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Physiological role of stalk lengthening in *Caulobacter crescentus*

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The Gram-negative bacterium *Caulobacter crescentus* forms a thin polar stalk, which mediates its attachment to solid surfaces. Whereas stalks remain short (1 µm) in nutrient-rich conditions, they lengthen dramatically (up to 30 µm) upon phosphate starvation. A long-standing hypothesis is that the *Caulobacter* stalk functions as a nutrient scavenging "antenna" that facilitates phosphate uptake and transport to the cell body. The mechanistic details of this model must be revisited, given our recent identification of a protein-mediated diffusion barrier, which prevents the exchange of both membrane and soluble proteins between the stalk extension and the cell body. In this report, we discuss the potential of stalks to facilitate nutrient uptake and propose additional physiological roles for stalk elongation in *Caulobacter* cells.

The Gram-negative bacterium *Caulobacter crescentus* develops a polar stalk that is formed by local extension of the cell body. The stalk mediates permanent attachment of cells to biotic and abiotic surfaces via an adhesive organelle (holdfast) that is established at its tip.[1] *Caulobacter* is an oligotrophic species which has evolved to survive in environments with very poor nutrient availability.[2] In response to low phosphate concentrations, *Caulobacter* lengthens its stalk dramatically (**Fig. 1A**).[3] While this phenomenon has been observed for nearly 45 y,[4] the physiological role of stalk elongation has remained elusive. Our recent discovery of protein (StpABCD)-mediated diffusion barriers which compartmentalize the stalk from the cell body[5] allows us to develop hypotheses regarding (1) the rationale behind stalk architecture and (2) the physiological function of stalk lengthening during phosphate starvation.

The Physiological Role of Stalk Diffusion Barriers

We have shown that diffusion barriers within the stalk of *Caulobacter* cells prevent exchange of proteins between the stalk and cell body as well as between intra-stalk compartments (**Fig. 1B**). Notably, these barriers do not segregate specific sets of proteins; rather, proteins present in the newly extruded stalk segment are coincidentally trapped behind the barrier. We therefore hypothesized that the barriers do not play a role in protein localization and instead have either a structural or physiological function. Our first hypothesis was that the Stp complex may serve as a molecular plug to prevent the leakage of cytoplasmic or periplasmic proteins following stalk breakage. Using a blender to shear off stalks, we consistently found that the viability of diffusion barrier-deficient (Δ*stpAB*) cells was not significantly decreased compared with wild-type cells (**Fig. 1C**). Thus, while the Stp complexes function as bidirectional protein diffusion barriers in stalks,[5] the propensity of disrupted membranes to reseal themselves appears to be sufficient to prevent the loss of cellular material. However, when cultivating *Caulobacter* cells in nutrient-poor media simulating their natural environment, Stp complexes emerged to provide a significant growth advantage by limiting the effective volume of the cell body once the stalk increases in length.[5] While the discovery of intra-stalk barriers has revealed an efficient strategy for reducing the physiological cost of stalk lengthening, it does not further elucidate the role of stalk lengthening per se. In the following sections, we will discuss several potential advantages that elongation of the stalk could confer to *Caulobacter* cells in their native environment.

The Stalk as a Nutrient Antenna

A long-standing model has proposed that stalk elongation is an efficient method of increasing the cell surface available for phosphate uptake. In a purely diffusive environment, the uptake of nutrients by an attached bacterium scales with its length, rather than its surface area. Therefore, extruding a thin stalk appendage would allow for greater phosphate uptake while using far less resources than would be needed to lengthen the entire cell body.[6] This model

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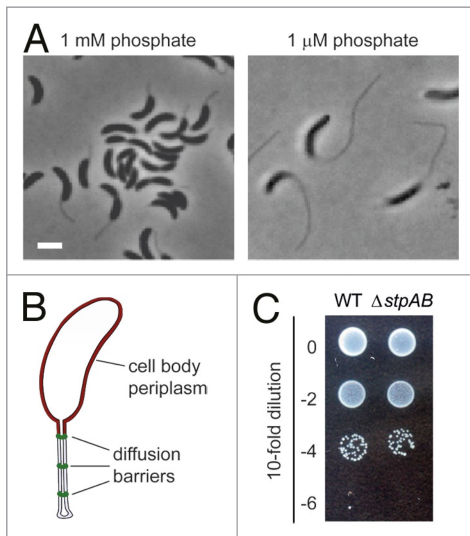

Figure 1. StpABCD complexes are not required for cell survival upon stalk detachment. (**A**) Stalk elongation upon phosphate starvation. *Caulobacter* cells were grown overnight in defined HIGG media[15] containing either 1 mM or 1 µM phosphate. Scale bar: 2 µm. (**B**) Schematic of a *Caulobacter* cell showing the physiological separation of the cell body from the stalk by protein diffusion barriers. (**C**) Mechanical removal of stalks does not affect viability. Stalks of wild-type (WT, CB15N)[16] and diffusion barrier-deficient (Δ*stpAB*) cells,[5] grown in low-phosphate medium (M2G^{-P})[5] for 24 h, were sheared off in a pre-cooled Waring blender for 3 min at maximum speed at 4°C. Successful removal of stalks was confirmed by DIC microscopy (data not shown). Suspensions of the resulting stalk-less cells were adjusted to equal optical densities, serially diluted, spotted onto PYE agar and incubated at 28°C.

requires that the phosphate captured by the stalk ultimately ends up in the cell body where it is metabolically processed. Proteomic analysis, however, suggested that the stalk only contained the periplasmic high-affinity phosphate binding protein PstS, whereas it lacked key components of the corresponding inner-membrane phosphate transporter complex (PstCAB).[6] Thus, the model was adapted to suggest that PstS shuttled phosphate from the stalk to the cell body, where it could be transported to the cytoplasm by PstCAB. However, this scenario is incompatible with our recent finding that PstS diffusion is restricted by the Stp diffusion barrier (**Fig. 2A**, red pathway).[5] Based on calculations using published values for the periplasmic protein diffusion[7] and PstS binding constants,[8] we estimate that a PstS-phosphate complex can only diffuse ~0.7 µm before it dissociates (**Fig. 2B**). Even in the absence of protein diffusion barriers, individual PstS-phosphate complexes would thus be unlikely to deliver phosphate from the stalk to the cell body, given that stalks often measure tens of microns in phosphate-limited conditions. However, PstS may shuttle phosphate within the intra-stalk compartments and occasionally release its cargo in the vicinity of a Stp complex. Upon dissociation, phosphate could diffuse through the barrier structure into the adjacent stalk compartment where it would then be re-captured by PstS, provided that the barriers are permeable to small molecules. However, since free phosphate ions readily diffuse through the outer-membrane porins, they would probably be more likely to leave the cell rather than travel down the length of the stalk.

Although the presence of diffusion barriers rules out longitudinal shuttling of PstS-phosphate complexes as an efficient means of phosphate acquisition, it does not necessarily rule out the nutrient antenna model per se. It is possible that phosphate captured by PstS in the stalk periplasm is transported into the cytoplasmic core of the stalk, from where it could diffuse into the cell body (**Fig. 2A**, green pathway). A previous study disfavored this model because the PstCAB phosphate transporter complex was not detected in the stalk.[6] However, proteomic analysis revealed that the stalk does contain PstB, the cytoplasmic ATPase of the transporter complex.[6] Additionally, our experiments show that PstA can in fact be detected in the stalk (**Fig. 2C**). This discrepancy is likely due to differences in growth conditions, since PstA accumulates to higher levels under the phosphate-limiting conditions used in our analysis.[3] Additional experiments will be required to determine whether the stalk contains fully assembled and functional PstCAB complexes. Importantly, the transport of phosphate to the stalk cytoplasm requires ATP, which would have to be (re-)generated in the stalk compartments or diffuse in from the cell body. Moreover, phosphate needs to be capable of traversing the StpABCD complex in order to reach the cell body. However, thus far, our studies have not been able to determine whether small molecules such as phosphate and ATP are compartmentalized by the StpABCD diffusion barriers. In addition, it remains to be clarified how long proteins encapsulated in the stalk remain functional, as degraded or denatured proteins cannot be replaced in intra-stalk compartments.

Stalks as Storage for Damaged Proteins

Recently, Baldi and Barral speculated that the generation of discrete stalk compartments might facilitate the asymmetric segregation of aging factors, thereby establishing a mechanism for cellular rejuvenation.[9] Inspired by this idea, we used a fluorescently tagged version of the protein aggregate-processing small heat-shock protein IbpA, which associates with inclusion bodies, to identify the subcellular localization of damaged or misfolded proteins.[10] In *Caulobacter*, IbpA (CCNA_03706) has previously been observed to form bright foci at variable positions in cells grown in high-phosphate medium.[11] Our analyses showed that this localization pattern was unchanged after exposing wild-type or diffusion barrier-deficient cells to a heat-shock (**Fig. 2D**). In particular, we did not observe any aggregates in the stalk, suggesting that the stalk and its compartmentalization from the cell body are unlikely to participate in segregating inclusion bodies. However, we cannot exclude an effect on hypothetical aging factors that might not be incorporated into inclusion bodies.

Caulobacter as an Expert in Fluid Mechanics

The holdfast at the tip of the *Caulobacter* stalk promotes attachment to surfaces. One disadvantage for surface attached cells is the limited nutrient flux in that environment. Under laminar flow, the fluid velocity is zero at the surface (referred to as the no-slip boundary condition), which means that nutrients can only reach cells on the surface by passive diffusion, which is far less efficient than convective transport. As previously proposed,[6] by

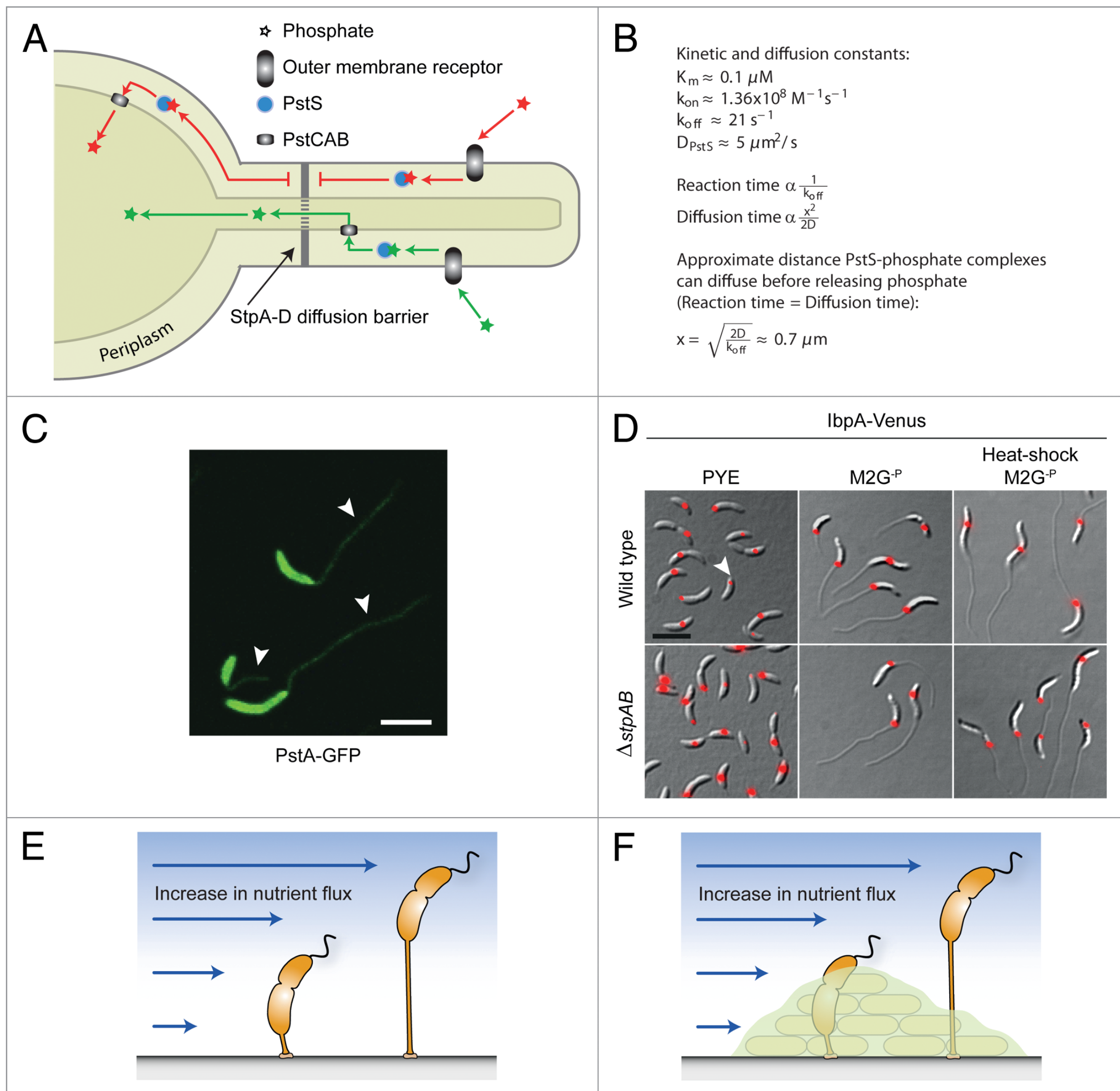

Figure 2. Models for the physiological role of stalk elongation. (**A**) The red pathway describes the previously held model of phosphate uptake, in which PstS shuttles phosphate from the stalk to the cell body, where it is imported by the PstCAB transporter.[6] Our discovery of a diffusion barrier which blocks PstS shuttling contradicts this model.[5] An alternative model, shown as the green pathway, assumes that previously undetected PstCAB complexes do exist in the stalk, allowing the uptake of phosphate into the cytoplasmic core of the stalk and its subsequent diffusion to the cell body. (**B**) Using kinetic and diffusion parameters, the distance that PstS-phosphate complexes can diffuse before phosphate release is approximately 0.7 µm. This distance is far shorter than the stalk length under phosphate-limiting conditions. (**C**) Localization of PstA-GFP in the stalk. Strain YB4062 (CB15N pMR10-P_{pst}-*pstCA-gfp*) was grown for 36 h in HIGG medium containing 30 µM phosphate. PstA-GFP was visualized by fluorescence microscopy. Arrowheads indicate stalks containing the fusion protein. Scale bar: 2 µm. (**D**) Subcellular localization of Heat Shock Protein 20 (HSP20, IbpA homolog) in wild-type and Δ*stpAB* cells grown in high-phosphate (PYE) and in low-phosphate ($M2G^{-P}$) medium. To test for the segregation of damaged proteins into the stalk, cells of strains SS419 (CB15N P_{xyl}::P_{xyl}-*ibpA-venus*) and SS420 (CB15N Δ*stpAB* P_{xyl}::P_{xyl}-*ibpA-venus*) were first grown in $M2G^{-P}$ for 12 h. Production of IbpA-Venus was induced by adding 0.3% xylose for 1 h prior to a heat shock. Cells were shifted to 40°C for 1 h, followed by a growth period of 8 h at 28°C. Untreated cells were cultured at 28°C for 9 h. Images show overlays of DIC and false-colored fluorescence images. Scale bar: 3 µm. (**E**) Stalk elongation may function to elevate single cells away from surfaces. As the cell distances itself from the surface, fluid velocity (blue gradient) and nutrient flux (blue arrows) increase. Thus, stalk elongation may ensure greater nutrient availability. (**F**) *Caulobacter* cells may co-colonize surfaces with other organisms. By distancing themselves from the surface, they may have greater access to nutrients relative to nearby surface-associated species, thereby increasing their competitiveness.

lengthening the stalk, *Caulobacter* cells may be distancing themselves from the surface in order to increase the mass flux of nutrients to the cell body. Indeed, within the boundary layer of fluid flow (roughly the first 1 mm from the surface) the fluid velocity increases linearly. While the velocity increases linearly, the flux of nutrients increases at an even faster rate, as shown in the seminal work by Berg and Percell.[12] Conservative estimations allow us to calculate that stalk elongation from 1 µm to 10 µm would provide a ~10% increase in nutrient flux. Therefore, *Caulobacter* may use stalk elongation to gain a nutrient scavenging advantage (**Fig. 2E**).

The Tallest Tree in the Forest

The ecology of *Caulobacter*'s freshwater environment is not well defined. We do not know which species, if any, co-colonize surfaces with *Caulobacter*. The previous model relating elongated stalks to increased nutrient flux focused on the uptake by isolated cells. However, in an ecological environment where *Caulobacter* cells may be competing with other surface-associated species, this height discrepancy may offer a significant growth advantage (**Fig. 2F**). Whether in the presence or absence of flow, the taller *Caulobacter* cells would have greater access to environmental nutrients relative to the other co-colonizing organisms. Moreover, stalk elongation may help elevate *Caulobacter* cells to the surface of a multi-species biofilm, thereby facilitating the release of motile swarmer cells into the surrounding aqueous environment. It is important to note that there are relatives of *Caulobacter*, such as *Asticcacaulis biprosthecum*, whose stalks elongate in nutrient-limiting conditions although they do not feature an adhesive holdfast at their tip. Because in these species stalk length does not affect the spacing between the cell body and the attached surface, their stalks must have a physiological role that is, at least in part, different from that in *Caulobacter* cells. They may, for instance, function as nutrient scavenging antennae or help increase cell size in order to prevent ingestion by predatory protozoa.[13]

Cell Shape as a Selective Advantage

Bacteria exist in an extraordinary variety of shapes and sizes,[14] but very little is known about how particular shapes provide a selective advantage. Stalk formation is common among a variety of α-proteobacteria and is also observed in evolutionary distant lineages such as planctomycetes and verrucomicrobia. For *Caulobacter,* the relationship between phosphate availability and stalk length has been known for decades, yet the evolutionary advantage of stalk elongation has remained elusive. Our recent identification of an intra-stalk diffusion barrier has provided insight into the physiology of the *Caulobacter* stalk. In the future, we hope to test the proposed hypotheses in order to clarify the physiological role of this peculiar structure and better understand the correlation between bacterial cell shape and fitness.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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