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MicroReview

Out on a limb: how the *Caulobacter* stalk can boost the study of bacterial cell shape

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Summary

Understanding the mechanisms underlying the establishment of different bacterial cell shapes and the advantage that a particular shape imparts is one of the most fascinating and challenging areas of study in microbiology. One remarkable example of bacterial morphogenesis is the elaboration of long, tubular extensions of the cell envelope of certain aquatic bacteria. These appendages (also called prosthecae or stalks) possess features that make them particularly amenable models for experiments designed to uncover general principles of cell morphogenesis and of cell shape function. Recent evidence supports the hypothesis that stalk synthesis in *Caulobacter crescentus* is a specialized form of cell elongation that confers to the cell substantial advantages in nutrient uptake. Further insights into the mechanisms and function of stalk synthesis will require a multidisciplinary systems biology approach using principles and methodologies from ecology and evolutionary biology to biophysics and mathematical modelling.

Introduction

The last decade has seen a renewed and invigorated interest in bacterial cell shape. This interest has been stimulated by a number of factors, including the emergence of antibiotic-resistant bacteria and the need to find new drug targets, increased research into the area of bacterial development involving cell shape changes (Brun and Shimkets, 2000), and improved methodologies for imaging bacteria. It is now apparent that bacterial cells are more organizationally complex than was previously appreciated. For example, an ever-increasing number of macromolecules are found to localize to specific subcellular sites, often in a dynamic manner (Shapiro and Losick, 1997; Errington, 2003; Janakiraman and Goldberg, 2004). The targeting cues for proteins and other molecules must somehow be tied to the organization of the bacterial envelope, and therefore to cell morphology, but we know very little about how the envelope is organized or, for that matter, why bacteria generate and perpetuate different shapes in the first place. How do bacteria co-ordinate the mechanisms for growth, division and molecule targeting? What selective pressures or cues led to the faithful reproduction of particular shapes in progeny or to the divergence of shape over evolutionary time?

One approach to tackling these tough questions is to begin with an 'unusual' case study. *Caulobacter crescentus* is one such example because this organism synthesizes a tubular extension of the cell envelope (comprising inner and outer membranes, peptidoglycan and periplasmic space) at one cell pole as part of its life cycle (Fig. 1 and 2). This structure, generically called a prosthecum or appendage, is in *C. crescentus* referred to as a stalk. Prosthecae are synthesized by a number of bacteria besides *C. crescentus*, including *Asticaccaulis biprosthecum* and *Hyphomonas neptunium* (Fig. 1) (Brun and Janakiraman, 2000).

Caulobacter crescentus is a vibriod, Gram-negative bacterium with a dimorphic life cycle that produces one swimming cell and one sessile cell at each cell cycle (Poindexter, 1964; Brun and Janakiraman, 2000). Prior to cell division, *C. crescentus* possesses a stalk at one cell pole and a flagellum at the other. Following cytokinesis, the flagellated daughter cell (swarmer) swims for about one-third of the cell cycle. The swarmer then sheds its polar flagellum, and from the same pole produces an adhesive holdfast. The holdfast, to date the strongest measured biological glue, cements *C. crescentus* to surfaces in aquatic environments (Tsang *et al*., 2006). Following holdfast secretion, the stalk is synthesized at the holdfast pole and as a result, the holdfast is pushed outward from the cell body and found at the tip of the stalk.

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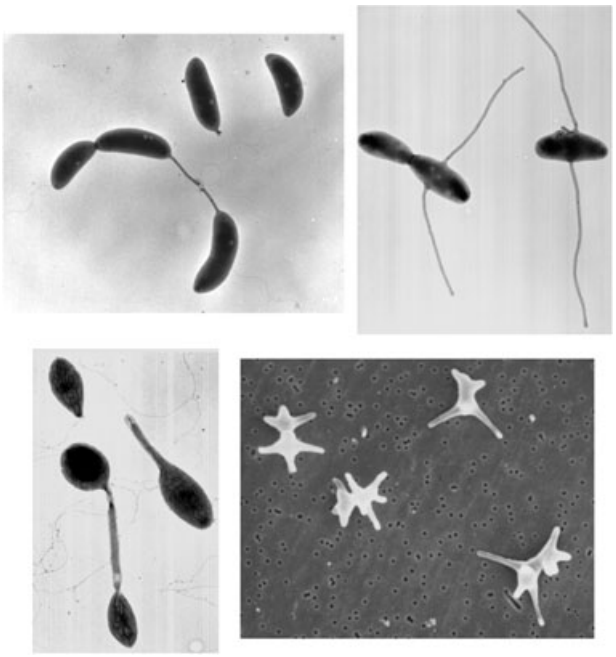

Fig. 1. Cell shape variation in stalked bacteria. Top left, *Caulobacter crescentus* (photo Y.V. Brun, from *Journal of Bacteriology* **181**: 1118–1125, reproduced with permission); top right, *Asticaccaulis biprosthecum* (photo E.M. Quardokus, from *Prokaryotic Development*, ASM Press, p. 297–317, reproduced with permission); bottom left, *Hyphomonas neptunium* (photo E.M. Quardokus, from *Prokaryotic Development*, ASM Press, p. 297–317, reproduced with permission); bottom right, *Ancalomicrobium adetum* (photo courtesy of A. Van Neerven, B. Oakley and J.T. Staley).

Caulobacter crescentus *stalk ultrastructure*

Stalk morphogenesis is fascinating because it occurs specifically at one cell pole, the elongation is directional (stalks do not grow into the cell body), and it culminates in the formation of a tube that is approximately 100 nm in diameter, or about one-fifth the diameter of the cell body cylinder. Stalks are synthesized by incorporation of new envelope materials at the junction between the cell body and the stalk (Schmidt and Stanier, 1966; Smit and Agabian, 1982; Seitz and Brun, 1998). As new cell envelope is inserted, the older material is displaced and becomes increasingly distal to the cell body (Fig. 2).

The stalk is free of ribosomes, DNA (Poindexter and Bazire, 1964) and many cytoplasmic proteins (Ireland *et al*., 2002; Wagner *et al*., 2006). The stalk is transected perpendicularly at irregular intervals by lysozyme-sensitive structures called crossbands (Schmidt, 1973). Crossbands isolated from *C. crescentus* are approximately the same width as a stalk and are synthesized temporally with a statistical correlation to each cell cycle, allowing the age of a stalked cell to be determined (Poindexter and Staley, 1996). Each crossband consists of concentric rings of electron-dense material that might or might not contain a central pore (Jones and Schmidt, 1973; Schmidt and Swafford, 1975). Interestingly, the stalks of some prosthecate bacteria lack crossbands and, while it is not known how or why crossbands are synthesized, it has been suggested that they might stabilize and/or compartmentalize the stalk structure (Jones and Schmidt, 1973).

Rationale for the study of cell shape in C. crescentus

Several features make the *C. crescentus* stalk an excellent model for studying questions related to bacterial morphogenesis. First, *C. crescentus* swarmer cells can be synchronized prior to differentiation into stalked cells; thus, it is possible to obtain a pure population of cells that do not yet have stalks (Evinger and Agabian, 1977). This population of cells is ideal for determining what proteins

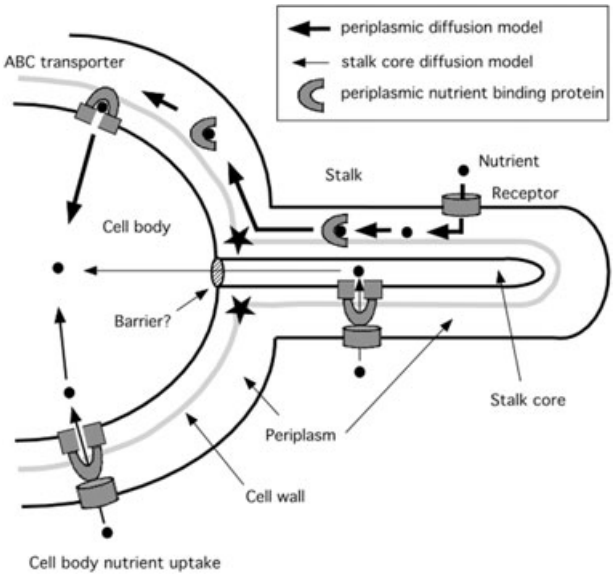

Fig. 2. Models of nutrient uptake by the stalk and cell body. Nutrient uptake by the cell body is shown as occurring through a hypothetical complex between an outer membrane nutrient receptor or pore and an ABC transporter. The existence of such complexes would prevent inner membrane proteins from diffusing to the stalk because the complex would be unable to move relative to the cell wall, and might therefore explain the observed protein compartmentalization between the cell body and the stalk. Alternatively or in addition, a barrier might exist at the base of the stalk. There are two major models for nutrient uptake by the stalk. The periplasmic diffusion model (top part of the diagram and thick arrows) is consistent with data obtained to date. Nutrient molecules bind to a periplasmic nutrient-binding protein and the complex diffuses from the stalk periplasm to the cell body periplasm before being transported into the cell body cytoplasm by an ABC transporter. The stalk core uptake model (bottom part of the diagram and thin arrows) would require nutrient uptake into the stalk core in a manner similar to the cell body, followed by its diffusion into the cell body cytoplasm. The site of stalk synthesis at the base of the stalk is indicated by stars. Note that the stalk-to-cell body transition is more tapered than what is shown in this diagram (see Fig. 1).

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are required for the elaboration of the stalk structure. Second, the rate of stalk elongation per cell cycle can be manipulated either genetically or by growing cells in low phosphate media, which stimulates stalk elongation (Gonin *et al*., 2000). Thus, it is possible to design experiments that identify and characterize genes or proteins involved in regulating stalk elongation by examining differences between wild-type cells and those that are generating stalks at a faster rate. Third, as the synthesis of the stalk is known to occur at the stalk–body junction (Schmidt and Stanier, 1966; Smit and Agabian, 1982; Seitz and Brun, 1998), the proteins required for establishing the site for and dimensions of the stalk must be localized to this region prior to and/or during stalk synthesis. This observation could be utilized in several ways. For example, as there are mutants that release stalks and adjacent polar material following an erratic constriction event (Poindexter, 1978), it is theoretically possible to use proteomic approaches to determine if certain proteins are enriched at the stalk–body junction. Finally, as explained in the next section, stalk synthesis is a special type of cell elongation.

The features mentioned above make stalk synthesis a highly tractable model for testing current models for cell shape determination, providing us with general information concerning the production of cell shape in any bacterium.

Stalk synthesis: a special type of cell elongation

Stalk morphogenesis and elongation utilizes many of the same proteins that are required for elongation of the cell body in *C. crescentus* and other Gram-negative bacteria, namely penicillin-binding protein 2 (PBP2) (Seitz and Brun, 1998), RodA (Wagner *et al*., 2005) and MreB (Wagner *et al*., 2005). Depleting or inactivating the function of these proteins leads to an increase in the diameter and an elongation defect not only of the cell body, but also of the stalk. However, as the stalk structure is distinct from the cell body, the mechanisms that determine the characteristic location, shape and size of the stalk cannot be inherent to the cell wall elongation proteins themselves, but must instead be conferred by other proteins and/or processes that are stalk specific.

Theoretically, it should be possible to obtain mutants of *C. crescentus* that do not synthesize stalks (for example, by enrichment – see Poindexter, 1978). Determining the nature of these mutations will help to determine how stalk synthesis is controlled spatially and temporally. Thus far, all mutants that do not make stalks contain mutations in regulatory genes that also affect processes other than stalk synthesis. Furthermore, the stalk synthesis defect is always overcome by growing cells in low phosphate medium (Brun and Shapiro, 1992; Wang *et al*., 1993; Quon *et al*., 1996; Seitz and Brun, 1998; Gonin *et al*., 2000; Biondi *et al*., 2006). The identification of bona-fide 'stalkless' mutants would be a boon not only because they would provide clues about how a bacterial shape is determined, but also because a non-pleiotropic stalkless mutant could be utilized in experiments testing the relative fitness of cells with and without stalks under various selective pressures. Thus, we could test hypotheses concerning the possible function of a specific bacterial shape under different environmental conditions. At present, screens for stalkless mutants have not been saturating due to the burden of visually screening for the phenotype.

Stalks are not involved in surface adhesion

The function of the stalk in *C. crescentus* and other prosthecate bacteria is mysterious. From the placement of the adhesive holdfast at the tip of the *C. crescentus* stalk, it is often deduced that stalked cells have a selective advantage in the process of adhesion. However, the *C. crescentus* stalk is not synthesized until after holdfast secretion and occurs even in the absence of surface adhesion (Bodenmiller *et al*., 2004; Levi and Jenal, 2006), consistent with an adhesion-independent function. Furthermore, in the closely related bacteria *Asticaccaulis excentricus* and *A. biprosthecum*, the holdfasts are associated with the cell body, not the stalks (Poindexter and Bazire, 1964), indicating that stalks confer an advantage that is adhesion independent. Therefore, the association of the stalk and holdfast in *C. crescentus* is probably a red herring with regard to stalk function, although this does not mean that adhesion and the development of stalks are not intertwined in the context of evolution.

Getting away from the surface?

Even in rich media, where phosphate is not limiting, stalk elongation is a constitutive process for a growing cell (Haars and Schmidt, 1974). This suggests either that the mechanisms required for stalk growth cannot be 'shut-down' (except at developmental checkpoints), without also stopping growth of the cell itself, and/or that there might be a nutrition-independent advantage to stalk elongation. In the specific case of *C. crescentus*, stalk elongation elevates the cell body away from the site of holdfast-surface attachment. The ability of the cell body to 'move' away from a surface but still remain in the boundary layer (where nutrients sediment) could be advantageous when there is a surface biofilm and therefore a concomitant competition for nutrients. In this same vein, stalk elongation could also aid in dispersal and colonization of new surfaces, as newly divided swarmer cells would be released away from the established biofilm. In each of these instances, inorganic phosphate levels might

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serve as a cue to stimulate stalk elongation, not because *C. crescentus* stalks are specifically involved in phosphate acquisition, but rather because phosphate is the most frequent limiting factor for microbial growth in the environment (Karl, 2000). Notably, prosthecae elongation is stimulated by phosphate limitation in other bacteria (Poindexter, 1984). As these bacteria adhere to surfaces via holdfasts that are associated with the cell body rather than the stalk, stalk elongation in these bacteria would not relocate the cell body, but growth of these stalks could allow the cell to extend the 'reach' of its nutrient uptake surface into new microenvironments. It should be noted that in addition to phosphate starvation, some other bacteria elongate their prosthecae in response to limitation for other nutrients, such as carbon (Whittenbury and Dow, 1977). Therefore, we hypothesize that stalks can take up a variety of nutrients but that the cues that can trigger stalk elongation may depend on the specific environmental conditions encountered by each species during evolution.

The nutrient uptake hypothesis

In the oligotrophic (low nutrient) environments where *C. crescentus* thrives, the stalks are amazingly long, sometimes over 20 times the length of the cell body (Poindexter, 1964; Brun and Janakiraman, 2000). The rate of stalk elongation is significantly stimulated by inorganic phosphate limitation or activation of the Pho regulon that controls phosphate uptake (Schmidt and Stanier, 1966; Gonin *et al*., 2000). This observation, coupled with the obvious increase in surface-to-volume ratio that the stalk provides and the ubiquity of stalked bacteria in oligotrophic environments, has led to the hypothesis that stalks directly enhance nutrient uptake by increasing the surface of the cell (Poindexter, 1981).

There is a growing abundance of direct and indirect evidence that supports a nutrient uptake role for stalk function. Stalks sheared from cell bodies of *A. biprosthecum* are able to deplete glucose and amino acids from growth medium (Larson and Pate, 1976; Tam and Pate, 1985) and possess alkaline phosphatase activity (Jordan *et al*., 1974). Purified *C. crescentus* stalks are able to take up and hydrolyse an organic phosphate source, and contain periplasmic nutrient-binding proteins (Wagner *et al*., 2006). Furthermore, in environments where nutrient uptake is diffusion-limited, such as those where *C. crescentus* is generally found, the rate of uptake is proportional to the length of the object rather than its surface area (Berg and Purcell, 1977; Berg, 1993; Wagner *et al*., 2006). Therefore, the long, thin shape of the stalk is highly optimized from a biophysical standpoint for contacting diffusive nutrients while minimizing increases of both cell volume and surface area (Wagner *et al*., 2006). Mathematical modelling of diffusive nutrient uptake by *C. crescentus* indicates that a 1 μm cell with a 2.4 μm stalk has a maximum rate of nutrient uptake 1.8 times greater than that of a 2 μm-long stalkless cell with the same surface area. On the other hand, under conditions of nutrient flow past the cell (as opposed to diffusion), a stalked cell and a stalkless cell of identical surface areas have identical rates of nutrient uptake. However, as the dimensions of the stalked cell contribute less to volume than an elongated cell body shape, stalk synthesis is still highly economical from a bioenergetics standpoint (Wagner *et al*., 2006).

If stalks are involved in nutrient uptake, then the repertoire of molecules found in stalks should be consistent with this function. The relative composition of *A. biprosthecum* stalks and cell body envelopes is not significantly different with respect to total lipid, carbohydrate and protein levels; however, the profile of proteins present in each fraction is different (Jordan *et al*., 1974). Specifically, the number of different proteins present in stalk fractions is reduced compared with the envelopes of cells. This result has been mirrored in *C. crescentus* using a combination of proteomic profiling and protein localization studies (Ireland *et al*., 2002; Wagner *et al*., 2006). *C. crescentus* stalks are relatively devoid of cytoplasmic material. Even though the inner diameter of the stalk is around 40 nm (Wagner *et al*., 2006), large enough for ribosomes (~20 nm diameter) to enter, microscopy experiments have shown that stalks are devoid of ribosomes (Poindexter and Bazire, 1964), and cytoplasmic GFP (< 5 nm diameter) (J.K. Wagner and Y.V. Brun, unpubl. results). Furthermore, proteomic analysis suggests that the stalk is relatively devoid of cytoplasmic and inner membrane proteins (Ireland *et al*., 2002; Wagner *et al*., 2006), although we note that the inability to identify a protein by mass spectrometry does not necessarily mean that it is absent from the sample. In contrast, the stalk does possess periplasmic proteins for binding and utilizing a variety of nutrients, including phosphate (Ireland *et al*., 2002; Wagner *et al*., 2006). The reduced complement of proteins in the stalk is consistent with the hypothesis that the stalk is an organelle streamlined for nutrient uptake (Ireland *et al*., 2002; Wagner *et al*., 2006). The question then becomes, how is the compartmentalization achieved and how is it physiologically significant with respect to stalk function?

We envision three ways that the compartmentalization between the stalk and cell body might be established and maintained. First, there could be a physical barrier at the stalk–body junction, preventing cytoplasmic and inner membrane proteins from diffusing freely into the stalk (Fig. 2). Some electron micrographs of *C. crescentus* are consistent with the presence of membrane at the stalk to cell body junction (Poindexter and Bazire, 1964;

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Briegel *et al*., 2006), but the presence of a membrane has not been demonstrated formally. Second, there might be a sorting mechanism at the level of protein synthesis and secretion that targets proteins to the stalk structure. Third, many proteins might not enter the stalk because they are associated with protein complexes in the cell body and are therefore not free to diffuse along the stalk (Fig. 2).

At present, it is not clear how the stalk compartmentalization is achieved but assuming the stalk is a nutrient uptake organelle, it would be advantageous for the cell to eliminate the cost of equipping the stalk with inner membrane and cytoplasmic proteins if they are not necessary for stalk function. Inner membrane and cytoplasmic proteins would be required for active transport of nutrients, so their absence from the stalk suggests that nutrient acquisition by the stalk might be diffusion-limited (Wagner *et al*., 2006). Once nutrients have entered the periplasmic space of the stalk, they probably bind to periplasmic nutrient-binding proteins and diffuse through the stalk periplasm to the cell body periplasm, at which point the nutrient could be actively transported across the inner membrane and into the cytoplasm (Fig. 2). Mathematical modelling indicates that steady state in the periplasm would be established relatively fast compared with the length of the cell cycle, and that under these conditions, even diffusion-limited nutrient uptake by the stalk would provide a significant advantage to the cell (Wagner *et al*., 2006).

Conclusion

Antony van Leeuwenhoek documented the first observations of bacterial cell morphologies over 300 years ago, in his letters to the Royal Society in London. Despite the immense expansion of knowledge and improvement of tools that has occurred since Leeuwenhoek's time, we still know very little about how and why bacteria make different shapes. Given the large diversity of shapes and sizes of bacteria, there are obviously selective pressures that make various morphologies advantageous in different genera of bacteria. However, even a typically rod-shaped *Escherichia coli* cell has the capacity to form branches, spirals and stalk-like structures under certain conditions (Nelson and Young, 2001), indicating that the genetic differences that give rise to shape divergence might be subtle. Furthermore, the advantage a given shape change confers is not necessarily intuitive, and even when a hypothesis is made, it is difficult to test experimentally as size and shape are clearly a function of a large number of factors (Young, 2006), including nutrient availability, turgor pressure and even circadian rhythms (Mori and Johnson, 2001).

One approach to unearthing the molecular mechanisms behind bacterial morphogenesis could be to apply selective pressure to bacteria, observe what happens to the cellular morphology over time, and then identify the genetic change(s) that led to the adaptation(s). This type of approach has the potential to become increasingly high throughput as genomic and proteomic tools continue to improve. Furthermore, as we learn more about the molecular mechanisms of morphogenesis, it will be possible to genetically manipulate organisms to produce specific shapes of interest: for example, a stalkless *C. crescentus* cell or an *E. coli* cell with a stalk. The relative fitness of the stalked and stalkless cells could be tested under different selective pressures experimentally, and then compared with predictions made by biophysical modelling.

Even though there is a growing abundance of data suggesting that the stalk enhances nutrient uptake to the cell, there is a need for more sophisticated experiments to assay nutrient uptake by the stalk in a direct manner. This might be possible using microfluidics to deliver nutrients to precise locations in the cell. For example, microfluidics devices could be used to determine if *C. crescentus* cells are capable of growth when nutrients are directed only at stalks, if the stalk absorbs particular nutrients, or whether the stalk is competent to deliver nutrients to the cell body at all.

The information we gain from studying the basic process of bacterial morphogenesis will increase our basic understanding of how bacterial cells are organized, but will also be useful for environmental, industrial, agricultural and medical applications. One possible use of this knowledge would be to determine the optimal shape for a bacterium involved in a particular type of bioremediation. It is truly an exciting time to study the biology of form and function at the cellular level.

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