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Polarity and cell fate asymmetry in *Caulobacter crescentus*

Christos G Tsokos¹ and Michael T Laub^{1,2}

¹Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, United States

²Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02139, United States

Abstract

The production of asymmetric daughter cells is a hallmark of metazoan development and critical to the life cycle of many microbes, including the α -proteobacterium *Caulobacter crescentus*. For *Caulobacter*, every cell division is asymmetric, yielding daughter cells with different morphologies and replicative potentials. This asymmetry in daughter cell fate is governed by the response regulator CtrA, a transcription factor that can also bind and silence the origin of replication. CtrA activity is controlled by a complex regulatory circuit that includes several polarly localized histidine kinases. This circuit ensures differential activation of CtrA in daughter cells, leading to their asymmetric replicative potentials. Here, we review progress in elucidating the molecular mechanisms regulating CtrA and the role of cellular polarity in this process.

Introduction

Bacteria were long thought to be symmetric and to divide through simple binary fission, creating identical daughter cells. Every rod-shaped bacterium is, however, inherently asymmetric, with a new pole created during the previous round of cell division and an old pole created during an earlier cell division (Figure 1a). There is growing recognition that many species exploit this polarity to support directional motility, generate variable growth rates and cell sizes, or produce daughter cells with distinct fates. The molecular mechanisms that underlie the formation and exploitation of cellular asymmetry remain incompletely understood and an active area of research.

The α -proteobacterium *Caulobacter crescentus* divides asymmetrically to produce two different daughter cells, a stalked cell and a swarmer cell, that have distinct morphologies and replicative potentials (Figure 1b). The sessile stalked cell contains a polar stalk and produces an exopolysaccharide that facilitates adhesion to various surfaces; this cell type also initiates DNA replication shortly after birth. By contrast, the daughter swarmer cell is motile and chemotactic through use of a polar flagellum, and it cannot initiate DNA replication. Given sufficient nutrients, the swarmer cell will differentiate into a stalked cell by ejecting its polar flagellum and constructing a stalk at the same site. After initiating DNA replication, stalked cells enter a predivisional phase during which they complete DNA replication and prepare the division septum. They also execute a developmental program that creates a new swarmer pole opposite the stalk, synthesizing a polar flagellum and the membrane-proximal portions of pili, which are extended into full pili after cell division.

CtrA, the enforcer of replicative asymmetry

The generation of *Caulobacter* daughter cells with different morphologies and replicative potentials is a complex and tightly regulated process. The replicative asymmetry is governed predominantly by CtrA, an essential response regulator and two-component signaling protein [1*]. CtrA is a transcription factor that, upon phosphorylation, can activate the expression of nearly 100 genes, many of which are critical to polar morphogenesis and cell division [2,3]. In addition, CtrA directly regulates DNA replication by binding to and silencing the origin of replication [4**,5]. CtrA is abundant and phosphorylated in swarmer cells to maintain the G1 state, and then dephosphorylated and degraded in stalked cells to permit the onset of S phase [6]. Following replication initiation, CtrA is synthesized *de novo* and again phosphorylated, allowing it to activate target genes in predivisional cells. Cell division then yields a new swarmer cell that retains active CtrA, and hence cannot initiate replication, and a stalked cell that inactivates and degrades CtrA, permitting another round of replication (Figure 2a).

The differential activity of CtrA in daughter cells is essential in determining their different replicative capacities. Not surprisingly then, CtrA activity is tightly controlled, temporally and spatially, through a combination of proteolysis, phosphorylation, and protein–protein interaction. The transcription of *ctrA* is also cell cycle-regulated [7], but cells constitutively expressing *ctrA* show no major cell cycle or asymmetry defects [6]. Instead, regulated proteolysis and phosphorylation play the dominant roles, and cells producing a non-degradable, constitutively active version of CtrA fail to progress through the cell cycle, arresting in G1 [6].

The cell type-specific stabilization and phosphorylation of CtrA is ultimately regulated by two phosphorelays [8], each initiating with CckA, an essential hybrid histidine kinase [9]. After autophosphorylating, CckA transfers the phosphoryl group intramolecularly to its receiver domain. The histidine phosphotransferase ChpT then shuttles the phosphoryl group from CckA to either CtrA (Figure 2b) or another response regulator called CpdR. The phosphorylation of CtrA enhances DNA-binding [10], enabling the activation of target genes in predivisional cells and silencing of the origin in swarmer cells. The phosphorylation of CpdR, first identified in a systematic search for two-component cell-cycle regulators [11], prevents CtrA degradation by the protease ClpXP [8,12–14]. Unphosphorylated CpdR was postulated to drive localization of ClpXP to the stalked pole where it would degrade CtrA [12], but whether the polar localization of ClpXP or CtrA is required for degradation is unresolved [15]. Regardless, it appears that unphosphorylated CpdR directly promotes the degradation of a c-di-GMP phosphodiesterase called PdeA [16]. The proteolysis of PdeA helps trigger accumulation of c-di-GMP, a second messenger that induces the swarmer-to-stalked cell morphological transition [17,18]. c-di-GMP activates CtrA degradation through a protein called PopA [19**], but the underlying mechanism remains unclear.

In sum, the activation of CckA as a kinase in swarmer and predivisional cells [20] drives CtrA phosphorylation and stabilization in these cell types (Figure 2a). In stalked cells, CckA is inactivated leading to the dephosphorylation and degradation of CtrA. Like most histidine kinases, CckA is bifunctional, harboring both kinase and phosphatase activity [21]. Consequently, when not stimulated to autophosphorylate (and phosphotransfer), phosphoryl groups are siphoned back through the phosphorelays to the receiver domain of CckA where they are actively eliminated by hydrolysis [21].

Although CtrA accumulates in swarmer cells to silence replication, it is prevented from activating most of its target genes in this cell type. A small (~11 kDa) protein called SciP accumulates specifically in swarmer cells and binds directly to CtrA to inhibit

transcriptional activation [22**]. *SciP* does not, however, prevent CtrA from binding DNA, allowing it to repress target genes and silence the origin in swarmer cells [22**,23]. Although *sciP* is not essential for viability, cells lacking *SciP* grow slowly and exhibit a variety of cell-cycle phenotypes, as the inappropriate expression of CtrA-dependent genes in swarmer cells disrupts subsequent cell cycle progression [22**].

Regulation of the histidine kinase CckA

Given that CtrA and replicative asymmetry are ultimately regulated by CckA, how is CckA activity controlled in a cell type-specific manner? Early fluorescence microscopy studies found that a fusion of CckA and green fluorescent protein (GFP) often localizes to the cell poles, but the relationship between localization and activity was initially unclear [9]. CckA is primarily delocalized in swarmer cells, localized to the stalked pole in some stalked cells, and localized either bipolarly or only at the swarmer pole in predivisional cells [21,24]. Thus, the localization of CckA does not correlate well with its activity as a kinase (Figure 2a).

New insight into the regulation of CckA has come with characterization of DivL, an atypical histidine kinase [25,26*]. DivL is required for maximal activation of CckA and, consequently, the phosphorylation of both CtrA and CpdR [27–29]. DivL typically localizes only to the swarmer pole of predivisional cells and is required to recruit CckA to this pole [27,28**,30] (Figure 2a). DivL is unorthodox as it bears strong homology to canonical histidine kinases but contains a tyrosine in place of the conserved histidine, and it does not require its ATPase domain to perform essential cell cycle functions [31]. Instead, DivL probably modulates CckA activity through a regulated protein–protein interaction.

The ability of DivL to stimulate CckA kinase activity is regulated by the essential single-domain response regulator DivK [32]. Cells lacking DivK arrest in G1 with stable, phosphorylated CtrA [33], and epistasis experiments indicate that DivK acts upstream of DivL and CckA [28**]. When phosphorylated, DivK binds directly to DivL to inhibit CckA kinase activity [28**]. Conversely, in the unphosphorylated state, DivK does not inhibit DivL, allowing DivL to promote CckA kinase activity (Figure 2b). That DivK and DivL interact in a phosphorylation-dependent manner is not unexpected given that they are two-component signaling proteins. However, it is unusual that DivK, the response regulator, modulates DivL, the histidine kinase. Precisely how DivK affects DivL's ability to promote CckA activity is an open question.

DivK phosphorylation is controlled by a cognate kinase, DivJ, and phosphatase, PleC [34–37,38*,39,40]. Swarmer cells harbor PleC that dephosphorylates DivK, thereby allowing DivL and CckA to maintain active, phosphorylated CtrA. Upon differentiating into stalked cells, PleC is replaced by DivJ, which phosphorylates DivK. Phosphorylated DivK allosterically activates DivJ resulting in a positive feedback loop that further accelerates DivK phosphorylation [34] and the consequent downregulation of CtrA, a prerequisite for replication initiation. The activities of DivK and CtrA are thus inversely related in swarmer and stalked cells: when DivK is phosphorylated, CtrA is not, and when DivK is dephosphorylated, CtrA is phosphorylated (Figure 2).

The relationship between DivK and CtrA is more complicated in predivisional cells where both regulators are phosphorylated. In this cell type PleC is located at the swarmer pole and DivJ at the stalked pole (Figure 2a). Importantly, CckA and DivL are colocalized with PleC at the swarmer pole. Within this polar microenvironment, DivK is dephosphorylated by PleC to prevent, or somehow change, its binding to DivL, thereby activating CckA specifically at this pole [28**]. PleC acts locally and cannot overpower DivJ to dephosphorylate DivK across the entire cell; hence, bulk measurements indicated that DivK

is phosphorylated in this cell type [37]. The rapid inactivation of PleC using a temperature-sensitive allele causes the immediate inactivation of CckA and CtrA, as phosphorylated DivK accumulates throughout the cell, including within the swarmer pole microenvironment, and downregulates CckA via DivL [28**].

In sum, this recent work indicates that a primary reason for CckA localization in predivisional cells is to bring it, and DivL, into close proximity with the phosphatase of DivK [28**]. Polar localization is not, however, always required for CckA kinase activation. CckA is also active in swarmer cells [20] where it and DivL are often delocalized (Figure 2a); at this stage, PleC is more abundant than DivJ, and, consequently, DivK phosphorylation levels are low across the whole cell. DivL is still required to activate CckA in swarmer cells, indicating that DivL does not just shield CckA from DivK, but probably also promotes an active conformation of CckA.

As noted, in predivisional cells, CckA is usually bipolarly localized and so present at the stalked pole without PleC. This pool of CckA probably functions as a phosphatase because DivL is usually absent from the stalked pole; even if DivL is present, DivJ is at this pole to ensure high local levels of phosphorylated DivK and a consequent downregulation of CckA kinase activity.

The fact that CckA is a kinase at the swarmer pole and a phosphatase at the stalked pole suggests that phosphorylated CtrA could form a gradient across the cell (Figure 2a). In *Caulobacter*, the origins of replication, which are silenced by CtrA, are anchored at the cell poles [41]. Hence, the CtrA gradient model predicts that *Caulobacter* predivisional cells that are allowed to reinitiate DNA replication (by blocking cytokinesis) will initiate a new round of DNA replication at the stalked pole where levels of phosphorylated CtrA are lowest, but not at the swarmer pole. This prediction was verified by examining DNA replication in individual cells in which the polarly localized origins were fluorescently marked [42*]. The replicative asymmetry observed depended on CtrA as cells lacking active CtrA, or containing fewer CtrA binding sites in the origin, replicated from both poles simultaneously or from one pole with equal likelihood [42*]. These results indicate that the asymmetric fates of daughter cells are established before cytokinesis and, more generally, they provide a striking example of the sophisticated spatial patterns of protein activity that can be generated inside bacterial cells.

Importantly, the heterogeneous distribution of phosphorylated CtrA in predivisional cells occurs despite a mostly uniform distribution in this cell type of CtrA-GFP [42*,43] and ChpT-GFP (M. Laub, unpublished data), which do not distinguish between phosphoforms. Mathematical models indicate that a gradient of phosphorylated CtrA requires only (i) a localized kinase and/or phosphatase, which is the case, and (ii) that the kinase and phosphatase operate on time-scales faster than diffusion. Although diffusion within bacterial cells is fast, the time-scale of CtrA phosphorylation and dephosphorylation is faster [42*].

Sources of polarity and asymmetry

DivL ultimately functions to couple two signaling pathways (Figure 2b). One, comprising the CckA-based phosphorelays, controls CtrA activity. The other, comprising DivJ/PleC and their cognate regulator DivK, ensures cell type-specific activation of these phosphorelays. The localization of DivJ and PleC to opposite poles and their differential inheritance by daughter cells is thus critical to the asymmetric replicative fates of daughter cells. What, then, targets DivJ and PleC, and DivL, to specific poles?

For DivJ, stalked pole localization and kinase activity depend on a membrane protein called SpmX [44*], but precisely how SpmX gets localized to the stalked pole is unclear. The

cytoplasmic, polar scaffolding protein PopZ is necessary for SpmX localization [45], but whether it binds SpmX directly and whether other factors are required to localize SpmX is unknown. Intriguingly, the periplasmic domain of SpmX has homology to a muramidase-like peptidoglycan binding protein [44*], and this domain is required for localization suggesting it might recognize stalked pole-specific cell wall material.

SpmX is synthesized in swarmer cells shortly before it localizes to the stalked pole. The transcription of *spmX* depends on the σ^{54} -dependent response regulator TacA [44*], which is activated by a multi-step phosphorelay [46]. Interestingly, TacA is abundant in swarmer and predivisional cells [44*], but *spmX* transcript levels are much higher in swarmer cells suggesting that TacA could be phosphorylated specifically in that cell type [3]. TacA is also specifically cleared from stalked cells. These observations suggest that TacA may be asymmetrically regulated in daughter cells and, in turn, contribute to replicative asymmetry by activating *spmX* transcription specifically in swarmer cells.

The swarmer pole localization of PleC depends on a polarly localized membrane protein called PodJ [47,48]. Like SpmX, PodJ has a periplasmic domain that may directly bind peptidoglycan. Cells lacking *podJ* have morphological phenotypes similar to a *ΔpleC* strain, but whether PodJ directly binds PleC and precisely how it affects PleC phosphatase activity is unknown. Localization of PleC specifically to the swarmer pole also depends on TipN, a membrane protein that marks the new pole in predivisional cells [49]. Moreover, cells overproducing TipN form ectopic poles that recruit PleC, indicating that TipN is sufficient to initiate the polar development required for PleC localization.

The swarmer pole localization of DivL in predivisional cells also depends, to some extent, on PodJ [50]. However, cells lacking *podJ* frequently still localize DivL suggesting that other factors may be involved. The localization of DivL and, consequently, CckA also depend somehow on replication initiation but the mechanism involved is, as yet, uncharacterized [51]. Conversely, DNA replication does not depend on CtrA. In fact, cells depleted of active CtrA continue to replicate and, importantly, do so with a periodicity similar to that of wild-type stalked cells [52]. The timing of replication is ultimately dictated by an independent regulatory circuit involving the conserved replication initiator DnaA. This circuit ensures cell cycle-dependent fluctuations in DnaA activity, but does not significantly impact the replicative asymmetry of daughter cells [52].

Conclusions

Our understanding of the molecular circuitry governing cell fate asymmetry in *Caulobacter* has progressed significantly in recent years. At the heart of this circuitry are two signaling pathways: (1) the CckA-based phosphorelay that regulates CtrA phosphorylation and (2) the DivJ/PleC-based pathway that regulates DivK phosphorylation. These pathways are connected by an atypical kinase, DivL, with phosphorylated DivK downregulating CckA via DivL specifically in stalked cells to permit DNA replication in this cell type. The colocalization of CckA, DivL, and PleC (the DivK phosphatase) in predivisional cells drives maximal activation of CtrA and its target genes. This spatial arrangement of kinases at the swarmer pole of predivisional cells highlights the remarkable ability of bacteria to create specialized, subcellular environments without the use of membrane-enclosed compartments. Precisely how cell cycle kinases get to, and remain at, specific poles remains largely unclear. Some, such as CckA and PleC, rely on other proteins for localization, but how do those proteins localize and what are the ultimate polar anchors in the cell? There are several possible mechanisms for achieving polar localization. First, as noted, the peptidoglycan left over at the poles from a prior cell division could be specialized and recruit proteins, such as PodJ and SpmX, that harbor peptidoglycan-binding domains. Second, the geometry or

higher membrane curvature of the poles could serve as a localization or recruiting signal [53]. Third, the polar regions of a cell have less DNA than the rest of the cell, which could drive localization of proteins that are repelled by DNA or DNA-associated factors. Understanding the mechanisms and function of protein localization in bacteria remains a major challenge. *Caulobacter*, and the histidine kinases, that coordinate cellular asymmetry will continue to serve as an important model system for such studies.

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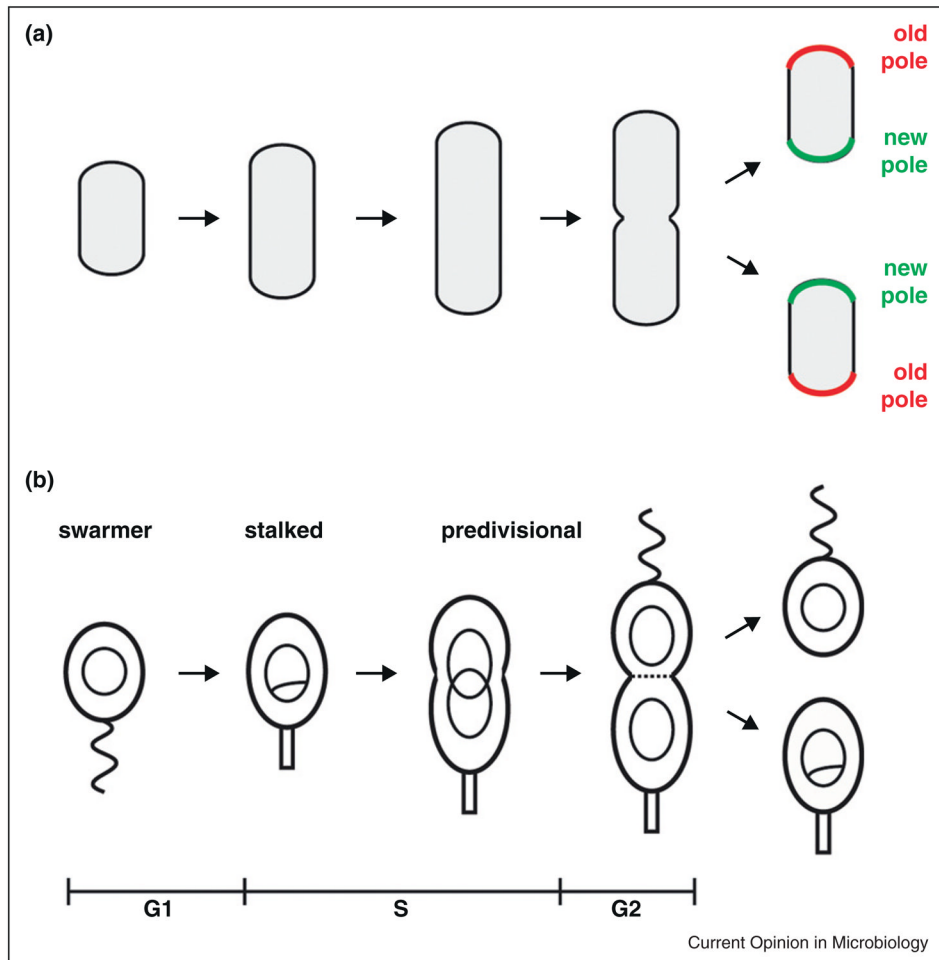
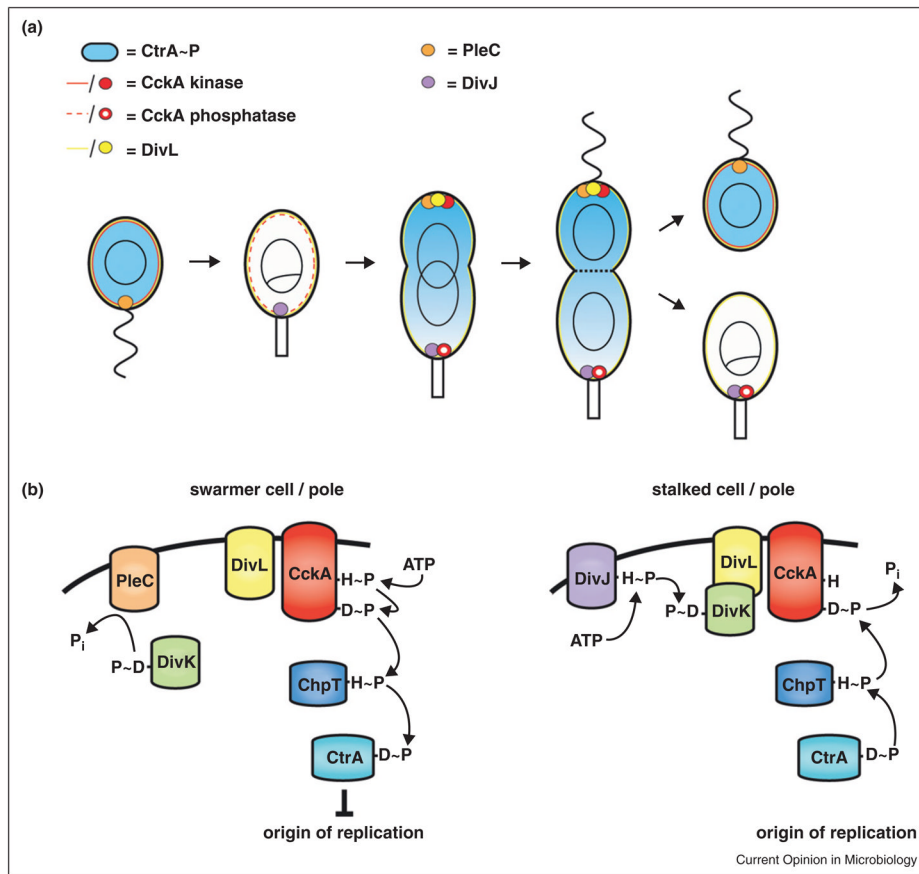


Figure 1.

Asymmetric cell division in bacteria. (a) For all rod-shaped bacteria, cell division yields daughter cells with poles of different ages, one created by the most recent division and one created by an earlier division. (b) Schematic of the *Caulobacter crescentus* life cycle indicating the asymmetric division that produces daughter cells with different polar morphologies and replicative capacities. Morphological and cell cycle stages are indicated above and below, respectively, the diagram.

**Figure 2.**

Regulation of CtrA and replicative asymmetry in *Caulobacter*. **(a)** Summary of the regulatory circuitry that controls CtrA in a cell type-specific manner, including the subcellular localization patterns of the histidine kinases CckA, DivL, PleC, and DivJ. **(b)** Model of the two-component signaling pathways and protein–protein interactions that regulate CtrA. CckA initiates a phosphorelay that culminates in phosphorylation and activation of CtrA. The activation of CckA as a kinase depends on DivL. Phosphorylated DivK can inhibit the CckA–DivL complex. DivK phosphorylation is controlled by a cognate kinase, DivJ, and phosphatase, PleC, which localize to opposite poles of the predivisive cell and to opposite daughter cells, thereby imposing cell type-specificity on CtrA activation.

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