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REGULATION OF ASYMMETRY AND POLARITY DURING THE *CAULOBACTER* CELL CYCLE

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I. Introduction

Cell division events resulting in dissimilar progeny are integral to differentiation processes in many prokaryotes and single-celled

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eukaryotes, and are essential for development of metazoans. The dimorphic bacterium *Caulobacter crescentus* has proven to be a useful model system for studying the generation of asymmetry during the cell cycle, as every division results in two morphologically and functionally distinct progeny cells (Fig. 1). These dramatic differences are first expressed in the predivisional cell and result in microscopically detectable polarity, in that the poles of the two cell halves

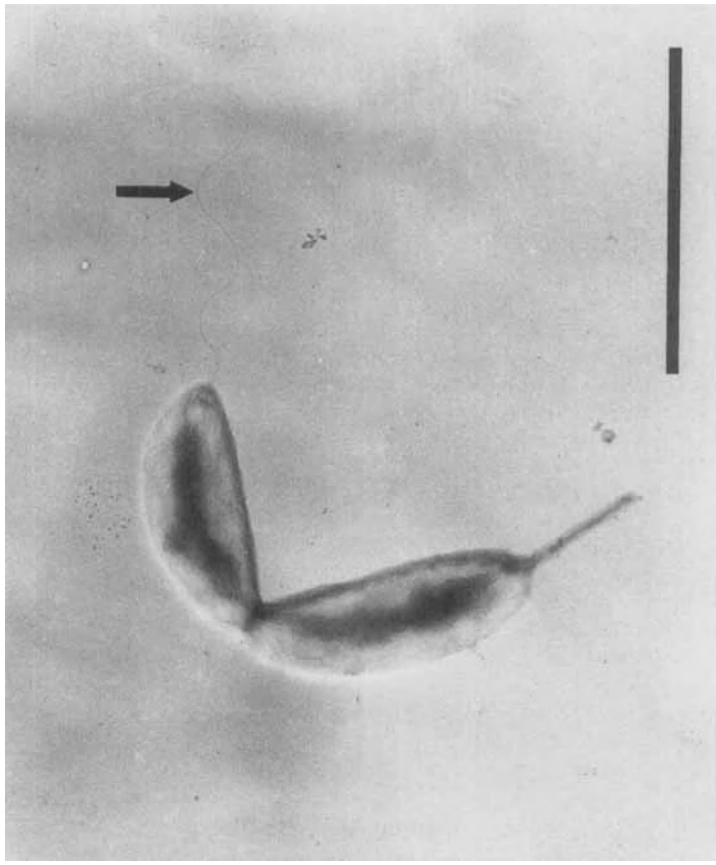

Figure 1. *Caulobacter crescentus* predivisional cell. Electron micrograph of a dividing *Caulobacter* cell with the two polar appendices: the stalk and the flagellum (arrow). The bar represents 2 μm.

are occupied by different morphological markers: the stalk and the newly synthesized flagellum (Fig. 1). We will focus in this chapter on the regulation and assembly of polar structures in *Caulobacter* and on two general mechanisms involved in generating polarity during *Caulobacter* differentiation: asymmetric transcription from the newly replicated chromosomes of the predivisional cell and differential localization of proteins to the poles of the predivisional cell.

II. The Cell Cycle

The *Caulobacter* cell cycle is typically depicted as beginning with the swarmer cell (Fig. 2), as this is the cell type that can be specifically isolated for experiments requiring synchronous cultures. Pure populations of swarmer cells can be obtained easily by density gradient centrifugation (1). Isolated swarmer cells, when incubated in fresh growth medium, proceed synchronously through the cell cycle (Fig. 2). The swarmer cell is motile by virtue of a single polar flagellum. The flagellum, in combination with a chemosensory apparatus, allows the swarmer cell to perform directed movements in response to attractants or repellents. The chemosensory apparatus is localized to the flagellated pole of the swarmer cell (2), as are multiple pili (3) and an adhesive holdfast (4). The swarmer cell is unable to replicate its chromosome and initiate cell division until it differentiates into a sessile stalked cell (Fig. 2). During differentiation of the swarmer cell into a stalked cell the flagellum is ejected, the cell loses its polar pili and chemoreceptors, and a stalk is synthesized at the same pole (see below). The adhesive holdfast is now located at the tip of the stalk and anchors the cell to available surface structures. The portion of the *Caulobacter* cell cycle designated to the swarmer cell phase is a fixed fraction, one-third of the entire cell cycle, independent of the generation time and environmental changes (4, 5). Thus, the signal that initiates the swarmer-to-stalked-cell transition is believed to be an internal cell cycle cue rather than an environmental stimulus, as is the case in two other procaryotes that exhibit cell differentiation: *Bacillus subtilis* sporulation [reviewed by (6)] and *Myxococcus xanthus* fruiting body development [reviewed by (7)].

Coincident with the loss of motility the structure of the nucleiod changes and chromosome replication is initiated. The nucleoid is a supramolecular complex consisting of the chromosome and associ-

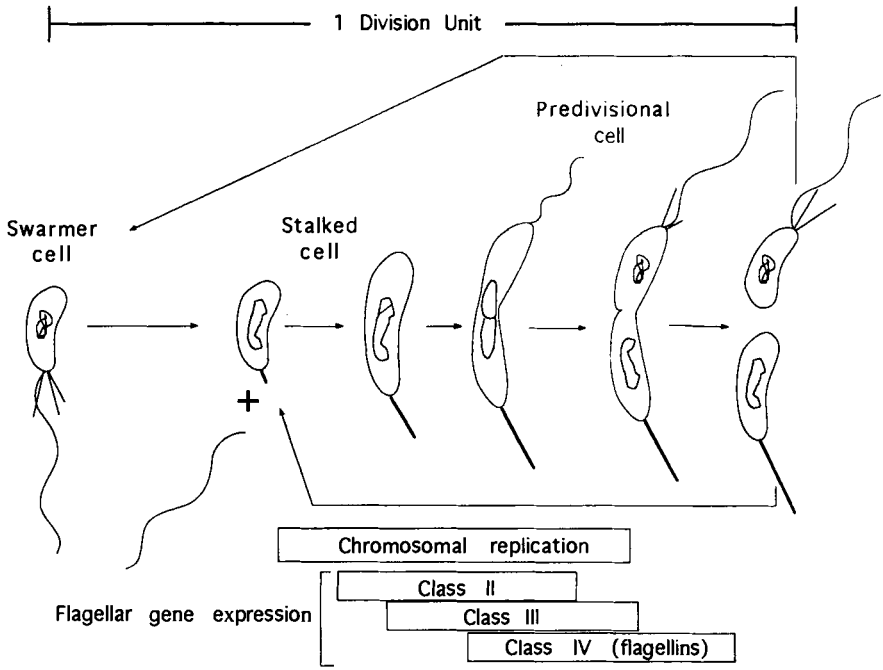

Figure 2. The *Caulobacter* cell cycle. The biogenesis and loss of polar appendages (flagellum, pili, and stalk) described in the text are shown. The nucleoid is condensed and nonreplicating in the swarmer cell, then decondenses and initiates replication upon differentiation into a stalked cell. The period of chromosomal replication and flagellar gene expression are indicated beneath the cell cycle. The full length of the cell cycle is referred to as one "division unit."

ated proteins, including DNA and RNA polymerases, nascent RNAs, and polysomes. The fast-sedimenting, more highly condensed swarmer nucleoid undergoes an abrupt transition into a slow-sedimenting, relaxed configuration present in the stalked cell (8–10; Fig. 2). The precise temporal and regulatory relationship of the nucleoid transition to morphological changes and initiation of DNA replication are not known. After replication, the new chromosomes are partitioned to the swarmer or the stalked pole of the elongating predivisional cell. The late predivisional cell contains a slow-sedi-

menting chromosome at the stalked pole and a fast-sedimenting chromosome at the swarmer pole (1; Fig. 2). Upon cell division, the progeny stalked cell immediately initiates DNA replication, whereas the chromosome that partitions to the progeny swarmer cell is unable to initiate DNA replication until later in the cell cycle when the swarmer differentiates into a stalked cell (Fig. 2). It has been demonstrated that after DNA replication the new and the old DNA strands segregate randomly (11, 12). This suggests that the two newly formed chromosomes are not marked prior to segregation. Rather, two developmentally identical, slow sedimenting chromosomes are moved to the poles and become differentially restructured by specific polar signals in the predivisional cell. The nucleoid in both cell types is membrane bound (1, 9, 10). It is not clear when a barrier forms that divides the predivisional cell into two compartments with different developmental programs, but it is likely that this occurs well before cell division actually takes place. The presence of two structurally distinct chromosomes in the predivisional cell, having differential replicative potentials, is one of the fundamental examples of inherent asymmetry in the *Caulobacter* life cycle.

While the chromosome is replicating and the cell division process is initiating, a new flagellum is synthesized at the pole opposite the stalk. Flagellar assembly is a highly regulated process coupled tightly to the *Caulobacter* cell cycle. It is the best understood example of how the developmental program of *Caulobacter* results in cell polarity and will be discussed in detail later. After synthesis and assembly of the polar appendages and chromosome segregation have been completed, cell division results from progressive pinching of the cell wall at the division site.

III. Stalk Formation

The stalk is a thin cylindrical extension of the cell surface. The cell wall and membranes of the stalk are continuous with the surface layers of the cell body (13). The core of the stalk contains cytoplasmic material that seems to be continuous with the cell's cytoplasm but is devoid of DNA and ribosomes (14, 15). Studies with cell-free extracts demonstrated that separated stalk compartments possess a glucose uptake system, but the growth substrate accumulates and is not metabolized (16, 17). From this, and the absence of

several other metabolic enzyme activities in stalks (17, 18), it was concluded that the stalk simply functions as an extension of the cell's absorptive surface, presumably an advantage to these organisms in an environment of low nutrient concentration. This is in agreement with the observation that dilute nutrient environments promote stalk elongation in *Caulobacter* (14). The stalk is traversed at intervals by dense rings known as cross bands, which may provide rigidity to the stalk by attaching the inner and the outer membrane (13).

Little is known about the mechanisms involved in stalk biogenesis. It has been proposed that formation of the stalk is the equivalent of a polar cell division event (19). Stalk biosynthesis is localized to a confined area at the juncture of the stalk with the cell (20). Growth of the surface array, a hexagonally packed periodic surface layer that covers the entire cell including the stalk, is also restricted to the stalk–cell body junction (21). Surface array biogenesis also takes place at the site of cell division late in the division process (21). If cell wall biosynthesis operative in stalk growth and in cell division has a common molecular basis, the two processes must be regulated separately. This can be concluded from the observation that low phosphate concentrations cause an up to 10-fold increase in stalk length while cell division is still proceeding in an apparently normal fashion (20). Phosphate limitation thus derepresses synthesis of cell wall material at the site of stalk formation but does not detectably modify the regulation of wall synthesis leading to cell division.

One possible difference in cell wall synthesis at the division site and at the site of stalk growth emerged from comparison of penicillin-binding proteins (PBPs) contained in isolated stalks and in the rest of the cell body. PBPs were originally identified by their specific covalent binding to labeled penicillin. They are membrane proteins that have enzymatic activities involved in bacterial cell wall synthesis [transpeptidase, transglycosylase; reviewed by Waxman and Strominger, (22)]. *C. crescentus* possesses between 15 and 20 different PBPs (23–25). To study the involvement of PBPs in stalk biogenesis, the distribution of PBPs in stalks shed from a stalk abscission mutant was compared with that of total cell envelopes. The stalks lacked both PBP 1A and PBP 3, which are located in the inner membrane of growing cells (26). PBP X and PBP Y, which are minor PBPs in the total cell envelope fraction, were greatly enriched in the isolated stalks (26). In addition to that, a mecillinam-resistant, stalk-

defective mutant lacked both PBP X and PBP Y (26). This findings suggest that PBP X and PBP Y are involved in cell wall synthesis at the stalk-cell body junction. Recently, a *C. crescentus* mutant has been isolated that is blocked at the swarmer-to-stalk cell transition, and has a defect in stalk growth (P. J. Kang and L. Shapiro, in preparation). The mutated gene encodes a protein with similarity to PBP 2 of *E. coli*. There is evidence that the *Caulobacter* PBP 2 homolog and PBP Y are the same protein. The predicted size of the PBP 2 homolog agrees well with the size of PBP Y. Furthermore, the isolated mutant also shows an increased cell diameter. In *E. coli*, PBP 2 is a specific target of mecillinam, a β-lactam antibiotic that causes an increase in cell diameter. In *C. crescentus*, mecillinam not only causes an increase in cell diameter, but also inhibits stalk biosynthesis (25, 26). Since the isolated mutant is not viable at the restrictive temperature, it is possible that PBP Y is also required for general cell wall biosynthesis in *C. crescentus*.

Another possible candidate playing a role in *Caulobacter* stalk biosynthesis is the tubulin-like FtsZ protein. FtsZ is an essential guanosine 5′-triphosphate (GTP)-binding protein required for the initiation of cell division and in *E. coli* and *B. subtilis* this protein has been shown to be localized to the site of cell division (27). A model has been proposed in which FtsZ molecules form a cytokinetic ring at the division site through a reversible self-assembly regulated by a GTP/GDP cycle (29). It has, in fact, been demonstrated that purified FtsZ polymerizes into macroscopic filaments *in vitro* (28). The gene coding for the FtsZ homolog in *C. crescentus* has recently been cloned (Y. Brun and L. Shapiro, unpublished results). It has been shown that the FtsZ protein is absent in swarmer cells and appears at the time of initiation of stalk biosynthesis, reaching its highest concentration during cell division. The expression of *ftsZ* from a constitutive promoter resulted in stalk defects (Y. Brun and L. Shapiro, unpublished results). The exact role of FtsZ in *Caulobacter* stalk formation and the identity of other components involved in the cellular division process remain to be determined.

IV. Flagellar Structure and Assembly

Flagellar biogenesis is the most intensively studied and best understood developmental event in the *Caulobacter* life cycle. The

synthesis of this macromolecular structure presents us with two questions of fundamental interest in developmental biology. The first involves timing: How is the flagellar cascade initiated in response to signals from the cell cycle and how is flagellar gene expression limited to the predivisional stage of the cell cycle? The second involves spatial distribution: How are all the components of the flagellum targeted to a specific place in the cell, the pole opposite the stalk?

Over the past decade, these questions have begun to yield to molecular genetic analysis. An estimated 50 genes are required for motility in *Caulobacter* (29). This number is similar to that required for flagellar synthesis and function in enteric bacteria [reviewed by (30)]. Currently, about one-half of these genes have been cloned in *Caulobacter,* most of them by complementation of nonmotile mutants. The identity of the isolated genes was resolved in most cases by comparison with homologs involved in flagellar assembly in other bacteria. Epistasis experiments were used to examine the regulation of expression of the isolated flagellar genes. This analysis revealed that most of these genes can be organized into a three-tiered hierarchy (31–33; Figs. 2 and 3), conceptually similar to the flagellar hierarchies of *E. coli* and *Salmonella typhimurium* (30). Disruption of any member of a gene class results in the disruption of the expression of genes placed lower in the hierarchy. Class II genes code for proteins known to reside in or at the inner membrane (Fig. 3). Two important exceptions to this rule are transcription factors required for expression of Class III and IV genes (see below). Class III genes code for structural components of the basal body and hook and Class IV genes code for the flagellin subunits of the flagellar filament (Fig. 3). By analogy to *S. typhimurium* flagellar biogenesis, it is likely that morphological features of the developing flagellum serve as regulatory cues for the transcriptional hierarchy (43). The fact that the genes coding for the basal body-hook complex of *C. crescentus* are divided into two regulatory classes (Classes II and III; Fig. 3), compared to a single class in enterics (Class 2; 30), suggests that there is an additional structural checkpoint (44) controlling flagellar gene expression in *Caulobacter.*

A second important distinction between these systems is that in *Caulobacter* induction of the flagellar hierarchy does not seem to be under environmental control, but is instead dependent on cell cycle

signals. In enterics the control of motility is very complex. Expression of the Class I master operon is sensitive to catabolite repression and is positively regulated by the cAMP–CAP complex (45, 46). The master operon is also positively regulated by the H-NS protein, a major component of bacterial chromatin (47, 48), and the heat shock proteins DnaK, DnaJ, and GrpE are also required for motility (49). In addition, the organization of the bacterial membrane affects swarming properties of *E. coli*. Flagellum formation is impaired in lipopolysaccharide-deficient strains (50) and mutations disrupting phosphatidylethanolamine biosynthesis lacked flagella and showed reduced transcription of the Class 1 master operon (51). Synthesis of flagella is also shut off when *E. coli* cells are grown under a number of adverse conditions, most likely due to transcriptional repression of the master operon (52, 53). Interestingly, there is increasing evidence that the flagellar regulon in enterics is also under cell cycle control (54, 55); there may be more parallels between regulation of the flagellar hierarchy in *Caulobacter* and enterics than we are currently aware of.

Class I of the *Caulobacter* hierarchy has been reserved for the unidentified factor(s) that link the initiation of the flagellar cascade to cell cycle cues. Recently, a temperature sensitive mutant has been isolated based on an altered transcription pattern of Class II flagellar genes that is not viable at the restrictive temperature (56). The mutation is located in a gene that has now been designated *ctrA* (*c*ell cycle *t*ranscriptional *r*egulator) encoding a member of a family of response regulators. Response regulators are part of a simple bacterial signaling system that consists of two components: a sensor, often located in the cytoplasmic membrane, which monitors some environmental parameter, and a response regulator that mediates changes in gene expression or motility in response to a sensor signal [reviewed by (57)]. The signal is transmitted from the sensor, a histidine kinase, to the response regulator by transfer of a phosphoryl group that activates the response regulator. The *ctrA* gene is essential for cell growth and appears to regulate transcription of Class II flagellar genes, either directly or indirectly (56). The identity of the signal and the sensor responsible for CtrA activation is unknown.

The flagellar structure, consisting of three major components [the basal body, the hook, and the filament (Fig. 3)], is highly conserved in *Caulobacter* (34–36, 58, 59) and the enterics (37, 60, 61). The

basal body anchors the flagellum in the cell envelope and acts as a rotor. It consists of a pair of rings in the inner membrane and a pair of rings in the peptidoglycan-outer-membrane layer. These pairs of rings are connected by the rod. Attached to the basal body on the outside of the cell is a flexible hook that serves as a connecting element to the helical filament. The composition and temporal expression patterns of the genes in Classes II, III, and IV reflect the order of assembly of the gene products into the flagellar structure (Fig. 3). Flagellar structural proteins encoded by Class II are laid down at the inner membrane followed by the assembly of Class III and Class IV structural proteins in a proximal to distal progression.

In *S. typhimurium*, radiolabeling studies with temperature sensitive mutants (62), as well as electron microscopic analysis of flagellar structural intermediates in flagellar mutants (63, 64), revealed a defined morphogenetic pathway for the biogenesis of the flagellum (30). Several of the earliest expressed Class II flagellar genes have been cloned in *C. crescentus*. The *fliF* gene encodes the protein that forms the compound MS-ring of the basal body and is the first gene in an operon that also contains *flbD*, a gene encoding a transcriptional activator for Class III and Class IV genes (65–68; 68a). Insertion of the FliF protein into the membrane is the initial event of flagellar biogenesis in *S. typhimurium* (62, 63). It seems likely that localized membrane insertion of FliF is one of the critical steps in targeting the assembly of the *Caulobacter* flagellum to the swarmer pole of the predivisional cell. Another Class II operon, *fliLM*, encodes FliL, a protein with a general motility function in *Caulobacter* (42). The *fliL* gene is required only for the function of the flagellum, but not for flagellar assembly (42). The *fliM* gene encodes a switch protein. The switch apparatus consists of three proteins, FliM, FliN, and FliG, which are located at the cytoplasmic face of the MS-ring (37, 69; Fig. 3). The switch proteins are essential elements of the motor and control the direction of flagellar rotation in response to chemotactic signals (70–72). Chemotactic signals are transduced from the polar membrane receptors to the flagellar motor via a phosphorylation cascade involving the receptor-coupled sensor kinase CheA and the response regulator CheY [reviewed by (73)]. The phosphorylated form of CheY interacts *in vitro* with the FliM protein (74).

Several *Caulobacter* Class II flagellar genes including *flhA*, *fliQR*,

and *fliP* encode proteins that are probably not part of the flagellar structure, but are required for flagellar biogenesis. The predicted amino acid sequence of each of these proteins suggests that they are integral membrane proteins, and FliQ and FliR have been shown to be associated with the cell membrane (41). There is indirect evidence that these proteins might be involved in the export of flagellar structural proteins. Flagellar components like flagellins or hook protein subunits, which are assembled at the distal end of the growing flagellar structure, lack signal sequences and are postulated to be exported to the site of polymerization by a flagellum-specific export apparatus (30, 75). The proteins are believed to be directed through the core of the nascent structure itself. Such a core has been observed in the case of the filament and the hook (10a, 59, 76) and is also predicted to exist in the rod. The product of the *Caulobacter flhA* gene (formerly called *flbF*) is homologous to the LcrD protein of *Yersinia* spp. (40). The *lcrD* gene is part of the low calcium response regulon of *Yersinia*, which controls the expression of virulence genes (*yops*) in response to the ambient calcium concentration [reviewed by (77)]. Mutations in *lcrD* affect both the transcription and the export of virulence factors (78, 79). The FlhA/LcrD family now contains several members from pathogenic species that are postulated to be part of a novel signal sequence-independent secretion pathway that includes HrpO from *Pseudomonas solanacerum* (80), InvA from *S. typhimurium* (81), and MxiA from *Shigella flexneri* (82). By analogy to a possible LcrD function in export of virulence factors, a role for FlhA in export of flagellar proteins has been proposed (30).

The *Caulobacter fliQR* operon encodes two very hydrophobic membrane proteins that belong to another family of proteins, which was also implicated in the export of virulence factors (41). These include the MopD and MopE proteins from *Erwinia carotovora* (83), the Spa9 and Spa29 proteins from *S. flexneri* (84), the SpaP and SpaQ proteins from *S. typhimurium* (85), and the YscS protein from *Y. pestis* (86). The *fliP* gene of *Caulobacter* has recently been sequenced (38) and sequence comparison has shown that it encodes a homolog of virulence factors in *Erwinia*, *Xanthomonas*, *Salmonella*, and *Shigella* (83–85, 87). The early expression of these Class II genes is consistent with a potential role in the subsequent export of the rod, hook, and filament monomers. However, experimental data

demonstrating a role for these proteins in the export of flagellar components has not yet been obtained.

The genes encoding the flagellar P-ring (*flgI*) and L-ring (*flgH*) have been cloned and sequenced (88, 89). In addition to the MS-, P-, and L-rings the *Caulobacter* basal body has a ring (E-ring) that is not present in the enteric flagellar structure (34; Fig. 3). In contrast to all the other exported flagellar proteins, the E-, L-, and P-ring proteins have cleavable leader sequences and are thought to be exported by the primary SecA-dependent cellular export pathway (90, 91). This presents an interesting question: Are these proteins exported to the periplasm by a localized general secretory apparatus, or are they randomly translocated to the periplasm and positioned to the pole by movement in the periplasmic compartment? The rod upon which the rings are mounted has two parts, a proximal rod and a distal rod (Fig. 3), and two *Caulobacter* genes (*flgG* and *flgF*) encoding the homologs to the enteric rod proteins have been isolated and characterized (92).

The external components of the flagellum are assembled next. The hook is composed of monomers of a single polypeptide encoded by the *flgE* gene (58, 56, 93, 94). In enterics, several proteins have been shown to be involved in the polymerization of FlgE monomers (95), the control of hook length (96), and the formation of the hook–filament junction (97). Although a *C. crescentus* polyhook mutant has been described (94) no genes have been isolated thus far that encode homologs of the proteins that control hook length. Hook-associated proteins that, by analogy to enterics, form the hook–filament junction in *Caulobacter*, are encoded by the *flaQN* operon (98). The *Caulobacter* flagellar filament is formed by three subunits of distinct molecular mass: 25, 27, and 29 kDa (35, 99–101). This formation is in contrast to the single subunit of the enteric filament (30). *S. typhimurium* has two serotypically distinct flagellins that are expressed in a mutually exclusive fashion due to phase variation (102). The *Caulobacter* 25 kDa flagellin is the most abundant of the flagellins and is encoded by homologous genes located in at least two distinct regions of the chromosome (103, 104). One of the genes encoding a 25 kDa flagellin, *fljK*, is located in a cluster with *fljL* and *fljJ*, the genes coding for the 27 and 29 kDa flagellins (105, 104). Immunological studies have demonstrated that the three flagellin

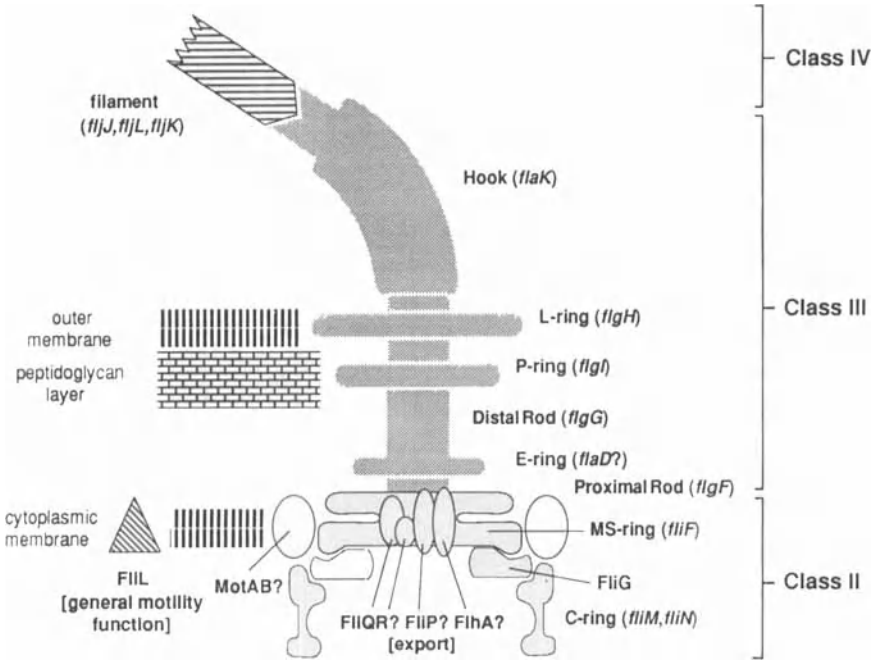

Figure 3. The *C. crescentus* flagellum. The *C. crescentus* structure is adapted from Stallmeyer et al. (34), Driks et al. (35), and Trachtenberg and DeRosier (36). The organization of the basal body attachments in the cytoplasm are adapted from that proposed for the *S. typhimurium* basal body (37). The positioning of the proteins FliQ, FliR, FliP, and FlhA is speculative and based on the assumption that all four are membrane proteins and might take part in the flagellar specific export pathway (38–41). Function and location of FliL is according to Jenal et al. (42). Flagellar structures composed of proteins encoded by Class II (stipled), Class III (shades of gray), and Class IV genes (cross-bars) are indicated on the right.

subunits are assembled in a distinct order (35, 106). The 29 kDa flagellin is assembled proximal to the hook, followed by the 27 kDa flagellin and finally, at the distal end of the filament, by the 25 kDa flagellin. Genetic studies have shown that all three flagellin types are required for normal motility in *Caulobacter* (104).

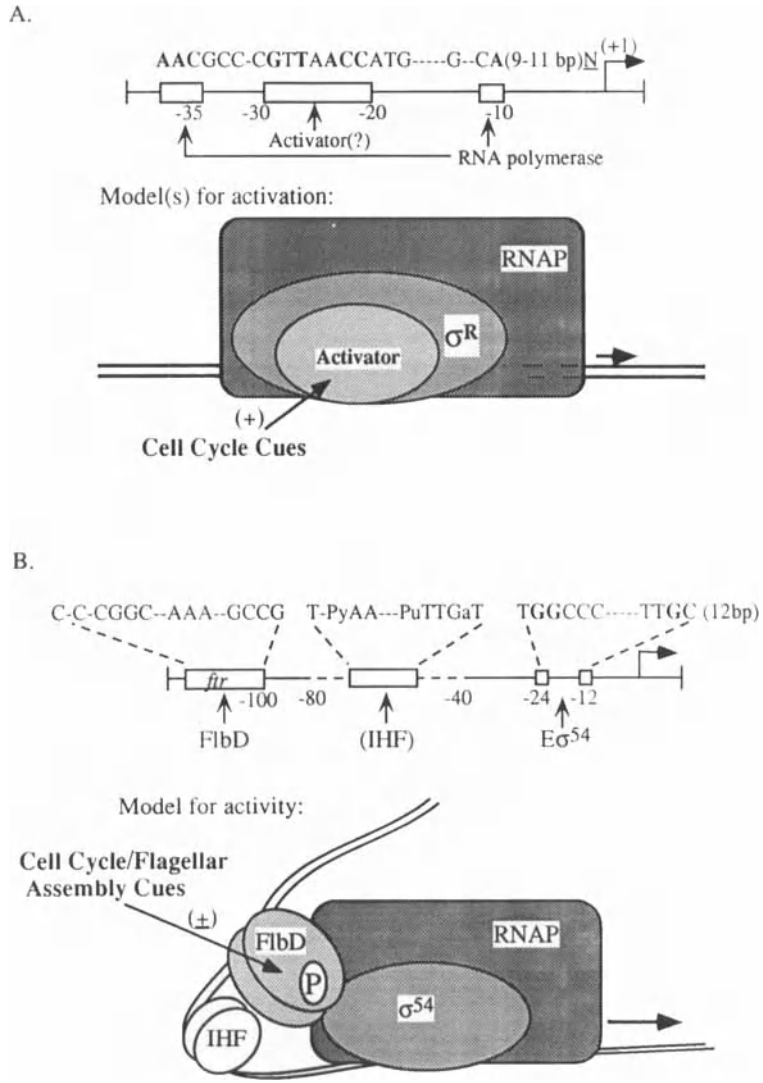

Figure 4. Models for activation of Class II and FlbD-dependent Class III and Class IV flagellar promoters. (A) Class II promoter structure. The consensus sequence is based on comparison of the *fliLM*, *fliQR*, *fliF*, and *ccrM* promoters (68, 107–110). The nucleotides indicated in bold are conserved in all four promoter sequences, those not in bold are conserved in three of the four promoters. The schematic beneath the