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Review

Cell division control in *Caulobacter crescentus*☆

Justine Collier

Department of Fundamental Microbiology, Faculty of Biology and Medicine, University of Lausanne, Quartier UNIL/Sorge, Lausanne, CH 1015, Switzerland

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ABSTRACT

Caulobacter crescentus is a free-living *Alphaproteobacterium* that thrives in oligotrophic environments. This review focuses on the regulatory network used by this bacterium to control the levels of cell division proteins, their organization inside the cell and their activity as a function of the cell cycle. Strikingly, *C. crescentus* makes frequent use of master transcriptional regulators and epigenetic signals, most likely to synchronize cell division with other events of the cell cycle. In addition, cellular metabolism and DNA damage sensors emerge as central players regulating cell division in response to changing environmental conditions.

1. Introduction

Caulobacter crescentus is a free-living Gram-negative *Alphaproteobacterium* that lives in oligotrophic aquatic environments such as lakes, rivers or oceans [1]. It always divides asymmetrically into two distinct cell types: a swarmer cell that explores its environment searching for resources, but which cannot replicate itself before it differentiates into the second cell type, called the stalked cell, if conditions are favorable (Fig. 1). The stalked cell immediately initiates the replication and the segregation of its chromosome, a process during which the cell approximately doubles its size. Soon after the beginning of chromosome replication, a multi-protein complex called the divisome assembles towards mid-cell, to orchestrate cytokinesis. *C. crescentus* is commonly seen as one of the best models to study regulatory networks controlling the bacterial cell cycle, notably because its original asymmetry can be used to synchronize its cell cycle [1,2]. Indeed, nearly pure populations of swarmer cells can be isolated by simple density centrifugation. Once introduced into fresh medium, swarmer cells will then all differentiate and divide mostly synchronously for a complete cell cycle. This unique feature makes it an ideal model to study the temporal control of the bacterial cell cycle.

The divisome is composed of both essential and non-essential proteins [3]. Most of the essential divisome proteins are highly conserved, not only in Gram-negative bacteria, but sometimes also in Gram-positive bacteria. In most bacteria, including *C. crescentus*, the first protein that assembles at the future division site is the essential homolog of eukaryotic tubulin FtsZ. It is a GTPase that can self-assemble into protofilaments, which in turn organize themselves into bundles forming a ring-like structure (Fig. 1) stabilized and anchored underneath the cytoplasmic membrane by the conserved actin homolog FtsA and by FzlC in *C. crescentus* [4,5]. Although conventional fluorescence microscopy depicts this *Z*-ring as a closed ring, the recent development of super-resolution microscopy methods revealed that it is not always a continuous ring [6,7]. Indeed, photoactivated localization microscopy (PALM) was used to visualize the spatial organization of FtsZ molecules in hundreds of *C. crescentus* cells as a function of the cell cycle [8]. This study indicates that the *Z*-ring is a rather patchy mid-cell band until the late pre-divisional stage of the *C. crescentus* cell cycle. Consistent with this observation, recent electron cryotomography images indicated that a complete *Z*-ring is not necessary for cell division and that constriction and peptidoglycan (PG) synthesis initiate asymmetrically [9]. Once assembled, the Z-ring acts as a scaffold for the recruitment of other divisome components required for membrane fission, peptidoglycan cell wall remodeling and cell separation. In addition, the *Z*-ring appears to be directly linked to cell wall metabolism independently of its capacity to recruit other divisome components [5,10]. In *C. crescentus*, more than a dozen different essential proteins sequentially assemble into the divisome in a FtsZ-dependent manner [3]. Among these, several proteins such as FtsW and FtsI play a key role in PG synthesis and remodeling, while others are thought to stabilize interactions within the divisome such as FtsQ, FtsL or FtsN, or to play dual roles during the initiation but also during late stages of cytokinesis like the FtsEX complex [3,11].

Obviously, cytokinesis is a highly controlled process to make sure that it takes place at the right place within the cell and at the right time of the cell cycle in a manner that must be coordinated with other events of the cell cycle such as chromosome replication or cell growth. In addition, the cell must take into account its available resources and dangers in case of environmental stresses in order to decide or not to divide. The mechanisms that control the division of *C. crescentus* in

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E-mail address: Justine.collier@unil.ch.

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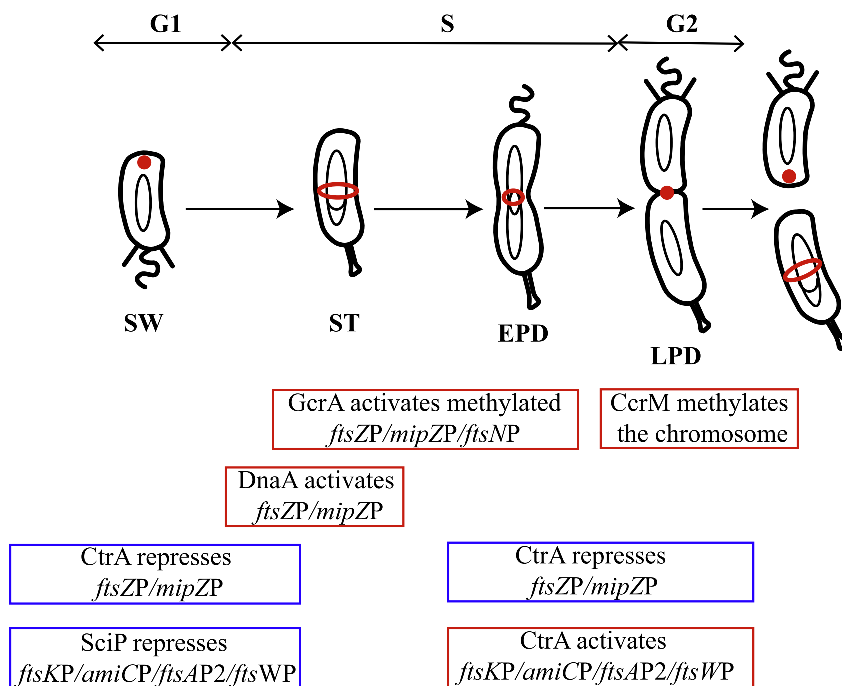

Fig. 1. Schematic of the *Caulobacter crescentus* cell cycle showing the assembly of the *Z*-ring and the role of master regulators controlling the on-time expression of cell division genes. SW, ST, EPD and LPD indicate swarmer, stalked, early pre-divisional and late pre-divisional cells, respectively. G1, S and G2 correspond to cell cycle stages. Black circles or theta structures inside cells represent the non-replicating and replicating chromosomes, respectively. The subcellular localization of FtsZ is indicated in red: it forms a ring-like structure towards mid-cell after the onset of DNA replication. A model for the regulation of promoters (P) controlling the transcription of several cell division genes is represented under the schematic. CtrA, GcrA, DnaA, SciP and CcrM are master transcriptional regulators of the *C. crescentus* cell cycle.

favorable or less favorable growth conditions are the focus of this review highlighting the most recent discoveries.

2. Transcriptional regulation of cell division genes during cell cycle progression

One level of control lies in the transcriptional regulation of genes encoding proteins required for cell division in *C. crescentus*. Indeed, whole genome transcriptome studies evaluating the levels of every transcript as a function of the cell cycle demonstrated that many of these genes are cell cycle regulated [12–14]. Genes encoding proteins required early during the division process, such as *ftsZ*, *ftsA* or *ftsN*, tend to be the most expressed in stalked or early pre-divisional cells, while those encoding proteins required later, such as *ftsW, ftsI* or *ftsL*, tend to be the most expressed in late pre-divisional cells [15]. In addition, a hyper-saturated transposon mutagenesis coupled with high-throughput sequencing experiment suggested that many of these essential open-reading frames are preceded by unusually long essential promoter elements [16]. The minimal *ftsZ* promoter region required for *C. crescentus* survival, for example, spans approximately 150 base pairs upstream of the transcription start site, which is far beyond the core promoter interacting with a Sigma factor to load the RNA polymerase and induce transcription. Overall, this indicates that the control of the transcription of division genes is often complex, notably to ensure that these are expressed in sufficient quantities for cell division and at the right time of the cell cycle.

To coordinate the expression of cell division genes with other events of the cell cycle, *C. crescentus* makes extensive use of so-called master regulators. Each of these transcription factors are the most abundant at a particular moment of the cell cycle and control the expression of many genes at the same time, with or without the participation of accessory proteins modulating their activity (Fig. 1). The CtrA response regulator and DnaA are such examples, coordinating gene expression with DNA replication [17,18]. Indeed, these two regulators act not only as transcription factors binding to many promoter regions, but also as a repressor or an activator, respectively, of the initiation of chromosome replication by binding to the chromosomal origin. CtrA is the most abundant and active in swarmer and pre-divisional cells, where it notably represses *ftsZ* transcription (Fig. 1) [19]. CtrA sometimes works with SciP (small CtrA inhibitory protein) to narrow down the time window when a given protein is produced, as is seen for the FtsK or the AmiC proteins for example, contributing to the ordered synthesis of cell division proteins necessary later during the division process [17,20–22]. In addition, it has been suggested that CtrA participates in a checkpoint-like mechanism to delay the expression of cell division genes if the initiation of chromosome replication is delayed [20]. Concerning DnaA, it is the most abundant and active during the swarmer-to-stalked cell transition, when it notably contributes to synchronize the transcription of *ftsZ* with the onset of chromosome replication (Fig. 1) [18,23].

Interestingly, recent discoveries showed that the transcription of several cell division genes is also regulated by an original epigenetic control mechanism involving the global cell cycle regulator GcrA and the cell cycle-regulated DNA methyltransferase CcrM (Fig. 1) [24]. This latter enzyme methylates adenines in 5′-GANTC-3′ motifs, which are enriched in intergenic regions of the *C. crescentus* chromosome that contain most promoters [25,26]. It is tightly regulated during the *C. crescentus* cell cycle, so that it can only methylate the newly replicated chromosome in late pre-divisional cells (Fig. 1). As a consequence, and since DNA replication is semi-conservative, the methylation state of GANTC motifs fluctuate as a function of the cell cycle: they are fully-methylated (both DNA strands are methylated) before their replication and in late pre-divisional cells, but they are hemi-methylated (only the old DNA strand is methylated) between the time when they get replicated (dependent on their position on the chromosome) and the time when they get re-methylated by CcrM in late pre-divisional cells [25]. The activity of certain promoters can be modulated depending on the methylation state of GANTC motifs that they carry, most likely by affecting the binding or the activity of specific regulators recruited to these promoters. Three of these promoters control the transcription of *ftsZ*, *ftsN* and *mipZ* (encoding the spatial regulator of *Z*-ring assembly) in *C. crescentus* [27–29]. Strikingly, the activation of *ftsZ* transcription through the methylation of the *ftsZ* promoter has been shown to be the most important function of CcrM in *C. crescentus*, since the severe cell division defect of cells lacking CcrM can be suppressed by increasing *ftsZ* transcription [27,30]. It was further observed that the CcrM regulon presented a significant overlap with the predicted GcrA regulon [26,31], suggesting that the activity of the unusual GcrA master

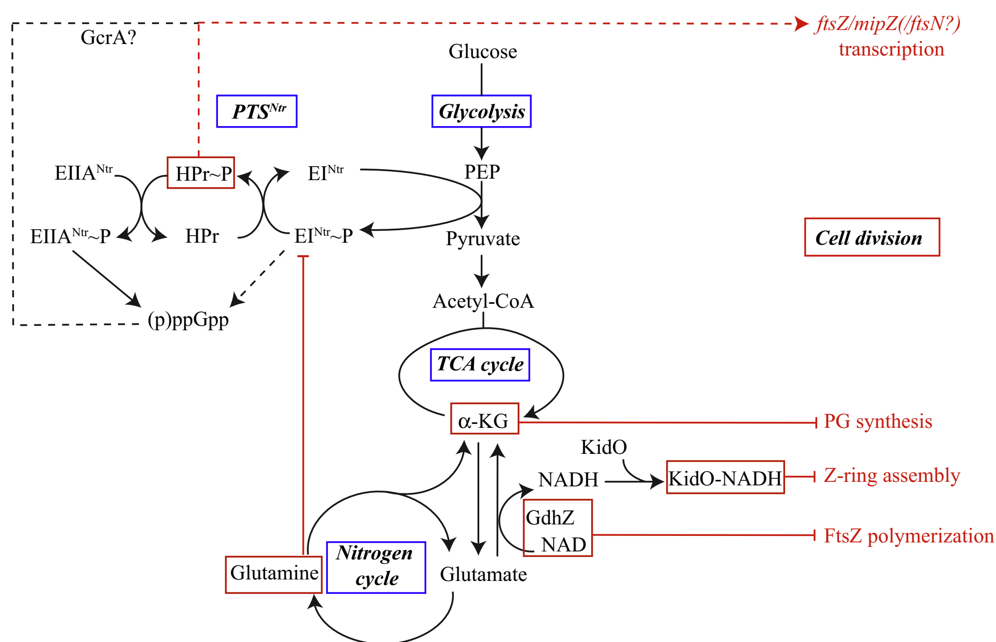

Fig. 2. Metabolic control of *C. crescentus* division. A simplified overview of identified connections between metabolic pathways (framed in blue) and cell division is shown. Enzymes or metabolites directly (plain lines) or indirectly (dashed line) involved in cell division control are framed in red. Abbreviations: α-KG, α-ketoglutarate; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid; PTS, phosphotransferase system; (p)ppGpp, guanosine (penta) tetra-phosphate.

regulator of the cell cycle might be modulated by the methylation state of the promoters that it regulates. Indeed, GcrA was found to bind to some of the promoters that it regulates in a manner that seems dependent on their methylation, including the ones controlling *mipZ* and *ftsN* transcription [28,29]. GcrA appears to promote the recruitment of the RNA polymerase and the initiation of transcription at methylated promoters through a direct interaction with the housekeeping Sigma 73 factor [31]. Overall, this epigenetic control mechanism appears as essential for the successful division of *C. crescentus* when it is cultivated in fast growing conditions [27,29].

3. Transcriptional regulation of cell division genes in response to environmental cues

In addition to being regulated in response to internal cues through the use of master cell cycle regulators, the transcription of several cell division genes is also modulated by environmental cues. Transcription of the *ftsZ* and *mipZ* genes, for example, is directly or indirectly controlled by the nitrogen-related phosphotransfer system (PTS^{Ntr}) of *C. crescentus* (Fig. 2) [30]. Interestingly, its EI^{Ntr} component binds to glutamine, which inhibits its autophosphorylation [32], indicating that *ftsZ* and *mipZ* transcription will be dependent on glutamine availability in the cell (Fig. 2). Considering that the PTS^{Ntr} also promotes the accumulation of the (p)ppGpp alarmone in response to nitrogen starvation, notably through interactions between its EI^{Ntr} and EII^{Ntr} components and the SpoT enzyme responsible for (p)ppGpp synthesis and hydrolysis in *C. crescentus* [32,33], it is possible that (p)ppGpp modulates *ftsZ* and *mipZ* transcription in response to nitrogen availability (Fig. 2). Consistent with this proposal, it was recently shown that the activity of several GcrA-dependent promoters is modulated by (p)ppGpp levels [33]. Overall, this regulatory network is most likely used by *C. crescentus* to regulate cell division and other events of the cell cycle in response to nitrogen/carbon availability in its natural oligotrophic environments.

4. Post-transcriptional control of cell division proteins

A second interesting connection between the metabolic status of the cell and its capacity to divide has been uncovered recently. It connects the central metabolism to PG synthesis through a regulation of α-ketoglutarate (α-KG) homeostasis by Hfq (Fig. 2) [34]. Beyond being a key intermediate of the TCA (Krebs) cycle, high α-KG levels can inhibit PG synthesis in *C. crescentus* cells, thereby affecting cytokinesis and morphogenesis. Interestingly, α-KG levels appear to be controlled by the conserved Hfq RNA chaperone. This chaperone is often involved in the regulation of mRNA translation by small non-coding RNAs (ncRNA) in bacteria. This finding, together with the recent discovery of the existence of many ncRNAs [35], including several that appear essential for the survival of *C. crescentus* [16], suggest that original post-transcriptional mechanisms may control cell wall remodeling during cytokinesis in response to nutrient availability.

The *ftsA* and the *ftsQ* genes belong to the same cell cycle-regulated operon, which is the most transcribed in pre-divisional cells, coincident with the end of *ftsZ* transcription [36]. This timely expression of all three genes is notably mediated by their co-regulation by the CtrA master regulator of the cell cycle (Fig. 1). In addition, the three proteins encoded by these genes are unstable proteins degraded by specific ATP-dependent proteases in swarmer cells, ensuring that protein levels also fluctuate as a function of the cell cycle according to the needs of the cell [37,38]. FtsZ appears to be degraded by the ClpAP and ClpXP proteases, while FtsA is most likely degraded by ClpAP [39]. The transient FtsZ-dependent localization of the ClpXP complex at mid-cell during the last steps of the cytokinesis process may play a role in activating the asymmetric degradation of FtsZ in the swarmer progeny but not in the stalked progeny after cell separation [39]. As for FtsQ, the protease responsible for its cell cycle-dependent degradation remains unidentified. It is nevertheless worth noting that the FtsQ protein appears to be SsrA-tagged [40]. The SsrA-tagging system is usually recruited to stalled ribosomes during translation and it plays two roles: it releases the stalled ribosomes for their recycling and it tags incomplete proteins for their degradation by ATP-dependent proteases [41]. Ribosome stalling notably happens at the 3′ end of truncated mRNA that do not carry a stop codon. Then, it is possible that the *ftsQ* mRNA gets cleaved by an endonuclease, leading to the recruitment of the SsrA RNA at stalled ribosomes to induce FtsQ degradation through an SsrA degradation tag added to its C-terminus. Since the SsrA RNA is the most abundant during the swarmer-to-stalked cell transition [42], it is tempting to speculate that such a system might be used to prevent the premature accumulation of FtsQ during the division process.

Although the stability of most other cell division proteins has not yet been evaluated, it is likely that many other ones are subject to cell cycle-dependent or constitutive degradation, so that transcriptional

regulation results in variations in intracellular protein levels as a function of the cell cycle.

5. Control of divisome assembly and cytokinesis

As other bacterial cells, *C. crescentus* needs a robust control mechanism to ensure that the *Z*-ring is assembled on time and at the right place in the cell so that the bacterium divides into two nearly equally-sized cells that each have a complete chromosome. For this, *C. crescentus* uses the MipZ protein, which coordinates the initiation of the replication of the chromosome with the assembly of the Z-ring at mid-cell [43]. MipZ is an inhibitor of FtsZ polymerization and it interacts with the ParB partition protein that itself interacts with *parS* sequences located near the chromosomal origin. This region of the chromosome is localized at the flagellated pole of swarmer cells before the initiation of DNA replication. Due to the capacity of the MipZ ATPase to dimerize, it forms a gradient being the most concentrated near the flagellated cell pole [44]. Thus, FtsZ molecules tend to accumulate towards the opposite cell pole at that step of the cell cycle (Fig. 1). During the swarmer-to-stalked cell transition, the replication of the chromosome initiates and one of the two newly replicated origins migrates to the opposite cell pole in a ParB-dependent manner. This relocation of the origin immediately affects the MipZ gradient, which now becomes bipolar in stalked cells. Then, the sub-cellular location where MipZ becomes the least abundant in stalked cells is a zone near mid-cell, where FtsZ can now polymerize into a *Z*-ring (Fig. 1). Unlike Min-like systems that are frequently used in bacteria, the MipZ-dependent control system not only defines where the Z-ring is assembled, but also delays its assembly until the time when the replication of the chromosome is initiated.

Bacterial cells also need to coordinate the timing of cell division with cell growth in other to give birth to progenies of constant size in given growth conditions. For this, the use of metabolic sensors controlling cell division may appear as an attractive option. The GdhZ enzyme of *C. crescentus* seems to play such a role (Fig. 2) [45]. This is a dual-function protein that is both a NAD-dependent glutamate dehydrogenase (converting glutamate into α-KG) and an inhibitor of FtsZ polymerization. Interestingly, this enzyme requires its metabolic substrates to inhibit the GTPase activity of FtsZ and is tightly controlled so that it is only detectable in G1 and G2 cells. The model is then that GdhZ prevents the premature assembly of the *Z*-ring in swarmer cells and promotes Z-ring constriction by limiting the formation of new FtsZ protofilaments in pre-divisional cells in response to glutamate and NAD intracellular levels. In addition, the NADH by-product of the metabolic activity of GdhZ appears to be used by the KidO protein to reinforce its inhibitory effect on cell division (Fig. 2) [45,46]. Indeed, KidO is present at the same time of the cell cycle as GdhZ and it destabilizes lateral interactions between FtsZ protofilaments in the presence of NADH, suggesting a functional synergy between GdhZ and KidO to modulate cell division in response to the metabolic or the redox status of the cell (Fig. 2). Noteworthy, it was recently shown that the intracellular redox state oscillates as a function of the *C. crescentus* cell cycle, the cytoplasm being reduced in G1 swarmer cells and the most oxidized in early S-phase cells [47]. This finding suggests that NADH may be more abundant in swarmer cells and in pre-divisional cells when KidO requires NADH to regulate cytokinesis.

Besides controlling cell division so that it occurs at the right time of the cell cycle, many bacteria display mechanism(s) blocking cell division in response to DNA damage, most likely to leave more opportunities to repair the damaged DNA by homologous recombination between the two daughter chromosomes. In *C. crescentus*, two such systems have been identified. The first one is mediated by the short SidA protein that interacts with the FtsW late cell division protein and thereby inhibits the final constriction of the *Z*-ring [48]. Like the *sulA* gene encoding the well conserved FtsZ inhibitor in many other bacteria, the *C. crescentus sidA* gene is a member of the LexA regulon induced during the SOS response. The second one is the DidA protein that interacts with the FtsN late cell division protein to inhibit cell constriction [49]. Contrarily to *sidA*, *didA* is not regulated by the SOS regulator LexA. Instead, its expression is induced by the DriD transcription factor in response to DNA damage. Although these two systems significantly slow down cell division in response to DNA damage and thereby increase survival, other systems may still need to be identified since cells lacking SidA and DidA are still significantly elongated when exposed to DNA damage [49]. It is possible that one of these additional regulators may target FtsZ more directly by affecting lateral interactions between FtsZ protofilaments or bundles, since the *Z*-ring appears as thicker and continuous in response to DNA damage when observed by PALM [8].

6. Cell size control

Cell size control is a universal feature of the cell cycle reflecting the coordination between growth and division. The observation that the size distribution of each *C. crescentus* cell type is very narrow indicates the presence of intrinsic mechanisms controlling cell size [50,51]. Single-cell microscopy was recently used to track a large number of individual cells over multiple cell cycles in highly controlled conditions to better appreciate this phenomenon [50,51]. Surprisingly, high precision measurements showed that *C. crescentus* cells elongate, on average, by a constant amount between two division events, irrespective of cell length at birth, resulting in robust cell size homeostasis [50]. Then, *C. crescentus* does not commit to divide only once a size threshold is reached, like what happens in yeast, for example. The molecular players involved in this constant extension model (also called the "adder" model) remain unclear, notably to understand how *C. crescentus* can sense how much it has grown before it decides to divide. The regulation of several steps of the cell division process by metabolic cues (Fig. 2) may contribute to this robust control system. A follow-up study looking more carefully at cell wall growth indicates that this model is even more complex than initially thought in *C. crescentus*, which now appears to display a bi-phasic mode of growth [52]. The newest "mixer" model is that cell growth is first correlated to its initial size at birth (relative "timer" phase) before the onset of cell constriction, and is then constant (pure "adder" phase) during cell constriction. The control mechanisms involved in that process seem very robust since errors in partitioning of volume among daughter cells appear as the main source of variations in cell size [53]. It is tempting to speculate that the switch from the relative "timer" phase to the pure "adder" phase may coincide with the time when the new flagellum is assembled at the pole opposite the stalked pole in early pre-divisional cells (Fig. 1) and, thus, that the FlbD regulator of flagellum assembly may play a role in this switch [54].

It is worth noting that the "adder" model also seems applicable to *Escherichia coli* cells [50], despite the fact that these two bacteria diverged more than one billion years ago. The difference between a pure "adder" model applicable to *E. coli* cells and the "mixer" model of *C. crescentus* may be connected to the fact that *C. crescentus* usually never initiates multiple rounds of DNA replication within the same cell cycle or that it divides asymmetrically.

7. Conclusion

This review highlights the diversity of mechanisms that are used by *C. crescentus* to control its division. Interestingly, many of these are conserved in other *Alphaproteobacteria* [2,55] or even in more distant bacteria [7,50] and *C. crescentus* just served as a useful model system to discover them or just to confirm their existence. Still, a few control systems appear to be more specific to *Caulobacterales*, such as the SidA-dependent division arrest that takes place when *C. crescentus* cells are exposed to DNA damaging conditions.

The development of novel methods to analyse bacterial genomes (including epigenetic marks) and their expression, and to visualize

complex structures such as the Z-ring at the nanoscale, already greatly accelerated discoveries over the last decade. There is no doubt that future developments in super-resolution, multi-colour and/or single-molecule microscopy and in synthetic biology to reconstitute regulatory systems will soon revolutionize this field. One hope is that a better understanding of the fundamental process of cell division helps to identify novel antibiotics targeting bacterial proliferation.

Transparency document

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