Cell cycle control of cell morphogenesis in *Caulobacter*

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In *Caulobacter crescentus*, morphogenic events, such as cytokinesis, the establishment of asymmetry and the biogenesis of polar structures, are precisely regulated during the cell cycle by internal cues, such as cell division and the initiation of DNA replication. Recent studies have revealed that the converse is also true. That is, differentiation events impose regulatory controls on other differentiation events, as well as on progression of the cell cycle. Thus, there are pathways that sense the assembly of structures or the localization of complexes and then transduce this information to subsequent biogenesis or cell cycle events. In this review, we examine the interplay between flagellar assembly and the *C. crescentus* cell cycle.

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Abbreviations
Cori *Caulobacter crescentus* origin of replication

Introduction

Many bacteria undergo a program of development in response to varied external cues. In contrast, for the aquatic bacterium *Caulobacter crescentus*, morphogenesis is an intrinsic part of the cell cycle, in which every cell division yields a motile swarmer cell and a sessile stalked cell (Figure 1) [1]. The stalked cell resembles a stem cell, which can immediately initiate DNA replication after cell division. The swarmer cell, with its single polar flagellum, can be considered the dispersal phase of the life cycle. This cell type is unable to initiate DNA replication until it differentiates into a stalked cell, which occurs after approximately one-third of the cell cycle. At this time, the flagellum is shed and the biosynthesis of the stalk and adhesive holdfast begins at the former flagellar pole. As the cell continues through the cell cycle, DNA replication, cell division and biogenesis of a new flagellum occur in a precisely orchestrated sequence of interconnected events, resulting in a new swarmer cell and a new stalked cell [2–4]. Thus, in the polar predivisional cell, a single round of DNA replication must be completed and the two chromosomes partitioned into the two nascent cells before cytokinesis occurs. In addition, the protein components of a new flagellum, chemotaxis machinery and pili must be synthesized, targeted to the swarmer pole and assembled before cell division. How are these morphogenic and cell cycle events coordinated? *C. crescentus* is an ideal organism with which to resolve such questions, because it is possible to isolate homogenous populations of swarmer cells using density gradient centrifugation. Cell cycle events can then be monitored as these isolated, synchronous populations continue to grow. In addition, the complete genome sequence of *C. crescentus* is now available [5••].

Several general mechanisms are known to be involved in the regulation of morphogenesis during the cell cycle. The importance of the activity and localization of phosphorelay proteins has been extensively covered in several recent reviews [4,6•,7]. In addition, microarray analysis and proteomic studies have revealed global patterns of cell-cycle-controlled gene expression and proteolysis [8••,9•]. In this review, we primarily focus on the regulation of flagellar biogenesis, the best understood aspect of *C. crescentus* development [2]. In particular, several recent studies have revealed exciting new progress in understanding the controls that link flagellar biogenesis, DNA synthesis, chromosome partitioning and the initiation of cell division in the *C. crescentus* predivisional cell.

Flagellar biogenesis

Approximately 50 genes are required for biogenesis of the *C. crescentus* polar flagellum [2]. These genes have been placed into different classes on the basis of results of epistasis experiments. The temporal expression of these genes is regulated by a complex trans-acting hierarchy that integrates sensory inputs regarding the progression of the cell cycle and the assembly of the flagellar structure itself [2,10–12]. The initiation of flagellar biogenesis occurs in the predivisional cell with the transcription of early, class II flagellar genes. The temporal transcription of these genes is activated by the response regulator, transcription factor CtrA. Class II genes encode the earliest components of the flagellum to be assembled: the MS ring (*fliF*), the flagellar switch (*fliG*, *fliM* and *fliN*) and elements of the flagellum-specific type III secretion system (*flhA*, *fliI*, *fliJ*, *fliO*, *fliP*, *fliQ* and *fliR*). In addition, class II genes encode two known trans-acting transcription factors, FlbD and FliX, and the alternative sigma factor, σ^{54}. Class III and class IV flagellar gene products comprise the outer parts of the basal body and hook, and the filament, respectively.

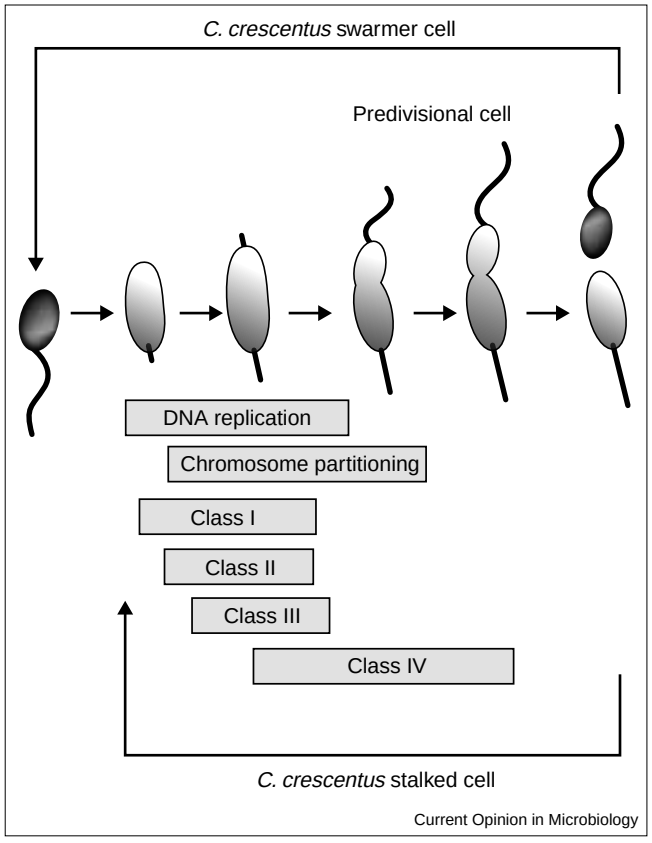
Transcription of early flagellar genes is regulated by the cell cycle

The only identified class I gene to date, *ctrA*, is intimately involved in the timing of DNA replication, cell division and polar organelle development [2,3]. The phosphorylated, active form of the CtrA protein is present in high concentrations in swarmer cells, where it represses the initiation of DNA replication through binding at specific sites in the origin [13–15]. It also suppresses expression of the early cell division gene *ftsZ* [16,17]. Degradation of CtrA during the swarmer→stalked cell transition prefaces the onset of DNA replication and prepares the cell for cell division [18].

Subsequently, through autoregulation of transcription, CtrA levels again increase once replication has commenced [19]. Once activated through phosphorylation, CtrA controls the transcriptional expression of many genes later in the cell cycle in addition to the class II genes, including those involved in biogenesis of the pili [20], the septum [16,17] and the chemotaxis apparatus [21•]. In the stalked compartment of the predivisional cell, CtrA degradation following cell division allows re-initiation of DNA replication, and a new cell cycle begins. CtrA activation of class II flagellar gene transcription thus coordinates the timing of *de novo* flagellar biogenesis with the onset of DNA replication and the new cell cycle, by restricting early flagellar gene expression to a narrow, temporal window. Interestingly, class II transcription has also been found to require DNA replication initiation [22,23]. It is unclear whether or not this is dependent on CtrA or is mediated by a different mechanism.

Temporal transcription of class III and class IV flagellar genes requires σ^{54}-containing RNA polymerase, FlbD, the DNA-binding protein integration host factor (IHF), and FliX [24–28,29••,30••]. The FlbD trans-acting transcription factor is a member of the large family of σ^{54} transcriptional activators that contain a conserved receiver domain of bacterial two-component regulatory systems [26,31]. Typically, these proteins are phosphorylated on a conserved aspartate residue within the receiver domain. Phosphorylation activates ATPase activity in the conserved central domain, which in turn stimulates oligomerization at enhancer sequences and transcriptional activation. Cell-cycle transcriptional activation of class III and class IV genes is accomplished through the temporal phosphorylation of FlbD [27]. The class II gene, *flbE*, was once thought to encode the FlbD kinase. FlbE is required for FlbD-dependent transcription of class III and class IV genes and exhibits the ability to phosphorylate FlbD *in vitro* [32]. However, *flbE* alleles with mutations in residues predicted to be critical for kinase activity were able to restore wild-type levels of class III and class IV gene transcription in a Δ*FlbE* strain [30••]. These alleles were also able to restore motility. Subsequent analysis of amino-terminal FlbE deletion mutants showed that FlbE is either an unknown structural component of the flagellum or the flagellum-specific secretory apparatus, or is otherwise required for proper assembly of early flagellar structures [30••]. Thus, the kinase that catalyzes the phosphorylation of FlbD remains unknown, but is hypothesized to respond to a cell cycle cue. In addition to functioning as an activator of class III and class IV genes, FlbD also represses the transcription of many of the early class II genes ([25,33]; RE Muir, JW Gober, unpublished data). Late in the cell cycle, the transcription of class III and class IV genes is restricted to the swarmer compartment of the predivisional cell [27,34]. Conversely, the transcription of *fliF*, an early class II operon, is repressed in the swarmer pole [33]. This program of polar transcription is accomplished through the swarmer-compartment-specific activation of FlbD [27].

Figure 1



Schematic of the *C. crescentus* cell cycle. For simplicity, the swarmer→stalked cell transition (left) is considered the 'beginning' of the cycle. The flagellated swarmer cell differentiates into a stalked cell after a defined period of time, roughly one-third of the life cycle. The flagellum is then shed and synthesis of a stalk begins at the former flagellar pole. At this time, the major cell cycle processes are initiated. These include DNA replication, chromosome partitioning and cell division, as well as expression of the class I genes in the hierarchy that ultimately leads to a new flagellum at the swarmer pole of the predivisional cell. Cytokinesis culminates (right) with the formation of a new swarmer cell and a new stalked cell; the latter immediately begins a new growth cycle. The wavy line indicates the flagellum and the straight line indicates the stalk. The timing of DNA replication and chromosome partitioning are shown by the bars below the corresponding developmental stages, as is the temporal transcription pattern of the flagellar gene class hierarchy.

Flagellum morphogenesis regulates cell-cycle gene expression

In addition to receiving sensory input from the cell cycle, the expression and assembly of class-II-encoded gene products is also required for the transcription of class III genes and one class IV gene (*fljL*) [2,10,12,35]. For example, epistasis experiments have demonstrated that strains containing a mutation in any one of the class II genes encoding the early flagellar structure fail to transcribe class III genes. Mutant strains (called *bfa* mutants) that relieve this dependency on class II assembly have been isolated [36]. Recently, the *bfa* mutations have been found to reside in the gene encoding FlbD [29••]. Therefore, FlbD activity is regulated, in part, by the progression of flagellum

assembly. Interestingly, strains containing a *flbD–bfa* mutation exhibit an altered pattern of temporal flagellar gene transcription [36]. This result suggests that flagellar assembly influences the temporal activity of FlbD. How is information regarding the status of flagellum assembly transduced to FlbD? One critical regulator in this regard is the product of the *flix* gene, which encodes a 14 kD protein of unknown biochemical function [28,29••]. Strains containing a null mutation in *fliX* are non-motile and fail to express class III and class IV flagellar genes, suggesting that FliX is required for FlbD activity [28]. Motile suppressors of *fliX* null mutations can be readily isolated and map to *flbD* [29••]. These motile suppressor mutants are phenotypically identical to the previously isolated gain-of-function *bfa* mutations in *flbD*. Although these experiments indicate that FliX functions as a positive regulator of FlbD activity, overexpression of *fliX* suppresses class III and class IV gene expression, indicating that FliX also functions as a negative regulator of FlbD [29••]. Recently, a *bfa*-like mutation in *fliX* was isolated [29••]. This mutant, which contains a frameshift near the end of the *fliX*-coding sequence, bypasses the class II assembly requirement for class III and class IV transcription in a fashion similar to *bfa* mutations in *flbD*. The simplest model to account for these results is that FliX transduces information regarding the status of early flagellar assembly to FlbD. How FliX 'senses' morphogenesis or influences FlbD activity is currently unknown, but it is possible that it influences the phosphorylation state of, and/or interacts directly with, FlbD. Interestingly, the genes *flgB* and *flgC*, which encode the *C. crescentus* homologues of proximal rod proteins, and *fliE*, a conserved gene of unknown function, are expressed outside the flagellar hierarchy [37•]. These genes are repressed by CtrA, but are transcribed slightly before other class II genes. In addition, deletions within the operon do not significantly affect expression of later flagellar genes. Because at least two of these proteins are known structural components of the flagellum, their existence outside of the hierarchy remains a mystery. It is possible that the assembly checkpoint mediated by FliX and FlbD occurs at a point before the assembly of the proximal rod.

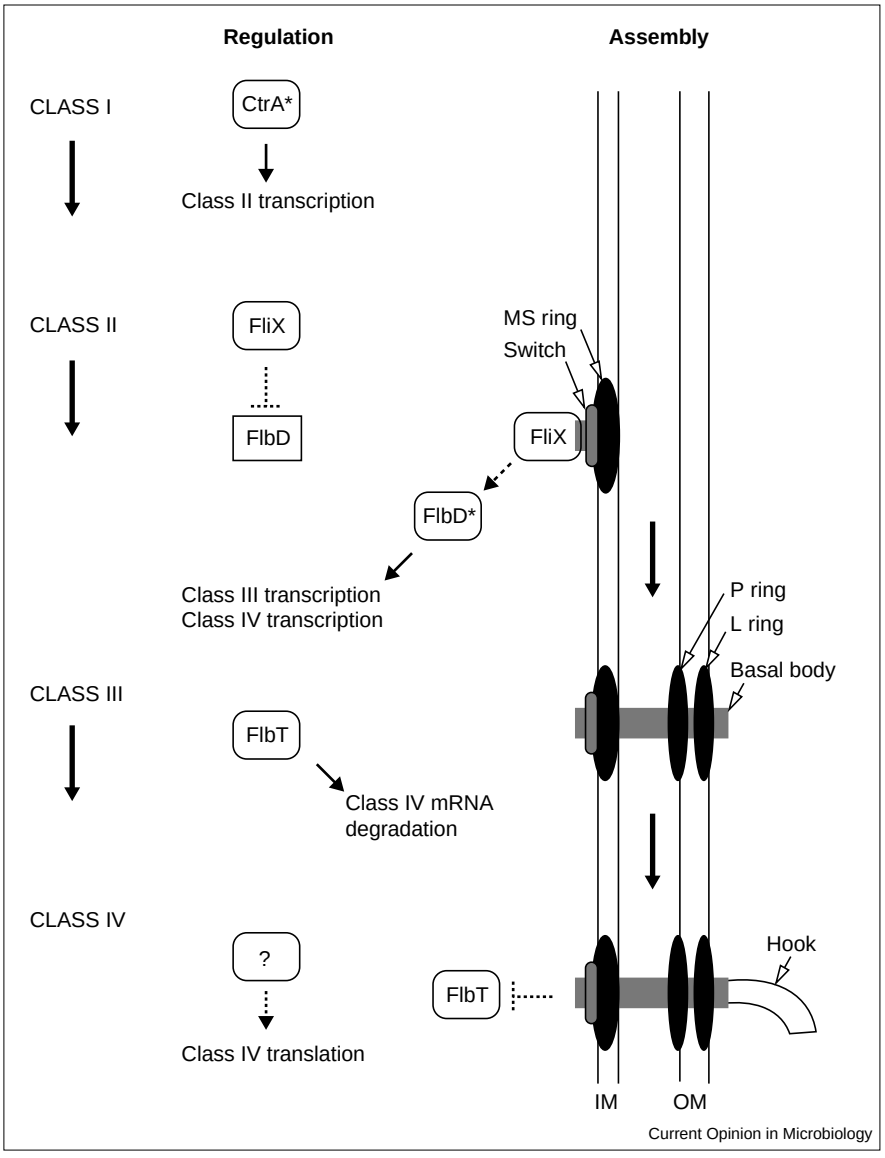
The final assembled flagellum component is the filament, which is comprised of flagellin protein [2,10,12,35]. The transcription of the genes encoding two of the major flagellins, *fljL* and *fljK*, requires FlbD activity [27]. The *fljK* gene, which encodes the most abundant 25 kD flagellin, is transcribed in the swarmer compartment of the late predivisional cell [27]; in the progeny swarmer cell, the *fljK* mRNA continues to be translated after transcription has ceased [38]. The persistence of *fljK* mRNA in the swarmer cell after division is a consequence of an unusually stable message [39]. The measured half-life of *fljK* mRNA in unsynchronized populations exceeds 15 minutes [39]. When the swarmer cell eventually differentiates into a stalked cell, the *fljK* mRNA is degraded [39]. This cell-type-specific degradation of *fljK* mRNA is accomplished through the activity of the *flbT* gene product, FlbT [39,40••]. The half-life of *fljK* mRNA in strains with mutations in *flbT* is greater than 45 minutes [39]. FlbT promotes degradation of mRNA by binding directly to the message, and *in vitro* experiments have demonstrated that this binding activity requires at least one other protein present in *C. crescentus* cell extracts [40••]. In addition to extending the half-life, *flbT* mutants also continue to express *fljK* in stalked cells [39]. Thus, FlbT can be considered as another regulator of cell-cycle gene expression. Although FlbT appears to function at a specific time, its cellular levels remain constant throughout the cell cycle [39]. Flagellum assembly apparently regulates the activity of FlbT. Indeed, in class III flagellar mutants, which do not assemble a hook structure, flagellin genes are transcribed but the mRNA is degraded in a FlbT-dependent fashion [39]. In wild-type cells, FlbT activity is probably activated in the stalked cell following the completion of flagellum assembly. In summary, the regulation of the class IV flagellar components occurs at many levels: cell-cycle cues and assembly of early flagellar structures regulate the transcription of class IV genes in a FlbD-dependent manner, and assembly of later flagellar structures controls the cell-type-specific translation of at least one class IV mRNA (that of *fljK*) through the activity of FlbT. Figure 2 illustrates a model for assembly-mediated regulatory events during flagellar biogenesis.

Flagellar assembly and chromosome partitioning regulate cell division

The earliest known event in bacterial cell division is assembly of the tubulin-like FtsZ protein into a polymeric ring (FtsZ ring) at the middle of the cell [3,4,41,42,43•]. The FtsZ ring is required throughout cytokinesis; it is necessary for constriction at the site of cell division and for recruitment of other cell division proteins, including FtsA, FtsK, FtsQ and FtsI [3,4,41,42,43•]. One might expect the formation and maintenance of the FtsZ ring to be important in the regulation of cell division. Indeed, several control points of cell division are known to directly or indirectly involve FtsZ. These include the initiation of DNA replication, chromosome partitioning and, surprisingly, flagellar assembly. The *ftsZ* gene is expressed shortly after the swarmer→stalked cell transition, when CtrA degradation relieves repression [16,44]. An FtsZ ring assembles at the midcell site of the stalked cell shortly after DNA replication commences. Following cell division, FtsZ is cleared from both the swarmer cell and the stalked cell, probably because of the instability of the unpolymerized protein [16,44].

How is assembly of the FtsZ ring timed to occur at the proper point in the cell cycle? One possibility is that formation of the FtsZ ring depends on the CtrA-dependent increase in the level of FtsZ protein present in the cell. However, when *ftsZ* is expressed inappropriately in swarmer cells, FtsZ rings are not visible until the swarmer cell differentiates into a stalked cell [45•]. This overexpression results in the

Figure 2



Regulation of flagellar gene expression by flagellar assembly. Flagellar biogenesis events proceed sequentially from top to bottom, representing temporal occurence during the cell cycle. Temporal expression of genes within the flagellar gene class hierarchy (class I–class IV) is shown on the left. Similarly, the corresponding progression of flagellar assembly is indicated on the right. Depicted in the center is a model of the regulation that coordinates assembly of flagellar structures with later steps in flagellar biogenesis. Class II transcription is activated by phosphorylated CtrA (top). The class II gene product FliX directly or indirectly inhibits FlbD activity; by an unknown mechanism, FliX 'senses' the assembly of class II flagellar structures, allowing activation of FlbD and transcription of class III and class IV genes. The class III gene product FlbT promotes degradation of class IV messenger RNA until assembly of the class III flagellar structures is complete (bottom), at which time, the class IV flagellin mRNA is translated. Large arrows reflect the temporal sequence of events. Light arrows indicate positive activity. Crossed bars indicate negative activity. Dashed symbols are used when the nature of the interaction is unknown. Likewise, please note that there is no evidence for direct interaction between FliX or FlbT and the assembled flagellar structures. Stars indicate active, phosphorylated proteins. The question mark is a hypothetical positive factor. IM, inner membrane; OM, outer membrane.

appearance of additional FtsZ rings clustered around the midcell site in the stalked cell and constriction initiates at the appropriate time, although the completion of cell division is somewhat delayed [45•]. This suggests that formation of the FtsZ ring is controlled by a distinct cell-cycle cue or by a specific property of a cell that is undergoing DNA replication.

Influence of flagellum assembly on cell division

One possibility is that this cue is, at least in part, the proper partitioning of the newly replicated chromosomal origins of replication to the poles of the predivisional cell. Efficient chromosome partitioning in *C. crescentus* requires ParA and ParB [46], cellular homologues of plasmid partitioning proteins [47–49]. ParB is a sequence-specific, DNA-binding protein that binds to sequences adjacent to the *C. crescentus* origin of replication (Cori) [46]. ParA possesses ATPase activity that is stimulated by ParB (JJ Easter, JW Gober, unpublished data). ParA and ParB are synthesized in stoichiometric amounts during the cell cycle, and depletion or overexpression of one or the other causes partitioning defects; both are essential for viability [46]. Immunolocalization experiments have revealed that ParB forms one focus at the stalked pole early in the cell cycle, and then forms two opposing polar foci later, in the predivisional cell. Polar ParA foci have also been observed in unsynchronized cultures [46]. The ParB pattern of localization mirrors that of Cori localization during the cell cycle ([50]; R Figge, JW Gober, unpublished data). Visualization of replication origins in *Bacillus subtilis* reveals a similar pattern of localization in that organism [51,52]. What function, if any, ParA and ParB may have in actively partitioning the chromosomes is unknown. However, investigation of the lethality of a

ParB depletion strain has revealed that these proteins may function in a signal transduction mechanism that links partitioning with assembly of the FtsZ ring and cell division. As cells are depleted of ParB, they form an increasing number of smooth filaments, and immunolocalization experiments revealed a concurrent decrease in the number of visible FtsZ rings (DA Mohl, JW Gober, unpublished data). DNA synthesis and transcription of *ftsZ* and *ftsQ* remain unaffected, suggesting that the lack of ParB most likely affects formation of the FtsZ ring at the level of assembly (DA Mohl, JW Gober, unpublished data). Overexpression of ParA gave an identical phenotype to depletion of ParB, reinforcing the idea that stoichiometric amounts of these proteins are necessary for proper function (DA Mohl, JW Gober, unpublished data). A proposed model is that ParB, bound to Cori, interacts with ParA at the poles of the cell, once the first steps of chromosomal partitioning have been completed. This interaction with ParA, perhaps by activating its ATPase activity or by stimulating exchange of ADP for ATP at the nucleotide-binding site, would act as a signal for cell division to commence (DA Mohl, JW Gober, unpublished data). The transduction of this signal could be through transcriptional activation of an unknown cell division gene (or genes) or through inactivation of a cell division inhibitor. The appearance of polar ParB foci precedes the appearance of FtsZ rings by approximately 20 minutes, suggesting that polar localization of a partitioning complex serves as a checkpoint that coordinates cell division with chromosome partitioning (DA Mohl, JW Gober, unpublished data).

An additional regulatory restriction placed on the cell division machinery is the assembly of the flagellum in the swarmer pole of the predivisional cell. Class II flagellar mutants display a filamentous phenotype in mid- to late-log phase, which may represent a checkpoint that delays cell division until the completion of flagellar assembly [53–55]. Two amino-terminal deletion mutants of FlbE, encoded by a class II gene, result in a dominant-negative, class-II-mutant phenotype when expressed in an otherwise wild-type strain [30••]. In the FlbE mutants, and in several other class II mutants, immunofluorescence revealed the dearth of (or, in most cases, the lack of) FtsZ rings in the filamentous cells [30••]. The cell division defect was reversed by the same *flbD–bfa* mutation that restores class III and class IV transcription in these mutants (see above) [30••]. Thus, the pathway that couples late flagellar development with assembly of early structures also regulates cell division. One proposed explanation for the regulation of cell division by assembly of early flagellar structures involves the role of FlbD in transcription of early flagellar genes [30••]. As discussed above, active FlbD is present exclusively in the swarmer compartment of the predivisional cell, after the cell division plane forms, where it represses class II genes [27,33]. Thus, delaying cell division until the early flagellar structures are assembled ensures that transcription continues from class II genes until their products are no longer needed. It is still unclear which step of the cell division process is affected by FlbD-mediated sensing of flagellar assembly, although the appearance of 'pinches' in class II mutant filamentous cells indicates that it is after the initiation of FtsZ ring assembly. Therefore, it is possible that either stability of the FtsZ ring or a later, FtsZ-ring-associated cell division gene is affected.

The precise mechanisms by which chromosome partitioning and flagellar assembly regulate assembly of the FtsZ ring remain unknown. Several *ftsZ* mutants have been identified that perturb FtsZ function at different points in cytokinesis, from assembly to constriction [56•]. Analysis of these mutants will be important tool not only in defining the activity of FtsZ and its interaction with other cell division proteins, but also in determining how different signals regulate cell division, through assembly of the FtsZ ring or through other steps in the process.

Conclusions

The levels of regulation involved in coordinating morphogenic events with the cell cycle in *C. crescentus* is astounding, and is becoming more intricate as new discoveries are made. Not only is the synthesis of a polar organelle (the flagellum) controlled by the cell cycle, but the cell cycle is, in turn, controlled by different steps in flagellar biogenesis. Furthermore, this inverse control occurs in conjunction with other regulatory mechanisms, such as the positions of the newly replicated chromosomes. Several questions remain. How is assembly of flagellar structures sensed? How does the partitioning complex arrive at and recognize the cell poles? And how are these signals transduced to FtsZ and the cell division machinery?

Acknowledgements

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- •• of outstanding interest

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This work reports the completion of sequencing of the genome (4 Mb) of *C. crescentus*. In addition to genes that encode proteins necessary for survival in the nutrient-poor aquatic environment of *C. crescentus*, the genome also contains genes that encode more than 100 known or predicted two-component signal-transduction protein members. This is more than has been found in any genome currently being sequenced, and reflects the importance of these proteins in cell cycle control.

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Using the complete *C. crescentus* genome sequence, the authors constructed DNA microarrays containing 3000 genes (approximately 80% of the genome) with which to study expression patterns during the cell cycle [5••]. Roughly 20% of the open reading frames in the genome are cell-cycle-regulated. The results indicate a correlation between temporal transcriptional patterns and the time at which gene products are required during the cell cycle. Genes that encode complexes are co-expressed, whereas genes that encode components of multiprotein structures are expressed in cascades. The global response regulator, CtrA, seems to contribute to the regulation of 26% of the genes found to be under cell cycle control.

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