Regulation of late flagellar gene transcription and cell division by flagellum assembly in *Caulobacter crescentus*

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**Summary**

Biogenesis of the single polar flagellum of *Caulobacter crescentus* is regulated by a complex interplay of cell cycle events and the progression of flagellum assembly. The expression of class III/IV flagellar genes requires the assembly of an early flagellar basal body structure, encoded by class II genes, and is activated by the transcription factor FlbD. Previous experiments indicated that the class II flagellar gene, *flbE*, encoded a trans-acting factor that was required for FlbD activity. Here, using mutant alleles of *flbE* we have determined that FlbE is either a structural component of the flagellum or is required for flagellar assembly and does not, as originally proposed, function as a trans-acting factor. We also demonstrate that two deleted derivatives of *flbE* have a dominant negative effect on the transcriptional activation of class III/IV flagellar genes that can be relieved by a gain-of-function mutation in *flbD* called *bfa*. This same mutation in *flbD* has been shown to restore class III/IV transcription in the absence of early class II flagellar assembly. These deleted mutants of *flbE* also exhibited a filamentous cell phenotype that was indistinguishable from that previously observed in class II flagellar mutants. Introduction of a *flbD–bfa* mutation into these cells expressing the deleted alleles of *flbE*, as well as several class II mutant strains, restored normal cell division and FtsZ localization. These results suggest that class III/IV transcription and a step in cell division are coupled to flagellar assembly by the same genetic pathway.

**Introduction**

*Caulobacter crescentus* undergoes an asymmetric cell division producing two distinct progeny cells, a motile swarmer and a sessile stalked cell. These two cell types differ in their global programs of gene expression and DNA replication (reviewed in Brun et al., 1994; Gober and Marques, 1995; Wu and Newton, 1997; Gober and England, 2000). The capacity to reinitiate DNA replication is repressed for a defined period of time in the swarmer cell, after which the flagellum is lost, a stalk grows at the former location of the flagellum and DNA replication initiates. In contrast, after division, the stalked cell immediately reinitiates a single round of DNA replication. As the cell cycle progresses, flagellar components are synthesized and a single polar flagellum is assembled at the pole opposite from the stalk.

Flagellar biogenesis is regulated by a relatively complex trans-acting hierarchy that is influenced by cell cycle events as well as the progression of flagellar assembly. The earliest flagellar structures synthesized are encoded by class II genes and include the MS-ring, the flagellar switch and the flagellum-specific secretory apparatus (reviewed in Brun et al., 1994; Gober and Marques, 1995; Wu and Newton, 1997). Synthesis and successful assembly is required, in turn, for the transcription of class III genes that encode the rod, the outer rings of the flagellar basal body, the external hook structure and a class IV flagellar gene (*fljL*) that encodes the 27 kd flagellin (Bryan et al., 1984; Ohta et al., 1985, 1991; Champer et al., 1987; Minnich and Newton, 1987; Newton et al., 1989; Xu et al., 1989; Ramakrishnan et al., 1994; Mangan et al., 1995).

Class III and IV flagellar promoters share conserved sequence elements (Minnich and Newton, 1987; Mullin et al., 1987; Mullin and Newton, 1989; 1993; Dingwall et al., 1990; 1992a; Gober et al., 1991a, 1991b; Khambaty and Ely, 1992; Gober and Shapiro, 1992; Wingrove et al., 1993; Benson et al., 1994a; Marques and Gober, 1995) including binding sites for σ54-containing RNA polymerase holoenzyme and integration host factor (IHF) (Gober and Shapiro, 1990; 1992; Gober and Marques, 1995). Transcriptional activation is accomplished through the binding of the response regulator, transcriptional activator, FlbD (Ramakrishnan and Newton, 1990; Wingrove et al., 1993; Wingrove and Gober, 1994; Benson et al., 1994a; 1994b; Mullin et al., 1994) to conserved enhancer sequences (ftr) that are located approximately 100 base pairs (bp) from the transcription start site (Minnich and
negative regulatory mechanism related to the activation of FlbD.

Results

Role of FlbE as a sensor histidine kinase

In vitro experiments using purified protein demonstrated that FlbE possessed autophosphorylation activity (Wingrove and Gober, 1996). Phosphorylated FlbE was also shown to be capable of transferring phosphate to purified FlbD at a slow rate. These observations, combined with epistasis experiments that showed that FlbD-dependent promoters were not expressed in flbE mutant strains, prompted the hypothesis that FlbE was a probable candidate to function as the cognate histidine kinase for FlbD (Wingrove and Gober, 1996). We tested this idea by examining the effect of mutations in the region of FlbE that would be predicted to be critical for functioning as a histidine kinase on the ability to complement the motility defect in a ΔflbE strain. Site-directed mutagenesis was performed on the putative ‘H’ region of FlbE. The altered amino acid residues are listed in Fig. 1. Based on comparisons with other sensor histidine kinases, histidine residue 83 would be predicted to be the site of autophosphorylation (see Parkinson and Kofoid, 1992). We have previously demonstrated that a mutant FlbE with an alteration of amino H83 does not possess autophosphorylation activity in vitro (Wingrove et al., 2001).

Fig. 1. Summary of FlbE mutants used in this study. Shown is a schematic diagram of FlbE. The proposed sensor domain as defined previously represents the amino terminal 52 residues. This is followed by a flexible linker and then a domain which shares some similarity to sensor histidine kinases. The conserved motifs in this transmitter domain are denoted ‘H’, ‘N’, ‘G1’ and ‘F’ according to nomenclature of Parkinson and Kofoid (1992). Deletions in the localization domain are indicated below. See Experimental procedures for details. Note that the schematic for deletion Δ10 is not shown. Site-directed mutagenesis was performed on the transmitter domain in order to create deletions or point mutations in the critical ‘H’ region, which is proposed to be important for kinase activity.

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and Gober, 1996). Surprisingly, mutant flbE alleles ΔHEH, ΔH83, ΔH85 and E84Q, which should all be defective in autophosphorylation activity, were able to restore motility in the ΔflbE strain (data not shown). In addition, these mutations had little effect on the expression levels of either fliF– or fljL–lacZ fusions (data not shown). These experiments suggest that the kinase activity of FlbE, observed in vitro, is dispensable for class III/IV gene expression and motility.

**Effect of mutations in the amino terminus of FlbE on motility**

Previous experiments have demonstrated that the 52 amino terminal residues of FlbE are required for motility (Wingrove and Gober, 1996). To define the minimal sequences that are required for motility, a series of deletions were created (Fig. 1). These deleted derivatives of flbE were then introduced on a multicopy plasmid into either wild-type cells (LS107) or a strain containing a deletion in the chromosomal copy of flbE (JG1141), and assayed for motility both by light microscopy and by inoculation into semisolid PYE agar media (Fig. 2). The results indicated that most of the amino terminal region of FlbE is critical for motility. Only the plasmid containing a deletion of amino acids 2–4 (flbEΔ4) could restore normal motility to the ΔflbE mutant strain JG1141 (Fig. 2A). All other deletions were unable to complement motility (Fig. 2A). The expression of all of these fusions was confirmed using immunoblot (Fig. 2C). This result is consistent with previous observations, which demonstrated that deletion of the entire 52 amino acid amino terminal domain failed to complement motility in a ΔflbE strain. We tested whether any of these deleted derivatives of FlbE could interfere with motility in wild-type C. crescentus cells (Fig. 2B). All of the deletions resulted in swarms of slightly smaller diameter than wild-type cells, but did not significantly interfere with motility. One possibility is that the smaller swarms observed in cells expressing the amino-terminal deletions of flbE resulted from increased expression of these mutant proteins. In order to test this, we performed immunoblot analysis with anti-FlbE antibody on these strains. All of the mutant-deleted derivatives of flbE were expressed at significantly higher levels than wild-type FlbE.

![Fig. 2.](image_url)

**Fig. 2.** The flbE deletion mutants were introduced into either a ΔflbE strain (JG1141) (A) or wild-type cells (LS107) (B) on a multicopy plasmid and assayed for motility by inoculation into semisolid PYE medium.

A. Expression of deleted flbE alleles in ΔflbE cells. ΔflbE are cells that do not contain plasmid and exhibit a non-motile phenotype. Cells expressing the flbEΔ4 allele were motile as they form a swarm in semisolid media. The alleles flbEΔ7, flbEΔ10, flbEΔ13 and flbEΔ26 are unable to complement motility in this strain.

B. Expression of deleted flbE mutant alleles in wild-type cells. All of the strains in this case are motile, although strains expressing the deleted alleles of flbE form swarms of slightly smaller diameter.

C. Expression of deleted alleles of flbE. Immunoblot analysis using anti-FlbE antibody was performed. Lane 1, extract prepared from wild-type cells. FlbE is indicated with an arrow. The band of abundant protein below FlbE is caused by a contaminating antibody in the FlbE antiserum. Lane 2, extract prepared from JG1140, a strain containing a deletion in flbE. Lanes 3–8, extracts prepared from strain JG1140 (ΔflbE) containing derivatives of flbE on a multicopy plasmid (see Table 2). Lane 3, wild-type flbE; lane 4, flbEΔ4; lane 5, flbEΔ7; lane 6, flbEΔ10; lane 7, flbEΔ13; lane 8, flbEΔ26. Lanes 9–12, overexposed images of lanes 4, 5, 7 and 8 respectively. The mutant alleles are indicated. Note that FlbEΔ26 protein is present as a band just above the dark band reacting with the contaminating antibody.

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lower levels than the strain expressing wild-type flbE from the multicopy plasmid (Fig. 2C), indicating that their effect on motility was not a consequence of overexpressed protein.

Effect of FlbE N-terminal deletions on the expression of FlbD-regulated promoters

Previous experiments have demonstrated that the amino terminal domain of FlbE has an important function in regulating the activity of FlbD (Wingrove and Gober, 1994). Therefore, we examined the effect of FlbE amino-terminal deletions on FlbD-mediated regulation of transcription. To accomplish this, two different terminal deletions on FlbD-mediated regulation of transcription. Of note, and consistent with this idea, the deletion mutant, flbEΔ14, which was capable of complementing motility in a ΔflbE strain, generated the lowest levels of β-galactosidase among the mutants tested (Table 1). When expressed in wild-type cells, full-length flbE and the deletions flbEΔ4, flbEΔ7 and flbEΔ10 had no significant effect on flIF–lacZ expression (Table 1). This is in contrast to strains bearing deletions flbEΔ13 and flbEΔ26, which exhibited a modest increase in flIF–lacZ expression relative to the other strains, indicating that these mutant alleles may be interfering with the FlbD-mediated repression of flIF (see below).

The flbE deletion alleles had an analogous effect on the class III/IV fljL–lacZ fusion. In wild-type cells, the fusion generated 7370 units of β-galactosidase activity and was not expressed in the ΔflbE strain (443 units) (Table 1). The single deletion mutant allele of flbE that could complement motility in a ΔflbE strain, flbEΔ4, was the only mutant that resulted in fljL–lacZ expression in this strain. When this mutant gene was expressed in the ΔflbE strain, the fljL–lacZ fusion generated 6709 units of β-galactosidase activity (Table 1). When expressed in wild-type cells, full-length flbE and the deletions flbEΔ4, flbEΔ7 and flbEΔ10 had no significant effect on fljL–lacZ expression (Table 1). However, mutant flbE alleles, flbEΔ13 and flbEΔ26 exhibited a significant decrease in β-galactosidase levels, generating only one-third to one-half that of the other strains. Therefore, these deleted derivatives of flbE have a dominant negative effect on two promoter activities that are regulated by FlbD.

In order to establish whether the alteration in flIF and fljL expression in strains containing the deleted derivatives, flbEΔ13 and flbEΔ26, was attributable to a reduction in FlbD activity, we assayed flIF–lacZ and fljL–lacZ expression in a bfa mutant strain (Table 1). The bfa alleles of flbD contain gain-of-function mutations that restore

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<th>Table 1. Effect of flbE and amino-terminal deletion alleles of flbE on FlbD-regulated gene expression.</th>
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a. The reported values are the mean of assays performed in triplicate on three independently grown cultures. The mean standard deviation for all reported values was less than 5%. ND; not determined.
class III/IV gene expression in the absence of class II-encoded flagellar components (Mangan et al., 1995; Muir et al., 2001; R. Muir and J.W. Gober, unpublished). The introduction of the flbD–bfa1204 allele into strains containing both deleted derivatives of flbE led to a decrease in fliF–lacZ expression to levels resembling wild-type cells (Table 1). Conversely, the flbD–bfa1204 allele resulted in an increase in the expression of the class III/IV reporter, fliJ–lacZ. This pattern of flagellar gene expression was also observed when the flbD–bfa1204 mutation was introduced into a ΔflbE strain. These results indicate that a null mutation in flbE or expression of these two dominant-negative alleles of flbE perturb flagellar gene expression by affecting FlbD activity.

Effect of a flbE deletion on flagellin expression

Interestingly, the flbD–bfa1204 allele could not restore motility in the ΔflbE strain as assayed using light microscopy and motility agar (data not shown). This is in contrast to strains containing a null mutation in the flagellar trans-acting factor, fliX. In ΔflX cells, motility is restored by the introduction of a flbD–bfa allele, indicating that fliX does not encode a flagellar structural gene (Muir et al., 2001). In the data presented here, the lack of motility in ΔflbE/flbD–bfa double mutants indicates that flbE probably does not encode a trans-acting regulatory factor as originally proposed, but rather a novel structural component of the flagellum or flagellum secretion system. In order to provide an additional test of this idea, we assayed for the presence of flagellin protein in a ΔflbE strain containing a flbD–bfa1204 allele. In bfa/class II double mutant strains, which do not assemble early flagellar structures, the genes encoding the 27 kd and 25 kd flagellins are transcribed, but the mRNA is degraded (Mangan et al., 1995; 1999; Anderson and Newton, 1997; Anderson and Gober, 2000). Therefore, the synthesis of these flagellin proteins serves as an indicator of flagellum assembly. In wild type and flbD–bfa1204 cells, both the 27 kd and 25 kd flagellin species were present (Fig. 3, lanes 1 and 2). In contrast, in a strain containing a deletion in the gene encoding the trans-acting factor, FliX, minor amounts of the 25 kd flagellin were produced (Fig. 3, lane 5). The introduction of the flbD–bfa1204 allele into this strain restores flagellin assembly and consequently wild-type levels of both 27 kd and 25 kd flagellins (Fig. 3, lane 6). In ΔflbE mutant cells only the minor 29 kd flagellin was present (Fig. 3, lane 3). In the ΔflbE strain containing a flbD–bfa1204 allele, the expression of the 27 kd and 25 kd flagellin species was only partially restored to levels far less than wild-type cells (Fig. 3, lane 4). Because the major flagellin species were poorly expressed, it is probable that the primary defect in ΔflbE and ΔflbE/flbD–bfa1204 cells is a lack of flagellar assembly.

Fig. 3. Effect of flbE on flagellin synthesis. Depicted is an immunoblot using antiflagellin antibodies. The genotype of each strain is indicated. Caulobacter crescentus synthesizes flagellins of three distinct molecular weights which are indicated. Lane 1, strain LS107 (wild type for motility); lane 2, strain JG1181, containing a gain of function mutation in flbD (bfa1204); lane 3, strain JG1140, containing a deletion in flbE; lane 4, strain JG1193, containing a deletion in flbE and the flbD–bfa1204 mutation; lane 5, strain JG1172, containing a deletion in fliX; lane 6, strain JG1177, containing a deletion in fliX and the flbD–bfa1204 mutation.

Effect of FlbE amino-terminal deletions on cell division

An interesting aspect of flagellar biogenesis in C. crescentus is the effect of the assembly of class II-encoded flagellar components on cell division. Class II flagellar mutants divide in a relatively normal fashion in early log phase. However, when they enter mid- to late-log phase, long filamentous cells accumulate in culture, indicating that cell division is inhibited to some extent (Yu and Shapiro, 1992; Gober et al., 1995; Zhuang and Shapiro, 1995). It has been suggested that this represents a checkpoint that delays cell division until a functional flagellum is assembled (Gober et al., 1995). Because two different amino-terminal deletions of FlbE affected motility and gene expression, we examined their effect on cell division in wild-type cells (Fig. 4). The allele that could restore motility in JG1141, flbEΔ4, had little discernible effect on the cell morphology of wild-type cells (data not shown). However, the expression of flbEΔ13 and flbEΔ26 resulted in the production of filamentous cells in a wild-type strain (Fig. 4C,D). This result indicates that these FlbE amino-terminal deletion mutants are also functioning as dominant negative alleles with respect to cell division. We next wanted to test whether cell division was regulated by the same genetic pathway as class III/IV gene expression. As shown above, class III/IV gene expression can be restored to greater than wild-type levels in strains expressing flbEΔ13 and flbEΔ26 by introducing a flbD–bfa1204 mutation. Interestingly, flbD–bfa1204 cells that were expressing either flbEΔ13 or flbEΔ26 exhibited no cell division defect (Fig. 4E,F). This result suggests that the cell division defect in strains expressing flbEΔ13 and flbEΔ26 is attributable to a decrease in FlbD activity. In support of this idea, cultures of cells containing a Tn5 insertion in flbD also exhibited a cell division defect (Fig. 4B). This observation raises the possibility that the cell division defect seen in class II flagellar mutants is also a consequence of a lack of FlbD activity. We therefore tested whether the flbD–bfa mutation could
also ameliorate the cell division defect in class II flagellar mutants. We tested this in strains containing mutations in flbE (Fig. 4G), fliQR (Fig. 4H) and fliX (Fig. 4I). In all cases, the majority of cells in culture were long and filamentous, containing cell division constrictions ranging from one to several, along the length of the filament. The introduction of flbD–bfa1204 into any one of these strains completely restored normal cell morphology (Fig. 4 J–L). We have determined that the bfa alleles of flbD can restore normal cell division in several other class II flagellar mutants including fliF, flhA, fliP and fliM (data not shown). These results indicate that FlbD may play a role in regulating cell division as well as flagellar biogenesis.

**Effect of mutant flbE alleles and flagellar assembly on FtsZ localization**

The results presented here suggest that a relationship exists between cell division and flagellar gene expression. We sought to characterize the nature of the cell division defect in class II flagellar mutants. To accomplish this we assayed the subcellular localization of the cell division protein, FtsZ. FtsZ has been shown to localize as a ring that girdles the circumference of the cell at the midcell location in several bacteria (Bi and Lutkenhaus, 1991; Ma et al., 1996) including C. crescentus (Kelly et al., 1998). The localization of FtsZ to the midcell is the earliest known event in the initiation of cell division in bacteria (reviewed in Lutkenhaus and Addinall, 1997). In order to assay the location and assembly of FtsZ rings, immunofluorescence microscopy was performed on both wild type and class II flagellar mutants (Fig. 5). As shown previously (Kelly et al., 1998), a fraction of wild-type cells exhibited bands of fluorescence, indicative of FtsZ ring formation at the midcell (Fig. 5A). The bands of fluorescence were present in older stalked and predivisional cells because FtsZ ring formation is under cell cycle control. As shown above, cultures of wild-type cells expressing the dominant negative flbE D26 allele contained both filamentous cells and a population of cells with normal morphology. In the filamentous cells of this strain, FtsZ rings were frequently absent (Fig. 5B). In those aberrant cells possessing FtsZ rings (29% of the total filamentous cells), up to 27% of the rings were mislocalized to the cell pole. These results are consistent with the idea that either the completion of cell division is uncoupled or delayed relative to the elongation of the cell. Similar results were obtained when FtsZ localization was assayed in the ΔflbE, flf::Tn5 and ΔfliX strains (Fig. 5C–E). As with the strain expressing flbEΔ26, these cultures contained numerous filamentous cells with...
Fig. 5. Effect of flagellar assembly on FtsZ ring formation and localization. FtsZ ring formation was assayed using immunofluorescence microscopy.

A. Localization of FtsZ in wild-type cells (LS107). Bright bands or foci indicate FtsZ localization.

B. Localization of FtsZ in LS107 cells expressing flbED26. 26% of the filamentous cells contained FtsZ foci. 27% of the filamentous cells with FtsZ staining had foci at the poles.

C. Localization of FtsZ in JG1140 (ΔflbE). 22% of the filamentous cells contained FtsZ foci. 11% of the filamentous cells with FtsZ staining had foci at the poles.

D. Localization of FtsZ in the class II flagellar mutant, UC1042 (flif::Tn5). 24% of the filamentous cells contained FtsZ foci. 33% of the filamentous cells with FtsZ staining had foci at the poles.

E. Localization of FtsZ in strain JG1172 (ΔflifX). 10% of the filamentous cells contained FtsZ foci. 10% of the filamentous cells with FtsZ staining had foci at the poles.

F. Localization of FtsZ in strain JG1177 (ΔflifX flbD–bfa1204).

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either absent or mislocalized FtsZ rings (see Fig. 5). We then assayed the subcellular localization of FtsZ in the ΔflfX strain containing a flbD–bfa1204 allele. The presence of the flbD–bfa1204 mutation in this strain resulted in a restoration of a normal FtsZ ring localization (Fig. 5F). These results suggest that class III/IV transcription and the completion of cell division both require the activation of FlbD.

**Mechanism of FlbD-mediated regulation of cell division**

FlbD has been demonstrated to regulate flagellar gene transcription by two distinct mechanisms. The transcription of class III/IV, ρ54-dependent flagellar promoters is activated by FlbD and the transcription of the fliF operon is repressed by FlbD. Both modes of regulation require that FlbD be activated, presumably via phosphorylation. The results presented here show that the perturbation in cell division seen in class II flagellar mutants is a consequence of their negative effect on FlbD activity. We envision three possible mechanisms whereby a decrease in activated FlbD in class II flagellar mutants could result in a cell division defect: (i) an increase in transcription of the fliF operon, which would result in the increased expression of five early flagellar genes (fliF, flfG, flbE, fln, flbD), may be inhibiting cell division (ii) FlbD may be a repressor of transcription of an unknown cell division inhibitor or (iii) FlbD may activate the transcription of a critical cell division gene.

In order to test the first possibility we used a complete fliF operon on a plasmid containing either a wild-type allele of flbD, a flbD–bfa1204 allele, or a mutant operon (OM-6) containing the flbD–bfa1204 allele with two point mutations in the promoter rendering it resistant to FlbD repression (Wingrove and Gober, 1994). When all three operons were introduced into wild-type cells there was no effect on cell morphology (Fig. 6A–C). This result indicates that overexpression of the fliF operon (Fig. 6C) does not perturb cell division in otherwise wild-type cells.

As a control to test whether, in each case, the operon was Fig. 6. Effect of fliF operon expression, flbD–bfa and rpoN on cell division. Cultures were grown to an O.D600nm = 1.0 and the cells were mounted on poly-L-lysine coated slides and examined by phase-contrast microscopy. A. Strain LS107 (wild type) containing a plasmid with the entire wild-type fliF operon. B. Strain LS107 (wild type) containing a plasmid with a fliF operon bearing a flbD–bfa1204 allele. C. Strain LS107 (wild type) containing a plasmid with a fliF operon bearing a flbD–bfa1204 allele and a mutant promoter (OM-6) (Wingrove and Gober, 1994) that cannot be repressed by FlbD. D. Strain JG1140 (ΔflbE) containing a plasmid with the entire wild-type fliF operon. E. Strain JG1140 (ΔflbE) containing a plasmid with a fliF operon bearing a flbD–bfa1204 allele. F. Strain JG1140 (ΔflbE) containing a plasmid with a fliF operon bearing a flbD–bfa1204 allele and the mutant promoter (OM-6) (Wingrove and Gober, 1994) that cannot be repressed by FlbD. G. Strain JG1172 (ΔflfX) containing a plasmid with the entire wild-type fliF operon. H. Strain JG1172 (ΔflfX) containing a plasmid with a fliF operon bearing a flbD–bfa1204 allele. I. Strain JG1172 (ΔflfX) containing a plasmid with a fliF operon bearing a flbD–bfa1204 allele and the mutant OM-6 promoter. J. Strain SC1055 (rpoN::Tn5) containing a plasmid with the entire wild-type fliF operon. K. Strain SC1055 (rpoN::Tn5) containing a plasmid with a fliF operon bearing a flbD–bfa1204 allele. L. Strain SC1055 (rpoN::Tn5) containing a plasmid with a fliF operon bearing a flbD–bfa1204 allele and the mutant OM-6 promoter.
expressed all three versions were introduced into a strain containing a deletion in \( \text{flbE} \). In each instance, introduction of the \( \text{fliF} \) operons restored normal cell morphology (Fig. 6D–F) and motility (data not shown) to the \( \Delta \text{flbE} \) strain. We next tested the effect of these operons on cell morphology in a class II \( \Delta \text{fliX} \) mutant strain. Introduction of the operon containing the wild-type allele of \( \text{flbD} \) into the \( \Delta \text{fliX} \) strain could not restore normal cell morphology (Fig. 6G). In contrast, introduction of the operon containing a \( \text{flbD–bfa1204} \) allele resulted in cells that were morphologically indistinguishable from wild type (Fig. 6H). Introduction of the \( \text{fliF} \) operon containing the mutant OM-6 promoter and a \( \text{flbD–bfa1204} \) allele into \( \Delta \text{fliX} \) cells also resulted in the restoration of normal division (Fig. 6I). Because this operon cannot be repressed by \( \text{FlbD} \) and thus is overexpressed, this result indicates that the aberrant cell division phenotype observed in class II flagellar mutants is not the result of increased expression of the \( \text{fliF} \) operon.

In order to test whether \( \text{FlbD} \) either repressed an inhibitor of cell division or activated the expression of a critical cell division gene, these \( \text{fliF} \) operons were introduced into a strain that contained a Tn5 insertion in the gene encoding the \( \sigma^{54} \) subunit of RNA polymerase (\( \text{rpoN} \)). These cells possess a filamentous morphology characteristic of class II flagellar mutants (Brun and Shapiro, 1992). In all cases, the \( \text{fliF} \) operons, whether they contained a wild type or a \( \text{bfa} \) allele of \( \text{flbD} \) were unable to restore normal cell division to the \( \text{rpoN} \) mutant (Fig. 6J–L). This suggests that \( \text{flbD} \) functions to influence cell division via activating transcription and not through repression.

**Discussion**

In this report we have investigated the role of \( \text{FlbE} \) in regulating the transcription of class III/IV flagellar genes and its relationship to flagellar assembly and cell division. It has been demonstrated that \( \text{flbE} \) is required for the \( \text{FlbD} \)-dependent transcription of class III/IV flagellar genes (Wingrove and Gober, 1996; Anderson and Newton, 1997). Furthermore, *in vitro* experiments suggested that \( \text{FlbE} \) might function as the cognate kinase for \( \text{FlbD} \) (Wingrove and Gober, 1996). Here, we have determined that \( \text{flbE} \) probably encodes either a novel component of the flagellum, or is required for flagellum assembly, and does not function as a trans-acting regulatory factor as originally proposed (Wingrove and Gober, 1996). This conclusion is based on the following evidence: (i) We tested the role of \( \text{FlbE} \) kinase activity in regulating flagellar biogenesis, by mutating the residues in \( \text{FlbE} \) that would be predicted to have a critical role in kinase activity. Surprisingly, all of the mutations tested, including deletions within the critical ‘H’ region, were able to complement motility and restore class III/IV flagellar gene transcription to wild-type levels in a \( \Delta \text{flbE} \) strain. As we have demonstrated that residue H83 is critical for *in vitro* autophosphorylation activity (Wingrove and Gober, 1996), it is unclear what role, if any, this activity serves in flagellar biogenesis. (ii) Gain-of-function mutations in the class III/IV flagellar transcription factor, \( \text{FlbD} \) cannot restore motility in a strain bearing a deletion in \( \text{flbE} \). (iii) In the double mutant \( \Delta \text{flbE}/\text{flbD–bfa} \) strain, flagellin transcription was restored, but flagellin protein expression was greatly reduced. Because flagellin translation is regulated by flagellar assembly, this result indicates that the primary defect in \( \text{flbE} \) mutant strains is a lack of a fully assembled flagellum.

**Coupling of flagellar assembly, cell division and class III/IV gene expression**

Progression through the cell cycle regulates the expression of flagellar genes in *C. crescentus*. For example, DNA replication is required for the transcription of early class II flagellar genes. Inhibition of replication shortly after initiation results in cells which are unable to transcribe class II promoters (Dingwall *et al.*, 1992b; Stephens and Shapiro, 1993). Class II flagellar promoters share a common conserved sequence (Mullin *et al.*, 1987; Dingwall *et al.*, 1992b, Yu and Shapiro, 1992; Stephens and Shapiro, 1993; Van Way *et al.*, 1993; Gober *et al.*, 1995; Mohr *et al.*, 1998) and are activated by the response regulator transcription factor, CtrA (Quon *et al.*, 1996; Reisenauer *et al.*, 1999). Interestingly, CtrA regulates not only the expression of early flagellar promoters, but has a global role in controlling cell cycle events. These include the initiation of DNA replication, through binding both to the origin of replication (Quon *et al.*, 1998) and the *hemE* promoter (Quon *et al.*, 1996) and regulating the expression of a critical DNA methyltransferase (ccrM) (Quon *et al.*, 1996), *ftsZ* (Kelly *et al.*, 1998) and *ftsQA* (Wortinger *et al.*, 2000). Thus, the expression of class II flagellar promoters is regulated coordinately with other cell cycle events such as the initiation of DNA replication and cell division.

The link between flagellar biogenesis and cell cycle events raises the question of whether there is any feedback regulation of flagellum synthesis on the progression of the cell cycle. The most readily apparent effect is the accumulation of filamentous cells in mid-logarithmic phase in strains that fail to assemble class II encoded structures. This filamentous cell phenotype was also observed in wild-type strains expressing the dominant negative \( \text{flbE} \) derivatives, \( \text{flbE}_{\Delta 13} \) and \( \text{flbE}_{\Delta 26} \), providing additional evidence that \( \text{flbE} \) encodes either a structural flagellar component or is required for flagellar assembly.

We have investigated the molecular nature of the cell division block in class II mutants by assaying for the localization of FtsZ rings. FtsZ ring formation is the earliest known cytoplasmic event in the initiation of cell division in
bacteria (reviewed in Lutkenhaus and Addinall, 1997). The assembly of an FtsZ ring then recruits other early cell division proteins to the midcell. The experiments presented here show that FtsZ rings are either absent or few in number in the filamentous cell population. We have determined that the level of FtsZ protein in class II flagellar mutants is similar to that found in wild-type cells (data not shown). From the experiments presented here, it is not possible to determine whether the effect on cell division is a result of a defect in the assembly or stability of FtsZ rings or one of the other cell division proteins. For example, strains with conditional mutations in flbD have been demonstrated to have unstable FtsZ rings (Pogliano et al., 1997).

The gain-of-function flbD–bfa mutation completely reversed the cell division defect in class II mutants and restored normal FtsZ ring formation. These results demonstrate that the timing of cell division and class III/IV gene expression share, at least with respect to flagellar assembly, a common regulatory pathway. We envision that the regulation of class III/IV gene expression and cell division represents a novel signal transduction pathway with the morphogenesis of the flagellum representing the stimulating cue. The output signal would be the activation of FlbD. Consistent with this idea, strains containing a Tn5 insertion in flbD also exhibited a filamentous cell division phenotype. The influence of FlbD on cell division is likely to be exerted at the level of transcriptional activation. In support of this idea, strains containing mutations in rpoN, the gene encoding the σ54 subunit of RNA polymerase also exhibit a cell division defect in mid-logarithmic phase that cannot be restored by a flbD–bfa mutation. This observation raises the possibility that the transcription of a critical cell division gene or regulator is activated by FlbD (Fig. 7).

It has been hypothesized that the cell division phenotype observed in class II mutants hints at a regulatory pathway that functions to delay the completion of division until the correct stage of flagellum assembly is achieved. Interestingly, the accumulation of filamentous cells is greatly reduced upon the addition of glucose to the medium (data not shown), indicating that regulation of carbon metabolism also influences this pathway. Therefore, this regulation is probably important in natural settings because Caulobacter often occupies nutrient-limited ecological niches (Poindexter, 1964). Why does the assembly of the class II flagellar structure function to regulate cell division and not later stages such as rod or hook assembly? For example, strains bearing mutations in the hook or basal body rod genes exhibit normal cell division. We hypothesize that this pathway operates at an early stage of assembly as a consequence of the timing of class II flagellar gene transcription. For example, CtrA-activated transcription of class II genes occurs before the initiation of cell division. After formation of the cell division plane, CtrA is found solely in the swarmer compartment of the predivisional cell, in which it is hypothesized to function as a repressor of DNA replication and class II gene expression (Domian et al., 1997). Any delay in the assembly of class II gene products without a parallel delay in cell division, would then result in a cessation of class II transcription in the swarmer compartment of the predivisional cell. This, in turn, may result in an insufficient level of class II gene products to complete flagellum assembly. We hypothesize that the coupling of flagellum assembly to cell division exists to ensure that this situation cannot occur. Therefore, the fine-tuned regulation of these multiple genetic networks serving to couple the cell cycle to flagellum biogenesis, and vice versa, ensure the formation of a motile swarmer cell at every cell division.

**Fig. 7.** Model depicting the regulatory relationships between flagellar assembly, class III gene expression and cell division. The master regulatory protein CtrA activates the transcription of early Class II flagellar genes (Quon et al., 1996; 1998). The assembly of these proteins into a nascent flagellar structure is required for the transcription of class III flagellar genes and, under certain growth conditions (e.g. reduced carbon availability), normal cell division. The trans-acting factor FlIX couples flagellar assembly to the activation of the σ54-dependent transcription factor, FlbD (Muir et al., 2001). A gain-of-function mutation in flbD (bfa) restores both class III gene expression and normal cell division in the absence of a class II flagellar structure. Strains containing a mutation in rpoN (σ54) also possess a cell division defect (Brun and Shapiro, 1992) that cannot be bypassed by the flbD–bfa1204 mutation. This suggests that FlbD plays a role in activating the transcription of a gene(s) in the cell division pathway.

**Experimental Procedures**

**Bacterial strains and plasmids**

Bacterial strains and plasmids used in this study are listed in Table 2. A *C. crescentus* synchronizable strain that was ampicillin sensitive (LS107) was used as host for all strain constructions. *Caulobacter crescentus* cells were grown at 31°C in either PYE medium (Poindexter, 1964) or minimal M2-glucose medium (Johnson and Ely, 1979). Motility was
Table 2. Bacterial strains and plasmids used.

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17–1</td>
<td>Rp4–2, Tc::Mu Km::Tn7</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Caulobacter crescentus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA1000</td>
<td>syn-1000</td>
<td>Evinger and Agabian (1977)</td>
</tr>
<tr>
<td>LS107</td>
<td>syn-1000 bla-6</td>
<td>Johnson and Ely (1979)</td>
</tr>
<tr>
<td>SC508</td>
<td>ΔflfIQR</td>
<td>Johnson and Ely (1979)</td>
</tr>
<tr>
<td>PC7070</td>
<td>recA526 zzz::Tn5 str-30</td>
<td>Ohta et al. (1990)</td>
</tr>
<tr>
<td>UC1040</td>
<td>bfa-1204 ΔflfIQR</td>
<td>Mangan et al. (1995)</td>
</tr>
<tr>
<td>JG1139</td>
<td>syn-1000 bla-6 recA526 zzz::Tn5</td>
<td>This work</td>
</tr>
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<td>JG1140</td>
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<td>This work</td>
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<tr>
<td>JG1141</td>
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</tr>
<tr>
<td>JG1177</td>
<td>syn-1000 bla-6 ΔflbX bfa-1204</td>
<td>Muir et al. (2001)</td>
</tr>
<tr>
<td>JG1181</td>
<td>syn-1000 bla-6 bfa-1204</td>
<td>Muir et al. (2001)</td>
</tr>
<tr>
<td>JG1193</td>
<td>syn-1000 bla-6 ΔflbE2 bfa-1204</td>
<td>This work</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>Bluescript KS + pMR4</td>
<td>Broad-host-range vector, tetR</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pfIIF-lacZ290</td>
<td>fliI–lacZ transcriptional reporter</td>
<td>Wingrove and Gober (1994)</td>
</tr>
<tr>
<td>pBBR1MCS</td>
<td>Broad-host-range vector (cmT)</td>
<td>Kovach et al. (1994)</td>
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<td>pREM70</td>
<td>flbEΔ4 in pBBR1MCS</td>
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<td>pREM71</td>
<td>flbEΔ17 in pBBR1MCS</td>
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<td>pREM72</td>
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<td>pREM73</td>
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<tr>
<td>pREM76</td>
<td>flbI operon in pRK290/20R</td>
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<td>pREM77</td>
<td>flfI operon with flbD–bfa1204</td>
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<tr>
<td>pREM78</td>
<td>flfI operon with flbD–bfa1204</td>
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</tr>
<tr>
<td></td>
<td>and OM-6 promoter in pMR4</td>
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</table>

The assay by inoculating cells into 0.2% peptone–0.1% yeast extract (PYE) media containing 0.3% agar. Motility plates were incubated at 31°C for 72 h. In order to construct pREM75, a 6.9 kb BamHI restriction fragment containing the entire fliIF operon was subcloned into pRK290/20R, pREM77, which contains the entire fliI operon with a flbD–bfa1204 allele, was constructed by subcloning the 6.9 kb BamHI fragment containing this operon from strain UC1040 into pMR4. To construct a fliI operon with a flbD–bfa1204 allele and the mutant OM-6 promoter, a 6.6 kb BamHI–HindIII restriction fragment from pREM77 was subcloned into a Bluescript KS plasmid that contained the 283 bp OM-6 fliI operon. This entire plasmid was then subcloned into pMR4 to create pREM78. The fliI– and fliJ–lacZ transcription fusions in plasmid placZ290 (Gober and Shapiro, 1992) were described previously. β-Galactosidase activity was assayed as described previously (Miller, 1972). The reported β-galactosi- dase values represent the mean value from assays performed in triplicate on three separately grown cultures. Flagellin protein was assayed using immunoblot (Towbin et al., 1979) with antiflagellin antibodies.

To construct a C. crescentus strain with a deletion in fliE (JG1141), a 796 bp SalI–EcoRI restriction fragment containing the entire fliE coding region (Wingrove and Gober, 1996) was subcloned into Bluescript KS. The resulting plasmid was digested with NcoI, filled in with Klenow fragment, digested with NotI and EcoRV and re-ligated. This results in a 490 bp internal deletion within the fliE coding region. The deleted fliE was then subcloned into the sac counterselection (Swisscher, 1992) vector pDELI3, electroporated LS107 and the transformants were selected for resistance to kanamycin (50 μg ml⁻¹). Kanamycin-resistant cells were grown in PYE liquid media overnight and then plated onto PYE containing 2% sucrose resistance to select against the integrated plasmid sequences. Sucrose-resistant colonies were tested for sensitivity to kanamycin and for motility. Confirmation of gene replacement was accomplished using Southern hybridization and PCR. This strain was then made recA deficient by transducing (Ely and Johnson, 1977) the Tn5 from PC7070 (Ohta et al., 1990).

**Site-directed mutagenesis of fliE**

The 796 bp SalI–EcoRI restriction fragment containing the entire fliE coding region was subcloned into M13-BM20 and site-directed mutagenesis was performed on single-stranded DNA template as described (Kunkel and Roberts, 1987). To construct mutant E84Q, codon 84 was changed from GAG to GAG to CAG. To create deletions in histidine residues 83 and 85 (H83 and H85 respectively), we used mutagenic oligonucleotides lacking these codons (see Fig. 1). Likewise, for deletion of codons 83–85 (HEH) (Fig. 1), a mutagenic oligonucleotide lacking these codons was used as a primer. All mutations were confirmed by DNA sequencing using the dyeoxy chain termination method (Sanger et al., 1977). The mutant alleles of fliE were subcloned as SalI–EcoRI fragments into the multicopy, broad-host-range plasmid.
pBBR1-MCS (Kovach et al., 1994), and subsequently introduced into Caulobacter crescentus JG1141 (ΔflbE) by conjugation with strain S17–1 (Simon et al., 1983).

To create flbE alleles with deletions in the amino terminus, polymerase chain reaction (PCR) was used. The 5’ primers contained either an EcoRI or PstI restriction site as well as an ATG start codon followed by DNA sequences that were complementary to the end-point of the deletion. The 3’ primer contained a BamHI restriction site as well as sequence complementary to that after the flbE translation stop codon. All PCR products were subcloned initially in pGEM T-easy (Promega) and sequenced. Each allele was then subcloned into pBBRI-MCS and introduced into either LS107 or JG1141 by conjugation. The expression and stability of all deleted derivatives of flbE was confirmed by immunoblot using antibodies directed against FlbE (data not shown).

Immunofluorescence microscopy

The localization of FtsZ was assayed using immunofluorescence microscopy as described previously (Maddock and Shapiro, 1993; Wingrove and Gober, 1996; Kelly et al., 1998). Rabbit anti-FtsZ antibodies were raised against purified His-tagged FtsZ (G.C. Draper, unpublished) and were used at a 1:200 dilution of serum. The secondary antibody used was conjugated to CY3 (Jackson Immunoresearch). To enhance the visualization of FtsZ localization, the images presented in Fig. 5 represent the fluorescent image overlayed onto the image from phase microscopy using Adobe Photoshop.

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References


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