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MicroCommentary

Understanding the shapes of bacteria just got more complicated

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Summary

The paper by Briegel *et al*. in this issue of *Molecular Microbiology* uses advanced cryo-transmission electron microscopy (cryoTEM) techniques to reveal four separate locations of cytoplasmic filament bundles in *Caulobacter crescentus*. Intuitively, these filaments should be rather rigid protein structures and composed of previously identified shape-forming proteins, such as crescentin or MreB. Yet, deletion mutants lacking these proteins still possessed filaments and still possessed wild-type morphology. These results suggest that a complex combination of protein structures, including those of crescentin, MreB and these newly identified bundles, in combination with the cell envelope help maintain the complicated shape of *C. crescentus*. Other bacteria might have similar architectural proteins to assist in maintaining the cell contours during growth and division.

Introduction

The maintenance of shape and form in bacteria throughout their growth and division has been a constant enigma to structural microbiologists since the early days of light and electron microscopy. Ever present were two salient problems: (i) light microscopy lacks the high resolution necessary to detect fine detail in prokaryotes (especially when phase-contrast is used on living bacteria) (Beveridge *et al*., 2006a) and (ii), transmission electron microscopy requires dehydrated samples, (often) fixation, and heavy metal stains for contrast (Beveridge *et al*., 2006b). It is difficult to see intimate structural details in bacteria, *especially* in their hydrated state. In light microscopy, we are still limited by the physics involved with photonic wavelengths and the precision of glass lenses, but the advent of confocal laser scanning microscopy (CLSM) using a coherent light source and the ability to position focal planes precisely within the specimen has provided us with increased resolving power plus the ability to visually 'section' through a specimen (Beveridge *et al*., 2006a). In addition, the fluorescent labelling of specific proteins distinguishes them from background, provides reasonable cellular location and, for dividing cells, has identified division-assisting proteins associated with the division annulus at septal (Gram-positive) or constricting (Gram-negative) sites (Weiss, 2004). Fluorescent labelling has also located proteins important for cell wall synthesis (Scheffers and Pinho, 2005). CLSM studies of both Gram-positive and Gram-negative bacteria have convincingly shown that a multitude of proteins are involved with growth and division, some with good molecular similarity to tubulin and therefore capable of forming scaffolding-like filamentous assemblies. Two structural proteins, crescentin and MreB, have recently been implicated as scaffolds responsible for maintaining shape within *Caulobacter crescentus* (Ausmees *et al*., 2003; Dye *et al*., 2005).

A stalked bacterium

Caulobacter belongs to the prosthecate group of bacteria and, when sessile, possesses a stalk attached to a slightly curved cell body (Fig. 1). The stalk is often devoid of cytoplasm and is continuous with the cell wall of the cell body. The terminus of the stalk is studded with thin proteinaceous fibrils that are used for attachment to the substratum (one of Nature's strongest 'glues') and the stalk can lift the cell body a substantial distance from the attachment site. In natural environments, the stalk lifts the bacterium above most other attached microorganisms into a region rich in nutrients for growth. Many caulobacters possess an S-layer that is situated above a typical Gram-negative envelope with the lipopolysaccharide O-side chains of the outer membrane presumably sticking out through the holes in the S-layer subunits. CB15N, the strain used by Briegel *et al*. (2006), has a hexagonally arranged (*p6*) S-layer that is self-assembled once the

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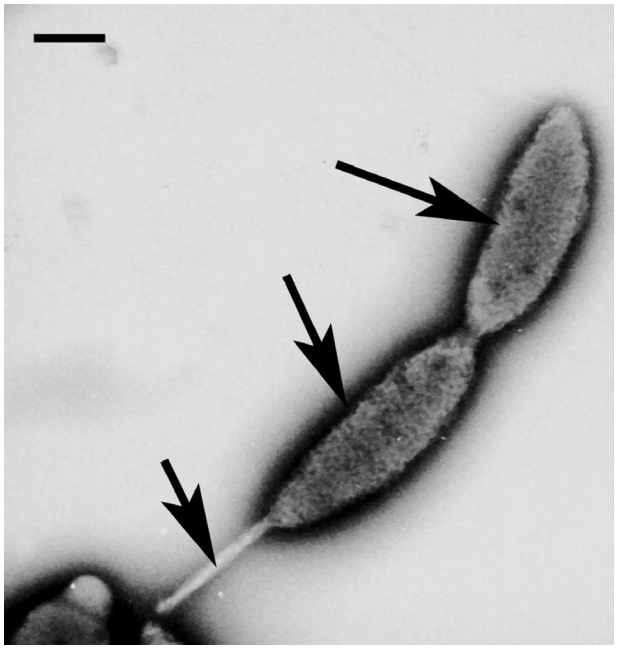

Fig. 1. Negative stain of a dividing caulobacter isolated from a natural freshwater environment. The arrows point (from bottom left to top right) to the stalk, cell body and swarmer cell. Scale bar is 500 nm.

~100–190 kDa S-layer protein is exported to the surface. After division, via constriction, a motile 'swarmer' cell swims from the sessile daughter and searches for its own substratum for attachment. This is a complex cell cycle for a complicatedly shaped bacterium and Briegel *et al*. suggest that it could be even more complicated than originally presumed because there might be at least four types of filament bundles!

Cryo-electron microscopy

Briegel *et al*. used a technique, cryo-transmission electron microscopy (cryoTEM), which is available only in specialized research facilities and underexploited in microbiology. Cells are snap-frozen within milliseconds so that they are physically fixed in a non-crystalline 'glass' of amorphous ice. The beauty of the technique is that the cells remain hydrated and that the snap-freezing ensures that molecules and their structures remain positioned as they were in the vital state. Most frozen cells come back to life when thawed. . . . this is as close to the living bacterium as possible!

Imaging cryo-specimens

Imaging is difficult because heavy metal contrasting stains are not used. Somehow the cell has to be distinguished from the surrounding ice. This is done by defocusing and using phase contrast for imaging; it relies on the difference in densities between the ice (H_2O) and organic components of the bacterium (i.e. protein, DNA, phospholipid, etc.). Some microscopes are equipped with special energy filters that can aid imaging by contributing differential contrast. Because Briegel *et al*. used frozen foils that are at least as thick as a single bacterium, high electron penetrating power is required and inelastic scattering of electrons, as they pass through such a thick specimen (which detracts from accurate imaging), is a problem. The authors use a 300 kV microscope equipped with an energy filter and a field emission gun (which produces a coherent electron beam and minimizes inelastic scattering) with high penetrating power. Furthermore, their microscope is equipped with a state-of-the-art cryo-stage and cryo-specimen holder for long holding times with little contamination allowing sufficient time to do tomography (imaging while tilting the specimen about a single axis) so that 3-D imaging is possible with the proper software. This sophisticated technology allowed breath-taking, elegant and intriguing images of *Caulobacter* to be obtained (e.g. Fig. 2).

Other evidence of internal layers

There are few cytoplasmic layers in bacteria to which a role in helping form cellular shape can be attributed. Most that have been visualized by electron microscopy have been in conventionally fixed and embedded samples and have been associated with polar flagella, such as polar membrane or concentric membrane rings (Fig. 3). These

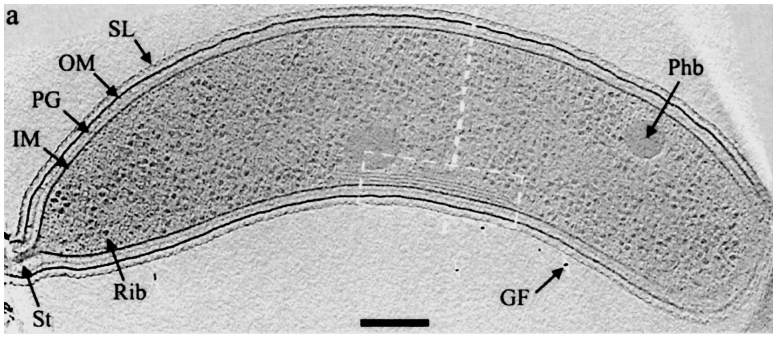

Fig. 2. *C. crescentus* CB15N embedded in a frozen thin foil (Briegel *et al*., 2006) showing the S-layer (SL), outer membrane (OM), peptidoglycan layer (PG), inner (plasma) membrane (IM), stalk (St), ribosomes (Rib), probable poly-β-hydroxybutyrate granule (Phb), and gold particle (used to align images). White box shows a filament bundle. Scale bar is 200 nm.

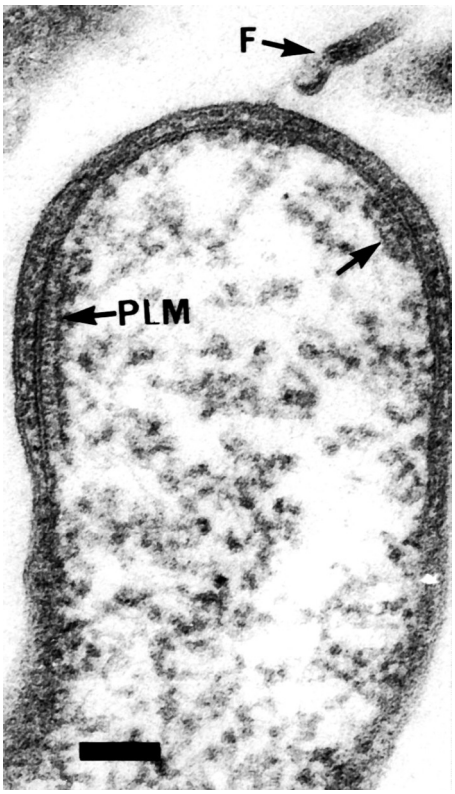

Fig. 3. Thin section of *Vibrio cholerae* from a conventionally fixed embedding showing the polar membrane (PLM and arrows) as an example of another type of a putative cytoplasmic structural layer. F denotes a small region of the bacterium's sheathed polar flagellum. Scale bar is 100 nm.

are thought to provide extra strength to a region of the cell that will be subjected to tremendous stress because of the torque produced as a long polar flagellum rotates. These structures are not seen in all bacteria that have polar flagella. Other structures such as mesosomes, thylakoids, membrane-bound vacuoles, etc. do not have a shape-forming function.

Complications of an S-layer

The *C. crescentus* studied by Briegel *et al*. has an S-layer that could complicate the shape issue considerably. S-layers are planar crystals of (glyco)protein that self-assemble (in this case) into donut-shaped hexamers that are held together by H-bonds, electrostatics or salt linkage to form a single subunit (Sleytr and Beveridge, 1999). Subunits are arranged in a similar manner, forming a *p6* lattice held together by similar linkages. Individually the bonds that anneal S-layers together are much weaker than covalent bonds but in concert they are substantial. Are they strong enough to contribute to the shape-forming ability? (Lattice defects in an S-layer always exist so that the S-layer can curve itself around a cell. On the other hand, caulobacters lacking the S-layer have the typical caulobacter shape of CB15N.) Indeed, because S-layer proteins have inherent self-assembly capacity, could certain S-layer strains use these same proteins to form intracellular filaments such as those seen within CB15N and could these same filaments act as scaffolds to contribute to shape, as the authors suggest?

Are filament bundles seen in other bacteria?

It is unfortunate that cryoTEM is rarely used in structural microbiology. So far, only a few systems have been looked at and most did not have filament bundles. These systems include *Bacillus*, *Enterococcus*, *Staphylococcus*, *Deinococcus*, *Escherichia* and *Pseudomonas*. *Caulobacter* has been examined before but the authors did not mention filament bundles (Judd *et al*., 2005). *Spiroplasma melliferum*, which is a member of the Mollicutes, does have filament bundles (Kürner *et al*., 2005).

Cell walls and shape

It has been difficult to attribute shape to a single structural entity in bacteria. For a long time, the easy solution was to attribute this role to the cell wall (Beveridge, 1981). This was simple and convenient because cell walls obtained by breaking even Gram-negative bacteria like *Caulobacter* retain the shape of the bacterium. Thus, cell shape is ingrained within the fabric of the cell wall. The protoplast with its high turgor pressure is constrained within the cell wall and is forced into a specific cellular form. Yet we also know that the strongest, most resilient wall component, the peptidoglycan, has great elasticity and reasonable deformability (Yao *et al*., 1999). Ancillary structures, such as a rudimentary cytoskeleton, might help in shaping the cell. Gram-positive bacteria have thick, sturdy walls that should readily withstand high turgor pressures and should mould a protoplast into a specific shape. But, Gram-negative bacteria have thinner peptidoglycan layers that, alone, might not be able to retain their shape under turgor pressure (even if lipoproteins are used to anchor the outer membrane and peptidoglycan together for added strength). This is the intriguing aspect of the work by Briegel *et al.* because the bundled filaments might constitute a rudimentary cytoskeleton. Many questions remain, however. For example, do the cells lose shape when the filament bundles are not present? How are they distributed during a complicated event such as division? Are they necessary in both swarmer and stalked cells? The fact that the filaments can be visualized only by cryoTEM makes it likely that future progress will be slow.

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