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Signal transduction mechanisms in *Caulobacter crescentus* development and cell cycle control

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Abstract

The life cycle of the aquatic bacterium *Caulobacter crescentus* includes an asymmetric cell division and an obligate cell differentiation. Each cell division gives rise to a motile but replication inert swarmer cell and a sessile, replication competent stalked cell. While the stalked progeny immediately reinitiates DNA replication and cell division, the swarmer cell remains motile and chemotactically active for a constant period of the cell cycle before it differentiates into a stalked cell. During this process, the cell looses motility by ejecting the flagellum, synthesizes a stalk and eventually initiates chromosome replication and cell division. The link of morphogenic transitions to the replicative cycle of *Caulobacter* implies that the developmental programs which determine asymmetry and cell differentiation must be tightly connected with cell cycle control. This has been confirmed by the recent identification of signal transduction mechanisms, which are involved in temporal and spatial control of both development and cell cycle. Interestingly, the cell has recruited two-component signal transduction systems for this internal control, a family of regulatory proteins which usually are involved in the information transfer between the environment and the inside of a cell. The response regulator protein CtrA controls several key cell cycle events like the initiation of DNA replication, DNA methylation, cell division, and flagellar biogenesis. The activity of this master regulator is subject to complex temporal and spatial control during the *C. crescentus* cell cycle, including regulated transcription, phosphorylation and degradation. Three membrane bound sensor kinases have been proposed to control the phosphorylation status of CtrA. Two of these, CckA and DivJ, exhibit specific subcellular localization and, in the case of CckA, dynamic rearrangement in the course of the cell cycle. These findings support the idea that the developmental program of *C. crescentus* is controlled at least in part by localized cues that act as checkpoints for the control of morphological changes and cell cycle progression.

Keywords: Bacterial cell cycle; Cell differentiation; Flagellum; Two-component signal transduction; *Caulobacter crescentus*

Contents

1. Introduction

Unlike most other bacteria, *Caulobacter crescentus* divides asymmetrically, generating two progeny with distinct developmental programs and behavior (Fig. 1). The stalked cell is sessile and attaches to surfaces by means

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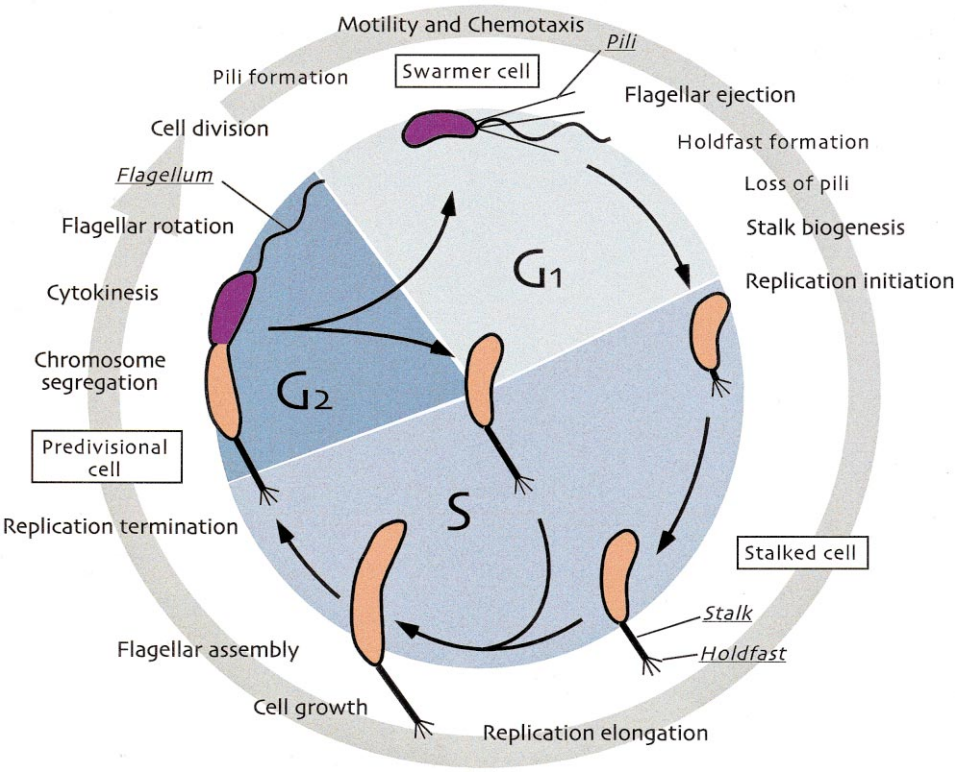

Fig. 1. Schematic diagram of the *C. crescentus* cell cycle. The three different cell types of *C. crescentus*, the stalked cell, the swarmer cell, and the predivisional cell are depicted with polar appendices underlined. The clockwise orientation of the cell cycle is indicated by arrows. The developmental and cell cycle events described in the text are highlighted at the time when they take place during the life cycle. The *Caulobacter* cell cycle is divided into three distinct phases: The G1-phase includes the motile stage and the swarmer-to-stalked cell transition; the S-phase defines the period of active chromosome replication; the G2-phase outlines cytokinesis and cell division stages. The length of the S-phase is identical for both the newly differentiated (unexperienced) stalked cell and the (experienced) stalked cell emerging from cell division.

of an adhesive holdfast located at the tip of the polar stalk [1–3]. The stalk is an extension of the cell envelope, containing the outer and inner membrane as well as the peptidoglycan layer [4,5]. Directed motility of the swarmer cell is mediated by a single flagellum and chemoreceptors located at the same pole. The two daughter cells also differ with respect to their potential for DNA replication. While the newborn stalked cell begins to duplicate its chromosome immediately and starts a new cell division cycle, the chromosome inherited by the swarmer progeny remains replication inert for an extended period. The swarmer cell regains the ability to replicate and divide only after it has differentiated into a stalked cell (Fig. 1). During this transition, the cell sheds the flagellar structure, looses the pili and the chemotaxis apparatus, and assembles the holdfast and the stalk at the pole previously occupied by the flagellum. Regardless of the doubling time, the duration of the motility period (G1-phase) is a constant fraction of the entire cell cycle (G1+S+G2, Fig. 1). This suggests that at least some of the signals that trigger the swarmer-to-stalked cell transition must be internal cues constituting a biological clock that controls development.

Once the swarmer cell has differentiated into a stalked cell, replication initiates and the cell elongates to double its size (S-phase, Fig. 1). At this stage of the cell cycle, a single flagellum is assembled at the swarmer pole, the pole opposite of the stalk. The two newly formed chromosomes are partitioned to the poles of the cell and in a subsequent step, cytokinesis leads to septum formation and ultimately cell division (G2-phase, Fig. 1). The separation of the two compartments fully establishes the different developmental programs of the two cell halves of the predivisional cell. The two polar appendices, stalk and flagellum, provide visible evidence of the inherent asymmetry of the predivisional cell at this stage. The flagellum has reached its fully assembled configuration and starts rotating immediately before the two daughter cells separate. Pili are formed at the flagellated pole of the swarmer cell after cell division and are lost coincident with the ejection of the flagellum during the swarmer-to-stalked cell transition (Fig. 1). The significance of the pili for the SW cell is not obvious but they could be facilitating reattachment to surfaces when swarmer cells end their adventurous phase and differentiate into sessile stalked cells.

Each *Caulobacter* cell cycle contains two obligate cell differentiation events, the first generating a swarmer cell during division and the second directing the swarmer cell back into the replicative cycle. Cell differentiation in *Cau-*

lobacter is thus intimately connected with cell proliferation, suggesting that a single regulatory system may control both development and the cell cycle. Both cell cycle and development can easily be examined in *Caulobacter*. Pure populations of swarmer cells can be prepared and their progression through the cell division cycle can be monitored microscopically and explored with molecular techniques. The basic questions of temporal and spatial control of development and the cell division cycle can therefore be readily addressed in this system. How is asymmetry generated in the predivisional cell? What controls the swarmer-to-stalked cell differentiation? How is the cell cycle interconnected with development and which signal transduction mechanisms are used by *Caulobacter* to control and coordinate these events?

Bacteria generally live in changing environments and in order to survive, they have evolved the ability to quickly adapt to a wide range of different external conditions. This is accomplished by sophisticated signal transduction strategies which employ two-component regulatory systems [6–8]. Two-component systems allow signal transduction across biological membranes and use phosphorylation as a means of information transfer. Sensing of outside signals is accomplished by a sensor kinase, which in most cases is membrane-located. In general, the N-terminus of the sensor functions as an input domain and, upon stimulation, a transmitter domain at the C-terminus of the sensor kinase is able to autophosphorylate at a conserved histidine residue using the γ-phosphoryl group of ATP. The phosphate residue is then transferred from the histidine of the sensor kinase to a conserved aspartate residue in the N-terminal receiver domain of the response regulator protein. The phosphorylation state of the receiver domain modulates the activity of a unique C-terminal output domain of the response regulator, in most cases a transcriptional regulator, to elicit an adaptive response to the stimulus. By combining a wide range of input and output domains with conserved transmitter and receiver modules, evolution has provided a variety of different signal transduction circuits.

Recent results have indicated that bacteria not only use two-component signal transduction systems to respond to the outside world, but also to control their internal organization and to direct and coordinate the periodic processes of cellular replication. Here, I discuss recent studies in *C. crescentus* in which two-component regulatory systems have been identified as key components of signal transduction pathways involved in controlling cell differentiation and cell cycle progression (for recent reviews on similar topics, see also [9,10]). One of the most exciting findings is that several of the sensory systems are localized to specific sites of the cell, indicating that development is controlled by specifically positioned information and local cellular cues. The review will focus on the mechanisms involved in temporal and spatial control of the biogenesis of *C. crescentus* polar structures and in the developmental control of chromosome replication and cell division.

2. Polar morphogenesis

An intrinsic property of *C. crescentus* development is the substantial remodeling of the cell poles during the cell cycle. Cell division gives rise to two different progeny cells with one new pole each. Both new poles will acquire flagella in their extant cell cycles and will become stalked poles in the consecutive round of division. Once a pole is occupied by a stalk, it does not further change its identity. The first visual event of pole development is the assembly of a single flagellum at the swarmer pole (the pole opposite the stalk) of the predivisional cell followed by the assembly of pili at the same site after cell division. Both the pili and the flagellum are removed during the transition of a swarmer into a stalked cell followed by the formation of a stalk at the same pole. *Caulobacter* mutants with a defective flagellar ejection process often carry a flagellum at the tip of a newly formed stalk [11–13]. In these mutant cells, the flagellum is not shed during cell differentiation and, as a consequence, is pushed out by the growing stalk. This illustrates that flagellar assembly, flagellar ejection and stalk biogenesis are sequential developmental processes that take place at the same subcellular location.

2.1. Flagellar assembly

2.1.1. Control of flagellar gene expression by assembly checkpoints

Flagellar biogenesis is the best understood and most intensively studied aspect of cellular differentiation in *C. crescentus*. A number of flagellar genes have been cloned by complementation and the structure of the *Caulobacter* flagellum has been studied by electron microscopy [14–16] (Fig. 2). The MS-ring (FliF) in the cytoplasmic membrane anchors the flagellum in the cell envelope and forms the platform for the assembly of the entire structure. On its cytoplasmic site, the MS-ring interacts with proteins of the switch apparatus (FliG, FliM, FliN) and on the outside, it is connected to the rod proteins (FlgG, FlgF). The switch controls the direction of flagellar rotation in response to environmental stimuli. The rod is stabilized by two additional rings in the planes of the peptidoglycan layer (P-ring, FlgI) and the outer membrane (L-ring, FlgH). Attached to the cell distal portion of the rod is a flexible hook (FlgE), which connects the basal body to the long rigid flagellar filament (FljJ, FljK and FljL). The axial proteins are translocated out of the cell to their site of assembly by a flagellar-specific export apparatus (FliP, FliQ, FliR, FlhA, FliI) (see Fig. 2).

Epistasis experiments have grouped most of the flagellar genes into three classes of a complex regulatory hierarchy, which are expressed periodically in the cell cycle in a se-

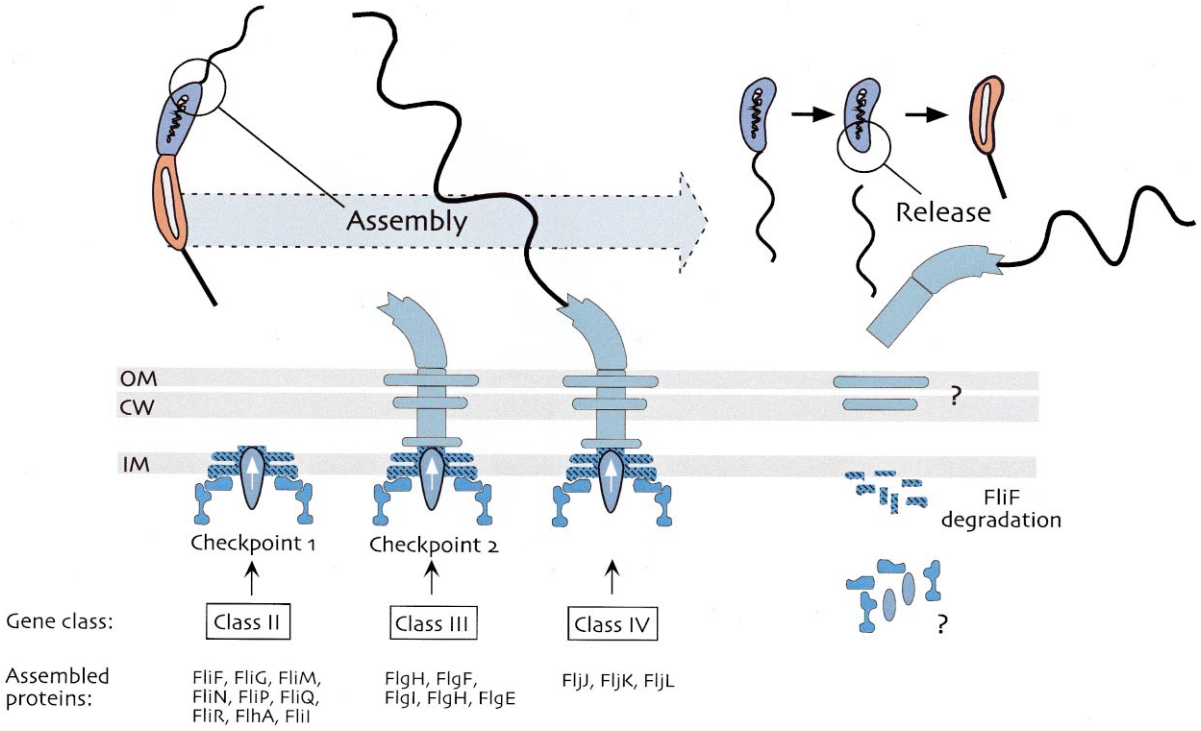

Fig. 2. Assembly and ejection of the polar *C. crescentus* flagellum. Flagellar assembly at the swarmer pole of the predivisional cell occurs in three consecutive steps in a proximal to distal way (left). Products of class II flagellar genes are assembled first in and around the inner membrane where they form the MS-ring-switch-export intermediate structure. This structure acts as a checkpoint for the expression of class III flagellar genes. The corresponding proteins are assembled next into rod, outer rings and hook. The hook-basal body structure then acts as a regulatory checkpoint for the expression of class IV flagellar genes. Class IV genes encode filament proteins which complete the assembly of the structure. The proteins encoded by the three gene classes are discussed in the text and are indicated below the substructures into which they are assembled. The process of flagellar release is shown schematically on the right. The entire axial part of the flagellum, including rod, hook and filament, is ejected into the medium during the swarmer-to-stalked cell transition. Degradation of the MS-ring anchor protein FliF is shown schematically. Question marks point to the unknown fate of outer ring, switch and export components after flagellar ejection.

quence that corresponds to the order of assembly of the gene products into the growing flagellum (Figs. 2 and 3) [17–21] (reviewed in [22]). All class II genes are required for the expression of class III genes, which in turn are necessary for the expression of class IV flagellar genes. This has led to the proposal that structural intermediates of the flagellum function as morphological checkpoints, which control the expression of the next set of proteins (Fig. 2). The class II genes, which are near the top of the hierarchy, encode the MS-ring subunits, the switch and the flagellar export components [23–29]. Class III genes encode the rod, the outer rings, the hook and hook-associated proteins [18,30–33], while class IV comprises two genes, *fljK* and *fljL*, which encode filament subunits [19,34]. The gene encoding the third filament protein, *fljJ*, is also transcribed periodically in the cell cycle [19] but does not seem to be part of the flagellar hierarchy. Class I has been reserved for genes required for the expression of class II genes and thus for the correct cell cycle timing of the flagellar hierarchy.

The expression of flagellar genes is thus coordinated with flagellar assembly by two distinct checkpoints of structural intermediates (Fig. 2). In contrast to *Caulobacter*, a single checkpoint exists in *Salmonella typhimurium* where the expression of flagellin genes is inhibited by the anti-sigma factor FlgM [35]. Transcription of the flagellin genes in *S. typhimurium* requires the sigma factor σ^{28}. FlgM directly binds to σ^{28} and in this way destabilizes the interaction between the sigma factor and core RNA polymerase [36]. Completion of the hook-basal body complex allows FlgM to be exported by the flagellar export apparatus resulting in the release of the FlgM inhibitory effect inside the cell [37]. In *Caulobacter*, the molecular basis for the coupling of assembly of the flagellum with gene expression is not clear yet. Mutations that bypass the requirement of the first flagellar checkpoint (products of class II genes) for the transcription of class III genes and the *fljL* flagellin gene have been isolated and termed *bfa* (bypass of flagellar assembly) [38]. Both *fljL* and *fljK* are transcribed in *bfa* mutants but the corresponding flagellin proteins are not synthesized, suggesting that both genes are also subject to post-transcriptional control [38]. Both genes are also transcribed in all class III flagellar mutants tested but their mRNA is not translated in the absence of any of the known class III proteins [39]. This indicates that the second flagellar assembly checkpoint (basal body-hook complex) regulates the expression of the *fljL* and *fljK* flagellin genes post-transcriptionally. Re-

cently, a novel regulatory component, FlbT, has been implicated in the control of stability of *fljK* mRNA [115]. Mutations in *flbT* can restore flagellin synthesis in class III flagellar mutants, suggesting that FlbT couples flagellin expression to the assembly of the hook-basal body complex [115].

A unique feature of flagellar assembly in *Caulobacter* is that strains with mutations in class II flagellar genes also display striking cell division defects [24,26,29–32]. This indicates that the correct assembly of the MS-ring-switch complex is required for coupling of flagellar assembly to cell division and that this structure functions as developmental checkpoint in *C. crescentus* to delay cell division until an early step in flagellar assembly is completed. Thus, not only does the cell cycle control the timing of polar morphogenesis in *C. crescentus* but developmental cues also seem to control events of the division cycle. This underscores the tight connection between *C. crescentus* development and the cell division cycle.

2.1.2. Cell cycle control of flagellar gene expression

2.1.2.1. Control of early flagellar genes (class II).

Class II genes are transcribed early in the cell cycle after chromosome replication has initiated in the stalked cell (Fig. 3). The periodic activation of class II promoters is thought to be controlled by cell cycle cues. The nature of the signal is not known but DNA synthesis probably acts as a cell cycle coordinator. Inhibition of DNA replication results in the inhibition of hook and flagellin synthesis [40,41]. While inhibition of DNA replication with hydroxyurea results in a rapid decline of class II promoter activity, late flagellar promoter activity decreases more slowly [42]. In addition, treatment of cells with hydroxyurea near the time of initiation of DNA replication had a greater effect on class II promoter activity than treatment later in the cell cycle [42]. This is consistent with the idea that an early cell cycle event, possibly the start of replication itself, triggers activation of the class II flagellar promoters. But what is the regulatory link between the cell cycle and the flagellar cascade?

The unusual consensus sequence of class II promoters suggested that transcription of these genes and thus their temporal pattern of activity is controlled by a novel sigma factor [27,42,43]. However, in vitro activation of class II promoters by the $E\sigma^{73}$ RNA polymerase indicates that these promoters are in fact recognized by the *C. crescentus* housekeeping sigma factor [44]. Cell cycle control of class II promoters would thus be mediated by transcriptional regulators rather than alternative sigma factors. *ctrA*, a gene encoding such a regulator, has recently been isolated as the first member of the hypothetical class I of genes, which provide the initial signal for the initiation of the regulatory cascade [45]. CtrA is a response regulator protein with a C-terminal DNA-binding domain that controls transcription of several class II genes [44,45]. Growth of conditional *ctrA* mutants under restrictive conditions resulted in a decreased rate of transcription for both the *fliL* and the *fliQ* promoters, suggesting that CtrA is required for their activation early in the predivisional cell [45]. Initiation of *fliL* and *fliF* transcription in vitro was dependent on the presence of CtrA in the assay [44]. All CtrA-controlled promoters share a similar architecture with a CtrA-binding site (CtrA-box) overlapping the −35 region [45,46] and with nucleotides in the CtrA-box being essential for their transcriptional activity [27,42]. In vitro binding studies have shown that phosphorylation enhances the affinity of CtrA for the *fliQ* promoter approximately 50-fold [46]. This and the observation that conditions permitting phosphorylation of CtrA were required to initiate *fliF* transcription in vitro [44] suggest that the transcription of class II genes is activated by $CtrA \sim P$ (see Fig. 3).

Is CtrA part of the *C. crescentus* signal transduction pathway that connects flagellar assembly to cell cycle cues? Several observations support this idea. First, CtrA is not only responsible for the initiation of the flagellar transcription hierarchy but is also involved in controlling a number of crucial cell cycle events like DNA replication, DNA methylation and cell division (see below) [45,47,48]. Second, the *ctrA* gene is essential for *Caulobacter* growth, as expected for a global cell cycle regulator [45]. Third, CtrA is activated by essential signal transduction components which are believed to respond to cell cycle cues (Figs. 3 and 4) [44,49,50].

Three signal transduction pathways have been proposed to be involved in the activation of CtrA (Fig. 3). The first one includes the sensor protein kinase DivJ and the response regulator DivK [49–51]. Both genetic and biochemical evidence suggest that these proteins function to control the activity of CtrA. The purified kinase domain of DivJ is able to in vitro phosphorylate DivK and, to a minor extent, CtrA [44]. In addition, suppressor mutations that support growth of *C. crescentus* in the absence of the essential DivK response regulator map to a region of *ctrA*, which encodes the conserved C-terminal DNA-binding domain of the protein [44]. No phosphotransfer was observed in vitro between DivK and CtrA, indicating that DivJ, DivK and CtrA are elements of a multicomponent signal transduction pathway with one component still unidentified (Fig. 3). In these multicomponent systems, the phosphoryl group is typically transferred from a histidine residue (H1) of the kinase transmitter domain to an aspartate residue (D1) of the response regulator receiver domain. This phospho-enzyme intermediate then serves as a phosphodonor for the next component, a phosphotransfer protein, which again is phosphorylated at a histidine residue (H2). In the last step of the cascade, the phosphoryl group is transferred to an aspartate (D2) of the receiver domain of the regulator (reviewed in [7,52]). In its outline, the $DivJ \rightarrow DivK \rightarrow X \rightarrow CtrA$ signal transduction pathway is similar to the $KinA/KinB \rightarrow Spo0F \rightarrow Spo0B \rightarrow Spo0A$ phosphorelay that controls the onset of sporulation in

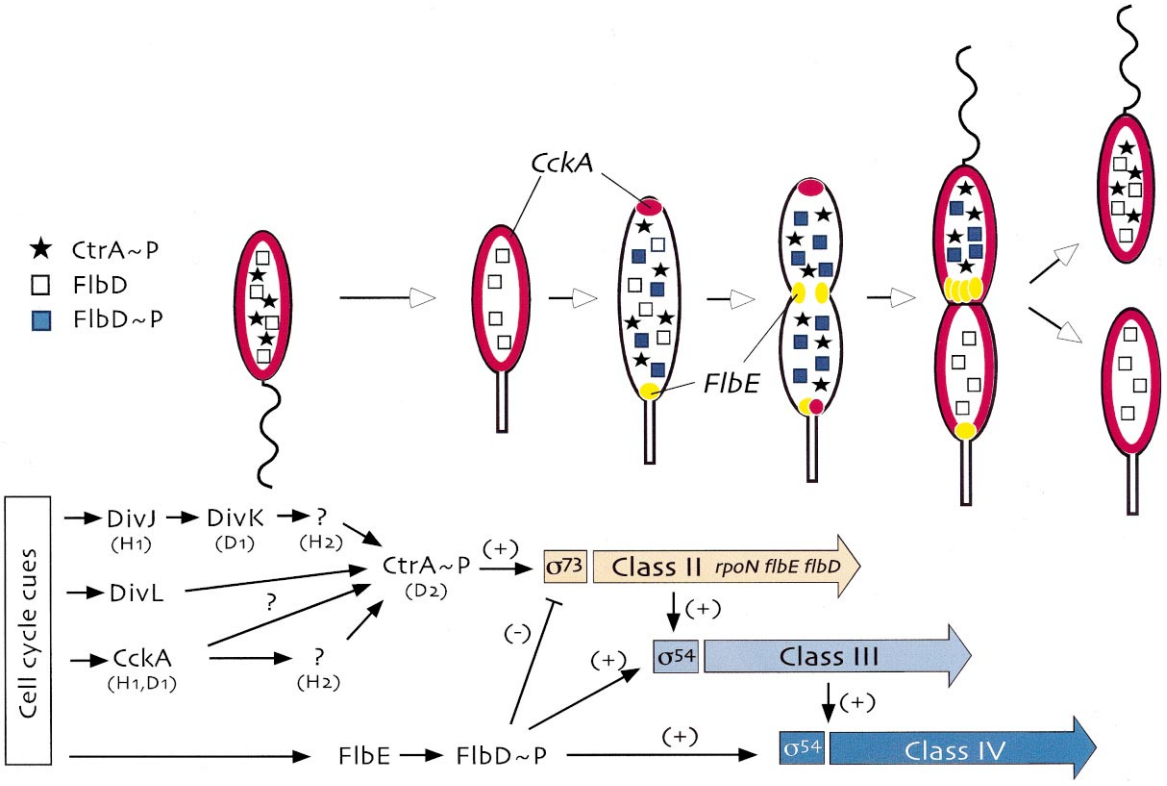

Fig. 3. Temporal and spatial control of the flagellar hierarchy by phosphotransfer signal transduction systems. The linear form of the *C. crescentus* cell cycle is shown, starting with the motile swarmer cell and proceeding from left to right until cell division has occurred. Chromosome replication initiates in the absence of CtrA in the stalked cell. Flagellar assembly takes place in the predivisional cell at the pole opposite of the stalk, beginning after replication has initiated and being completed before cells divide. The three gene classes (class II, class III and class IV) which define the flagellar hierarchy are indicated by arrows below the schematic of the cell cycle. The position and length of the arrows mark the approximate period of flagellar gene expression during the cell cycle. Boxed sigma factors (σ^{73} and σ^{54}) indicate the sigma factor requirement for the expression of each flagellar gene class. Both positive (+) and negative (−) control of class II, III and IV gene expression by transcription factors and by the preceding flagellar checkpoint are indicated. Signal transduction pathways that are proposed to regulate flagellar gene expression are outlined and discussed in the text. Question marks point to missing components or unknown phosphotransfer reactions of the regulatory pathways. The designation of histidine (H1, H2) and aspartate (D1, D2) residues of the signal transduction components is according to Appleby et al. [7]. The nature of the cell cycle cues which are sensed by the CtrA-activating kinases DivJ, DivL and CckA is still unknown. Subcellular localizations of the CckA (red) and FlbE (yellow) kinases are indicated. The presence of activated CtrA (blue stars), non-phosphorylated FlbD (open blue squares) and phosphorylated FlbD (filled blue squares) in the different cell types or compartments of the predivisional cell is indicated.

Bacillus subtilis in response to metabolic and cellular signals [53].

A two-step screen to isolate mutants with defects in polar development and with a temperature-sensitive (ts) lethal phenotype has recently led to the isolation of the gene for the CckA kinase (cell cycle kinase) [50]. CckA is required for CtrA phosphorylation in vivo, strongly suggesting that it is modulating CtrA activity through phosphorylation. In addition to the typical transmitter domain, CckA also has a receiver domain at the C-terminus implying an intramolecular phosphotransfer from H1 to D1. Such an architecture has been suggested for a number of histidine kinases including the *Saccharomyces cerevisiae* osmosensor Sln1p, which transfers a phosphoryl group to the phosphotransfer protein Ypd1p and eventually to the response regulator Ssk1p [54]. In accordance with this, one has to postulate an as yet unidentified phosphotransferase as the link between CckA and CtrA (Fig. 3). Alternatively, it is conceivable that the phosphoryl group is transferred directly from the CckA transmitter domain (H1) to CtrA (D2). Such a transfer mechanism has recently been suggested for ArcA/ArcB [55] of *Escherichia coli* and the VirA/VirB system of *Agrobacterium tumefaciens* [56]. Finally, the protein kinase DivL has recently been shown to be able to phosphorylate CtrA in vitro (see below) [57], suggesting that it might also contribute to the control of CtrA activity during the *Caulobacter* cell cycle.

It is not clear yet which signals are sensed by the DivJ, DivL and CckA kinases and transmitted to the flagellar cascade at the appropriate time of the cell cycle. One important observation suggests, however, that spatial information contributes to the temporal control of flagellar gene expression in the early predivisional cell. Like most sensor protein kinases, CckA is membrane bound and evenly distributed in the cell throughout most of the cell cycle. In the early predivisional cell, however, CckA protein concentrates at the swarmer pole, the pole opposite of

the stalk (Fig. 3) [50]. Polar localization of CckA is retained until immediately before cells divide, at which stage the protein delocalizes (Fig. 3). Deletion of the transmembrane domain results in the loss of CckA localization and activity, suggesting that polar localization leads to an active CckA and as a consequence, to the activation of the flagellar cascade through CtrA. The dynamic behavior of CckA suggests that its activation depends on a cell cycle cue located at the cell pole. CckA localization to the pole takes place after chromosome replication has initiated in the stalked cell. This is consistent with the observation that activation of the flagellar cascade is dependent, directly or indirectly, on active replication. One of the future challenges will be to identify the polar signal(s) that are sensed by CckA and uncover their link to DNA replication. One of several possibilities is that chromosome partitioning and in particular the movement of partition proteins associated with the newly replicated Cori regions to the cell poles [58] would constitute a localized signal for later events of the cell cycle.

Interestingly, the DivJ sensor kinase is localized exclusively to the stalked pole of either stalked or predivisional cells, implying that it recognizes and transmits a signal which is unique to this pole (Fig. 4) (A. Newton, personal communication). The finding that the protein kinases CckA and DivJ are specifically localized to swarmer and stalked pole, respectively, strengthens the view that CtrA activity, and by that the developmental program of *C. crescentus*, is controlled at least in part by the nature of the poles.

2.1.2.2. Control of late flagellar genes (class III and class IV). Class III and class IV flagellar genes are under both temporal and spatial control (Fig. 3). Their expression is sequential and becomes confined to the swarmer compartment in the late predivisional cell [59,60]. Swarmer pole-specific expression of these genes ensures that synthesis of the late flagellar components is confined to where they are needed. How does the cell restrict the expression of this class of genes to a specific time window and cellular compartment? Transcription of class III and class IV flagellar genes requires the alternative sigma factor σ^{54} [61–66]. While in many bacteria σ^{54} controls metabolic pathways in response to environmental signals, in *Caulobacter*, it is used to regulate several developmental processes like flagellar assembly and stalk formation (see below) in response to cell cycle cues. In a *C. crescentus* strain with a defective σ^{54}, class III and class IV genes are not expressed [65–67]. The *rpoN* gene, which encodes this alternative sigma factor, is under cell cycle control and has a class II-like temporal expression pattern (Fig. 3) [65]. Expression of σ^{54} peaks in the predivisional cell and this is thought to contribute to the temporal expression pattern of late flagellar genes. However, one of the characteristics of σ^{54}-containing RNA polymerase is that, although it can bind to σ^{54} promoters and form stable complexes, it can not catalyze the formation of an open complex unless it interacts with a transcriptional activator [68]. This suggests that temporal control of late flagellar genes would primarily be achieved by activator proteins. Mutational analysis has led to the identification of several *ftr* (flagellar transcription regulator) enhancer elements in the promoter regions of class III and class IV promoters that were postulated to promote binding of transcriptional activators [33,62–64,69,70,74]. In addition, σ^{54} promoters have a conserved binding site for the integration host factor (IHF) located between enhancer sequences and promoter [71,74,75]. Interaction of IHF with the conserved IHF-binding sequences increases activity of late flagellar promoters both in vitro and in vivo [71,72,74,75]. Like σ^{54}, IHF expression peaks in the predivisional cell coinciding with the peak of late flagellar gene transcription [76].

The class II *fliF* operon contains two genes, *flbE* and *flbD*, which do not encode structural components of the flagellum but regulatory proteins required for class III and class IV transcription. The FlbD protein is a homolog of the *E. coli* σ^{54} activator protein NtrC [77,78]. FlbD has a C-terminal DNA-binding motif which allows specific interaction with the enhancer sequences of class III and class IV flagellar promoters [67,72,73,79]. FlbD is strictly required for the activity of these promoters both in vitro and in vivo [66,67,72,73,78,79]. As a member of a class II flagellar operon, *flbD* expression is under cell cycle control (Fig. 3) [67]. However, FlbD steady state levels do not seem to change during the cell cycle and FlbD is evenly distributed in all *Caulobacter* cell types (Fig. 3) [67]. Temporal and spatial control of late flagellar genes must therefore be based on changes of FlbD activity rather then on changes of FlbD concentration. The FlbD homolog NtrC is activated through the phosphorylation of an aspartate residue (D54) in its amino-terminus [80]. The corresponding aspartate residue of FlbD (D52) is required for cell motility, consistent with the idea that activation of FlbD occurs through phosphorylation of D52 [67]. FlbD is phosphorylated in a cell cycle-dependent manner. While no phosphorylation activity is present early in the cell cycle, FlbD phosphorylation peaks in the predivisional cell coincident with maximal transcription of FlbD-dependent flagellar genes [67]. This observation strongly supported the model that cell cycle phosphorylation of FlbD regulates temporal transcription of late flagellar genes. Interestingly, cells with a mutant FlbD, which is active even in the absence of phosphorylation (S140F), retained the temporal expression pattern of flagellin gene transcription, indicating that factors other than FlbD phosphorylation have a role in regulating cell cycle transcription of these promoters [67].

Phosphorylation of FlbD is also involved in spatial control of late flagellar genes. The activity of late flagellar promoters is restricted to the chromosome partitioned to the swarmer compartment of the predivisional cell [60]. In addition, FlbD also functions as a pole-specific repressor

of its own synthesis by negatively controlling the activity of the *fliF* promoter in the swarmer compartment of the late predivisional cell [81]. Pulse-chase experiments demonstrated that cells expressing the constitutively active mutant form of FlbD (S140F) no longer exhibited swarmer compartment-specific activation of late flagellar genes or repression of the *fliF* operon [67,81]. This is indirect evidence that phosphorylation of FlbD is restricted to the swarmer pole of the predivisional cell (Fig. 3).

What are the signals and the regulatory components that are responsible for temporal and spatial control of FlbD activation? A potential kinase for FlbD is encoded by the *flbE* gene, which lies within the same operon as *flbD* and is required for activation of the σ^{54}-dependent class III and class IV promoters [25]. FlbE shares regions of homology with two-component histidine kinases and is able to phosphorylate FlbD in vitro [82]. In addition, a *flbE* mutant is unable to activate FlbD in a compartment-specific manner [82]. FlbE concentration does not fluctuate during the cell cycle but the FlbE protein has a very characteristic subcellular distribution. While FlbE is not localized to a specific site of the swarmer and the stalked cell, the protein concentrates at the stalked pole of the early predivisional cell and, after separation has occurred, between the two compartments, at mid-cell in the compartment opposite of the stalked pole (Fig. 3) [82]. The amino-terminus of FlbE is sufficient for localization and a fusion protein between the FlbE N-terminus and a reporter gene could successfully compete for localization with the full length protein [82]. The fact that cells containing the competing fusion protein exhibited a marked decrease of FlbD-dependent pole-specific flagellin promoter activity suggested that correct localization of FlbE is required for the activation of FlbD and thus for temporal and spatial control of late flagellar promoters [82]. Localization of FlbE to the swarmer pole-exposed side of the septum explains the swarmer pole-specific activation of FlbD and the compartmentalized expression of late flagellar genes (Fig. 3). FlbE localized to the stalked pole of the late predivisional cell is apparently not able to activate FlbD (Fig. 3). Whether this is due to the absence of an additional signal or signaling component at this pole or due to the presence of an inhibiting factor of FlbD activation in this compartment is not clear yet. The observed FlbE localization to the stalked pole and mid-cell is consistent with the idea that the N-terminus of the protein interacts with a component of the cell division machinery involved in either septum formation or stalk biogenesis. The FlbE → FlbD signal transduction system would thus be a device to couple flagellar assembly to early cell division events.

2.2. Flagellar ejection and stalk biogenesis

In addition to flagellar biogenesis, flagellar ejection and stalk biogenesis are also under cell cycle control (Fig. 1). During the swarmer-to-stalked cell transition, the axial components of the flagellar structure including rod, hook and filament are shed into the supernatant (Fig. 2) [16,83] and a stalk is synthesized at the same site [5]. How does the cell cycle control the loss of motility? Ejection of the flagellum coincides with the degradation of the flagellar anchor protein FliF, which forms the MS-ring in the cytoplasmic membrane (Fig. 2) [84]. Cell cycle-dependent degradation of FliF does not require any of the other structural flagellar components, strongly favoring the view that it is the initial event leading to the ejection of the flagellum [13]. Interestingly, FliF degradation also seems to be controlled by a two-component signal transduction system. Analysis of the polar development mutant *pleD* revealed that the *pleD* gene is required for FliF turnover during swarmer-to-stalked cell transition [13]. The *pleD* requirement for cell cycle-dependent FliF degradation is also not dependent on any other part of the flagellar structure, suggesting that PleD does not influence FliF degradation indirectly via the loss of the flagellum [13]. As a result of the increased FliF stability, a large fraction of *pleD*$^-$ cells do not eject the flagellum during cell differentiation, leading to the formation of stalked cells carrying a flagellum at their stalked pole and ultimately to the formation of biflagellated predivisional cells [13]. The *pleD* gene encodes a novel type response regulator with a duplication of the amino-terminal receiver domain and an unusual output domain [12]. Only the first receiver domain of PleD contains the predicted phosphorylation site, a conserved aspartate residue at position 53 (D53) [8]. The aspartate of the second receiver domain is replaced by an asparagine residue, suggesting that this domain is not phosphorylated. Moreover, the carboxyl-terminus of PleD does not contain a typical helix-turn-helix DNA-binding motif, but has homology to a large family of proteins from both Gram-negative and Gram-positive bacteria with as yet unknown functions [12,13]. Mutational analysis has shown that both an intact receiver and output domain are required for PleD function, suggesting that signals are transmitted from a sensor protein kinase via the first receiver domain to the PleD signaling domain [12,13]. The following questions remain to be answered: What is the significance of the second receiver domain? What is the cognate histidine kinase and what are the signals that it recognizes? What is the role of the novel signaling domain at the PleD C-terminus and how does it control FliF stability?

The *pleD* gene was identified through the isolation of mutations that bypass the motility defect of a pleotrophic *pleC* mutant [11]. PleC is a sensor kinase that controls polar morphogenesis in *C. crescentus* including the acquisition of motility late in the PD cell (Fig. 1) [85]. *pleC* mutants are able to assemble a flagellum but the structure remains paralyzed. In addition, *pleC* mutants are also deficient in flagellar ejection, in stalk biogenesis, holdfast formation and are resistant to the polar bacteriophage

φCbK [11,85,86]. Bypass of the motility defect of a *pleC* mutant by a mutation in the *pleD* gene implies that PleD, together with PleC, is involved in the control of flagellar rotation. The finding that both a *pleC* and a *pleD* mutant are also defective in the loss of the flagellum and in stalk biogenesis [11,13,85] further supports the idea that the PleC kinase and the PleD response regulator are part of the same pathway that coordinates polar morphogenesis in *C. crescentus*. This would imply that PleC, directly or indirectly, contributes to PleD phosphorylation, dephosphorylation, or both. The existing data are consistent with the view that the phosphorylated form of PleD negatively controls gain of motility in the late predivisional cell and positively controls flagellar loss and stalk biogenesis during the swarmer-to-stalk cell transition. While the PleD protein is not localized to a specific site of the cell (P. Aldridge and U. Jenal, unpublished observation), the subcellular distribution of the PleC sensor kinase remains to be examined. Since PleC is required for a number of developmental events that take part at the same pole during its transition form a flagellated to a stalked pole, it is conceivable that it is specifically localized to this site of the cell.

Stalk biogenesis involves a localized topological change in cell surface growth in a confined polar area at the base of the stalk [87–89]. Elongation of the stalk requires cell wall biosynthesis and can be specifically inhibited by the β-lactam antibiotic mecillinam [89,90]. It has been suggested that stalk biosynthesis and cell division might be dependent on similar processes with some components being involved in both events [91]. The correct developmental timing of stalk biogenesis in the *C. crescentus* life cycle requires the PleC kinase and the PleD response regulator [13,85]. In addition, cell cycle-dependent stalk biogenesis is also under the control of the response regulator CtrA [45] and of the alternative sigma factor σ^{54} [65]. Because no genes have been isolated so far which encode components of the stalk biogenesis machinery, it is not clear if PleC, PleD, CtrA or σ^{54} are directly or indirectly involved in the control of this process. For example, the requirement of CtrA for stalk biogenesis could be explained by the recent finding that the promoter of the *divK pleD* operon contains a CtrA-binding site [44]. Rather then being directly involved in the control of stalk biogenesis genes, CtrA could be required for the cell cycle-dependent expression of PleD [49], which in turn is a positive regulator of stalk biogenesis.

As a result of stalk elongation during each stalked cell cycle, stalk length increases linearly with increasing age of the cells. This relationship has recently been documented for over 150 consecutive divisions of individual stalked cells (M. Ackermann and U. Jenal, unpublished data). In addition to the developmental control, stalk biogenesis is also subject to environmental control. Stalk length in *C. crescentus* is inversely related to the phosphate concentration in the medium [87]. Interestingly, all mutants with a stalkless or short stalk phenotype isolated so far do form stalks when grown with limiting phosphate concentrations [13,45,65,85]. This indicates that the system which controls stalk elongation and maybe initiation in *C. crescentus* in response to the availability of phosphate is dominant over, or independent of, the control elements known so far. In *E. coli*, gene expression in response to the external phosphate concentration is governed by a two-component system, PhoR/PhoB, which senses the P-flux through the Pts high affinity phosphate uptake system [92]. The *C. crescentus* genes encoding the Pts and Pho homologs have recently been isolated and shown to be involved in the control of stalk length in response to phosphate [93]. Mutants with disrupted *pts* genes exhibit a long stalk phenotype, irrespective of the extracellular phosphate concentration. A strain with a mutation in the *phoB* gene was unable to efficiently elongate stalks when grown in low phosphate medium, suggesting that the response regulator PhoB controls stalk length in response to a signal emanating from the high affinity phosphate uptake system Pts [93]. Identification of components of the *C. crescentus pho* regulon should thus lead to the isolation of genes involved in stalk biogenesis.

3. Control of cell cycle events

Each bacterial cell cycle can be divided into several stages with a series of consecutive events: cell mass increase, replication of the chromosome, segregation of the newly replicated chromosomes to the cell poles, cytokinesis and cell separation. While some of these processes like cell growth and chromosome replication overlap, others like chromosome segregation and cytokinesis are clearly separated and take place in a sequential order. How are these events controlled and coordinated with each other to ensure the faithful distribution of complete copies of the genomic information during the division cycle? For example, how is replication initiation coordinated with the growth of the cell? This latter question is particularly intriguing to address in *Caulobacter* because not only has the cell to accomplish the correct timing and synchrony of replication initiation but it also has to coordinate it with development (Fig. 1).

Various genetic screens were used to isolate genes encoding essential control components of the *C. crescentus* cell cycle. All approaches made use of the observation that in *C. crescentus*, polar development and cell cycle control are interdependent (see above). In short, ts mutations were isolated that also specifically altered one or several developmental events. With the goal to isolate components which control both the earliest steps of the flagellar cascade and events critical for cell cycle progression, Quon et al. [45] identified ts lethal mutations that at the permissive temperature change the expression of class II flagellar genes and at the restrictive temperature disrupt functions

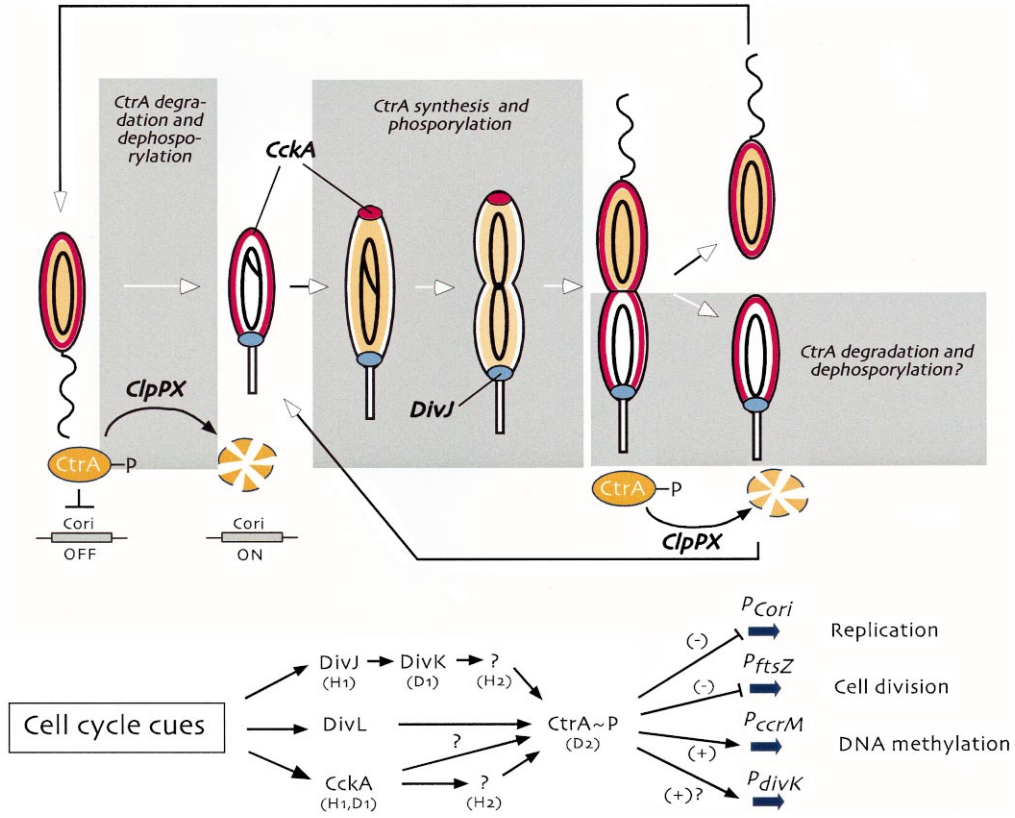

Fig. 4. A model illustrating the role of CtrA in the *C. crescentus* cell cycle and its temporal and spatial control by regulated synthesis, modification and degradation. The linear form of the *C. crescentus* cell cycle is depicted as described in Fig. 3. The chromosome is displayed as black circle and progression of chromosome replication during the cell cycle is indicated schematically. The time windows of CtrA synthesis, phosphorylation, degradation and postulated dephosphorylation are labeled accordingly and are indicated as gray boxes. CtrA-dependent negative control of replication initiation at the origin of replication (Cori) is shown schematically (OFF). Dephosphorylation and degradation of CtrA leads to the activation of replication initiation in the stalked cell (ON). Cells which contain CtrA are indicated by an orange fill, while cells without CtrA have a white fill. The cellular locations of the sensor kinases CckA (red) and DivJ (blue) are shown throughout the cell cycle. The proposed role of the ClpXP protease in cell cycle-dependent CtrA degradation is indicated. The bottom part of the graph shows the signal transduction pathways which have been proposed to activate CtrA by phosphorylation. The designation of histidine (H1, H2) and aspartate (D1, D2) residues of the signal transduction components is according to Appleby et al. [7]. CtrA-dependent negative (−) and positive control (+) of the strong promoter in the Cori region (P_{Cori}), and of the promoters of the *ftsZ* (P_{ftsZ}), *ccrM* (P_{ccrM}) and *divK* genes (P_{divK}) are indicated as discussed in the text.

essential for growth and viability. This screen led to the identification of the *ctrA401* allele, which at 28°C affects transcription of a number of class II flagellar genes (see above) and is lethal at 37°C [45]. With a similar screen, cold-sensitive (cs) suppressors of the motility defect of a *pleC* mutant (see above) were isolated [94]. Mutant alleles in three genes *divJ*, *divL* and *divK* were identified, which partially or fully restored motility of the *pleC* mutant at 37°C but were unable to support growth and division at 24°C. Finally, a general screen for ts mutants with a defect in polar organelle development (flagellar assembly, resistance to polar phage ϕCbK) at the permissive temperature resulted in the isolation of additional *ctrA* mutant alleles and the *cckAts1* allele [50]. The corresponding gene products are part of a signal transduction network which not only controls development (Fig. 3) but also several cell cycle events like replication, DNA methylation and cell division in response to still unknown cell cycle cues (Fig. 4).

At present, the response regulator CtrA is the only known output molecule of this signal transduction pathway and seems to play a pivotal role in orchestrating the above cell cycle events. CtrA control takes place on three different levels (Fig. 4): (i) Synthesis of CtrA occurs only in the predivisional cell after replication of the chromosome has initiated [45–47,99]. (ii) CtrA is immediately phosphorylated after it is synthesized and genetic evidence suggests that it is dephosphorylated as cells undergo the transition from G1 to S-phase [95]. (iii) The CtrA protein is specifically degraded during the swarmer-to-stalked cell transition and in the stalked compartment of the late predivisional cell [95,96]. As a consequence of this complex control, CtrA activity is absent at the beginning of the S-phase when replication is initiated.

Phosphorylation of CtrA is controlled by the DivJ/DivK phosphorelay and the CckA protein kinase (see above, Fig. 4). The finding that the DivJ and the CckA sensor kinases localize to opposite poles of the predivisional cell (Figs. 3 and 4) strongly suggests that signals emanating at the two cell poles are integrated by these

signal transduction pathways and via phosphorylation of CtrA contribute to the temporal control of several cell cycle events. More recently, the *divL* gene, which had been isolated in a genetic screen together with *divJ* and *divK*, has been shown to encode an atypical bacterial kinase involved in CtrA phosphorylation. Although structurally similar to histidine kinases like DivJ or CckA, DivL seems to autophosphorylate on a tyrosine residue and transfers the phosphoryl group directly to CtrA [57]. Like CckA, DivK and CtrA, DivL is essential for *Caulobacter* growth and genetic evidence indicates that CtrA is a target of DivL. A mutation mapping to a region of *ctrA* which encodes the conserved C-terminal DNA-binding domain of the protein is not only able to support growth in the absence of the essential DivK response regulator [44] but also suppressed a cs *divL* allele [57]. From this, it has been postulated that DivL is also involved in integrating cell cycle and/or morphological cues by modulating CtrA activity.

The fact that three distinct protein kinases are proposed to control the activity of CtrA indicates that a number of different cellular signals must be identified and integrated before the decision is made to activate the pathway at a specific time in the cell cycle. Similar to the phosphorelay that controls sporulation in *B. subtilis*, it can be assumed that several phosphatases are involved in controlling the phosphate flux through this signal transduction pathway [97]. One of the main challenges in the future will be to isolate all the components of the machinery, which controls CtrA activity, and to identify the nature of the signals, which are sensed and integrated by these pathways.

In addition to this intricate control of activity, the CtrA protein is subject to cell cycle-dependent proteolysis (Fig. 4). The expression of a constitutively active and stable CtrA protein results in a G1 cell cycle arrest [95]. In order to enter S-phase and initiate chromosome replication, it is essential for *Caulobacter* cells to eliminate active CtrA [95]. This can either occur by dephosphorylation or by degradation of CtrA. CtrA has a turnover box at the very C-terminus that earmarks the protein for proteolysis during the swarmer-to-stalked cell transition and in the stalked compartment of the late predivisional cell [95]. Constitutive expression of *ctrA* during the cell cycle does not alter the distribution of the CtrA protein, demonstrating that regulation by proteolysis overrides synthesis control [95]. Initial characterization of *Caulobacter* ClpXP suggested that this ATP-dependent protease is involved in cell cycle-dependent degradation of CtrA [96,98]. The *clpX* and *clpP* genes are indispensable for growth and survival of *C. crescentus* and depleting cells for the protease also results in a G1 cell cycle arrest [96]. The ClpXP protease could thus be required for cells to proceed into S-phase through removal of the CtrA response regulator. The finding that the expression of a stable CtrA derivative by itself does not lead to a cell cycle arrest [95] suggests however that ClpXP has to act on additional substrates to allow transition into the replication phase. The protein levels of ClpP and ClpX do not fluctuate during the cell cycle and it is likely that temporal control is accomplished via accessory proteins controlling the proteolytic activity or substrate interaction of ClpXP. Equivalent mechanisms have been shown for the control of sigma factor degradation in *E. coli* [100–103].

How does CtrA control cell cycle events? CtrA binds to five sites within the chromosomal origin of replication and inhibits DNA replication [47]. The CtrA-binding sites in the *C. crescentus* Cori overlap an essential DnaA-box and the P_{Cori} promoter, a strong promoter that is essential for replication initiation [47]. The P_{Cori} promoter is only active in stalked cells, when replication is initiated [104]. Repression of P_{Cori} in the other cell types is dependent on CtrA [45,47,104]. Thus, CtrA negatively controls replication initiation by repressing the P_{Cori} activity. In addition, CtrA may also act by inhibiting DnaA interaction with the Cori [47]. In *E. coli*, DnaA is the critical positive control element of replication initiation but it is not clear exactly how the threshold level of active DnaA is reached at a particular time in the cell cycle [105]. In *C. crescentus*, *dnaA* is expressed throughout the cell cycle but doubles just prior to replication initiation [106]. DnaA could thus reach a critical concentration for replication initiation in stalked cells and in addition could actively be excluded from the Cori during the rest of the cell cycle by CtrA. The crucial role of CtrA in replication control is supported by the observation that decreased CtrA activity causes the accumulation of multiple chromosomes per cell while increased CtrA activity results in a replication block [47,95].

CtrA is also required for the cell cycle-dependent expression of the *ccrM* gene which encodes an essential DNA methyl transferase (Fig. 4) [45,107–109]. CcrM is only present during a short period of the cell cycle, late in the predivisional cell, where it methylates the newly replicated hemimethylated chromosomes [110]. Temporal control of CcrM levels is accomplished by a combination of cell cycle-dependent *ccrM* transcription and rapid degradation of the CcrM protein by the Lon protease [108,110]. Restriction of the CcrM activity to the late predivisional cell results in an extended period of chromosome hemimethylation after replication. This phase of chromosome hemimethylation seems to be important for replication control in *C. crescentus*. Mutations that result in CcrM being present throughout the cell cycle cause an immediate remethylation of hemimethylated sites after replication and a loss of normal timing of initiation of DNA replication [110]. The exact temporal control of *ccrM* transcription during the cell cycle is thus critical for synchronized replication initiation events. The *ccrM* promoter has an architecture similar to class II flagellar promoters and its activity is also dependent on CtrA [45]. However, class II flagellar genes and the *ccrM* gene are transcribed in sequence, with class II genes being activated early and *ccrM* being expressed late in the predivisional

cell. Recently, promoter-binding studies with purified CtrA∼P have revealed that the response regulator has a higher affinity for the class II *fliQ* promoter than for the *ccrM* promoter [46]. The different timing of these promoters during the cell cycle could thus at least in part be explained with changes in cellular levels of total CtrA protein. However, the observation that an increased level of CtrA did change the timing of the *fliQ* promoter activity but not the stringent temporal control of *ccrM* transcription indicates that additional factors contribute to the differential expression of these genes [46].

CtrA is also involved in the repression of cell cycle genes in the non-replicating swarmer cell. An example is the negative control of the *ftsZ* gene. The FtsZ protein is an essential component of the cytokinesis machinery being the earliest known protein to be assembled and subsequently recruiting other division proteins to the site of cell division [111]. In *C. crescentus*, FtsZ is not made in swarmer cells and synthesis starts in stalked cells at the beginning of S-phase [48]. The transcription rate of the *ftsZ* gene is low when CtrA levels are high during the cell cycle, indicating that CtrA is a repressor of *ftsZ* [48]. The *ftsZ* promoter contains a conserved CtrA-box that specifically binds CtrA [48]. *Caulobacter* promoters which are activated by CtrA have the CtrA-box positioned around −30 with respect to the transcription start site [45–47]. In contrast, the CtrA-box of the *ftsZ* promoter overlaps the transcriptional start site, which in general is the preferred repressor binding site in negatively regulated promoters. Strains with decreased CtrA levels have an increased *ftsZ* promoter activity and removal of the CtrA-box in the *ftsZ* promoter region results in an increased swarmer cell-specific transcription [48]. This is consistent with the idea that CtrA specifically inhibits the expression of FtsZ and possibly other components of the cell division machinery in the swarmer cell.

It has been proposed earlier that the key to understanding the expression and maintenance of asymmetry and the control of the *Caulobacter* life cycle may lie in the cell poles and that polar cues may be integral components of the *Caulobacter* cell cycle clock [112]. *Caulobacter* shows a clear succession pattern in pole development in that poles which are newly formed at cell division always develop into swarmer poles, and swarmer poles are destined to become stalked poles [112]. Markers could be positioned at mid-cell during cell division and could then function as organizational centers at the new poles [113,114]. The finding that signal transduction components involved in cell cycle and developmental control are specifically localized to the swarmer pole, the stalked pole or to mid-cell clearly strengthens this idea. It is possible that the specific nature of these sites generates a sequence of signals, which are then transmitted and integrated by the signal transduction machinery described above. By identifying these signals, it should be possible to unravel the nature of the biological clock that drives development in *C. crescentus*.

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