar gene expression in response to assembly. In this paper, we describe and characterize a mutation that uncouples the transcription of class III and IV flagellar genes from early events in flagellar biogenesis. In *bfa* mutant strains, class III and IV flagellar genes are expressed in the absence of all known class II gene products with the exception of those encoding transcription factors. The *bfa* mutation apparently has a general regulatory defect. Strains carrying a *bfa* mutation transcribe basal body, hook operon, and flagellin promoters in the absence of class II gene products.

The *bfa* mutation in *C. crescentus* is analogous to mutations in the *flgM* gene of the flagellar regulon in *Salmonella typhimurium* (17, 18). The regulatory hierarchy in enteric bacteria functions in the same fashion as in *C. crescentus* (34, 37; reviewed in references 31 and 39). One notable difference is that as there are four levels of the genetic hierarchy in *C. crescentus*, the enteric bacterial hierarchy operates with three levels of regulation. In *S. typhimurium*, class I is occupied by the master regulatory gene products. Class II contains the homologs of both class II and class III *Caulobacter* flagellar genes. For example, in the enteric bacterial scheme, early flagellar structural genes such as *fliF* (MS ring) and genes that encode the basal body rods, outer rings, and the hook structure are genetically classified as class II genes. In *S. typhimurium*, this middle set of genes is required for the expression of flagellins, chemotaxis, and motility genes, as well as genes that encode several late flagellar structures (class III) (34, 37). Class III genes are transcribed by RNA polymerase containing the alternative sigma factor, σ^{28} (reviewed in references 31 and 39). The *flgM* gene product negatively regulates the expression of class III promoters by inhibiting the ability of σ^{28} to participate in transcription (17, 18, 36, 49). FlgM-mediated negative regulation is alleviated only when the assembly of a basal body-hook complex is completed (27, 35). The formation of this structure permits the export of FlgM to the outside of the cell (27, 35).

In *C. crescentus*, apparently two distinct mechanisms couple flagellar assembly to transcription. The *bfa* system regulates transcription of class III and IV promoters in response to an absence of class II gene products. Experiments presented here also indicate that flagellin expression is regulated by a posttranscriptional mechanism. Flagellin protein is not synthesized in class II/*bfa* double mutant strains even though the promoter is transcribed. A protein fusion of the amino terminus (29 amino acids) of FlgK (25-kDa flagellin) to a *lacZ* reporter was also not expressed in this mutant strain, suggesting that the regulation is exerted either at the 5′ end of the *fljK* mRNA or the amino terminus of the newly translated protein. Experiments that test mRNA and protein stability are required to determine which of these mechanisms is occurring. Posttranscriptional regulation may function to couple class III gene expression to class IV gene expression. Recent experiments have shown that the *fljK*::*lacZ* protein fusion is also not expressed in class III mutant cells (40). This is the analogous level of the hierarchy that FlgM regulates in *S. typhimurium*. It will be interesting to determine whether *Caulobacter* posttranscriptional negative regulation is relieved by flagellum-specific export.

***bfa* regulates two classes of σ^{54} flagellar promoters.** The class III and IV flagellin promoters that are under *bfa* control possess similar *cis*-regulatory sequences. Both classes are transcribed by σ^{54}-containing RNA polymerase (11, 23, 33, 42, 43) and require the DNA-bending protein integration host factor for maximal levels of transcription (22, 23). Differential regulation of transcription is accomplished through the utilization of different enhancer sequences. The *flgF* basal body promoter contains a conserved upstream enhancer element known as RE-1 (previously known as RF3) (41). This enhancer sequence is also located upstream of the *flgI* gene, which encodes the P ring of the basal body (33). The hook operon, *fljL*, and *fljK* possess a different conserved enhancer element called *ftr* (24, 42, 44, 45). Late in the cell cycle, the transcription of *ftr*-containing promoters is restricted to the swarmer cell compartment of the predivisional cell (22, 23, 66). A flagellar gene-specific σ^{54} transcriptional activator encoded by the *flbD* gene is required for the transcription of both basal body and *ftr*-containing σ^{54} promoter types (3, 4, 46, 47, 52, 66). FlbD apparently does not directly activate the transcription of basal body promoters. In vitro binding experiments have demonstrated that FlbD does not directly bind to the RE-1 basal body enhancer (65). Pure FlbD does bind specifically to the *ftr* enhancer of the hook operon, *fljL*, and *fljK* promoters (3, 4, 47, 65, 66). DNA binding activity and transcriptional activation are abolished by the same mutation in the *ftr* enhancer, suggesting that FlbD directly activates transcription of these promoters (66). FlbD activity is regulated by spatial and temporal phosphorylation (66). Experiments using constitutive mutants of FlbD have indicated that the phosphorylated form of FlbD is restricted to the swarmer cell compartment of the late predivisional cell (66).

The fact that *bfa* functions to regulate both of these σ^{54} promoter types indicates that the target for regulation could conceivably be either the σ^{54} subunit of RNA polymerase, FlbD, or possibly the putative kinase that activates FlbD. A single exception to *bfa* regulation among the late flagellar genes is *fljK* transcription, which is insensitive to regulation by flagellar assembly (66). Transcription of *fljK* does however, require σ^{54} and *flbD*. Therefore, it is reasonable to conclude that *bfa* does not regulate transcription by directly inhibiting the activity of either σ^{54} or FlbD. A possible alternative model is that *bfa* regulates by directly binding to the promoter and therefore repressing transcription. This mode of regulation of σ^{54} promoters if it exists, would be unique. We are currently

testing whether conserved sequences within these promoters are required for negative regulation by flagellar assembly.

Role of *bfa* regulation within the cell cycle. The experiments reported here demonstrate that a strain containing a *bfa* mutation possesses an altered temporal pattern of *fljL* transcription. Transcription of *fljL* in the *bfa* mutant strain continued late in the cell cycle, with some expression occurring even after cell division in the progeny swarmer cell. The transcription pattern of *fljL* in a *bfa* mutant is similar to that of the *fljK* promoter in wild-type cells, which is normally not regulated by *bfa. fljK* expression in wild-type swarmer cells is a due to the translation of mRNA that was transcribed in the swarmer pole before cell division (20). It is possible that *fljL* expression in *bfa* swarmer cells is the result of the same mechanism. This suggests that the function of *bfa* in normal cells may be to regulate most middle and late flagellar promoters late in the cell cycle. This regulation ensures that progeny swarmer cells only express 25-kDa flagellin (FljK), the final assembled component of the flagellum.

Epistasis experiments have demonstrated that *bfa*-mediated regulation apparently operates in mutant cells that are lacking one or more class II flagellar components. The cell cycle experiments presented here indicate that *bfa* also exerts regulation late in the cell division cycle. Are there similarities between these two conditions when *bfa* is active? What are the possible internal cues that trigger *bfa* regulation? One plausible model may be that *bfa* regulation is initiated when there is a high intracellular concentration of unassembled class II gene products. This would presumably take place in class II mutants; the absence of one component of the flagellum may prevent the assembly of other components. The same type of situation may arise if class II gene expression continues after the early flagellar components are assembled into the nascent structure. This model would predict that the cell possesses mechanisms to efficiently down-regulate class II gene expression. One known example is the class II *fliF* promoter, which is repressed by FlbD, the activator of class III and IV promoters (3, 46, 64). Therefore, the repression of early gene expression is tightly coupled to the activation of late flagellar gene transcription. On the other hand, the *bfa* regulatory system apparently decreases the transcription of late genes in the absence of early class II gene expression. We hypothesize that the cell balances the activation of FlbD with *bfa* activity to ensure that the expression and assembly of flagellar components are coordinated with cell division.

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