Review

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Generating asymmetry in a changing environment: cell cycle regulation in dimorphic alphaproteobacteria

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Abstract: While many bacteria divide by symmetric binary fission, some alphaproteobacteria have strikingly asymmetric cell cycles, producing offspring that differs significantly in their morphology and reproductive state. To establish this asymmetry, these species employ a complex cell cycle regulatory pathway based on two-component signaling cascades. At the center of this network is the essential DNA-binding response regulator CtrA, which acts as a transcription factor controlling numerous genes with cell cycle-relevant functions as well as a regulator of chromosome replication. The DNA-binding activity of CtrA is controlled at the level of both protein phosphorylation and stability, dependent on an intricate network of regulatory proteins, whose function is tightly coordinated in time and space. CtrA is differentially activated in the two (developing) offspring, thereby establishing distinct transcriptional programs that ultimately determine their distinct cell fates. Phase-separated polar microdomains of changing composition sequester proteins involved in the (in-)activation and degradation of CtrA specifically at each pole. In this review, we summarize the current knowledge of the CtrA pathway and discuss how it has evolved to regulate the cell cycle of morphologically distinct alphaproteobacteria.

Keywords: *Caulobacter*; cell cycle regulation; cell differentiation; CtrA; phosphorelay; two-component signaling.

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Introduction

Whereas many bacteria grow and divide symmetrically, some bacteria have evolved more complex modes of reproduction. A prominent example are the *Alphaproteobacteria*, which include intracellular pathogens, plant-associated bacteria as well as free-living species. Various members of this class show a striking asymmetry, in which the physiological and reproductive state of the two siblings differ markedly after cell division (Figure 1). After birth, one of them is immediately ready to enter S phase and initiate a new round of DNA replication, whereas its sibling shows a pronounced lag (G1 phase) before it starts its reproductive cycle (Degnen and Newton 1972; Ehrle et al. 2017; Frage et al. 2016; Jung et al. 2019). These asymmetric, dimorphic life cycles appear to have arisen in the orders *Rhizobiales* and *Caulobacterales* after they had diverged from the *Rhodobacterales* (Muñoz-Gómez et al. 2019) (Figure 2). Interestingly, concomitant with the establishment of cellular asymmetry, multiple different growth modes have evolved within this lineage, such as polar growth in the *Rhizobiales* (Brown et al. 2012), stalk-terminal budding in the *Hyphomonadaceae* (Moore et al. 1984; Schlesner et al. 1990) and *Hyphomicrobiaceae* (Moore 1981), and binary fission combined with polar stalk formation in the *Caulobacteraceae* (Pointdexter 1964). To enable their complex life cycles, these species have taken up the challenging task to establish an asymmetry between the two poles of predivisional cells that translates into different morphologies and fates in the two daughter cells.

Generating asymmetry within a micron-scaled cell poses a significant challenge (Kiekebusch and Thanbichler 2014). Even in the crowded cytoplasmic environment, protein diffusion is so fast that gradients over such length scales are normally dissipated in a matter of seconds. However, various mechanisms have evolved to overcome this problem. Usually, gradients require a localized source that generates the gradient-forming species and/or a localized sink that removes it from the system. The source and sink can be localized protein synthesis and degradation. Alternatively, gradient formation can be limited to a

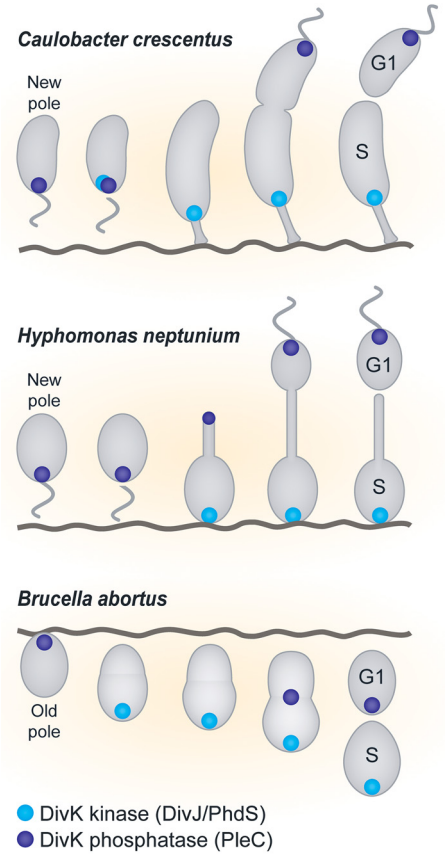

Figure 1: Multiple alphaproteobacteria have a dimorphic cell cycle, in which cell division produces two morphologically and physiologically distinct siblings.
Shown are the cell cycles of the dimorphic species *C. crescentus*, *H. neptunium* and *B. abortus*. The localization of the major phosphatase (dark blue circle) and kinase (light blue circle) of DivK, which together play an important role in establishing cell polarity in these cells, is indicated. One sibling enters the cell cycle in S phase and the other one starts in G1 phase.

modified (e.g. phosphorylated or dimerized) form of the protein with a lifetime much shorter than that of the protein itself that is formed and/or degraded by a localized enzymatic activity. To facilitate gradient formation, the gradient-forming species can, in addition, attach to a largely stationary cellular structure, such as the cytoplasmic membrane or the nucleoid, to limit its diffusion. Seminal work in *Caulobacter crescentus* has shown that dimorphic alphaproteobacteria have evolved a complex and robust regulatory network to establish this asymmetry, which, intriguingly, uses all of these mechanisms. At its core is the DNA-binding protein CtrA, which acts as a transcriptional regulator (Laub et al. 2002) controlling genes with critical roles in cell cycle progression and cell development. In some species, it further controls chromosome replication by binding at the origin of replication and preventing replication initiation (Quon et al. 1998). CtrA binds its chromosomal target sequences with significantly higher affinity when it is phosphorylated (Reisenauer et al. 1999; Siam and Marczynski 2000). Phosphorylation of CtrA (generating CtrA~P) takes place at the new cell pole, where its cognate kinase is localized and activated (Angelastro et al. 2009; Iniesta et al. 2010), whereas its dephosphorylation occurs throughout the cell (Mann and Shapiro 2018) (Figure 3). At the new pole, the cells harbor a microdomain made of a polymeric network of the protein PopZ, which slows down the diffusion of CtrA and the proteins involved in its phosphorylation, thereby promoting the local formation of CtrA~P (Lasker et al. 2020). As a consequence, a gradient of CtrA~P is established, with a concentration maximum at the new pole and a minimum at the old pole. This gradient is reinforced by the local degradation of CtrA at the old pole (McGrath et al. 2005) and the upregulation of *ctrA* transcription in the nascent daughter cell at the new pole (Domian et al. 1999). Gradient formation may be further promoted by the increased binding affinity of CtrA~P to DNA, which may slow down CtrA(~P) diffusion within the cell. The tight control of CtrA activity depends on an intricate regulatory network that integrates cell cycle and environmental cues and acts at the levels of CtrA phosphorylation, dephosphorylation and protein degradation.

Multiple alphaproteobacteria control their cell cycles in response to environmental conditions. Several free-living and symbiotic species were, for instance, shown to slow down cell cycle progression (Boutte et al. 2012) or (temporally) halt cell division (Heinrich et al. 2016; Robledo et al. 2018) in response to nutrient limitation or other stress conditions. Tight environmental control of the CtrA pathway is also critical in the context of symbiosis, e.g. for *Sinorhizobium meliloti*, which can live in close contact with plants, or intracellular pathogens such as *Brucella abortus*. In these species, CtrA has acquired control over cell cycle-related and unrelated processes that are relevant for symbiosis, such as the development of symbiotic cell types (Pini et al. 2015) or the synthesis of surface proteins that might be important during infection (Poncin et al. 2018). To modulate CtrA activity in response to environmental signals, a number of different mechanisms have evolved. These mechanisms can affect various nodes of the CtrA pathway and act at the level of transcription, protein stability or (local) phosphorylation and dephosphorylation.

This review discusses the mechanisms that underlie CtrA-dependent cell cycle regulation in alphaproteobacteria. It highlights conserved elements of the CtrA pathway and modifications that have evolved in response to different life cycles. In doing so, it illustrates the amazing diversity of

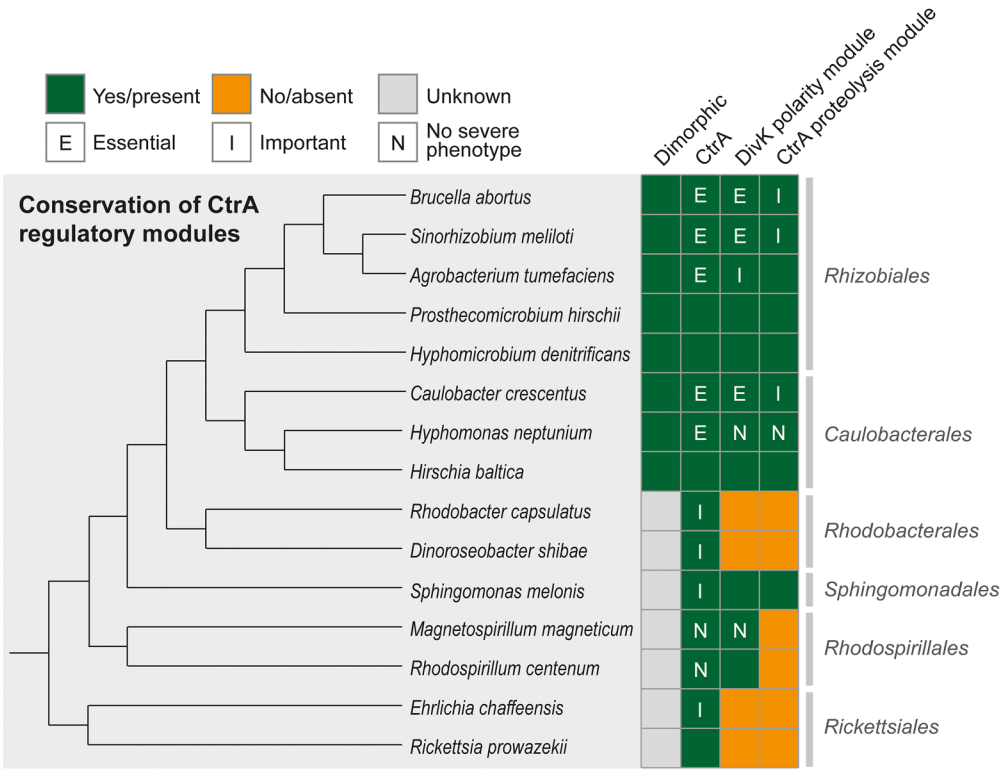

Figure 2: Dimorphism within the *Alphaproteobacteria* correlates with the essentiality of the CtrA regulatory network as well as the presence of the DivK polarity and the CtrA proteolysis modules. Shown is a stylized phylogenetic tree of several representatives of the *Alphaproteobacteria*. The graph on the right indicates whether the mentioned species has a dimorphic life cycle, as defined by a differential reproductive state and size of the two daughter cells. Moreover, it shows the genomic conservation of CtrA, the DivK polarity module (indicated by the presence of a DivK homolog and a homolog of DivJ and/or PleC) and the proteolysis module (indicated by the presence of both an RcdA and CpdR homolog). Homologs were identified by BLAST analysis (Altschul et al. 1990) using the *C. crescentus* protein sequences as queries. Proteins were referred to as homologs when they represented the best reciprocal BLAST hit of the *C. crescentus* homolog with an e-value of e^{-25} or lower. The importance of CtrA, the DivK polarity module and the CtrA proteolysis module as shown by experiments are classified as: essential (E), important (severe phenotype) (I) or no obvious phenotype (N). The tree is adapted from Muñoz-Gómez et al. (2019), and the phylogenetic relationships were determined using the Genome Taxonomy Database (Parks et al. 2018, 2020).

the alphaproteobacterial lineage as well as the astounding complexity and evolutionary plasticity of the regulatory network controlling CtrA activity.

The *C. crescentus* cell cycle and its regulation

The freshwater species *C. crescentus* is a major model system for bacterial development and, in particular, for the establishment of cellular asymmetry in bacterial cells. *C. crescentus* starts its life cycle as a flagellated swarmer cell that is motile and scavenges nutrients from its environment (Figure 1). Swarmer cells have a single circular chromosome and are arrested in a G1-like phase in which the chromosome is not replicated (Degnen and Newton 1972). The origin of replication (*oriC*) of the chromosome localizes to the flagellated pole, where it is tethered to a polar microdomain formed by a loose polymeric network of the intrinsically disordered protein PopZ (Bowman et al. 2008, 2013; Ebersbach et al. 2008). This polar microdomain is hypothesized to represent a biomolecular condensate that consists exclusively of PopZ as well as its specific interactors but excludes other proteins (Lasker et al. 2020). It thus concentrates a series of proteins with cell cycle-regulated functions (see below), limits their diffusion and thereby facilitates interactions between them. One of the proteins that associates with the PopZ microdomain is the chromosome partitioning protein ParB, which in turn binds centromeric sequences (*parS*) located in close proximity of *oriC* and thus positions the origin region at the polar microdomain (Bowman et al. 2008). As the cell cycle progresses, the protein composition of the PopZ microdomain changes, thereby enabling the developmental transition of the flagellated pole to a stalked pole (Bowman et al. 2010).

The development of the flagellated pole initiates with holdfast formation (Hardy et al. 2010; Hernando-Pérez

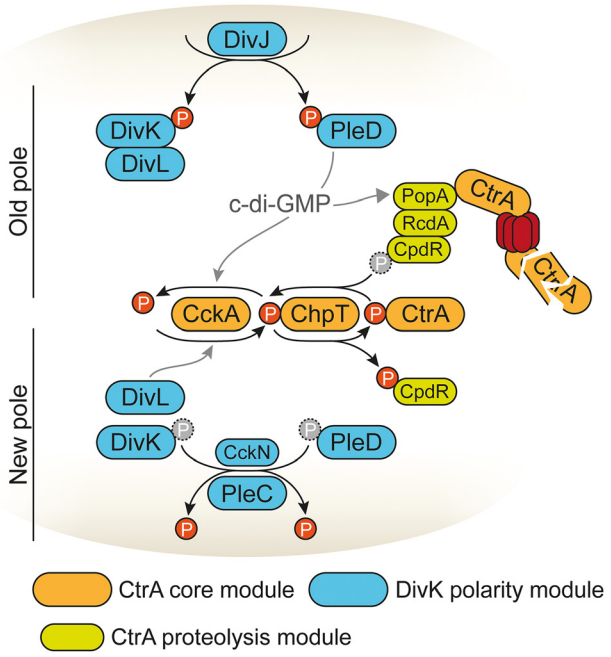

Figure 3: The activity state of the DNA-binding response regulator CtrA is controlled by a complex regulatory pathway.
The scheme illustrates the activation of CtrA at the new pole and the inactivation of CtrA at the old pole of predivisional cells in the well-studied *C. crescentus* system. At the old cell pole, DivJ phosphorylates both PleD and DivK. DivK~P interacts with DivL, and PleD~P synthesizes cyclic-di-GMP (c-di-GMP). These two events set CckA to the phosphatase mode, which then acts via ChpT to dephosphorylate CtrA at the old pole. In parallel, ChpT also dephosphorylates the proteolytic adapter CpdR, which in turn triggers the sequential binding of RcdA, PopA and, ultimately, CtrA to the protease ClpXP, inducing CtrA degradation. At the new cell pole, PleC and, to a smaller extent, CckN dephosphorylate DivK and PleD. As a consequence, DivK cannot interact with DivL, which is thus free to activate the kinase activity of CckA, leading to the phosphorylation of CtrA through the CckA-ChpT phosphorelay. In parallel, ChpT phosphorylates CpdR, thereby preventing the recruitment of RcdA, PopA and CtrA to ClpXP and inhibiting CtrA degradation.

et al. 2018; Hershey et al. 2019) and ejection of the flagellum. Subsequently, a stalk is synthesized at the same pole. The stalk lumen is separated from the cytoplasm by protein disks called cross-bands (Schlimpert et al. 2012). Shortly after stalk synthesis commences, the cells enter S phase by releasing the *oriC* region from the polar PopZ microdomain (Bowman et al. 2010) and initiating chromosome replication (Hottes et al. 2005). The initiation of chromosome replication and segregation, in turn, triggers the formation of a new PopZ microdomain at the pole opposite the stalk (the new pole), which captures the newly replicated, ParB-bound second copy of the *oriC* region. The positioning of ParB at both cell poles enables the cell division regulator MipZ to form a bipolar gradient within the cell with a minimum at the cell center (Kiekebusch et al. 2012; Thanbichler and Shapiro 2006). MipZ acts a negative regulator of the key cell division protein FtsZ and thus limits the assembly of the cell division apparatus to the midcell region, thus ensuring proper cell division. In the meantime, while replication continues, the sister chromosomes are segregated into the nascent daughter cells (Viollier et al. 2004). Concomitantly, one of the nascent daughter cells is preparing for its future life as a swarmer cell, for instance by synthesizing and assembling the chemotaxis machinery and the flagellum (Gomes and Shapiro 1984; Shapiro and Maizel 1973).

Once the cell has finished chromosome replication and is deeply constricted, it enters G2 phase (Jensen 2006). The predivisional cell then finishes cell division, thereby generating a swarmer cell as well as a stalked cell, which is larger in size than the swarmer cell. The stalked cell remains sessile and immediately enters S phase again, whereas the swarmer cell starts its cell cycle in G1 phase and then differentiates into a stalked cell as it initiates DNA replication.

The orchestration of this complex developmental program requires a tight spatiotemporal regulation of cellular processes. In particular, the differentiation of the two daughter cell compartments into distinct cell types poses a considerable challenge. To cope with this issue, *C. crescentus* has evolved a multifaceted cell cycle regulatory network, whose output is to a large extent mediated by the essential DNA-binding response regulator CtrA, a protein affecting the transcription of close to 100 genes (Laub et al. 2002) (Figure 3). In predivisional cells, CtrA upregulates cell division genes as well as genes involved in flagellar biogenesis and chemotaxis. In new-born swarmer cells, by contrast, it represses various genes (mostly of unknown function) and binds to the chromosomal origin of replication to delay replication initiation (Jonas et al. 2011; Laub et al. 2002; Quon et al. 1998). CtrA thus acts specifically at different phases of the cell cycle and, in late predivisional cells, in different cell compartments.

The first factor contributing to the cell cycle-dependent regulation of CtrA activity is the active degradation of CtrA at the stalked pole of predivisional cells. It is mediated by the ClpXP protease (McGrath et al. 2005), which recruits CtrA via the adaptors CpdR, RcdA and PopA (Joshi et al. 2015) (Figure 3). The second factor is its differential phosphorylation in the two daughter cell compartments, combined with the fact that it binds its specific target DNA sequences preferentially in the phosphorylated state (Reisenauer et al. 1999; Siam and Marczynski 2000). CtrA~P is present in swarmer cells and in the nascent swarmer cell compartment of predivisional cells (Domian et al. 1997;

Lasker et al. 2020). CtrA is phosphorylated by the essential phosphotransferase ChpT, which in turn is phosphorylated by the essential histidine kinase CckA (Biondi et al. 2006). It has recently been shown that the activation of CtrA by this phosphorelay is enabled by the concentration of its components in the PopZ microdomain at the new pole (Lasker et al. 2020). Importantly, ChpT does not only shuttle phosphoryl groups to CtrA, but also to the ClpXP-priming adaptor CpdR, thereby blocking its ability to recruit CtrA. Thus, the phosphorylation of CtrA goes hand in hand with its accumulation (Iniesta et al. 2006), leading to robust regulation of its activity at the level of both protein modification and stability. In the predivisional cell, this phosphorelay can also act in reverse to actively dephosphorylate CtrA and CpdR, thereby clearing the cell of CtrA~P (Mann and Shapiro 2018).

Asymmetry is introduced into this system by the localization and concentration of CckA to the new pole of predivisional cells and its subsequent transition to the kinase state. This process is mediated by the essential pseudokinase DivL (Mann and Shapiro 2018; Tsokos et al. 2011), which is, as CckA itself, recruited to the polar PopZ microdomain (Holmes et al. 2016). However, this recruitment of DivL only occurs when DivL is not bound by the phosphorylated essential single-domain response regulator DivK~P (Tsokos et al. 2011). DivK, in turn, is phosphorylated at the stalked pole by the histidine kinase DivJ and dephosphorylated at the flagellated pole by the phosphatase PleC (Tsokos et al. 2011) and the auxiliary phosphatase CckN (Coppine et al. 2020). These (de)phosphorylation processes, which switch CckA to its kinase or phosphatase mode and ultimately lead to CtrA activation and stabilization at the flagellated pole and CtrA inactivation and degradation at the stalked pole, are strictly localized through the recruitment of DivJ by SpmX (Radhakrishnan et al. 2008) and PleC by PodJ (Viollier et al. 2002) at their respective poles (Wheeler and Shapiro 1999). Interestingly, PopZ has been shown to bind to both PodJ (Zhao et al. 2018) and SpmX (Perez et al. 2017), thus creating microdomains of a different composition at each pole. In addition to its spatial regulation, this system is also controlled in time, as the two phosphatases PleC and CckN were found to be degraded in a ClpXP-dependent fashion at the entry into S phase (Coppine et al. 2020). Although DivJ and PleC have an important role in cell cycle regulation, they are not essential, in contrast to all factors downstream of them (DivK, DivL, CckA, ChpT and CtrA).

Another level of complexity is added in the form of the secondary messenger molecule c-di-GMP. It is distributed throughout the cell except for the PopZ microdomain at the flagellated pole. Importantly, c-di-GMP activates the phosphatase mode of CckA (Lori et al. 2015) and concomitantly triggers PopA to recruit CtrA to its ClpXP-adaptor RcdA at the stalked pole for proteolysis (Duerig et al. 2009), thereby clearing the cell of CtrA~P. The accumulation of c-di-GMP, which reaches its maximum at the G1-to-S transition, is mediated by its localized production at the stalked pole, mediated by the response regulator PleD after its phosphorylation by DivJ (Abel et al. 2013).

In addition to the master regulator CtrA, whose abundance peaks in early swarmer cells and the nascent daughter cell, several other regulators are required to ensure proper cell cycle regulation in *C. crescentus*. The two main additional factors are DnaA, which accumulates in swarmer cells just before the G1-S transition (Hottes et al. 2005), and GcrA, which reaches its peak in stalked cells (Holtzendorff et al. 2004). DnaA is a conserved protein that interacts with *oriC* to initiate chromosome replication. However, in *C. crescentus*, it also positively regulates the transcription of at least 40 genes, among them many genes related to DNA replication. Other functions that are affected by DnaA are cell division as well as flagellar motility. Furthermore, DnaA influences cell cycle progression by stimulating the transcription of genes encoding important factors in the CtrA pathway, notably PleC and its polar recruitment factor PodJ, as well as the cell cycle regulator GcrA. GcrA associates with RNA polymerase and activates transcription at methylated σ^{70}-dependent promoters (Haakonsen et al. 2015). Its regulon comprises 125 genes, involved in functions such as DNA replication, recombination and repair, motility and cell wall biogenesis (Holtzendorff et al. 2004). Like DnaA, GcrA is tightly integrated in the cell cycle regulatory network, as it blocks the transcription of *dnaA* and stimulates the transcription of *ctrA* as well as *pleC* and *podJ*. In addition to CtrA, DnaA and GcrA, *C. crescentus* contains various additional proteins that help to control gene expression in specific phases of the cell cycle. SciP, for instance, is thought to prevent the expression of multiple CtrA-activated genes during the G1 phase (Gora et al. 2010; Tan et al. 2010), while MucR prevents expression of CtrA-activated genes in predivisional cells, so that they are exclusively transcribed in G1 phase (Fumeaux et al. 2014). MucR activates *ctrA* transcription and inhibits *sciP* transcription, whereas SciP inhibits transcription of both *ctrA* and the gene for the DNA adenine methyltransferase CcrM (Panis et al. 2015). CcrM, after being expressed in a CtrA-dependent manner in S phase, in turn methylates chromosomal DNA at specific sites, thereby stimulating the activity of GcrA and ensuring the efficient transcription of many S phase genes (Fioravanti et al. 2013). Additionally, during S phase, CtrA triggers the synthesis of the σ^{54}-dependent transcription factor TacA, a protein that is activated through phosphorylation by the

hybrid histidine kinase ShkA (itself activated by c-di-GMP; Kaczmarczyk et al. 2020) and the phosphotransferase ShpA, controlling the expression of 30 genes involved in the swarmer-to-stalked cell transition (Biondi et al. 2005).

CtrA-dependent cell cycle regulation in stalked budding bacteria

Stalked budding bacteria are found in two distinct branches within the *Alphaproteobacteria*. The newly established model for stalked budding bacteria, *Hyphomonas neptunium* (Jung et al. 2015), and other species of this genus, as well as the genus *Hirschia*, belong to the *Caulobacterales* (Muñoz-Gómez et al. 2019) (Figure 2). Another group of stalked budding bacteria, comprising the genus *Hyphomicrobium*, has evolved within the *Rhizobiales*. Just like *C. crescentus* cells, stalked budding bacteria start their life cycle as a flagellated swarmer cell and grow a stalk after shedding their flagellum (Leifson 1964) (Figure 1). However, the stalk grows from the pole opposite the flagellum and does not include crossbands that separate the stalk lumen from the cytoplasmic volume of the cell. Furthermore, surface attachment is believed to be mediated by a holdfast and/or a capsule polysaccharide that surrounds the entire cell body (Badger et al. 2006), as was shown for other *Hyphomonas* species (Quintero and Weiner 1995; Quintero et al. 1998). In *H. neptunium*, whose growth mode and chromosome dynamics have been studied in some detail (Wali et al. 1980; Weiner and Blackman 1973), the stalk elongates from the base (Cserti et al. 2017), like in *C. crescentus*. As a next step, just after stalk synthesis has commenced, the chromosomal *oriC* region, which colocalizes with PopZ at the previously flagellated pole, is replicated (Jung et al. 2019). Following replication, one of the sister *oriC* regions is segregated towards the opposite pole, where it waits at the stalk base. Subsequently, the stalk elongates and its tip is remodeled into a daughter cell (bud) (Cserti et al. 2017). The chromosome, probably in an extended conformation, then rapidly moves through the stalk into the nascent daughter cell, where its *oriC* ultimately colocalizes with PopZ at the pole opposite the stalk (Jung et al. 2019). The nascent daughter cell forms a flagellum and becomes a motile swarmer cell upon division. The stalked mother cell, by contrast, immediately enters S phase, restores its stalk and then again starts to remodel the tip of the stalk to form the next daughter cell.

A recent publication has shed light on the CtrA pathway in *H. neptunium* and shown that it is also critical for cell cycle regulation in stalked budding bacteria (Leicht et al. 2020). *In vitro* experiments confirmed that the (de)phosphorylation of CtrA through the CckA–ChpT phosphorelay and of DivK through DivJ and PleC is conserved in this species. As in *C. crescentus*, the CtrA homolog of *H. neptunium* directly regulates ~100 genes and binds to the chromosomal origin region close to *oriC*. CtrA, the CckA–ChpT phosphorelay, and DivL were found to be essential in *H. neptunium*, and cells depleted of these factors accumulate multiple ectopic and elongated stalks, indicative of a block in cell division. As in the *C. crescentus* system, the histidine kinases DivJ and PleC are differentially localized to the old and new pole, respectively, in predivisional cells, suggesting that they are involved in activating the kinase activity of CckA at the one pole and its phosphatase activity at the other pole.

However, there are also striking differences between the CtrA regulatory networks of *C. crescentus* and *H. neptunium*. It appears that, in *H. neptunium*, so-far unidentified factors act in parallel to the canonical upstream pathway consisting of PleC, DivJ and DivK, because mutants lacking these proteins show (close to) normal cell growth and morphology. Moreover, the switching of CckA between the phosphatase and kinase states might be controlled in a fundamentally different manner. Whereas, in *C. crescentus*, CckA needs to accumulate within the polar PopZ microdomain to transition to the kinase state (Mann et al. 2016), its *H. neptunium* homolog does not concentrate into a polar focus that colocalizes with PopZ *in vivo*. In this respect, it is interesting to note that *H. neptunium* cells can tolerate the loss of PopZ without showing any obvious phenotype (Jung et al. 2019), which raises the question whether polar microdomains play a significant role in cell cycle regulation in this species. Another striking difference that might reflect a difference in the regulation of CckA is the lack of a phenotype for the loss of PleD, which could indicate that c-di-GMP may have no (major) role in CckA regulation (Leicht et al. 2020). A notable difference is finally also seen in the mechanisms that control CtrA stability. One of the mechanisms that establishes cellular asymmetry in *C. crescentus* is the cell cycle-dependent proteolysis of CtrA at the stalked pole. However, CtrA does not appear to be actively degraded in *H. neptunium*, suggesting that it may be largely regulated at the level of phosphorylation.

So far, only a single stalked budding bacterium has been studied in appreciable detail, which makes it difficult to determine whether the differences observed in the CtrA pathway represent a specific adaptation to the unusual developmental cycle of *H. neptunium* or are simply tolerated because of the specific morphology of this species. The

difference with the largest impact on CtrA-dependent cell cycle regulation between predivisional *C. crescentus* and *H. neptunium* cells might be that the two sister chromosomes remain closely associated through the entire cell cycle in *C. crescentus*. In *H. neptunium*, by contrast, the presence of the stalk spatially separates the sister chromosome that is segregated into the nascent bud from the maternal chromosome in the mother cell body. It is conceivable that CtrA~P formed in the bud is trapped through association with the proximal nucleoid, so that only a small amount of CtrA~P diffuses through the stalk to the mother cell compartment. This effect may obviate the need to limit CtrA phosphorylation to the pole or to degrade CtrA at the stalked pole to maintain the asymmetry between the nascent mother and daughter cells.

CtrA-dependent cell cycle regulation in polarly growing *Rhizobiales*

The *Rhizobiales* include many species that live in close association with eukaryotes, which form a phylogenetic cluster comprising the families *Bartonellaceae*, *Brucellaceae* and *Rhizobiaceae*. They also contain many free-living species, such as various families of methano- and methylotrophs as well as bacteria with complex morphologies belonging to the family *Hyphomicrobiaceae*. Multiple members of the *Rhizobiales*, including the animal pathogen *B. abortus*, the plant pathogen *Agrobacterium tumefaciens*, the plant symbiont *S. meliloti* and multiple species within the *Hyphomicrobiaceae*, have been shown to grow by polar extension of their cell bodies rather than by MreB-based lateral growth, as observed for the majority of bacteria (Brown et al. 2012). *B. abortus*, *A. tumefaciens* and *S. meliloti* are rod-shaped and stalkless. For these species, the asymmetry between the mother and daughter cell is therefore less obvious at first sight. However, careful studies have shown that, in all three species, cell division produces one larger and one smaller sibling (Ehrle et al. 2017; Hallez et al. 2004). Moreover, for *A. tumefaciens* and *S. meliloti*, the two siblings showed a difference in the timing of replication initiation (Ehrle et al. 2017; Frage et al. 2016). Whether this asymmetry in the timing of the cell cycle also applies to *B. abortus* is still an open question (de Bolle et al. 2015). It has not been explicitly studied whether, in the *Rhizobiales*, the mother cell consistently enters S phase, while the daughter cell enters G1 phase after division. However, some members of the *Hyphomicrobiaceae*, such as the stalked budding bacterium *Hyphomicrobium denitrificans* (Urakami et al. 1995) and *Prosthecomicrobium hirschii* (Williams et al. 2016), were shown to divide asymmetrically into a sessile mother cell as well as a motile daughter cell that later differentiates into a sessile cell. In the following paragraphs, we will first discuss cell cycle regulation in the eukaryote-associated rod-shaped bacteria *B. abortus*, *A. tumefaciens* and *S. meliloti*, as these species share many similar features. Subsequently, we will discuss cell cycle regulation in *P. hirschii*, the only example of a morphologically more complex non-stalked budding member of the *Rhizobiales* in which CtrA has been studied.

Newborn *B. abortus*, *A. tumefaciens* and *S. meliloti* daughter cells are rod-shaped and arrested in G1 phase (Deghelt et al. 2014; Ehrle et al. 2017; Frage et al. 2016). The newborn daughters of *A. tumefaciens* are motile by means of a (sub)polar tuft of flagella (Heindl et al. 2014). By contrast, no motility has been observed under laboratory conditions for *B. abortus* (De Bolle et al. 2015). *S. meliloti* cells have peritrichous flagella (Sourjik and Schmitt 1996), but it remains unknown if these are formed specifically in the daughter cell. Similar to *C. crescentus*, *A. tumefaciens* cells produce an adhesive, called unipolar polysaccharide (UPP), which mediates the attachment of one of their poles to surfaces (Tomlinson and Fuqua 2009; Li et al. 2012). It has been suggested that the UPP is produced by the mother cell, whereas flagella are formed at the new pole, that is by the incipient daughter cell (Heindl et al. 2014), but this hypothesis has not been verified experimentally. The UPP gene cluster is conserved in *S. meliloti* and was shown to be involved in biofilm formation in this species (Schäper et al. 2016). However, also in this case, it is unclear whether the UPP is limited to one of the cell poles and whether it is specially associated with the mother cell. Furthermore, it appears that other polysaccharides might be more important for *S. meliloti* biofilm formation (Schäper et al. 2019). *B. abortus* cells were shown to produce unipolar adhesins, and strikingly these adhesins were consistently detected at the new pole (Ruiz-Ranwez et al. 2013) rather than the old pole, as observed for *C. crescentus* and *H. neptunium*.

A notable feature that differentiates *B. abortus*, *A. tumefaciens* and *S. meliloti* from *C. crescentus* are their multipartite genomes: *B. abortus* has two chromosomes (Chain et al. 2005), *S. meliloti* has one main chromosome and two megaplasmids (Galibert et al. 2001) and *A. tumefaciens* has one circular and one linear chromosome, a megaplasmid and, in some strains, a virulence plasmid (Wood et al. 2001). At a certain point in the cell cycle, which is affected by the environmental conditions (Deghelt et al. 2014), the cells replicate and segregate their various replicons. The spatiotemporal control of the underlying processes has been studied in *B. abortus* and *S. meliloti*. Upon entry into S phase, both species first start to replicate their main chromosome in a manner dependent on the

replication initiator DnaA. As DNA synthesis proceeds, one of the newly synthesized chromosomal copies is segregated into the nascent daughter cell compartment, probably driven by the ParABS system (Deghelt et al. 2014; Frage et al. 2016). The replication and segregation of the other replicons initiates later, independent of DnaA (Frage et al. 2016) and coordinated by the RepABC system (Fournes et al. 2018). In both species, PopZ is important for proper chromosome segregation and appears to capture ParB, which binds close to the *oriC* of the main chromosome, at the new pole after segregation (Ehrle et al. 2017; Howell et al. 2017). Interestingly, as in *H. neptunium*, PopZ is localized predominantly to the new pole in *A. tumefaciens*, *B. abortus* and *S. meliloti*, suggesting the existence of another polar anchor that captures the *oriC* region of the chromosome located at the old pole in these organisms. The replicated *oriC* regions of non-chromosomal replicons move to a subpolar region in all three cases (Deghelt et al. 2014; Frage et al. 2016; Kahng and Shapiro 2003).

In addition to the vegetative, dimorphic cell cycle described above, *B. abortus* and *S. meliloti* undergo a specific developmental program upon infection of eukaryotic cells. In the case of *B. abortus*, infectivity is highest for daughter cells that are in the G1 phase (Deghelt et al. 2014). In this light, it is of advantage to attach the nascent daughter cell to surfaces (as opposed to the mother cell as seen in many other alphaproteobacteria), as attachment is the first step in pathogenesis. During the first hours of infection, *B. abortus* cells show a prolonged G1 arrest, concurrent with an arrest of growth. Later, they resume their cell cycle and proliferate inside host cells (Deghelt et al. 2014). The colonization of plant roots by *S. meliloti* is triggered by chemical cues from the plant root prior to invasion of the root and, subsequently, the growing nodules (Gibson et al. 2008). Inside the nodules, the bacteria enter plant cells and differentiate into so-called bacteroids, which fix nitrogen to assist in plant growth. These bacteroids lose the ability to divide, while they continue to grow and replicate their various replicons. As a consequence, they are significantly longer than free-living cells and show a branched morphology (Mergaert et al. 2006), as typically observed upon inhibition of cell division in polarly growing members of the *Rhizobiales*. *A. tumefaciens* cells sense signals secreted by wounded plant tissues and in response move chemotactically towards these tissues and express specific virulence genes (Hwang et al. 2017). After the bacteria attach to the plant cells, they produce a type IV secretion system, through which they translocate a linear single-stranded piece of DNA into the plant cell, which gets integrated in the chromosome of the plant cell. After several days of infection, the plant starts to develop the tumors that are characteristic of *A. tumefaciens* infections. Only little is known about the morphology and replicative status of the *A. tumefaciens* cells during infection, but studies suggest that the morphology of the cells does not fundamentally differ from that of free-living bacteria (Li et al. 1999; Matthysse et al. 1981).

As free-living *A. tumefaciens*, *S. meliloti* and *B. abortus* show dimorphic life cycles comparable to those of other alphaproteobacteria, it is not surprising that the CtrA pathway is essential for proper growth and development of the free-living stages (Bellefontaine et al. 2002; Figueroa-Cuilan et al. 2016; Francis et al. 2017; Pini et al. 2015; Willett et al. 2015). Its role has been studied in *B. abortus* and *S. meliloti* (Francis et al. 2017; Pini et al. 2015), where it was found to control processes such as cell division, cell growth, motility and cell cycle progression. In *B. abortus*, CtrA also appears to have an important role in the infection process, as its depletion leads to a significant reduction in cell viability after the invasion of host cells (Francis et al. 2017; Willett et al. 2015). In line with this finding, it was shown to regulate the production of multiple outer membrane proteins that could potentially affect the infectivity of *B. abortus* (Francis et al. 2017). Interestingly, however, CtrA is absent from *S. meliloti* bacteroids (Pini et al. 2013) and downregulated during infection in *A. tumefaciens* (González-Mula et al. 2018). Phosphotransfer in the CckA–ChpT–CtrA phosphorelay has been shown directly for *B. abortus* (Bellefontaine et al. 2002; Willett et al. 2015) and indirectly for *S. meliloti* (Pini et al. 2013). In addition, regulated proteolysis of CtrA by ClpXP is conserved and important in *B. abortus* (Willett et al. 2015) and *S. meliloti* (Pini et al. 2015). If CtrA proteolysis is important in *A. tumefaciens* remains to be clarified.

Interestingly, in all three organisms, the upstream pathway that mediates the (de)phosphorylation of DivK includes additional histidine kinases next to DivJ and PleC (Gibson et al. 2006; Hallez et al. 2007; Kim et al. 2013; Pini et al. 2013). These proteins are referred to as PdhS (PleC/DivJ homolog sensor) kinases, and some of them have taken over major roles in cell cycle regulation (Hallez et al. 2007; Kim et al. 2013; van der Henst et al. 2012). The increased importance of PdhS kinases over DivJ and PleC is also reflected in the localization of these factors. For instance, in *B. abortus*, DivJ does not show any polar localization, while the PdhS protein that has taken over the role of the major DivK kinase is detected at the old pole during the majority of the cell cycle and required to position DivK~P at the same pole (Hallez et al. 2007). Surprisingly, in *B. abortus*, PleC is not localized to the new pole in predivisional cells, although it is recruited to the nascent/newly formed poles around the time of cell division. This poses the question of how cellular asymmetry is

established in pre- and postdivisional cells. It has been observed that CtrA in *B. abortus* regulates the expression of many proteins that are involved in its own phosphorylation, such as DivL and DivK (Francis et al. 2017). It is conceivable that CckA is routinely switched to the kinase state, leading to high CtrA~P levels throughout the cell, so that it suffices to switch CckA to the phosphatase mode specifically at the old pole by PdhS-mediated phosphorylation (and localization) of DivK at this pole. The finding that the production of a CpdR variant that can no longer be phosphorylated and therefore constitutively facilitates CtrA degradation has a more severe phenotype than the lack of CpdR (Willett et al. 2015) could be seen as a support for the hypothesis that CckA kinase activity is the default state in *B. abortus* cells. The very brief localization of PleC in the newly divided cells could trigger a reset of CckA to the kinase state, which could then persist in the daughter cell until PdhS localizes to the old pole at a later point in the cell cycle.

As in *B. abortus*, the PdhS-like histidine kinase CbrA of *S. meliloti* acts, together with DivJ, as a major kinase of DivK (Pini et al. 2013) and localizes DivK~P to the old pole (Greif et al. 2010; Lam et al. 2003; Sadowksi et al. 2013). PodJ, which in *C. crescentus* positions PleC (Viollier et al. 2002), localizes to the new pole of the predivisional cell. By contrast, PleC, which is essential in *S. meliloti*, was observed to localize to the septum (as in *B. abortus*), but the significance of this result remains to be clarified (Fields et al. 2012). The localization patterns of the DivK kinases of *A. tumefaciens* resemble that of DivJ in *C. crescentus*. Both DivJ and PdhS1, which is thought to act as a second, less important DivK kinase (Kim et al. 2013), localize at the old pole (Ehrle et al. 2017). PdhS2, by contrast, which is thought to function as a DivK phosphatase, acting in parallel to PleC and through a CckA-independent pathway (Heindl et al. 2019), localizes to the (incipient) new poles just before and after cell division (Ehrle et al. 2017). Interestingly, in *A. tumefaciens*, DivK itself was found to be non-essential, although cells lacking DivK had a severe phenotype (Kim et al. 2013).

Although CtrA depletion or misregulation was shown to cause overreplication of the different replicons in *B. abortus* and *S. meliloti*, CtrA does not bind the chromosomal *oriC* region in these species (Francis et al. 2017; Pini et al. 2015). Instead, in *B. abortus*, the replication initiator DnaA as well as the *repABC* operon, involved in the replication and segregation of chromosome II, are regulated by CtrA (Francis et al. 2017). The mechanism controlling replication initiation of the multiple replicons in *S. meliloti* are still unclear (Pini et al. 2015).

Not all members of the *Rhizobiales* have simple, rod-like morphologies. A prominent example of a species with a more complex morphology is *P. hirschii*, which has two different cell types: cells covered with short stalks and cells that have between three and twelve stalks that are longer than the cell body (Williams et al. 2016). Whereas short-stalked mother cells give rise almost exclusively to short-stalked daughter cells, half of the long-stalked mother cells produce short-stalked daughter cells. The short-stalked cells have a dimorphic life cycle, in which the mother cell, which is sessile by means of a polar holdfast, gives rise to a motile daughter cell. Motile daughter cells form holdfasts shortly after sensing a surface. The replication states of the mother and daughter cell remain to be investigated. The cell cycle regulatory pathway of *P. hirschii* has so far only been inferred from *in silico* analyses, which suggest that CtrA regulates processes such as motility, chemotaxis, cell growth and division, and chromosome replication (Williams et al. 2016). Interestingly, these analyses further revealed similarities to other members of both the *Rhizobiales* and *Caulobacterales*. Like the rod-shaped members of the *Rhizobiales* described above, *P. hirschii* contains two PdhS proteins that likely act in parallel to PleC and DivJ. Future research will show the importance of these factors. However, unlike in its relatives, a predicted CtrA binding site was detected close to the predicted *oriC* locus of its single chromosome. This finding reflects the situation for *C. crescentus* and *H. neptunium* and supports the hypothesis that the CtrA binding site close to *oriC* was lost during evolution in species that have a multipartite genome.

Role of CtrA in non-dimorphic alphaproteobacteria

The *Alphaproteobacteria* comprise numerous orders besides the *Rhizobiales* and *Caulobacterales*. To our knowledge, these other orders do not include dimorphic bacteria. Nonetheless, CtrA appears to be conserved in the alphaproteobacterial clade (Brilli et al. 2010) (Figure 2). Moreover, the components of the phosphorelay that control its activity (Figure 3), including CckA and, to a lesser extent, ChpT as well as the pseudokinase DivL, involved in CckA activation, are also relatively well-conserved. However, both the DivJ-PleC-DivK module and also the proteolytic adaptors CpdR and RcdA, which are critical to control cellular asymmetry in the *Rhizobiales* and *Caulobacterales*, are barely present outside these lineages (Figure 2). This finding, together with the sporadic conservation of individual factors throughout the alphaproteobacterial lineage, suggests that the asymmetry determinants of the CtrA network, and possibly the dimorphic life cycles that they

enable, may have been present in the alphaproteobacterial ancestor but then gradually lost during the course of evolution.

The wide conservation of CtrA and, to some extent, its regulatory factors CckA and ChpT, suggests that these proteins also have important functions in non-dimorphic alphaproteobacteria. CtrA has been studied in various species, including members of the *Rickettsiales*, *Sphingomonadales*, *Rhodobacterales* and *Rhodospirillales*. In all seven cases in which a deletion of *ctrA* has been attempted to date mutants were readily obtained, indicating that CtrA is not essential in non-dimorphic species. Nonetheless, in the three species in which transcriptome studies were performed, CtrA emerged as a global transcriptional regulator that controls, directly or indirectly, between 191 and 452 genes (Greene et al. 2012; Mercer et al. 2010; Wang et al. 2014). A process that has been predicted or confirmed to be regulated by CtrA in all of the species investigated, except for a non-motile *Rickettsia* species (Cheng et al. 2011), is flagellar motility (Bird and MacKrell 2011; Francez-Charlot et al. 2015; Greene et al. 2012; Mercer et al. 2010; Miller et al. 2006; Wang et al. 2014; Zan et al. 2013). The omnipresence of this regulatory scheme has sparked the hypothesis that this is the ancestral function of CtrA (Greene et al. 2012). Apart from the regulation of motility, CtrA is often involved in the adaptation of cells to changing environments. It was found to control the development of cysts in response to starvation by *Rhodospirillum centenum* (Bird and MacKrell 2011), transition to an infective state in the obligate intracellular pathogen *Ehrlichia chaffeensis* (Cheng et al. 2011), or cell size and shape differentiation in response to high cell density in *Dinoroseobacter shibae* (Wang et al. 2014). As in the *Rhizobiales* and *Caulobacterales*, CtrA also controls chromosome replication in *Rickettsia prowazekii* (Brassinga et al. 2002) and *D. shibae* (Wang et al. 2014). Another functional category of genes that is overrepresented in the CtrA regulons of non-dimorphic alphaproteobacteria is 'signal transduction'. It thus appears that in the last common ancestor of the *Alphaproteobacteria*, the CtrA network might have served to integrate environmental signals in order to steer conditional developmental processes. Most likely, the process that this ancestral system controlled was motility, but it may potentially also have produced cellular asymmetry by differentially affecting replicative state of the daughter cells.

Concluding remarks

A key feature of the *Alphaproteobacteria* is the occurrence of complex life cycles. The knowledge accumulated to date shows that, from a cell biological perspective, the most important distinction within this bacterial class is between dimorphic and non-dimorphic species. The dimorphic species produce a smaller, often motile, daughter cell that is arrested in G1 phase and a larger, often sessile, cell that enters S phase immediately after cell division. How and why this complex cell cycle has evolved remains an open question. It is, however, striking that similar cell cycles have evolved within the *Planctomycetes* (Wiegand et al. 2018). Like some of the dimorphic alphaproteobacteria, including *C. crescentus* (Pointdexter 1964), *H. neptunium* (Leifson 1964) and *P. hirschii* (Staley 1984), several members of the *Planctomycetes* are abundant in low-nutrient aquatic environments (Delmont et al. 2018; Ivanova and Dedysh 2012; Lefort and Gasol 2013). The switching between sessile and motile cells may thus provide a fitness advantage in these specific growth conditions. It has been hypothesized to represent a bet-hedging strategy that enables a subpopulation of cells colonize the local environment, whereas another subpopulation can search the surroundings for new nutrient sources (Kysela et al. 2013). This strategy may be especially useful in environmental niches that are characterized by rapid changes in nutrient availability. Another advantage of asymmetric cell divisions can lie in the differential ageing of the two offspring. For instance, under certain conditions, *C. crescentus* stalked cells reproduce more slowly with increasing age, whereas the 'rejuvenated' swarmer cells show similar reproduction times independent of the age of their stalked mother cells (Ackermann et al. 2003). This differential ageing may provide a subset of the population with a fitness advantage under competitive growth conditions.

The DNA-binding response regulator CtrA is present in both dimorphic and non-dimorphic alphaproteobacteria, but only in the dimorphic species its function is essential. In these species, CtrA~P affects asymmetry by influencing the timing of replication, cell division and motility and attachment. In dimorphic species, a variety of different proteins are involved in CtrA (in)activation, and the complexity of its regulatory networks is still incompletely understood. The upstream module that controls the phosphorylation state of DivK, comprising DivJ and PleC, or their PdhS homologs, is the main driver of cellular asymmetry, as it controls the differential activity of CtrA in the two cell compartments. Consistently, this module is present in the dimorphic species but absent in most other alphaproteobacteria (Figure 2). As an additional module that makes the generation of cellular asymmetry more robust, most dimorphic species use a proteolytic cascade that degrades CtrA specifically at one of two cell poles. A possible explanation for the multi-layered regulation of CtrA is that cell cycle progression needs to be precisely adjusted to both the physiological state of the cell and environmental conditions. The complexity of the CtrA

regulatory network may allow the integration of various regulatory inputs and thus facilitate the fine-tuning of cell growth and differentiation.

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