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Advantages and mechanisms of polarity and cell shape determination in *Caulobacter crescentus*

Melanie L. Lawler and Yves V. Brun*

Department of Biology, Indiana University, Bloomington, Indiana 47405, USA

Summary

The tremendous diversity of bacterial cell shapes and the targeting of proteins and macromolecular complexes to specific subcellular sites strongly suggest that cellular organization provides important advantages for bacteria in their environment. Key advances have been made in the understanding of the mechanism and function of polarity and cell shape by studying the aquatic bacterium *Caulobacter crescentus*, whose cell cycle progression involves the ordered synthesis of different polar structures, and culminates in the biosynthesis of a thin polar cell envelope extension called the stalk. Recent results indicate that one important function of polar development is to maximize cell attachment to surfaces and to improve nutrient uptake by non-motile and attached cells. Major progress has been made in understanding the regulatory network that coordinates polar development and morphogenesis, and the role of polar localization of regulatory proteins.

Introduction

Ever since Antoni van Leeuwenhoek first observed them in his microscope, we have known that bacteria exhibit an amazing diversity of shapes and sizes that are precisely reproduced at every generation. The reproduction and evolutionary conservation of these shapes indicate that they play an important role in the lives of bacteria. Impressive progress has been made recently in our understanding of the basis of bacterial cell shape, with the discovery that bacteria possess a cytoskeleton; however, we are still very far from understanding how bacterial cells generate specific shapes or the function of those shapes. Many hypotheses have been proposed to explain the function of different cell shapes, but essentially no experimental evidence exists to support most of them [1–3].

One striking case of morphological differentiation occurs in a group of alpha-proteobacteria known as the prosthecate (or stalked) bacteria (Fig. 1). These bacteria synthesize a tubular extension of their envelope, called a prosthecum or stalk, which is thought to improve the nutrient uptake capability of the cell [4]. Most species in this group produce two different cell types at each cell cycle, a motile swarmer cell that does not replicate its chromosome and a chromosome replication-competent stalked cell (Fig. 2). Beyond their common dimorphic life cycle strategy, morphological diversity abounds in the stalked bacteria. *Caulobacter crescentus* is by far the best-studied member of this group. When one considers the intricate mechanisms that control polar development and cell shape discovered in *C. crescentus* in the last 15 years or so, one cannot help but hypothesize that these morphological changes must be extremely important for the bacterium. Recent studies of surface adhesion and stalk function

*To whom correspondence should be addressed: Yves V. Brun, Department of Biology, 1001 East Third Street, Bloomington, IN 47405-3700, Phone: 812/855-8860, Fax: 812/855-6705, E-mail: ybrun@indiana.edu.

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strongly suggest that the succession of polar morphological changes serves to optimize the efficiency of surface adhesion and endows the attached cell with optimized nutrient uptake capabilities. This review will focus on current models of the function of morphological change and the mechanisms that lead to these changes in *C. crescentus*.

Ordered polar development optimizes surface adhesion

C. crescentus holdfasts mediate surface attachment with impressive strength [5]. In addition, progression through the cell cycle is required for optimal surface attachment [6,7]. Attachment is most efficient during the swarmer phase of the cell cycle, and optimal attachment requires flagellar motility and pili [6–8]. The flagellum provides the propulsive force of motility to make the initial contact with the surface. The pili are present at the same pole as the flagellum in swarmer cells and provide additional contacts with the surface and retract to bring the cell pole in close proximity with the surface. Therefore, the presence of the flagellum and pili at the pole where holdfast synthesis will occur provides an efficient orientation mechanism that ensures that the holdfast-bearing pole contacts the surface. Whether the cell senses the surface environment and uses a mechanism such as chemotaxis to make the decision to settle or not is unknown. However, the fact that the diguanylate cyclase PleD is required for the proper timing of holdfast synthesis during development [7], combined with the well established role of cyclic-di-GMP (c-di-GMP) in the transition from the planktonic to the surface-associated existence of bacteria [9], suggests the intriguing possibility that the cell regulates the decision to settle based on environmental inputs.

Once the cell attaches to a surface, cell growth depends on nutrient availability at that surface and on nutrients that diffuse or flow past the cell. *C. crescentus*, and presumably other stalked bacteria, utilize a dedicated morphological change to optimize the uptake of nutrients essential for cell growth.

Optimization of nutrient uptake by morphological changes

Stalked bacteria are often found in nutrient-limited environments. The biosynthesis of a stalk provides many advantages for this oligotrophic lifestyle. For the past 40 years, it had been hypothesized that stalks improve the nutrient uptake capacity of cells, and results of nutrient uptake studies with *A. biprosthecum* were consistent with this model (reviewed in [4]). Stalks appear to be devoid of cytoplasmic and inner membrane proteins, but do contain outer membrane and periplasmic proteins [10,11]. The use of a fluorogenic substrate for alkaline phosphatase allowed the observation of organic phosphate transport and hydrolysis in the periplasm of purified stalks, yielding the first direct demonstration that the *C. crescentus* stalk is able to take up nutrients [11]. It is thought that nutrients taken up through the outer membrane and into the periplasm of the stalk bind to high-affinity periplasmic nutrient-binding proteins. Binding to these proteins should prevent their escape from the stalk. Nutrients bound to their nutrient-binding proteins would then diffuse from the periplasm of the stalk to that of the cell body. Once in the cell body periplasm, the nutrients can be taken up by inner membrane transporters.

The periplasmic diffusion strategy is advantageous from an energetic standpoint since it obviates the cost of adding inner membrane and cytoplasmic proteins to the stalk. By increasing the cell's surface-to-volume ratio, stalks improve nutrient uptake while consuming comparatively little energy, a distinct advantage for an oligotroph. Synthesizing a stalk becomes a particularly elegant solution to enhance nutrient uptake when considering the typical environment where *C. crescentus* lives. When cells are in a turbulent environment where there is flow past the cell, the rate of nutrient uptake is proportional to the cell surface area. However, *C. crescentus* cells are often trapped in the diffusion-limited laminar sublayer, attached to surfaces where there is essentially no flow past the cell. In such situations, nutrient molecules

depend on diffusion to contact their receptors and the rate of nutrient uptake is proportional to cell length rather than to its surface area [11]. Therefore, the stalk offers a biophysical advantage to the cell by acting as a nutrient-scavenging antenna. Mathematical modeling indicates that a 1 μm cell with a 9 μm stalk is more efficient at nutrient uptake than a 10 μm long cell with no stalk by ~ 7 -fold per unit volume or by ~ 3 -fold per surface area [11]. Finally, continued stalk elongation of up to tens of μm [12] “pushes” the cell body away from the surface to access to nutrients carried by the liquid flow outside the laminar sublayer. Pushing the cell body away from the surface also results in cell division and swarmer cell birth away from the surface, and therefore improves dispersal away from the mother cell during the obligatory dispersal period of the swarmer stage. In the next sections, we describe the newest findings in the study of stalk morphogenesis, in asymmetric cell division, and in the coordination of polar development with cell cycle progression of *C. crescentus*.

Stalk synthesis and its regulation

Stalk synthesis is regulated by the cell cycle and stalk length by both cell cycle and environmental cues. The cell cycle dependent control of stalk synthesis is regulated by the sigma factor σ^{54} [13] and by a phosphorelay composed of the hybrid histidine kinase, ShkA, the histidine phosphotransferase, ShpA, and the response regulator TacA [14]. A stalk synthesis regulon of 30 genes was identified using DNA microarray analysis, providing an entry point into the investigation of this fascinating morphological change [14]. Phosphate limitation has a striking stimulatory effect on stalk elongation. Since phosphate is the most frequent limiting nutrient for bacterial growth in the environment [15], this mechanism ensures that long stalks are synthesized in nutrient-limited environments. The phosphate limitation response is under the control of the response regulator PhoB and is regulated by a mechanism analogous to the Pho regulon in *Escherichia coli* [12].

Recent studies have indicated that stalk synthesis is a specialized form of cell elongation [4]. Stalk synthesis requires PBP2, RodA, and MreB [16-18]. It is unclear how the activity of these proteins is modulated for stalk synthesis, since they are also involved in general cell shape determination. Morphologically, the stalk contains interspersed dense rings known as crossbands, whose synthesis occurs at every cell cycle and requires FtsZ, like septum synthesis [18]. Crossbands are thought to provide structural support to the stalk and may be involved in compartmentalizing the stalk from the cell body.

The asymmetric degradation of a master regulator controls cell fate

The response regulator, CtrA, coordinates many aspects of the cell cycle and development by directly regulating the transcription of genes involved in polar development, cell division, and DNA replication [19]. CtrA's activity is controlled by phosphorylation and localized degradation. CtrA is present in swarmer cells, where it prevents initiation of DNA replication, and is degraded during swarmer to stalked cell differentiation. CtrA is resynthesized in predivisional cells, where it is phosphorylated by the CckA-ChpT phosphorelay [20], and is degraded in the stalked compartment of the predivisional cell just prior to cell division (Fig. 3). This regulated proteolysis of CtrA involves its localization together with the protease ClpXP to the new and mature stalked poles in swarmer and predivisional cells, respectively [21,22]. The formation of a polar complex composed of CtrA, ClpXP, the localization factor RcdA, and the unphosphorylated response regulator CpdR is required for CtrA degradation [22,23]. Phosphorylation of CpdR by the CckA-ChpT phosphorelay prevents its polar localization, and therefore prevents the degradation of CtrA while it is phosphorylated by the same phosphorelay. In turn, CckA activity is down-regulated by the single domain response regulator DivK, whose expression is activated by CtrA~P [20]. The phosphorylation of DivK and CtrA

and the bipolar localization of DivK are also regulated by the tyrosine kinase DivL, in a manner independent of the CckA-ChpT phosphorelay [24,25].

The asymmetric localization of two histidine kinases couples polar development to cell division

The connectivity of the regulatory proteins described in the previous section forms an integrated regulatory circuit that can explain the oscillation of CtrA activity during the cell cycle [20]. But how is spatial regulation achieved? A number of regulatory proteins that coordinate polar development with cell cycle progression display a distinct localization pattern that helps define different cell fates. Two members of the histidine kinase family, DivJ and PleC, provide the spatial component required to generate different cell fates by localizing to opposite poles of predivisional cells (Fig. 3). DivJ phosphorylates DivK at the stalked pole and PleC dephosphorylates it at the swarmer pole [26]. Since DivK regulates CtrA through its action on CckA, controlling the phosphorylation state of DivK is an important part of developmental and cell cycle regulation and helps to couple development with cell division [20,27,28]. DivJ and PleC also regulate the phosphorylation of the diguanylate cyclase PleD in a similar manner to DivK [29,30]. As PleD is involved in regulating motility and stalk synthesis [31], modulation of PleD by DivJ and PleC can control activation of motility and stalk synthesis at specific times during the cell cycle, presumably at a posttranslational level by modulating the pool of c-di-GMP. Therefore, by modulating the activity of DivK and PleD differently at the two poles of predivisional cells, DivJ and PleC impart both temporal and spatial context to gene expression (through the DivK-CckA-ChpT-CtrA circuit) and to post-expression activity (through c-di-GMP).

Establishing cellular asymmetry

How does the cell regulate the spatial distribution of regulatory proteins to set up different fates? The hypothesis that an “organizational center” exists at the site of cell division to mark the new cell poles after division [32] was confirmed by the identification of the TipN (for *tip* of *new* pole) protein [33,34]. TipN localizes to the division site of predivisional cells in a FtsZ-dependent manner and is therefore inherited at the new pole of both cells after division (Fig. 3). *tipN* mutants have a high proportion of cells with mislocalized flagella, and upon overexpression of TipN, cells produce ectopic poles competent for flagellar assembly and for the localization of PleC and DivJ [33,34]. Cells lacking *tipN* have an asymmetry defect that shifts the division plane so that daughter swarmer cells are larger than those of wild-type cells [34]. This shift in asymmetry is intriguing, considering the dependence of TipN on cell division for mid-cell localization. TipN is required for proper localization of MreB, suggesting that TipN may control polarity through its action on MreB [34]. MreB has been implicated in the localization of PleC and DivJ to the correct poles, and ectopic poles are produced when expression of the cell shape protein MreB is altered [17,35]. Since similar ectopic poles are also produced by altering the activity of PBP2 or the expression of RodA [17], two proteins involved in peptidoglycan synthesis, it is possible that TipN exerts its role in polarity through modification of the peptidoglycan [36].

Another protein that is critical for establishing cellular asymmetry is the polar localization factor, PodJ. PodJ is required for motility, flagellum release, and the synthesis of holdfast and pili [37], as well as the localization of PleC and the pilus assembly protein, CpaE [38,39]. PodJ is present in two forms – a full-length form (PodJ_L) produced in stalked cells and a shortened form (PodJ_S) that is the result of proteolytic cleavage of PodJ_L late in the cell cycle (Fig. 3). PodJ_L is processed by the aspartic protease PerP in the periplasm, reducing it to the membrane-bound PodJ_S. [40]. PodJ_S is then cleaved off of the membrane and released into the cytoplasm by the Rip (regulated intramembrane proteolysis) metalloprotease MmpA and subsequently

degraded by an as yet unknown protease [41]. PodJ is organized in modules, with domains required for the specific functions of PodJ found in distinct subcellular compartments [42]. Therefore, PodJ processing at the time of cell division and its degradation during swarmer cell differentiation probably provide molecular switches that regulate the progression of polar development. Furthermore, PodJ processing requires cell division [40], which is also required for polar development past flagellum synthesis [32]. Surprisingly, deletion of either *perP*, *mmpA*, or both does not produce a developmental phenotype, nor does it completely abolish processing or degradation of PodJ, suggesting the existence of redundant mechanisms for PodJ processing and degradation and/or the regulation of its activity [40,41].

Future perspectives

The last few years have seen major progress in our understanding of the regulatory network that coordinates polar development and the cell cycle in *C. crescentus*. The connectivity of many of the regulatory proteins is now known, as is the role of the polar localization of some regulators. A major remaining challenge is to understand the mechanism of polar localization of key regulators and how localization to, or the exclusion from, a specific pole relates to cell shape. The new poles of *C. crescentus* are reshaped after cell division [43]. Therefore, peptidoglycan modifications at the poles could provide structural cues for the localization of proteins. Alternatively, as in the case of TipN, localization to the division site could occur by interaction with the divisome, or by sensing some aspect of the newly discovered redirection of peptidoglycan synthesis at the site of division [43], followed by subsequent inheritance at the new pole of the cell. TipN could then play a role in targeting polarly localizing proteins such as PodJ to this pole. Since PodJ is required for PleC localization, TipN may in part set up the polarity of the cell through this localization pathway.

With the advent of high-throughput genome sequencing, it will be possible to assess the extent of conservation of the *C. crescentus* regulatory network in other stalked bacteria. The *H. neptunium* genome contains homologs of many *C. crescentus* developmental regulatory genes despite having substantial differences in its life cycle [44]. Many of these regulators and their polar localization are likely to play a role in asymmetric division, not only in stalked bacteria, but also in more distant alpha-proteobacteria that do not possess obvious asymmetry [45]. Indeed, polarly localized homologs of PleC and DivK are found in *Brucella abortus* [46]. Comparative and functional genomics analysis of stalked bacteria should provide clues about how a complex regulatory network can be modified by evolution to produce developmental outcomes with subtle or major differences. For example, what genomic changes are responsible for the evolution of the stalk as a conduit for the chromosome in budding bacteria from the stalk as a nutrient uptake organelle, or vice versa?

More in depth study of stalked bacteria will expand our understanding of the role of the stalk. While the stalk has been shown to take up organic phosphate molecules and mathematical modeling supports an important role for the stalk in nutrient uptake, there is still no direct evidence that nutrients taken up in the stalk find their way to the cell's cytoplasm and contribute to growth. Fluorescence recovery after photobleaching and microfluidics experiments will help to resolve these issues. It will also be interesting to determine if the stalk is specialized for phosphate uptake or if it is a general nutrient uptake organelle. Finally, determining how cell shape determination proteins such as MreB, PBP2, and RodA are modulated to direct both general cell shape and stalk synthesis should shed considerable light on the mechanisms of cellular morphogenesis.

We currently know more about the how of cell shape determination than about the why. A detailed investigation of the variations on the dimorphic life cycles and morphology of stalked

bacteria is certain to reveal general principles about the contribution of polarity and cell shape to the adaptation of bacteria to specific environmental challenges.

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Figure 1.

Cell shape variation in stalked bacteria. *Caulobacter crescentus* (top left) has a flagellum at one pole of the predivisional cell and a stalk tipped by an adhesive holdfast at the other pole. *Asticacaulis biprosthecum* (top right) has two stalks symmetrically placed on each side of its stalked and predivisional cells, with its flagellum and holdfast at the same respective poles as *C. crescentus*. In budding stalked bacteria such as *Hyphomonas neptunium* (bottom left), the daughter swarmer cell grows from the tip of the stalk and possesses a single flagellum at its distal pole. *Ancalomicrobium adetum* (bottom right), which may well be the most extreme case of stalked bacteria, appears to have neither flagella nor motility, grows by budding from the cell body, and produces up to a dozen prosthecae around the periphery of the predivisional cell. Reproduced from Molecular Microbiology [4] with permission.

Figure 2.

Life cycle of stalked bacteria. A) *Caulobacter crescentus* life cycle. A motile swarmer cell and a sessile stalked cell are produced at every cell division. The older pole of the swarmer cell has pili and a single polar flagellum. After a defined period in the swarmer phase of the life cycle, during which the cell is not competent for DNA replication, the cell differentiates into a stalked cell by retracting the pili, ejecting the flagellum, and synthesizing an adhesive holdfast at the previously flagellated pole. A stalk is synthesized at the holdfast-bearing pole, resulting in the extension of the holdfast away from the cell as the stalk grows. The stalked cell initiates DNA replication and elongates in preparation for division. The division septum is placed off center in the predivisional cell, and a new flagellum is formed at the pole opposite the stalk. After cell compartmentalization, the flagellum is activated, and the force generated by the flagellum helps to separate the daughter cells. After cell separation is complete, pili are synthesized at the same pole as the flagellum in the swarmer cell. The swarmer cell swims away, and the stalked cell remains attached to immediately initiate a new cell cycle. B) *Asticcacaulis biprosthecum* life cycle. *A. biprosthecum* synthesizes two stalks on the side of cells instead of one, but still has a flagellum and a holdfast at the same poles as *C. crescentus*. C) *Hyphomonas*

neptunium life cycle. *H. neptunium* is a budding bacterium in which the daughter swarmer cell grows from the tip of the stalk. Therefore, a copy of the chromosome must traverse the stalk at every cell cycle to be inherited by the swarmer cell.

Figure 3.

Localization of several *C. crescentus* developmental regulatory proteins during the cell cycle. Large dots indicate distinct protein localization at a particular location, while small dots represent diffuse cytoplasmic protein throughout the cell compartment. As PleC is a membrane bound protein, delocalization of PleC is illustrated as a ring around the cell body. In swarmer cells, CtrA is present throughout the cytoplasm. During swarmer to stalked cell differentiation, CtrA localizes to the incipient stalked pole, together with CpdR, RcdA, and ClpXP, resulting in CtrA degradation and allowing the initiation of DNA replication. CtrA is synthesized again in late stalked cells, and after compartmentalization, localizes to the stalked pole of the stalked cell, where it is degraded by the same complex described above. Therefore, CtrA is present in the daughter swarmer cell but not the stalked cell, allowing the stalked cell to immediately initiate a new round of DNA replication. The DivK protein is diffuse in swarmer cells, then localizes to the stalked pole after differentiation, where it is phosphorylated by the DivJ protein. Upon phosphorylation, DivK is released from the stalked pole and diffuses toward the nascent flagellar pole, where it is dephosphorylated by PleC. This shuttling of DivK between the two poles of the cell continues until the time of cell compartmentalization, when DivK remains phosphorylated in the stalked cell compartment and dephosphorylated in the swarmer cell compartment. DivJ localizes to the stalked pole in cells with a stalk. PodJ is present in two forms – a full-length form (PodJ_L) produced in stalked cells and a shortened form (PodJ_S) that is the result of proteolytic cleavage of PodJ_L late in the cell cycle. Newly-synthesized PodJ_L localizes to the nascent flagellar pole in stalked cells and is required for the localization of PleC at the same pole. Processing of PodJ_L and cell division results in the production of a swarmer cell with PodJ_S and PleC co-localized at the flagellar pole. During swarmer cell differentiation, PodJ_S is degraded, resulting in delocalization of PleC. The TipN protein is localized at the pole opposite the flagellum in swarmer cells, and remains there until cell division is initiated, which targets its localization to the division site. TipN is consequently inherited by both daughter cells after cell division, localized at the newly formed pole in both cell types.