

# The *trans*-acting flagellar regulatory proteins, FliX and FlbD, play a central role in linking flagellar biogenesis and cytokinesis in *Caulobacter crescentus*

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The FliX/FlbD-dependent temporal transcription of late flagellar genes in *Caulobacter crescentus* requires the assembly of an early, class II-encoded flagellar structure. Class II flagellar-mutant strains exhibit a delay in the completion of cell division, with the accumulation of filamentous cells in culture. It is shown here that this cell-division defect is attributable to an arrest in the final stages of cell separation. Normal cell morphology could be restored in class II mutants by gain-of-function alleles of FliX or FlbD, suggesting that the timely completion of cell division requires these *trans*-acting factors. In synchronized cultures, inhibition of cell division by depleting FtsZ resulted in normal initial expression of the late, FlbD-dependent *fliK* gene; however, the cell cycle-regulated cessation of transcription was delayed, indicating that cell division may be required to negatively regulate FlbD activity. Interestingly, prolonged depletion of FtsZ resulted in an eventual loss of FlbD activity that could be bypassed by a constitutive mutant of FlbD, but not of FliX, suggesting the possible existence of a second cell cycle-dependent pathway for FlbD activation.

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## INTRODUCTION

The dimorphic bacterium *Caulobacter crescentus* divides asymmetrically, forming a sessile stalked cell and a motile swarmer cell possessing a single polar flagellum. One consequence of this simple developmental process is the differing capacities of the progeny cells to reinitiate chromosomal DNA replication. In the newly divided stalked cell, replication initiates almost immediately following division, whereas, in the swarmer cell type, DNA replication is repressed for a defined period of time, after which the cell differentiates into a stalked cell and initiates DNA replication (Fig. 1). Then, through a series of temporally and spatially regulated molecular events, including cell cycle-regulated transcription and the polar positioning of signal-transduction proteins, a swarmer cell type arises at the pole opposite the stalk (reviewed by Ausmees & Jacobs-Wagner, 2003; Quardokus & Brun, 2003; Ryan & Shapiro, 2003).

A distinguishing characteristic of this simple program of cellular differentiation is the biogenesis of the flagellum that occurs once during every cell cycle. The timing of this process is coordinated with the cell-division cycle such that

all progeny swarmer cells possess a fully functional flagellum (reviewed by Gober & England, 2000; England & Gober, 2001). Flagellum synthesis requires nearly 50 genes, most of which are transcribed at a defined time in the cell cycle. Additionally, flagellar gene expression is influenced by a *trans*-acting regulatory hierarchy in which the expression and successful assembly of gene products that comprise early flagellar substructures are required for the expression of gene products incorporated later into the nascent flagellum (Newton *et al.*, 1989; Xu *et al.*, 1989; Ramakrishnan *et al.*, 1994; Mangan *et al.*, 1995). Epistasis experiments have demonstrated that this regulatory cascade consists of four hierarchical classes (Fig. 1). The cascade initiates with the temporal activation of the global transcription factor CtrA (class I), which, in turn, activates the transcription of the class II genes encoding the MS ring of the basal body, the flagellar switch and the flagellum-specific type III secretion system (TTSS) (Quon *et al.*, 1996; Domian *et al.*, 1997; Reisenauer *et al.*, 1999). The assembly of this class II-encoded structure is required for the transcription of the flagellar class III and IV genes, encoding the basal body-hook complex and the flagellin-containing filament, respectively (Newton *et al.*, 1989; Xu *et al.*, 1989; Ramakrishnan *et al.*, 1994; Mangan *et al.*, 1995). Thus, an assembly checkpoint functions to inhibit class III/IV gene expression until the class II-encoded structure is completed (Mangan *et al.*, 1995; Muir *et al.*, 2001; Muir & Gober, 2002, 2004).

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Abbreviation: TTSS, type III secretion system.



flagellar structure, FliX represses FlbD activity, and when a class II-encoded flagellar structure is completed, FliX activates FlbD. Genetic experiments have indeed shown that FliX functions as both a positive and negative regulator of FlbD activity (Muir *et al.*, 2001; Muir & Gober, 2002, 2004). Mutant strains containing a deletion in *fliX* are non-motile and fail to express class III and IV flagellar genes (Mohr *et al.*, 1998; Muir *et al.*, 2001). Gain-of-function mutations in *flbD* restore motility to *fliX* mutant strains, indicating that FliX functions in the same pathway as FlbD in the activation of late flagellar-gene transcription (Muir *et al.*, 2001; Muir & Gober, 2002). Consistent with this idea, a gain-of-function mutant *fliX* that permits class III/IV flagellar-gene expression in the absence of a class II-encoded structure has also been isolated (Muir *et al.*, 2001). FliX has recently been shown to interact with FlbD in a bacterial two-hybrid assay, indicating that they may form a complex in *Caulobacter* cells (Muir & Gober, 2004). Interestingly, FlbD and FliX are conserved in several species of alphaproteobacteria possessing polar flagella, thus constituting a regulatory pair in organisms that assemble flagella under cell-cycle control.

The FliX/FlbD regulatory pathway is also required for normal cell division (Muir & Gober, 2001). Strains containing mutations in class II flagellar-structural genes, *flbD* or *fliX* exhibit a cell-division defect characterized by the accumulation of filamentous cells in late exponential-phase cultures (Yu & Shapiro, 1992; Gober *et al.*, 1995; Zhuang & Shapiro, 1995; Muir & Gober, 2001). These filamentous cells often contain multiple constrictions along the cell length, indicative of a late-stage cell-division defect. Experiments examining the effects of overexpressing dominant-negative alleles of the early flagellar gene *flbE* demonstrated that the filamentous phenotype in class II flagellar-mutant strains was attributable to an absence of FlbD activity (Muir & Gober, 2001). The same gain-of-function mutations in *flbD* that restore late flagellar-gene transcription in class II flagellar mutants also completely ameliorated the cell-division defect in these mutant cells. Thus, the early flagellar-assembly checkpoint regulates both cell division and late flagellar-gene transcription by modulating FlbD activity. The simultaneous operation of these two checkpoints assures that progeny swarmer cells possess a fully functional flagellum at the time of cell division.

In this report, we examine the relationship between flagellum assembly, cell division and the FliX/FlbD regulatory pathway. We demonstrate that FliX-mediated negative regulation of FlbD activity is responsible for the cell-division defect in class II flagellar mutants. Additionally, we show that this effect is attributable to an absence of FlbD activity. By using a strain depleted of FtsZ, we also demonstrate that cell division is not required for the proper timing of the initiation of FliX/FlbD-dependent transcription of late flagellar genes. Prolonged inhibition of cell division resulted in the eventual loss of FlbD activity, which could be reversed by gain-of-function mutations in *flbD*, but not in *fliX*,

indicating the existence of a second cell cycle-dependent pathway for FlbD activation.

## METHODS

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this work are listed in Table 1. Transduction of the kanamycin-resistant, xylose-inducible *ftsZ* locus from strain YB1585 (Wang *et al.*, 2001) into strains JG1172 and JG1181 using bacteriophage  $\phi$ CR30 to create strains JG1216 and JG1217, respectively, was performed as described by Ely & Johnson (1977). Plasmids were introduced into *C. crescentus* by bacterial conjugation using *Escherichia coli* S17-1 (Simon *et al.*, 1983) as a donor and *C. crescentus* strains were grown at 31 °C in PYE medium (peptone-yeast extract medium; Poindexter, 1964) or M2 minimal medium (Ely & Johnson, 1977) either alone or supplemented with one or more of the following: chloramphenicol (2.5  $\mu\text{g ml}^{-1}$ ), tetracycline (2.0  $\mu\text{g ml}^{-1}$ ), kanamycin (20  $\mu\text{g ml}^{-1}$ ), nalidixic acid (20  $\mu\text{g ml}^{-1}$ ) and 0.2% xylose. The *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium (Miller, 1972) either alone or supplemented with one or more of the following: chloramphenicol (30  $\mu\text{g ml}^{-1}$ ) and tetracycline (12.5  $\mu\text{g ml}^{-1}$ ).

**Assay of gene expression.** Expression of the flagellar and cell-division gene transcriptional reporters was measured as described previously (Mangan *et al.*, 1995).  $\beta$ -Galactosidase activity was assayed in triplicate for each time point over the course of the experiment. Within one of these particular experiments, the variation in assayed  $\beta$ -galactosidase activity was <5% for each time point. Synchronizable YB1585 (*ftsZ* depletion), JG1261/*pfliX1* ( $\Delta$ *fliX ftsZ* depletion extragenic *fliX1*) and JG1217 (*flbD-1204 ftsZ* depletion) *C. crescentus* strains harbouring a *fliK-lacZ* transcriptional-reporter fusion were used for the cell-cycle gene-expression experiments and were performed essentially as described by Wingrove & Gober (1994) with the modifications detailed by Muir & Gober (2002). For the cell-cycle experiments involving the depletion of xylose-inducible *ftsZ*, swarmer cells isolated from xylose-replete M2 minimal medium were divided and inoculated into both xylose-replete and xylose-deficient M2 minimal medium at time 0. Immunoblots were prepared essentially as described by Towbin *et al.* (1979) and were analysed by using anti-FtsZ (Mohl *et al.*, 2001), anti-FlbD (Wingrove *et al.*, 1993 personal communication), anti-FliX (Muir & Gober, 2002) and anti-flagellin antisera.

**Microscopy.** For the visualization of live cells, grown cultures were concentrated by harvesting the cells in a microcentrifuge, removing 90% of the supernatant and suspending the cell pellet in the remainder of the supernatant. Cells (2  $\mu\text{l}$ ) from this suspension were mixed with 1  $\mu\text{l}$  FM4-64 (25  $\mu\text{g ml}^{-1}$ ; Molecular Probes) and the sample was covered with a poly-L-lysine-treated coverslip. Samples were visualized by using fluorescence microscopy. Images were captured and analysed by using the Resolve3D image-acquisition software package (Applied Precision). In order to visualize cells by using transmission electron microscopy, late exponential-phase cultures were grown in PYE medium; the cells were then harvested, washed once in 150 mM NaCl and then suspended in 150 mM NaCl. This cell suspension was applied to carbon-coated grids; the cells were allowed to adhere and were then stained with 1% uranyl acetate for approximately 45 s.

Visualization of the flagellum was done by using an immunofluorescence microscopy protocol adapted from that of Maddock & Shapiro (1993). YB1585 (*ftsZ* depletion) cells from synchronized cultures grown in the presence or absence of xylose to the late predivisional cell stage (starting culture OD<sub>600</sub>, 0.4) were fixed in a final concentration of 3.0% formaldehyde and 30 mM sodium phosphate, pH 7.5, for 45 min at room temperature; 37% formaldehyde and 1 M sodium phosphate were added directly to 1 ml culture. Fixed cells were

**Table 1.** Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype or description	Reference or source
<b>Strains</b>		
<i>C. crescentus</i> :		
LS107	<i>syn-1000 bla-6</i>	Stephens <i>et al.</i> (1997)
JG1172	<i>syn-1000 bla-6 ΔfliX</i>	Muir <i>et al.</i> (2001)
JG1176	<i>syn-1000 bla-6 ΔfliX fliP::Tn5</i>	Muir <i>et al.</i> (2001)
JG1180	<i>syn-1000 bla-6 fliP::Tn5</i>	Muir <i>et al.</i> (2001)
JG1181	<i>syn-1000 bla-6 flbD-1204</i>	Muir <i>et al.</i> (2001)
JG1182	<i>syn-1000 bla-6 flbD198::Tn5</i>	Muir <i>et al.</i> (2001)
JG1216	<i>syn-1000 bla-6 ΔfliX ftsZ::pBJM1</i>	This work
JG1217	<i>syn-1000 bla-6 flbD-1204 ftsZ::pBJM1</i>	This work
YB1585	<i>syn-1000 ftsZ::pBJM1</i>	Wang <i>et al.</i> (2001)
<i>E. coli</i> :		
DH5α	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 deoR [φ80dlacΔ(lac)M15]</i>	Life Technologies
S17-1	Rp4-2, Tc::Mu, Km::Tn7	Simon <i>et al.</i> (1983)
<b>Plasmids</b>		
pBBR1MCS	Broad-host-range, multicopy, chlor <sup>r</sup>	Kovach <i>et al.</i> (1994)
pX10	3.4 kb <i>Sau3A</i> fragment encompassing <i>fliX</i> in <i>Bam</i> HI site of pBBR1MCS	Muir <i>et al.</i> (2001)
p <i>fliX1</i>	3.4 kb <i>Sau3A</i> fragment encompassing <i>fliX1</i> in <i>Bam</i> HI site of pBBR1MCS	Muir <i>et al.</i> (2001)
p <i>fliF/lacZ</i> /290	294 bp <i>Bam</i> HI– <i>Hind</i> III <i>fliF</i> promoter fragment in <i>placZ</i> /290	Wingrove & Gober (1994)
p <i>fliK/lacZ</i> /290	1.3 kb <i>Pst</i> I– <i>Xho</i> I fragment containing the <i>fliK</i> promoter in <i>placZ</i> /290	Gober & Shapiro (1992)
pREM80	1.5 kb <i>Xho</i> I– <i>Xba</i> I (PCR-generated) fragment containing <i>flbD-1204</i> in pBBR1MCS	Muir & Gober (2002)

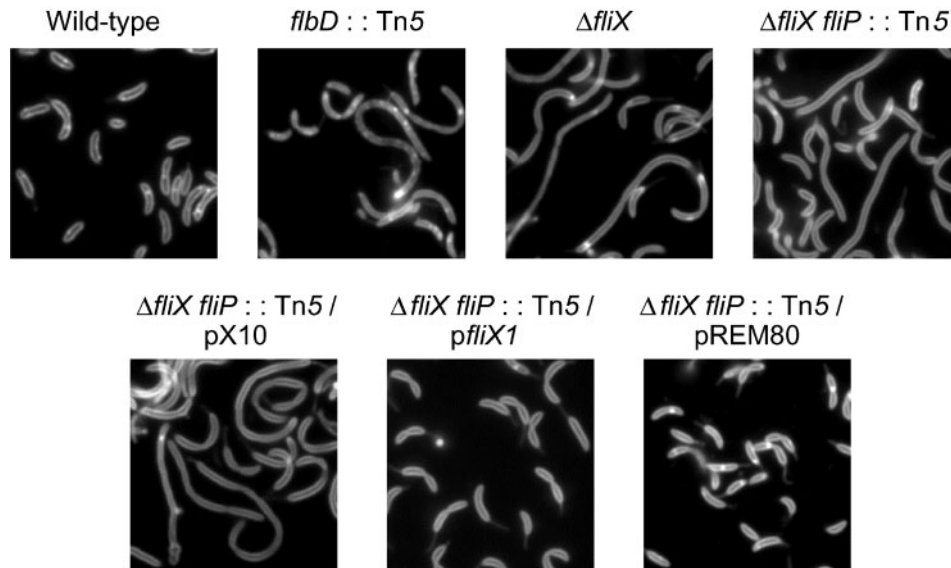
harvested and rinsed three times with cold PBST (140 mM NaCl, 3 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub> and 0.05 % Tween 20) in 1.5 ml microfuge tubes. Pelleted cells were suspended in 200 μl cold PBST and placed onto poly-lysine-coated slides. Cells were left to adhere to slides at room temperature for 5 min. Non-adherent cells were removed by aspiration and those remaining on the slide were incubated at room temperature in ice-cold PBST containing 2 % BSA for 15 min. Slides were then washed once with cold PBST and incubated with a 1 : 200 dilution of anti-flagellin antisera in PBST in a humid chamber at room temperature for 2 h. Slides were rinsed twice with cold PBST and incubated for 1 h in the dark, under the conditions described above, with a secondary antibody conjugated to CY3 (Jackson Immunoresearch). Slides were then rinsed twice with cold PBST and dried by aspiration. Immediately after drying, a 5–10 μl drop of 10 % glycerol was placed onto the treated cells, followed by a coverslip. Slides were either visualized immediately or stored at 4 °C in the dark after edges of coverslips had been sealed with nail polish in order to prevent dehydration.

## RESULTS

### FliX couples early flagellar assembly to cell division

In previous experiments, we demonstrated that the cell-division defect in class II mutants was attributable to a loss of

FlbD activity, as the presence of gain-of-function mutations in *flbD* (called *bfa* for bypass of flagellar assembly) resulted in a normal cell morphology in class II mutant strains (Muir & Gober, 2001). Here, we wanted to test whether the *trans*-acting factor FliX was responsible for regulating cell division in response to flagellar assembly. Cells containing a Tn5 insertion in *flbD* possessed a characteristic late cell-division defect (Fig. 2), with the majority of cells in culture exhibiting a filamentous morphology with multiple constrictions evident along the cell length. An identical phenotype was observed for cells containing a deletion in *fliX* ( $\Delta$ *fliX*), present singly or combined with a Tn5 insertion in the class II structural gene *fliP* ( $\Delta$ *fliX fliP::Tn5) (Fig. 2). In order to examine the role of FliX in regulating this late cell-division event, we compared the effect on cell division of having either wild-type *fliX* or the gain-of-function allele, *fliX1*, present *in trans* in the  $\Delta$ *fliX fliP::Tn5 strain. When wild-type *fliX* was expressed from a plasmid (pX10) in the  $\Delta$ *fliX fliP::Tn5 mutant strain, the filamentous cell-division phenotype was indistinguishable from that of other class II flagellar mutants (Fig. 2). Previous experiments have shown that, in this strain (i.e.  $\Delta$ *fliX fliP::Tn5), wild-type FliX expressed from a plasmid is a potent repressor of FlbD****



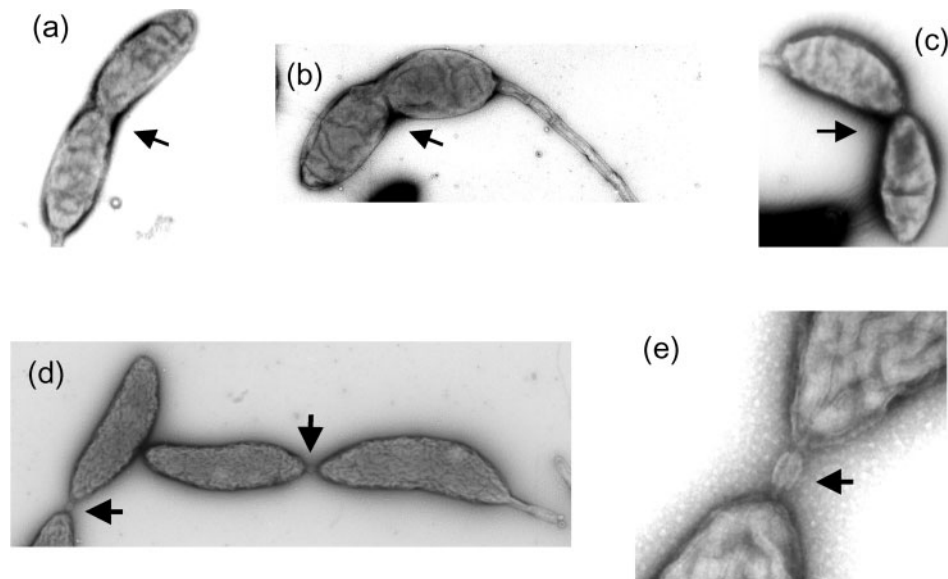
**Fig. 2.** The cell-division defect exhibited by class II flagellar-assembly mutants is the result of inactive FliX/FlbD. Fluorescence microscopy was performed on cells stained with FM4-64. Strains bearing mutations in *flbD* (*flbD*::Tn5), *fliX* ( $\Delta$ *fliX*) and *fliX* and *fliP* ( $\Delta$ *fliX fliP*::Tn5) exhibit a filamentous phenotype. The presence of the gain-of-function mutant *fliX1* allele (*pfliX1*) in the class II flagellar-assembly mutant  $\Delta$ *fliX fliP*::Tn5, but not the wild-type *fliX* allele (pX10), results in a normal cell-division phenotype, similar to that exhibited by the  $\Delta$ *fliX fliP*::Tn5 strain containing the gain-of-function *flbD-1204* allele (pREM80).

activity (Muir & Gober, 2002, 2004). We then compared the morphology of these cells to that of those expressing the constitutively active *fliX1* allele. This particular allele of *fliX*, like gain-of-function *bfa* mutations in *flbD*, restores late flagellar-gene transcription in class II flagellar mutants (Muir *et al.*, 2001). Likewise, the expression of FliX1 in the  $\Delta$ *fliX fliP*::Tn5 class II mutant strain resulted in the restoration of a completely wild-type cell-division phenotype (Fig. 2). This observation indicates that the filamentous cell-division phenotype of class II flagellar mutants is attributable to an inactive FliX/FlbD complex. In support of this view,  $\Delta$ *fliX fliP*::Tn5 cells containing a *flbD-1204 bfa* allele on a plasmid (pREM80) also exhibited a wild-type cell morphology (Fig. 2). In previous experiments, we demonstrated that this conversion of a class II flagellar mutant to wild-type morphology could only be observed with the *flbD-1204 bfa* allele on a plasmid and not wild-type *flbD* (Muir & Gober, 2001).

The presence of multiple constrictions spaced unevenly along the length of the filamentous class II flagellar mutants is probably attributable to an arrest at a late stage of cell division. Wild-type *C. crescentus* cells do not septate in a fashion morphologically similar to that of other rod-shaped bacteria, but rather appear to form a constriction at the midcell (Fig. 3a). This constriction gradually severs the predivisional cell into two compartments (Fig. 3b), an event ultimately culminating in the separation of the two daughter progeny cells (Fig. 3c). We have now performed transmission electron microscopy on an *flbD*::Tn5 mutant strain in order to examine the nature of the cell-division defect (Fig. 3). Predivisional cells of this mutant strain appeared

to possess a tube-like structure that connected the nascent progeny cells (Fig. 3d). This tube-like structure was also observed in the filamentous cells of this mutant strain (data not shown). A narrower rod, appearing to originate from the surface of each daughter cell, traversed the length of this tube (Fig. 3e). The cytoplasm, as well as the remainder of the cell envelope of each progeny cell, otherwise appeared not to be contiguous. Thus, the apparent cell-division defect in class II flagellar mutants occurs at the final stage of cell division in *Caulobacter*: the scission of cell-envelope material, possibly peptidoglycan, connecting two completely divided daughter cells.

Although the filamentous phenotype of class II flagellar mutants appears to involve an arrest at a late stage in cell division, previous experiments have shown that FtsZ rings, the earliest known cytological marker in the initiation of cell division (Bi & Lutkenhaus, 1991), are present in reduced frequency in these cells (Muir & Gober, 2001). We examined whether class II flagellum assembly and FliX/FlbD activity affected the cellular levels of FtsZ (Fig. 4). As a control in this experiment, we assayed the levels of FliX, FlbD and flagellin in each of these strains (Fig. 4). The steady-state level of FtsZ protein in the *flbD*::Tn5 strain was approximately twice that of wild-type cells. Interestingly, the level of FtsZ in the  $\Delta$ *fliX* and  $\Delta$ *fliX/fliP*::Tn5 strains was approximately one-half to one-third, respectively, of that present in wild-type cell extracts (Fig. 4). Introduction of wild-type *fliX* on a plasmid into the  $\Delta$ *fliX/fliP*::Tn5 strain had no effect on the level of FtsZ; however, when this strain expressed either a gain-of-function allele of *fliX* (*pfliX1*) or *flbD* (pREM80), the levels of FtsZ were elevated slightly

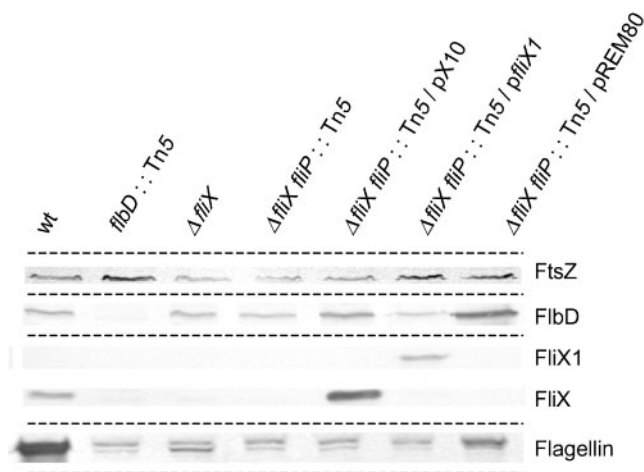


**Fig. 3.** Class II flagellar mutants exhibit an arrest in cell separation. Transmission electron micrographs of both wild-type predivisional cells (a–c) and *flbD*::Tn5 mutant filamentous cells (d–e) are shown. In wild-type cells of *C. crescentus*, cytokinesis is accomplished through an initial constriction at the midcell (a) (indicated by an arrow), followed by a steady progression of the constriction (b) (indicated by an arrow), eventually leading to complete separation of the nascent daughter cells (c). Note that these completely separated cells (c) may, in many cases, still be attached by material (dark staining, indicated by an arrow) derived from the cell envelope. In contrast, class II flagellar mutants and the *flbD*::Tn5 strain shown here (d) often fail to separate fully upon cytokinesis, leading to the accumulation of filamentous cells in culture (see Fig. 2). This arrest or delay in cell separation results in predivisional cells (d) that remain connected by what appears to be a rod of cell wall-like material (e). Note that this structure is not only observed in filamentous cells in class II mutant cultures, but also, as shown here, in those cells that are similar in length to wild-type cells.

compared with wild-type cells (Fig. 4). One possibility is that FtsZ may have a decreased stability in these filamentous flagellar-mutant strains.

### FtsZ ring formation is not required for the proper timing of FlbD-dependent transcription

The experiments presented here show that the activation of FlhX and FlbD via the assembly of a class II flagellar structure



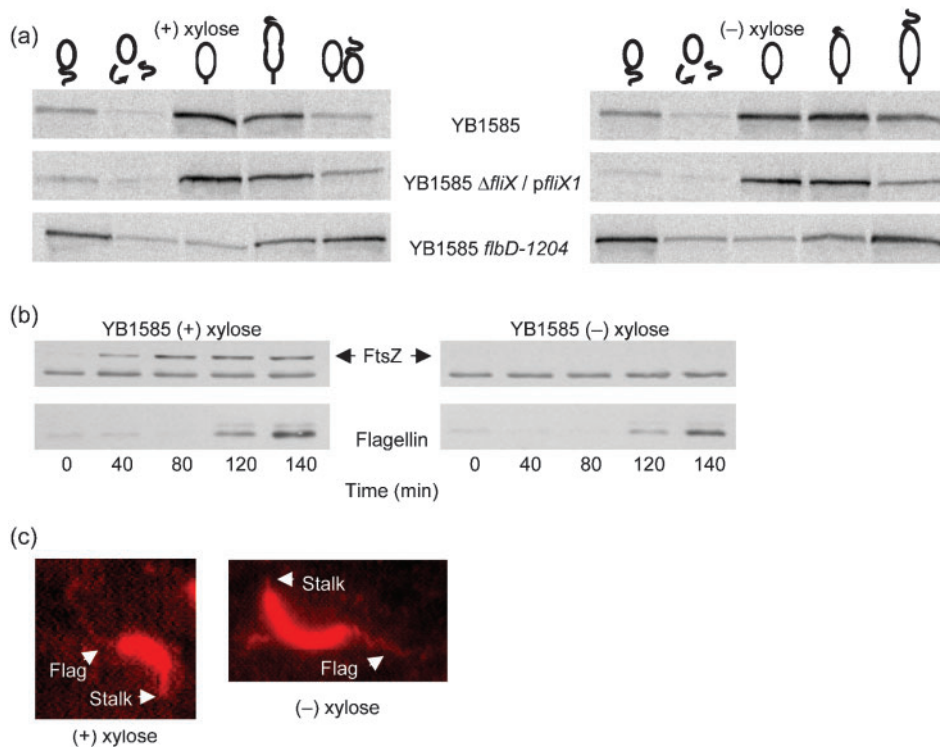
is required for a timely completion of cell division. This finding suggests that the timing of flagellar assembly is enmeshed with the progression of the cell-division cycle. Indeed, previous experiments have demonstrated that the inhibition of cell division, through the use of either cell-division inhibitors or conditional mutants, resulted in cells possessing an intact flagellum that failed to rotate (Terrana

**Fig. 4.** Effect of class II-encoded flagellum assembly on FtsZ protein levels. The cellular concentration of FtsZ in whole-cell extracts was determined by immunoblot analysis. These cell extracts were also subjected to immunoblot analyses using anti-FlbD, anti-FlhX and anti-flagellin sera. The effect of FlbD and FlhX activity on FtsZ levels was tested by introducing multi-copy plasmids pX10 (wild-type *flhX*), *pflhX1* (*flhX1*) and pREM80 (*flbD-1204*) into the  $\Delta flhX$  or the  $\Delta flhX flhP::Tn5$  mutant strains. When grown in PYE medium, the class II flagellar-assembly mutants  $\Delta flhX$  and  $\Delta flhX flhP::Tn5$  were found to accumulate FtsZ levels that were somewhat lower than those of wild-type (wt). The presence of either *flhX1* or *flbD-1204* was found to restore FtsZ in the  $\Delta flhX flhP::Tn5$  strain to levels that were comparable to those of wild-type cells. Note that the mutant *flhX1* allele possesses a frameshift that results in an encoded polypeptide of greater molecular mass than wild-type FlhX (Muir & Gober, 2004). The levels of FlhX are reduced in an *flbD* mutant strain and vice versa.

& Newton, 1976; Huguenel & Newton, 1982; Ohta *et al.*, 1997; Matroule *et al.*, 2004). Here, we wanted to determine whether early cell-division processes influenced, or were required for, the expression of FliX- and FlbD-dependent flagellar genes. In order to accomplish this, a FliX/FlbD-dependent class III reporter fusion, *fliK-lacZ*, was introduced into a strain (YB1585) in which *ftsZ* expression was dependent on the presence of xylose in the culture medium (Wang *et al.*, 2001). Swarmer cells were isolated from cultures grown overnight in the presence of xylose and were permitted to proceed synchronously through the cell cycle in either the presence or absence of xylose in the medium. At various time points, samples were removed, proteins were pulse labelled and labelled  $\beta$ -galactosidase was immunoprecipitated in order to assay the rate of *fliK* promoter activity. This experimental strategy takes advantage of the fact that FtsZ is proteolysed in swarmer cells (Quardokus *et al.*, 1996; Kelly *et al.*, 1998). Thus, when isolated swarmer

cells of strain YB1585 are cultured in medium lacking xylose, the cells progress through the cell cycle, but never initiate cytokinesis and, hence, do not divide.

When these isolated YB1585 swarmer cells were incubated in medium containing xylose and allowed to progress through the cell cycle, the *fliK-lacZ* reporter fusion had a peak rate of expression in late predivisional cells that declined following cell division (Fig. 5a). This pattern of expression was similar to that of flagellin protein (Fig. 5b), as well as that reported for *fliK* expression in wild-type cells of *C. crescentus* (Muir & Gober, 2002). FtsZ levels were monitored in this same experiment via immunoblot with an anti-FtsZ antibody. As has been reported previously (Quardokus *et al.*, 1996; Kelly *et al.*, 1998), FtsZ protein was below detectable limits in isolated swarmer cells and returned in stalked cells, with peak levels appearing in predivisional cells (Fig. 5b). We also assayed the effects of expressing the gain-of-function alleles



**Fig. 5.** (a) Temporal expression of the FlbD-dependent class III *fliK-lacZ* transcriptional-reporter fusion was assayed over the course of the cell cycle in the presence and absence of FtsZ. A schematic of the cell types present at each time point [see (b) for time in min] during the cell cycle, as determined by light microscopy, is shown above the phosphorimages of the dried electrophoresis gels. The parental strain, YB1585, contains a single functional copy of *ftsZ* that is controlled by a xylose-inducible promoter. The gain-of-function *fliX1* ( $\Delta fliX/pfliX1$ ) and *flbD-1204* alleles were also introduced into strain YB1585 and the temporal pattern of *fliK-lacZ* expression was assayed. These data indicate that FtsZ, and thus initiation of cell division, is not required for the activation of FlbD-dependent flagellar-gene expression during the *C. crescentus* cell cycle, but is required for the cessation of *fliK* transcription. (b) Immunoblot analyses of samples of synchronized cells of YB1585 from (a) suspended in medium either containing (+) or lacking (-) xylose, using anti-FtsZ and anti-flagellin sera, at the same cell-cycle time points as in (a) are shown; the data presented are representative of those obtained for the other strains. (c) Immunofluorescence microscopy using anti-flagellin antibody was performed on cells of YB1585 suspended in medium either containing (+) or lacking (-) xylose. The stalk and flagellar filament are indicated by arrows. Note that an apparently complete flagellum is present even in the absence of FtsZ and cell division.

of *FliX* (*FliX1*) and *FlbD* (*FlbD-1204*) on the timing of *fliK-lacZ* expression in YB1585 (Fig. 5a). In cells in which the *fliX1* allele was the sole copy of *fliX* in the cell, the temporal pattern of *fliK-lacZ* expression was almost identical to that of cells containing wild-type *fliX* (Fig. 5a). In contrast, YB1585 cells containing the gain-of-function *flbD-1204* allele exhibited aberrant timing of *fliK-lacZ* expression. In addition to having high levels of expression in predivisional cells, this strain also expressed the *fliK-lacZ* fusion late in the cell cycle and in swarmer cells (Fig. 5a). This pattern of expression is similar to that reported previously for strains carrying this *flbD-1204* allele in a wild-type genetic background (Muir & Gober, 2002).

We then performed this same basic experiment, instead suspending isolated swarmer cells of strain YB1585 in medium lacking xylose. In this case, when the population progressed through the cell cycle, *FtsZ* was not synthesized and was therefore not present at any time point throughout the course of the experiment (Fig. 5b). In the absence of *FtsZ*, the timing of *fliK-lacZ* expression was remarkably similar to that of the control cells, with the rate of promoter activity reaching a peak in predivisional cells (Fig. 5a). The lack of *FtsZ*, however, resulted in a delay in the cessation of *fliK-lacZ* expression, with new synthesis of  $\beta$ -galactosidase still occurring 140 min into the experiment. In control cells possessing *FtsZ*, expression of *fliK-lacZ* decreased markedly by this point in the experiment (Fig. 5a). This result indicates that cytokinesis may be required to reduce the activity of *FlbD* late in the cell cycle. Cells containing the *fliX1* allele depleted of *FtsZ* had a similar pattern of *fliK-lacZ* expression to the cells containing wild-type *fliX*, also exhibiting an increase in the rate of  $\beta$ -galactosidase synthesis late in the experiment (Fig. 5a). The absence of *FtsZ* had no effect on cells containing the constitutive *flbD-1204* allele, as the presence of this *FlbD* already results in delayed cessation of class III flagellar-gene expression in cells that are competent for cell division (Fig. 5a).

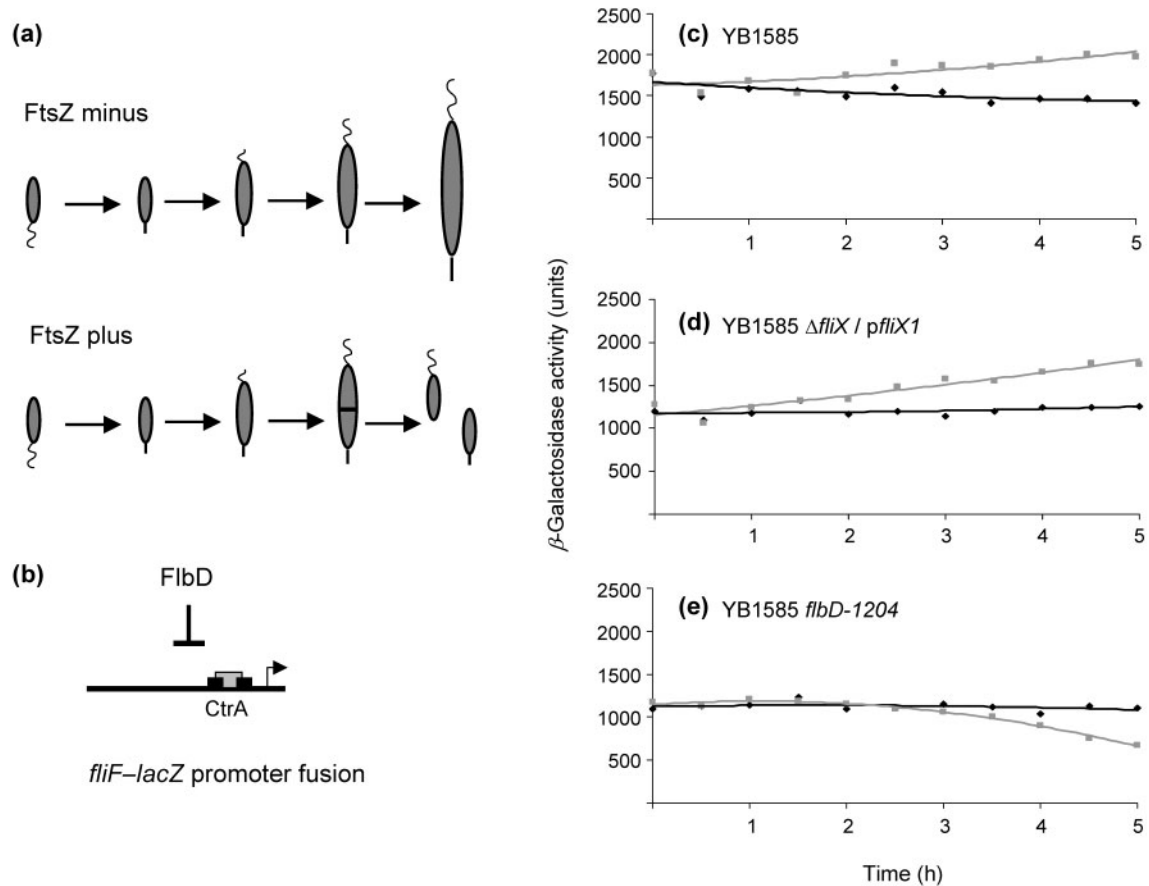
These results show that *FlbD* does not require the earliest known step in the cell-division cycle in order to undergo cell cycle-regulated activation. Although they expressed late flagellar genes (Fig. 5b), the cells lacking *FtsZ* were non-motile (data not shown). This is consistent with previous observations (Terrana & Newton, 1976; Huguenel & Newton, 1982; Ohta *et al.*, 1997; Matroule *et al.*, 2004) demonstrating that an inhibition of cell division resulted in non-motile cells possessing a completely assembled flagellum. Likewise, immunofluorescence microscopy employing an anti-flagellin antibody to visualize the flagellar filament revealed the presence of an intact flagellar filament in the synchronized cells lacking *FtsZ* (Fig. 5c).

### Cell division is required for reactivation of *FlbD* following flagellum assembly

*C. crescentus* cells depleted of *FtsZ* continue to lengthen and replicate chromosomal DNA for what would represent several generations (data not shown). We wanted to observe

the long-term, i.e. greater than one generation, effects of *FtsZ* depletion on *FlbD* activity. One hypothesis is that the persistent presence of an intact polar flagellum in *FtsZ*-depleted cells would eventually result in a repression of *FlbD* activity, as *C. crescentus* possesses regulatory mechanisms to shut off flagellar-gene expression following the completion of flagellum assembly (i.e. through the repressive effects of *FliX*) (Muir & Gober, 2002, 2004). In order to test this idea, YB1585 cells containing a *fliF-lacZ* reporter fusion were grown overnight in the presence of xylose, harvested, washed and suspended in fresh medium in either the presence or absence of xylose (Fig. 6a).  $\beta$ -Galactosidase activity was assayed over time, up to what would represent approximately 2.5 generations (5 h). As the *fliF* promoter is repressed by *FlbD* (Benson *et al.*, 1994a; Mullin *et al.*, 1994; Wingrove & Gober, 1994), an increase in  $\beta$ -galactosidase activity would be indicative of a loss of *FlbD* activity. Indeed, the gradual loss of *FtsZ* by this strain resulted in a loss of *FlbD* activity over time, as indicated by an increase in *fliF-lacZ* promoter activity (Fig. 6c). We next wanted to test the idea that the loss of *FlbD* activity was a consequence of the repressive effects of *FliX*. We assayed whether the loss of *FtsZ* would cause a decrease in *FlbD* activity in a strain bearing the gain-of-function *fliX1* allele. Previous experiments have demonstrated that this allele of *fliX* is locked as a positive activator and cannot repress *FlbD* activity, even following successful flagellum assembly or in class II mutant strains (Muir & Gober, 2004). Surprisingly, when cells carrying the *fliX1* allele were depleted of *FtsZ*, there was also a loss of *FlbD* activity, as indicated by the increase in *fliF-lacZ* promoter activity (Fig. 6d). This result indicates that the loss of *FlbD* activity under conditions of *FtsZ* depletion is not related to the presence of an intact flagellum in these cells and the accompanying repressive actions of *FliX*. Next, we determined whether the *flbD-1204* gain-of-function allele would also be subject to a loss of activity when cell division was inhibited. In this case, there was a gradual loss of *fliF-lacZ* promoter activity over time following *FtsZ* depletion, indicating that *FlbD-1204* activity slightly increased over the course of the experiment (Fig. 6e).

These results indicate that *FlbD* requires a cell-division event, possibly cell separation, in order to maintain activity or become reactivated following flagellum synthesis, as only the hyperactive *flbD-1204* allele could bypass the loss of activity associated with an inhibition of cell division. As the *fliF-lacZ* reporter fusion used to assay *FlbD* activity in these experiments is activated by the cell cycle-regulated, global transcription factor *CtrA*, it was important to determine that the observed changes in promoter activity were not a consequence of alterations in *CtrA* activity. In this regard, we repeated this experiment using an *fliK-lacZ* reporter fusion which is activated by *FlbD* (Fig. 7a). In this case, depletion of *FtsZ* led to a gradual decrease in  $\beta$ -galactosidase activity relative to control cultures in which *FtsZ* was present (Fig. 7b). This observation is consistent with the idea that *FlbD* activity is lost gradually following *FtsZ* depletion. Note, here, that the magnitude in the decrease in



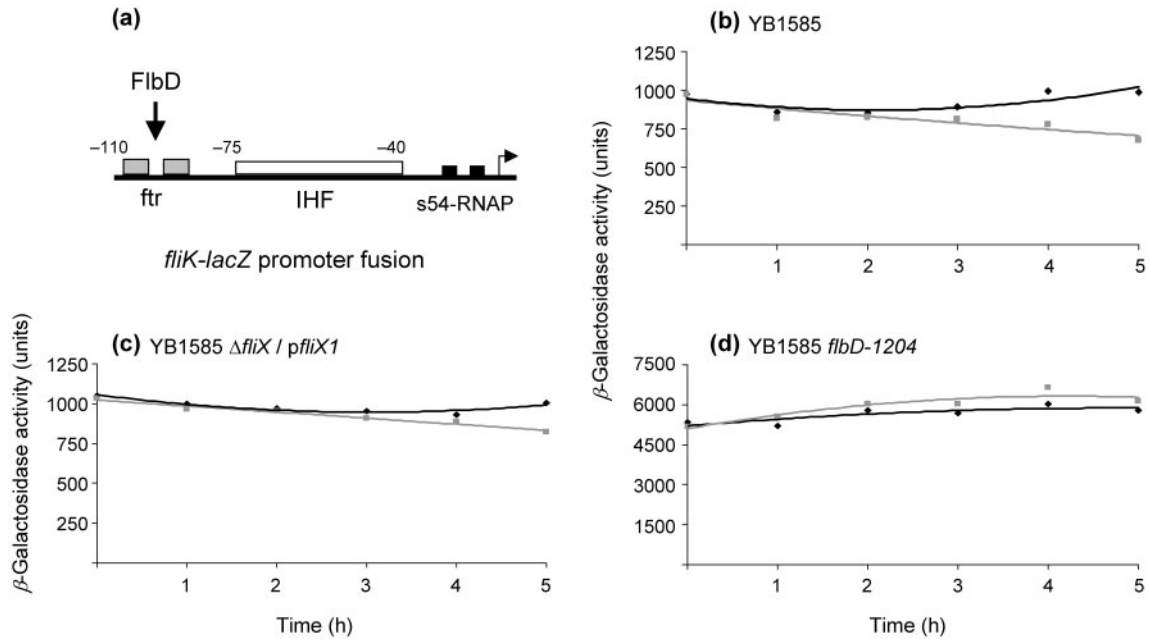
**Fig. 6.** FlbD-mediated repression of *fliF* promoter activity requires a round of cell division. (a) Shown is a schematic diagram depicting the effect of FtsZ depletion and the resulting inhibition of cell division. Derivatives of strain YB1585 were isolated from an overnight culture grown in the presence of xylose, which is required for the induction of *ftsZ* expression in all of the YB1585 strains assayed. The cells were washed with PYE medium lacking xylose and a portion of the washed cells were placed in both PYE medium (■) and PYE medium containing 0.2% xylose (◆) so that each liquid culture was at an  $OD_{600}$  of 0.15. The cells were permitted to grow at 31 °C and the expression of the early, FlbD-repressed flagellar gene *fliF* (b) was assayed. (c) The FlbD-dependent repression of the class II *fliF-lacZ* transcriptional-reporter fusion was assayed over time following the depletion of FtsZ (grey lines) from unsynchronized cultures. Gene expression in the same unsynchronized cultures was also assayed in the presence of FtsZ (black lines) for comparison. Promoter activity measured for the class II *fliF-lacZ* flagellar-reporter fusions is reported as  $\beta$ -galactosidase activity. The  $\beta$ -galactosidase activity was assayed in triplicate for each time point over the course of the experiment. The variation in assayed  $\beta$ -galactosidase activity was < 5% for each time point. The doubling time for all cultures that were FtsZ replete was between 3 and 4 h. For the FtsZ-depleted cells, a loss of motility was observed after this time (not shown). (d) Effect of FtsZ depletion on *fliF-lacZ* promoter activity in strain YB1585 expressing the gain-of-function *fliX1* allele. This strain, like the YB1585 strain with wild-type *fliX* (c), was found to lose FlbD activity over the course of FtsZ depletion, as indicated by an increase in *fliF* promoter activity. (e) Effect of FtsZ depletion on *fliF-lacZ* promoter activity in strain YB1585 expressing the gain-of-function *flbD-1204* allele. This strain maintains FlbD-dependent repression of the class II flagellar-reporter fusion *fliF-lacZ* during the course of FtsZ depletion.

$\beta$ -galactosidase activity is less than the corresponding increase measured in the previous experiment (Fig. 6c). This is a consequence of slow turnover of pre-existing  $\beta$ -galactosidase protein. Consistent with the results obtained by using the *fliF-lacZ* reporter, the presence of the gain-of-function *fliX1* allele could not maintain FlbD activity following FtsZ depletion (Fig. 7c), whereas FlbD-1204 exhibited no such decrease in activity upon loss of FtsZ (Fig. 7d). These data are consistent with the view that the loss of FlbD activity in these experiments is probably a direct

consequence of the inhibition of cell division, and is not due to the persistent presence of a fully assembled flagellum. We hypothesize that FlbD requires a round of cell division in order to reactivate following flagellum assembly.

## DISCUSSION

In *Caulobacter*, biogenesis of the single polar flagellum occurs once every cell cycle and is regulated by both



**Fig. 7.** FliB-dependent activation of the class III *fliK-lacZ* transcriptional-reporter fusion (a) was assayed during the depletion of FtsZ (grey lines) from unsynchronized cultures. Gene expression in unsynchronized cultures was also assayed in the presence of FtsZ (black lines) for comparison. The experiment was performed basically as described in the legend to Fig. 6. (b) Effect of FtsZ depletion on *fliK-lacZ* promoter activity in strain YB1585. Promoter activity measured for the class III *fliK-lacZ* flagellar-reporter fusion is reported as  $\beta$ -galactosidase activity. The  $\beta$ -galactosidase activity was assayed in triplicate for each time point over the course of the experiment. The variation in assayed  $\beta$ -galactosidase activity was  $<5\%$  for each time point. (c) Effect of FtsZ depletion on *fliK-lacZ* promoter activity in strain YB1585 expressing the gain-of-function *fliX1* allele. This strain, like the YB1585 strain with wild-type *fliX* (b), was found to lose FliB activity over the course of FtsZ depletion, as indicated by a decrease in *fliK* promoter activity. (d) Effect of FtsZ depletion on *fliK-lacZ* promoter activity in strain YB1585 expressing the gain-of-function *fliB-1204* allele. This strain maintains FliB-dependent activation of the class II flagellar-reporter fusion *fliK-lacZ* during the course of FtsZ depletion.  $\blacklozenge$ , Xylose present;  $\blacksquare$ , xylose absent.

cell-cycle landmarks and the progression of flagellar assembly itself. In this report, we have examined the relationship between cell division and flagellar biogenesis in *C. crescentus*. We have found that the progression of each of these critical processes has a complex interdependence on that of the others. Most notably, the complete assembly of an early class II flagellar structure is required for normal cell division. In the absence of a completed class II structure, filamentous cells accumulate in culture. We have found that this is a consequence of the repressive action of FliX on the global flagellar-transcription factor FliB.

FliX and FliB regulate the expression of late flagellar genes, encoding the distal rods and rings of the basal body, the hook (class III) and the filament (class IV). Previous experiments have shown that these genes are not transcribed in the absence of a class II-encoded flagellar structure (Newton *et al.*, 1989; Xu *et al.*, 1989; Ramakrishnan *et al.*, 1994; Mangan *et al.*, 1995). FliX regulates the activity of the FliB transcription factor in response to this early flagellar-assembly event, such that FliB activity is repressed prior to flagellar assembly and activated via FliX action following completion of the early basal-body structure

(Muir *et al.*, 2001; Muir & Gober, 2002, 2004). Therefore, the same regulatory pathway controls the transcription of late flagellar genes and the completion of cell division. We hypothesize that the filamentous phenotype of class II flagellar mutants is attributable to a regulatory cell-cycle checkpoint that couples the assembly of an MS ring-switch-TTSS complex (class II structure) to the completion of cell division. The experiments presented here demonstrate that this checkpoint operates through the regulation of FliX and FliB activity, as the presence of constitutive alleles of either *trans*-acting factor resulted in the formation of morphologically normal cells, even in the absence of a completed flagellar structure.

In *E. coli*, cell division initiates with the formation of a polymerized ring of FtsZ at the midcell (Bi & Lutkenhaus, 1991), followed by the recruitment of other early-acting cell-division proteins including, among others, FtsA, FtsQ, FtsI, FtsW, FtsN and ZipA (reviewed by Lutkenhaus & Addinall, 1997). The assembly of this complex at the midcell leads to a redirecting of peptidoglycan assembly with the eventual formation of a septum, followed ultimately by cell separation. In cultures of *Caulobacter* class II mutants,

the cells often possess multiple constrictions along the cell length, indicative of an arrest in a late stage of cell division. This arrest is only partial, as these mutant cultures achieve a final cell density similar to that of wild-type cells. Therefore, the separation of class II-mutant cells is delayed with respect to cell elongation, resulting in an accumulation of filamentous cells with constrictions. Many of the cells that we observed by using light microscopy were similar in appearance to those expressing dominant-negative alleles of FtsZ (Wang *et al.*, 2001), possessing elongated constrictions along the length of the filament. Electron microscopy reveals that these predivisional cells very often appear to possess two completely separated compartments that are connected by a narrow bridge of material with a relatively electron-dense rod traversing the length of the structure and apparently connecting the two nascent compartments (Fig. 3). We were unable to detect this unusual structure in predivisional cells from wild-type cultures. As *Caulobacter* cells pinch gradually at the midcell during cytokinesis, rather than septating (Poindexter & Hagenzieker, 1981), this narrow neck of cell envelope-derived material in class II mutants probably arises from a delay in the constriction process. Specifically, the presence of the structure connecting the two nascent compartments suggests that the final stage in the constriction process, cell separation, is arrested. It is also possible that the presence of this structure is indicative of an aberrant cell-division pathway in class II flagellar mutants.

The completion of cell division and the expression of late flagellar genes parallel each other, with both processes being regulated by a common mechanism. We hypothesize that these checkpoints exist in order to ensure that each progeny swarmer cell possesses a fully functional flagellum. Thus, the completion of cell division is delayed until an early MS ring-switch-TTSS complex is assembled, resulting in the activation of FliX and FlbD. FlbD, in turn, activates the transcription of late flagellar genes and, we speculate, a late cell-division gene. Motility is an essential aspect of the *Caulobacter* lifestyle: every sessile reproductive stalked cell gives rise to a temporarily non-reproductive swarmer cell. *C. crescentus* is an oligotrophic bacterium, often occupying nutrient-poor, freshwater environments (Poindexter, 1964). The obligatory asymmetrical cell division of this organism serves to disperse the nascent daughter cells via motility and chemotaxis. The existence of a checkpoint locking the completion of cell division and flagellar synthesis in step with one another underscores the importance of functional motility in the progeny swarmer cells.

We also examined the influence of cell division on the cell cycle-regulated activation of FlbD. The initiation of DNA replication has been shown to be a critical event in the activation of early flagellar-gene expression (Dingwall *et al.*, 1992; Stephens & Shapiro, 1993). This cell-cycle event is coupled to early flagellar-gene expression through the timed synthesis and phosphorylation of the global transcription factor CtrA (Quon *et al.*, 1996; Domian *et al.*,

1997; Reisenauer *et al.*, 1999). In addition to activating the transcription of early flagellar genes, CtrA also regulates as many as 95 genes directly (Laub *et al.*, 2002), including the early cell-division genes *ftsZ* (Kelly *et al.*, 1998) and *ftsQA* (Wortinger *et al.*, 2000). As early flagellar assembly and early cell division shared a common regulator, we examined whether initiation of cell division, like class II flagellar assembly, influenced the expression of late flagellar genes. We conducted these cell-cycle experiments in synchronized populations of cells that could not synthesize FtsZ and found that the initiation of cell division was not required for regulating the temporal activation of FlbD. Interestingly, subsequent cell division was required for the normal cessation of class III transcription in these experiments. FlbD, however, did not remain active for longer than what would represent one generation in these FtsZ-depleted cells. FlbD activity was lost gradually in FtsZ-depleted cells that did not divide. One possibility was that the presence of the completed flagellum was inhibiting FlbD activity via the action of FliX (i.e. one function of FliX is to shut off FlbD activity following the completion of flagellar assembly). Surprisingly, we found that the loss of FlbD activity was not attributable to FliX-mediated repression, as the presence of a constitutively active allele of *fliX* (*fliX1*) could not rescue FlbD activity under conditions of FtsZ depletion. However, there was no measurable loss of FlbD activity when the constitutive *flbD-1204* allele was expressed under these conditions. These results suggest that FlbD is activated by a second, distinct, cell cycle-regulated mechanism and that this regulatory action is required for its ensuing activation by early flagellar assembly via FliX activity. The data also indicate that this cell cycle-related activation of FlbD requires cell division, followed perhaps by a round of DNA replication. One possibility is that FlbD becomes unphosphorylated upon FtsZ depletion, and requires cell division in order to be rephosphorylated. Indeed, previous experiments have indicated that FlbD is phosphorylated in a cell cycle-regulated fashion (Wingrove *et al.*, 1993). Identification of the kinase(s) that phosphorylate FlbD will resolve whether this activity is regulated by cell division.

The depletion of FtsZ from synchronized cultures led to the synthesis of an intact, and apparently complete, flagellum that did not rotate. Therefore, early flagellar assembly regulates cytokinesis, which in turn regulates the acquisition of motility by the fully assembled flagellum. The results presented here suggest that FliX and FlbD have a central role in helping to orchestrate this ordered sequence of developmental checkpoints.

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