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A multidimensional study of streptomyces morphogenesis and development

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CHAPTER 2

A Multidimensional View of the Bacterial Cytoskeleton

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ABSTRACT

The perspective of the cytoskeleton as a feature unique to eukaryotic organisms was overturned when homologs of the eukaryotic cytoskeletal elements were identified in prokaryotes and implicated in major cell functions, including growth, morphogenesis, cell division, DNA partitioning, and cell motility. FtsZ and MreB were the first identified homologs of tubulin and actin, respectively, followed by the discovery of crescentin as an intermediate filament-like protein. In addition, new elements were identified which have no apparent eukaryotic counterparts, such as the deviant Walker A-type ATPases, bactofilins, and several novel elements recently identified in streptomycetes, highlighting the unsuspected complexity of cytostructural components in bacteria. *In vivo* multidimensional fluorescence microscopy has demonstrated the dynamics of the bacterial intracellular world, and yet we are only starting to understand the role of cytoskeletal elements. Elucidating structure-function relationships remains challenging, because core cytoskeletal protein motifs show remarkable plasticity, with one element often performing various functions and one function being performed by several types of elements. Structural imaging techniques, such as cryo-electron tomography in combination with advanced light microscopy, are providing the missing links and enabling scientists to answer many outstanding questions regarding prokaryotic cellular architecture. Here we review the recent advances made toward understanding the different roles of cytoskeletal proteins in bacteria, with particular emphasis on modern imaging approaches.

When the term 'cytoskeleton' was first coined in 1931 (Wintrebert 1931), cytoskeletons were thought to consist of fibrous structural elements within a cell which, like the bones in our body, exist to provide reinforcement. It gradually became clear, however, that the cytoskeleton is not so much a static structural system like spokes in a wheel but is rather a highly dynamic system responsible for major processes in the cell, including muscle contraction (Banga and Szent-Györgyi 1942), the beating of cilia (Gibbons and Rowe 1965), chromosome segregation (Inoué and Sato 1967), cell division (Schroeder 1972), phagocytosis (Kaplan 1977), and organelle transport (Brady 1985; Vale *et al.* 1985), besides providing cell structure.

Still, it was a widely held notion that the cytoskeleton, consisting of microtubuli, microfilaments, and intermediate filaments (IFs), with cross-linking and other associating proteins providing additional levels of complexity (Alberts *et al.* 2002), is a feature unique to eukaryotic cells. The existence of a multifunctional cytoskeleton in bacteria became generally accepted only in the last decade, when the concept of bacterial cells as sacculi of freely diffusible proteins was overturned, and it was established that they, in fact, contain homologs of all known eukaryotic cytoskeletal elements (Graumann 2007; Margolin 2009; Young 2010). FtsZ (a tubulin homolog [Bi and Lutkenhaus 1991]) and MreB (an actin homolog [Jones *et al.* 2001]) were the first to be characterized; later, crescentin, the first intermediate filament (IF)-like protein, was discovered in *Caulobacter crescentus* (Ausmees *et al.* 2003). Currently, there are also newly identified elements with no eukaryotic counterparts, namely, the deviant Walker Amotif ATPases (Koonin 1993) and bactofilins (Kühn *et al.* 2010), clear evidence of the complexity of the bacterial cytoskeleton, while many elements are likely still to be discovered.

On the cellular scale, much has been learned about the cytoskeleton based on fluorescence light microscopy (fLM) studies and, in recent years, also via atomic force microscopy (AFM), which has been applied for the study of live cells as well as of isolated membrane proteins or microtubules (Hamon *et al.* 2010), by measurement of surface properties. On the molecular scale, X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are providing valuable structural information. In fact, rather than sequence similarity analyses, the main methods used for identification of prokaryotic cytoskeletal elements have been based on a combination of crystal structures, *in vitro* properties, and *in vivo* functional behavior (Cabeen and Jacobs-Wagner 2010). Bridging the gap between cellular and molecular structural studies (Figure 1), cryo-electron tomography (cryo-ET) is taking its place as an important part of the imaging arsenal, providing structural information about protein complexes under conditions directly relevant to the native state of the cell (Subramaniam 2005; Koning and Koster 2009; Tocheva *et al.* 2010; McIntosh *et al.* 2005; Lucič *et al.* 2005). Combining tomography with the

aforementioned imaging methods provides the multiscale and multidisciplinary approach needed to understand how cytoskeletal proteins function within the context of the cell.

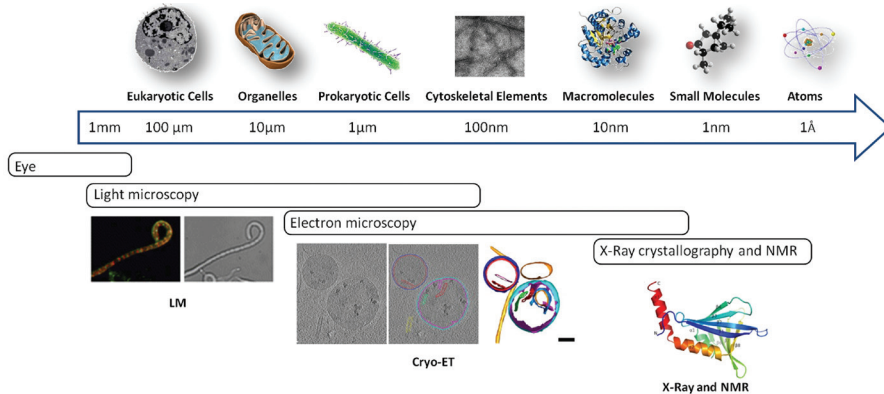


Figure 1. Resolution ladder demonstrating imaging techniques which can be used at different scales.

Light microscopy (LM) can be used to image the live localization of proteins tagged with fluorescent reporters to obtain dynamic information; at higher resolution, cryo-electron tomography (cryo-ET) can provide structural information about the localization of, for example, cytoskeletal elements within the cell. Crystal structures of the proteins of interest can be obtained via X-ray crystallography or nuclear magnetic resonance spectroscopy. LM images adapted from reference (Willemse et al. 2011) with permission from Cold Spring Harbor Laboratory Press; cryo-ET images adapted from reference (Koning and Koster 2009) with permission from Elsevier; X-ray and NMR images adapted from reference (Xu et al. 2009) with permission from the American Society for Biochemistry and Molecular Biology.

In this review, we focus on the different roles of the major cytoskeletal proteins and demonstrate ways in which multiscale imaging techniques have provided insight into the organization and spatial arrangement of cytoskeletal filaments. In addition, we highlight their function in the morphologically unusually complex mycelial *Streptomyces*, where many of the elements take on new and distinct roles. We believe that streptomycetes are a good illustration of the flexibility of core cytoskeletal protein motifs, where one type of element can perform various functions and one function can be performed by several types of elements (Graumann 2007; Cabeen and Jacobs-Wagner 2010). The review is nonexhaustive, and for detailed information on specific filament-forming proteins in bacteria we refer the reader to excellent reviews elsewhere (Graumann 2007; Cabeen and Jacobs-Wagner 2010; Shih and Rothfield 2006; Michie and Löwe 2006; Ingerson-Mahar and Gitai 2012).

DETERMINING (AND MAINTAINING) BACTERIAL CELL SHAPE

Bacteria have a wide variety of shapes, from the more common spherical and rod shapes to spiral, square, and filamentous shapes, and the molecular basis underlying cell shape is complex (Margolin 2009; Young 2010; Cabeen and Jacobs-Wagner 2005). Maintaining shape throughout cell growth and division is important if cells are to proliferate. In most bacteria, the external peptidoglycan (PG) wall, or murein sacculus, is responsible for maintaining cell shape by providing a rigid protection against osmotic pressure. Some bacteria, however, lack a cell wall and still maintain clearly defined shapes, ranging from the cocci of *Acholeplasmato* the tapered flask-like shape of some *Mycoplasma* species and the spiral shape of *Spiroplasma* species (Cabeen and Jacobs-Wagner 2005). It is little wonder that a wide variety of shape-defining and -maintaining cytoskeletal elements, forming a variety of superstructures (Pilhofer and Jensen 2013), must exist to enable this diversity.

MreB AND MreB-LIKE PROTEINS

In rod-shaped bacteria, mutation of *rodA*, *rodZ*, and *mreBCD* resulted in loss of shape, with formation of round cells that eventually die (Jones *et al.* 2001; Figge *et al.* 2004; Kruse *et al.* 2003; Defeu Soufo and Graumann 2003). Their structural analysis identified MreB as an actin homolog (van den Ent *et al.* 2001). Gram-negative bacteria apparently have a single *mreB* gene, typically in an operon with *mreC* and *mreD*, while genomes of Gram-positive bacteria encode up to three MreB-like proteins (MreB, Mbl, and MreBH in *Bacillus subtilis*). Imaging of MreB-green fluorescent protein (MreB-GFP) revealed patches localized in a spiral-like fashion along the long axis of cells, which was explained by MreB aiding in peptidoglycan deposition (Jones *et al.* 2001; Dye *et al.* 2005; Srivastava *et al.* 2007; Vats and Rothfield 2007) (Figure 2). Recent experiments, however, contradicted the notion of the existence of continuous helices and suggested rather a model whereby MreB moves circumferentially around the cell, perpendicular to its length, with synthesis complexes moving independently of each other in both directions (Garner *et al.* 2011). Inhibition of peptidoglycan synthesis blocks filament motion within 10 to 30 s, suggesting that PG synthesis drives the motion of MreB—and not vice versa. In fact, though previous studies have suggested that MreB dynamics are driven by its own polymerization, MreB rotation around the long axis of the cell requires the assembly of the peptidoglycan cell wall (van Teeffelen *et al.* 2011). Total internal reflection fluorescence microscopy visualized the dynamic relationship between MreB paralogs and the cell-wall synthesis machinery in *Bacillus* (Domínguez-Escobar *et*

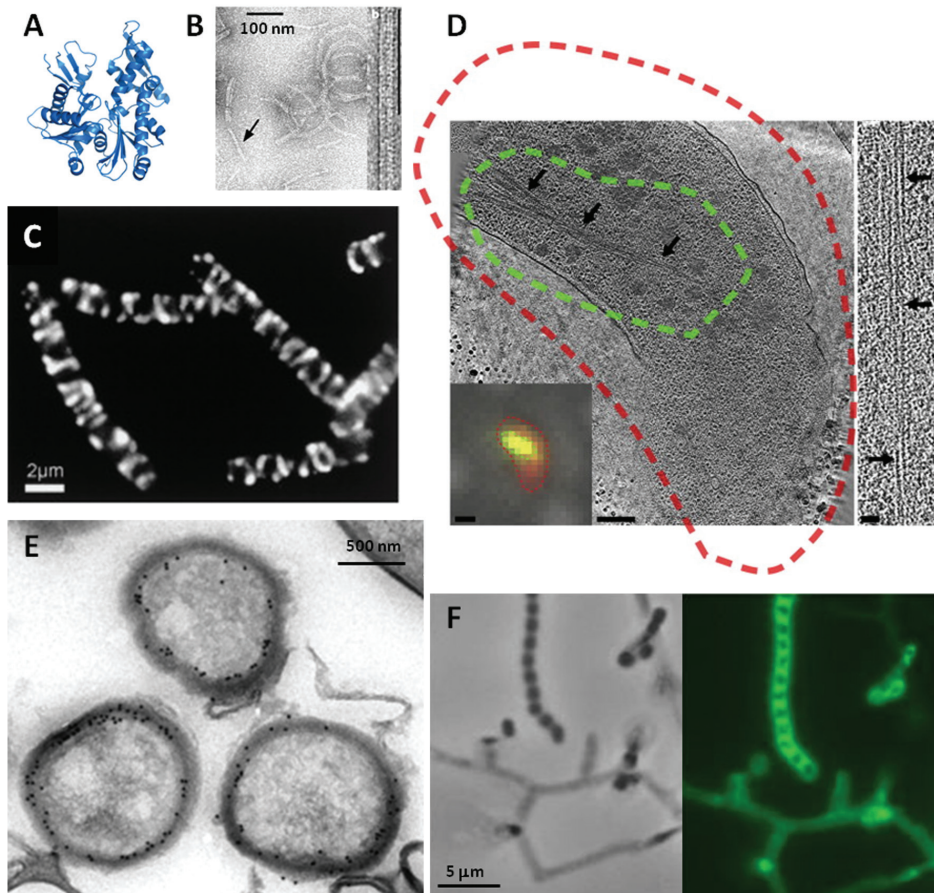


Figure 2. Structure and microscopy of MreB. (A and B) The fold of prokaryotic MreB is similar to that its eukaryotic counterpart, actin (A), and MreB assembles into actin-like filaments (B). (C) The seemingly helical localization of an (MreB-like) Mbl-GFP fusion protein in cells in rod-shaped *B. subtilis* is now called into question (Margolin 2012; Swulius et al. 2011; Swulius and Jensen 2012). (D) In fact, though cytoplasmic MreB filaments are visible in tomograms, no helical filaments were seen in several rod-shaped bacteria, and helical filaments seen in *E. coli* were shown to be an artifact of the N-terminal YFP tag. In panel D, a tomographic slice through a *Vibrio cholerae* cell overexpressing GFP-MreB is shown. The cryo-fLM inset shows the cell stained with membrane dye FM 4-64 (red) and expressing GFP-MreB (green); dashed lines in the main image represent the fluorescent signal boundaries. On the right side, a 15-nm-thick tomographic slice through an MreB bundle not fused to GFP is shown. The scale bars represent 1 μm in the fLM inset, 200 nm in the cryo-electron tomography slice, and 50 nm in the higher-magnification inset. In *Streptomyces coelicolor*, MreB is not essential for vegetative growth but is essential for spore integrity and has been shown by immunoelectron microscopy (E) and fluorescence microscopy (F) to localize to the spore envelope. Panel B adapted from (van den Ent et al. 2001) with permission from the Nature Publishing Group; panel C from (Jones et al. 2001) with permission from Elsevier Ltd.; panel D from (Swulius et al. 2011) with permission from Elsevier Ltd.; and panels E and F from (Mazza et al. 2006) with permission from John Wiley and Sons.

al. 2011; Vats *et al.* 2009), and similar patch-like localizations of MreB and dynamics were found in *Escherichia coli* and *Caulobacter crescentus*, suggesting that the behavior is widely conserved. These studies sparked a large debate. Is the helical organization the natural configuration that a filamentous structure on the inner surface of a cylinder will assume? Or are the localization patterns an artifact of images taken with long exposure times? Or is helical localization perhaps due to the effect of fluorescent fusions on tagged proteins? It is now becoming clear that different imaging techniques are required to corroborate the observed localization patterns (Margolin 2012). Cryo-electron tomography corroborated the absence of long (80-nm) MreB filaments near or along the inner membrane of six different rod-shaped bacteria, although use of correlative cryo-light and electron tomography of GFP-MreB allowed the identification of cytoplasmic MreB bundles, showing that MreB is indeed detectable by cryo-ET (Swulius *et al.* 2011). The same researchers later demonstrated that helical localization may be induced by fluorescent tagging: cryo-ET on *E. coli* cells producing native and yellow fluorescent protein (YFP)-tagged MreB demonstrated that MreB localizes in a helix when it is N-terminally tagged with YFP, while, when tagged with mCherry within an internal loop, it localizes in the same manner as native MreB (Swulius and Jensen 2012).

COILED-COIL PROTEINS: INTERMEDIATE FILAMENTS IN BACTERIA?

If MreB is mainly involved in maintaining the rod shape, some intermediate filament-like proteins act to control other bacterial shapes. Crescentin (CreS), bearing remarkable architectural and biochemical relatedness to eukaryotic IF proteins, was the first to be identified in this class; other IF-like proteins include CfpA (Izard *et al.* 2004; You *et al.* 1996), Scc (Mazouni *et al.* 2006), AglZ (Yang *et al.* 2004), and the four Ccrp proteins of *Helicobacter* (Specht *et al.* 2011; Waidner *et al.* 2009), as well as FilP (Bagchi *et al.* 2008) and Scy (Holmes *et al.* 2013) in *Streptomyces*. Because, unlike crescentin, the other proteins do not actually have a high degree of structural similarity to IF proteins and may in fact present a case of convergent evolution (Graumann 2009), they have also been termed coiled-coil rich proteins, or Ccrps.

Crescentin (CreS) forms a filamentous structure at the short axis of the curved bacterium *C. crescentus* (Ausmees *et al.* 2003). Deletion of the *creS* gene turns curved *C. crescentus* cells into straight rods, demonstrating that crescentin is required for the curved (crescent) cell shape. Cryo-electron tomography of *C. crescentus* revealed multiple filament bundles, which fall into four major classes based on their shape and location (inner curvature, cytoplasmic, polar, and ring-like [Ausmees *et al.* 2003]). Bundles, however, persisted in crescentinless

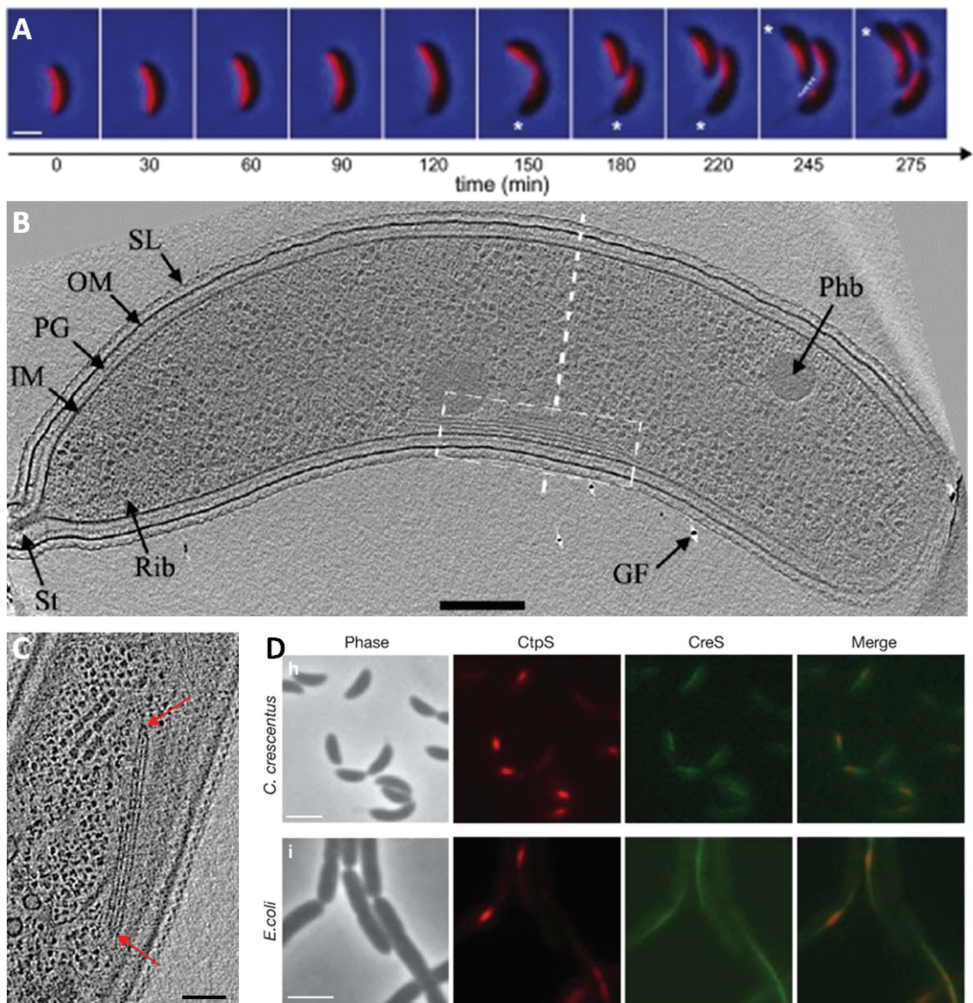


Figure 3. Discovery of metabolic enzyme CtpS as a novel filament-forming protein in *Caulobacter crescentus*.

(A) A time-lapse series of images of the GFP-labeled crescentin structure (red; laid over phase-contrast images) during the course of the cell cycle shows crescentin localizing to the short axis of curved *C. crescentus* cells. (B and C) Cryo-ET confirmed the presence of filament bundles (B), though bundles were still present in a crescentin deletion strain (C). This led to the discovery of metabolic enzyme CtpS as a novel filament-forming protein in *C. crescentus*. (D) CtpS and CreS colocalize. Bars, 1 μ m (A), 200 nm (B), 50 nm (C), and 2 μ m (D). Structural features in panel B: SL, surface layer; OM, outer membrane; PG, peptidoglycan layer; IM, inner membrane; St, stalk; Rib, probable ribosome; GF, gold fiducial used to align images; Phb, putative poly- β -hydroxybutyrate granule. Panel A adapted from (Charbon et al. 2009) with permission from Cold Spring Harbor Laboratory Press; panel B adapted from (Briegel et al. 2006) with permission from John Wiley and Sons; panels C and D adapted from (Ingerson-Mahar et al. 2010) with permission from the Nature Publishing Group.

mutants and in cells treated with A22, a compound which causes depolymerization of MreB filaments (Gitai *et al.* 2005), suggesting that they are composed of as-yet-unidentified cytoskeletal elements. This led to the identification of the metabolic enzyme CtpS as a novel filament-forming protein in *C. crescentus*, interacting dynamically with CreS to regulate cell curvature (Figure 3). The bifunctionality of CtpS filaments, which have a metabolic as well as a morphogenic role, demonstrates that protein polymerization may serve different functions within different cell contexts. Polymerization may have initially occurred for nonstructural, regulatory reasons— and the development of cytoskeletal and structural function of some proteins occurred later, during diversification and evolution. The four coiled-coil proteins of *Helicobacter pylori* (Ccrp48, Ccrp59, Ccrp1142, and Ccrp1143) have all been shown to be essential for the maintenance of proper (spiral) cell shape (Specht *et al.* 2011; Waidner *et al.* 2009). Deletion of these genes results in almost straight chained cells; in addition, though flagella are not affected, motility is reduced. All four Ccrps have different multimerization and filamentation properties and different types of smallest subunits and do not copurify, suggesting that the filaments have different though complementary roles (Yang *et al.* 2004).

The *Streptomyces* cytoskeletal element Scy was recently proposed to control polarized growth in existing hyphal tips, as well as to promote new tip formation during branching (Holmes *et al.* 2013). Deletion of *scy* affects polarized growth and, as a consequence, also hyphal geometry, resulting in irregular hyphal width, short hyphal length, and aberrant branching. FilP, encoded from a gene immediately downstream of *scy*, is important for the stability of filamentous hyphae and for correct DNA segregation (Bagchi *et al.* 2008), again underlining the versatile architecture of IF-like proteins, offering different solutions for a variety of cytoskeletal tasks. Characterization of these coiled-coil proteins was performed by analyzing protein architecture and sequence conservation and, in the case of FilP, by AFM to analyze the rigidity of wild-type hyphae and deletion mutants; cryo-ET should allow visualization of such filaments in situ. In fact, *Streptomyces* hyphae are ideally suited for study with whole-cell cryo-electron tomography, as they are thinner than 500 nm — almost half the width of unicellular bacteria such as *E. coli* and *B. subtilis* — and therefore within the range of permissible thickness for ET. Moreover, other than those already identified, streptomycetes may have many more cytostructural elements, and preliminary mutational analysis suggests specific functions for a number of the large coiled-coil proteins found in these organisms in the control of cell integrity, growth, development, protein secretion, and DNA segregation (our unpublished data). It will be interesting to see if cryo-ET can reveal these elements within the hyphae. Indeed, despite their expected significant width (eukaryotic IFs are around 10 nm wide), to date, no confirmed intermediate filament-like structures (of

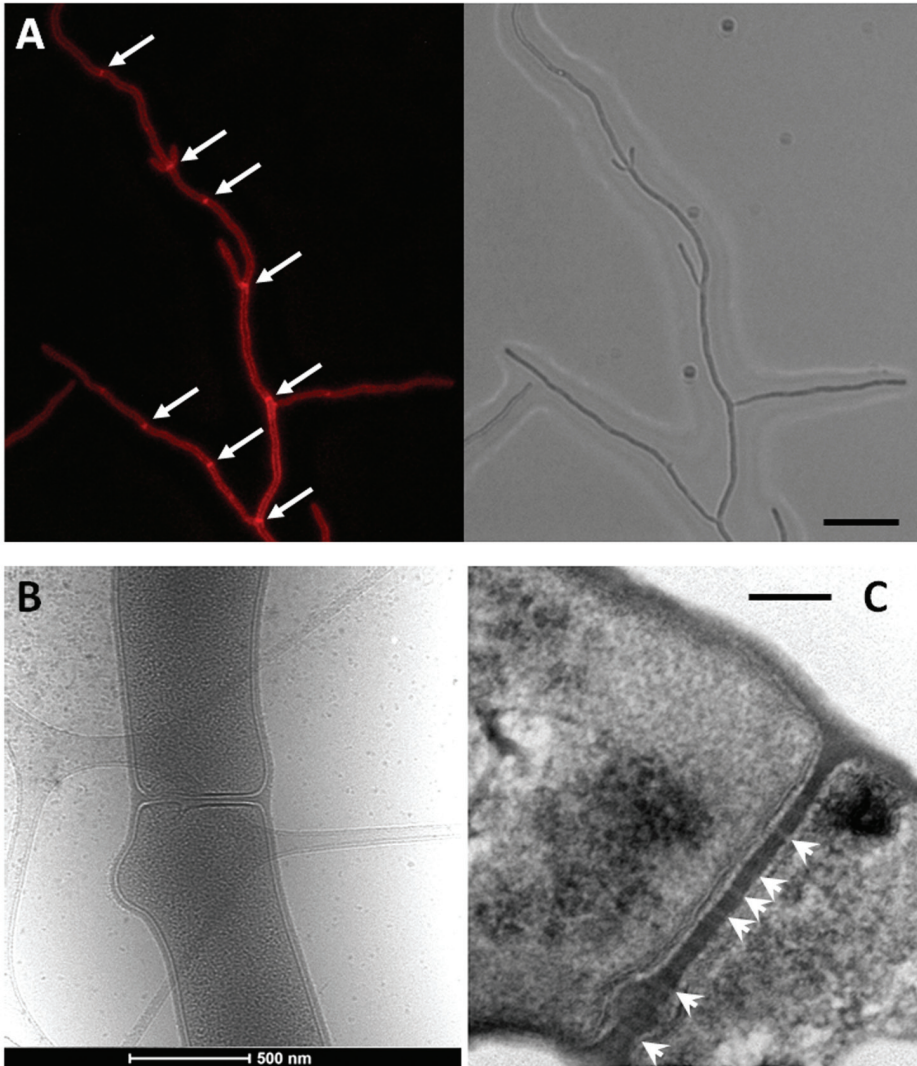


Figure 4. Cross-wall formation in *Streptomyces coelicolor*.

(A) Cross walls (arrows) are formed at irregular intervals in vegetative hyphae of *Streptomyces*, and their structure and control of their localization are still poorly understood. Left, fluorescence micrograph after staining with the membrane dye FM5-95; right, corresponding light image. Bar, 5 μ m. (B) Electron micrograph of a cross wall at higher resolution. (C) Transmission electron micrograph of a complete cross-wall with likely channels (arrowheads; apparent as lighter sections) and a bulge. Bar, 100 nm. It should be noted that cross walls may not all have channels. Figure 4C adapted from (Jakimowicz and van Wezel 2012) with permission.

CreS or others) have been unequivocally identified in tomograms of bacteria. Confirmation of the existence of prokaryotic IFs by, for example, high-resolution imaging combined with fluorescence microscopy is therefore eagerly awaited.

OTHER ROLES FOR MreB AND THE IF-LIKE PROTEINS

Difficult as it may be to group the intermediate filament-like proteins based on their structural similarity, it is even more difficult to classify them based on their function. Though several have an important role in shape control in non-rod-shaped bacteria, others have diverse roles in, for example, cell division (CfpA) or motility (AglZ). In a similar fashion, MreB and its homologs not only function to dictate rod shape but also play a role in motility (discussed below) (Mauriello *et al.* 2010; Kürner *et al.* 2005), chromosome segregation (Gitai *et al.* 2005; Defeu Soufo and Graumann 2005), establishing cell polarity (Gitai *et al.* 2004), and providing stability to the spore coat wall in *Streptomyces* (Mazza *et al.* 2006). In *Myxococcus xanthus*, filament-forming proteins AglZ and, interestingly, MreB are involved in (adventurous) A-type motility, a type of gliding for which the mechanism is not yet well understood. Many models were proposed (Wolgemuth *et al.* 2002; Mignot 2007), but the latest results indicate that A-motility involves distributed motors and focal adhesion complexes, involving up to 40 proteins in a large multiprotein structural complex (Nan and Zusman 2011; Mignot *et al.* 2007). Coiled-coil protein AglZ localizes in clusters at the leading cell pole and, as the cell moves, is transported toward the lagging cell pole, where clusters are disassembled (Mignot *et al.* 2007). These clusters are associated with A-motility motors that are hypothesized to power motility by coupling movement on a rigid cytoskeletal filament with adhesion complexes on the surface (Sun *et al.* 2011). Recently, MreB, in cooperation with MglA, a Ras-like GTPase, was shown to be critical for the proper positioning and stabilization of polar motility proteins and the focal adhesion complexes (Mauriello *et al.* 2010). A-type gliding motion of *M. xanthus* is thus actually remarkably similar to eukaryotic cell migration. In the mollicute *Spiroplasma melliferum*, propulsion is also assisted by the action of MreB filaments: two types of filaments were found arranged in three parallel ribbons underneath the cell membrane, with the two outer ribbons built of fibril protein and the inner ribbon suggested to be composed of MreB (Kürner *et al.* 2005). The structural data suggest a model explaining propulsion of helical mollicutes by means of coordinated length changes of the ribbons. In the filamentous actinomycetes, which grow by apical extension, MreB homologs occur only in species that differentiate by forming an aerial mycelium and spores, with a less pronounced and nonessential role during

vegetative growth (Mazza *et al.* 2006). The absence in other apically growing actinomycete genera such as corynebacteria and mycobacteria suggests that the function of MreB is directly related to the way bacteria elongate and divide (reviewed in reference Letek *et al.* 2012). In *Streptomyces*, the main function of MreB is to provide stability to the spore wall, which is corroborated by the dense crowding of the inner spore wall by MreB, as shown by immunoelectron microscopy (immuno-EM), suggesting that a large part of the spore wall is associated with MreB molecules (Mazza *et al.* 2006). Deletion of the paralogous *mbl* also compromises spore-wall integrity (Heichlinger *et al.* 2011). Detailed biochemical analysis of MreB and Mbl in streptomycetes should teach us more about the diverse roles these proteins can play in non-rod-shaped bacteria.

CELL DIVISION AND THE TUBULIN ANCESTOR FtsZ

Although some of the IF-like proteins assist in cell division processes, the major cell players in binary cell division are the Fts proteins, originally identified through analysis of temperaturesensitive mutants that fail to divide (the term fts, for filamentous temperature sensitive, was coined by Van de Putte and colleagues [Van de Putte *et al.* 1964]). Upon completion of chromosome segregation (influenced by MreB in *E. coli*, *Caulobacter*, and *B. subtilis* [Kruse *et al.* 2003; Gitai *et al.* 2005; Defeu Soufo and Graumann 2005]), the bacterial protein FtsZ directs the formation of the cytokinetic ring. A guanosine triphosphatase (GTPase) that is widely conserved and located within a cluster of genes involved in division and cell wall (dcw) synthesis, FtsZ polymerizes to form a scaffold of cell division proteins (the Z-ring) at the midplane of dividing cells. Interestingly, while in eukaryotes the cytokinetic ring is formed by actin, FtsZ is a structural homolog (and ancestor) of tubulin (Bi and Lutkenhaus 1991; Erickson 1995; Löwe and Amos 1998). The highly conserved FtsZ protein is found in virtually all bacteria and archaea (Michie and Löwe 2006), with only a few exceptions (Bernander and Ettema 2010).

CLUES FROM IMAGING OF CELL DIVISION

Cryo-ET imaging of bacterial cell division recently provided new insight into FtsZ localization. Cryo-ET images of dividing *C. crescentus* cells showed short, separated, arc-like filaments of FtsZ and not a complete ring or spiral (Li *et al.* 2007). In fact, the formation of FtsZ arcs was reported previously (Addinall and Lutkenhaus 1996) as a stage in ring maturation. Cryo-ET revealed irregularly spaced protofilaments of FtsZ, seemingly connected to the inner

membrane by other electron-dense protein complexes. Some were curved and others were straight, suggesting that, as speculated (Erickson *et al.* 1996; Lutkenhaus and Addinall 1997; Erickson 1997), FtsZ generates the constricting force for cell division itself through the nucleotide-hydrolysis-driven conformational change from straight to curved protofilaments.

Cross-wall formation in *Streptomyces* may, however, prove to be an exception to this model. Indeed, cell division in *Streptomyces* is remarkable. Not only is its division controlled in an entirely different manner, but it is also the only organism known to grow without cell division; the creation of a knockout mutant of *ftsZ* in *S. coelicolor* is an important event in cell biology (McCormick *et al.* 1994). Availability of null mutants for the canonical cell division genes such as *ftsEX*, *ftsI*, *ftsL*, *ftsQ*, *ftsW*, and *ftsZ* makes *Streptomyces* an important object for cell division studies (Jakimowicz and van Wezel 2012; McCormick 2009). Additionally, in *Streptomyces* two types of cell division occur: in aerial hyphae, septation results in formation of spores which can separate to disperse, and in vegetative hyphae, cross-walls form at irregular intervals, do not constrict, and do not result in cell fission (Figure 4). Amazingly, most canonical cell division proteins such as FtsI and FtsW are not even required for cross-wall formation. This suggests an entirely different cell division mechanism, whereby another pair consisting of a SEDS (shape, elongation, division, and sporulation) protein and a cognate class B penicillin-binding protein (PBP) may carry out septum synthesis (McCormick 2009; Bennett *et al.* 2009). This is something that has so far gained very little attention. One explanation is that because cross walls do not constrict but, rather, form semipermeable barriers that separate connecting compartments, the main function of the divisome is to mediate the activation of Z-ring contraction and that FtsZ does not generate a constricting force if other divisome components are absent. It will be interesting to see what role FtsZ plays in cross-wall formation.

OTHER TUBULIN HOMOLOGS

Although FtsZ is clearly the most common tubulin homolog, other bacterial tubulin homologs exist, including TubZ and RepX in *Bacillus* (Larsen *et al.* 2007; Tinsley and Khan 2006) and BtubA and BtubB in *Prostheco bacter* (Sontag *et al.* 2005; Schlieper *et al.* 2005). TubZ and RepX are plasmid-encoded proteins that play an important role in maintaining the stability of the plasmids that encode them and are therefore discussed in the following section. In contrast to TubZ, BtubA and BtubB are more closely related to eukaryotic alpha- and beta-tubulin than to any other bacterial protein, forming heterodimers which polymerize into protofilaments *in vitro*. Based on comparative modeling data, and because

microtubules were not found in thin EM sections, researchers initially predicted that BtubA and -B protofilaments are unlikely to form microtubule-like structures (Jenkins *et al.* 2002). When the ultrastructure of BtubA and BtubB was recently revisited using cryo-ET, however, it was shown that these proteins indeed assemble to form microtubules — consisting of five protofilaments instead of the 13 found in eukaryotes (Pilhofer *et al.* 2011) — but with the same basic architecture. Their existence suggests that microtubule organization may have originated in bacteria, although horizontal transfer of the eukaryotic tubulins cannot be ruled out.

POSITIVE CONTROL OF CELL DIVISION AND THE SsgA-LIKE PROTEINS

In bacteria that divide by binary fission, FtsZ is the first protein to localize at the midcell position at the onset of cell division, followed by the subsequent recruitment of the other cell division components (de Boer 2010). For details on prokaryotic cell division and the cell division machinery, we refer the reader to excellent reviews published elsewhere (for instance, references Margolin 2009; Young 2010; de Boer 2010; Adams and Errington 2009; Dajkovic and Lutkenhaus 2006). In *E. coli* and *Bacillus*, septum-site localization and stabilization of the Z-ring require, among others, FtsA and ZipA (Hale and de Boer 1997; Pichoff and Lutkenhaus 2002; RayChaudhuri 1999), ZapA (Gueiros-Filho and Losick 2002), and SepF (Hamoen *et al.* 2006), and the positioning and timing of septum formation involve the action of negative-control systems such as Min, which prevents Z-ring assembly at the cell poles (Marston *et al.* 1998; Raskin and de Boer 1997), and nucleoid occlusion (NOC), which prevents formation of the Z-ring over nonsegregated chromosomes (Bernhardt and de Boer 2005; Woldring *et al.* 1991; Wu and Errington 2004; Wu and Errington 2012).

Remarkably, division site selection during sporulation in *Streptomyces*, where up to a hundred septa are constructed almost simultaneously in the long aerial hyphae (Jakimowicz and van Wezel 2012; McCormick 2009), appears to be positively controlled. Similar positive control of cell division was also recently described in another multicellular bacterium, namely, the fruiting body forming *Myxococcus xanthus*, where FtsZ is recruited by the ParA-like protein PomZ (Treuner-Lange *et al.* 2013). In *Streptomyces*, division is mediated via the SsgA-like proteins, a family of small proteins that occur exclusively in morphologically complex actinomycetes and play a role in the control of morphogenesis (Traag and van Wezel 2008; Noens *et al.* 2005), with SsgA and SsgB required for sporulation (Keijser *et al.* 2003; van Wezel *et al.* 2000). During sporulation-specific cell division, FtsZ is actively recruited by the membrane-associated divisome component SsgB, which also stimulates

FtsZ polymerization *in vitro* (Willemse *et al.* 2011). The technique of Förster fluorescence resonance energy transfer combined with fluorescence lifetime imaging (FRET-FLIM), a powerful tool for the *in vivo* imaging and calculation of distances between proteins or between a protein and another cellular component such as the membrane, cell wall, or DNA (Alexeeva *et al.* 2010; Miyawaki *et al.* 1997), revealed that SsgB indeed interacts closely with FtsZ and with the membrane (Willemse *et al.* 2011). In turn, SsgB is guided to future septum sites by its paralog SsgA, a multifunctional protein that directly activates cell division (van Wezel *et al.* 2006) but also other events relating to cell-wall remodeling such as germination and branching (Noens *et al.* 2007). SsgB (and probably also SsgA) forms multimeric complexes, with the crystal structure of SsgB revealing a bell-shaped trimer (Xu *et al.* 2009). Whether or not SsgA-like proteins should be regarded as cytoskeletal elements themselves is yet unclear. Another interesting aspect of the control of division in *Streptomyces* is that reaching a threshold level of FtsZ expression appears to be the decisive step in the onset of division (Flärdh *et al.* 2000), and enhanced expression of FtsZ indeed overrules many sporulation (*whi*) mutants (Willemse *et al.* 2012). This again points at a different way of decision making toward the initiation of division. The concept of positive control of division apparently violates the general idea that in nature all major checkpoints are negatively regulated (Alberts *et al.* 2002). However, positive division control is probably less expensive in terms of ATP (e.g., not requiring the energy-consuming oscillation of the Min proteins). In the case of *Streptomyces*, occasional defective spores in a long spore chain are less consequential than mistakes during binary fission, which could be considered an advantage of a multicellular lifestyle (Jakimowicz and van Wezel 2012). Having said that, PomZ is required for binary fission in *M. xanthus* (Treuner-Lange *et al.* 2013), while FtsZ can also localize (though inefficiently) to division sites in the absence of Min and NOC in *B. subtilis* (Rodrigues and Harry 2012). It remains to be seen how widespread active FtsZ recruitment in unicellular bacteria actually is (Monahan and Harry 2013).

ON PAR: THE CYTOSKELETON AND CHROMOSOME SEGREGATION

As we have seen, components of the cytoskeleton play an important role in guiding the spatiotemporal dynamics that govern the assembly of cellular components into higher-order structures. Chromosome and plasmid segregation is a good case in point. Segregation is mediated by tripartite partitioning systems (Gerdes *et al.* 2010; Leonard *et al.* 2005), which consist of a cytoskeletal nucleotide triphosphatase that provides the energy (ParA, ParM, or TubZ), a DNA-binding protein that forms higher-order nucleoprotein complexes

with the DNA (ParB, ParG, or ParR), and a centromere site (*parC*, *parS*, or *parH*) close to the origin of replication (*ori*) that is recognized by dimers of the respective DNA binding proteins (Gerdes *et al.* 2010; Leonard *et al.* 2005). Interestingly, the cytoskeletal partitioning NTPases all have different structural folds, suggesting that convergent evolution resulted in these different elements (and solutions) for the general problem of DNA separation. ParM is an actin family ATPase (van Den Ent *et al.* 2002; Møller-Jensen *et al.* 2002), TubZ is a tubulin homolog (thus hydrolyzing GTP) (Larsen *et al.* 2007), and ParA is a deviant Walker A-type Cytoskeletal ATPase (WACA) protein (Koonin 1993), a bacterial cytoskeletal element that has no eukaryotic counterparts. ParM forms dynamic, actin-like filaments that segregate plasmids in a mitosis-like process. In *E. coli*, cryo-ET was used to identify small bundles of three to five intracellular ParM filaments located close to the nucleoid, confirming that plasmid-segregating ParM filaments are associated with the nucleoid (Salje *et al.* 2009). A recent model suggests that antiparallel ParM filaments work together to drive plasmid segregation (Gayathri *et al.* 2012). TubZ assembles into highly dynamic, linear polymers with directional polymerization that are involved in plasmid segregation and move by a process called treadmilling. This treadmilling has so far been observed only in eukaryotes and involves assembly at one end of the filament and disassembly at the other, with, as a result, the net movement of the filament. Yet, unlike the hollow cylinders formed by tubulin, TubZ forms a two-stranded doubly helical filament which much more resembles actin-like ParM, which is also doubly helical. ParA functions by fuelling ParB, which in turn forms higher-order nucleoprotein complexes at partitioning (*parS*) sites near the chromosomal origin of replication, or *oriC* (Hayes and Barilla 2006; Leonard *et al.* 2004). In rod-shaped bacteria, ParB complexes actively transfer the *oriC* proximal chromosomal region to the cell poles after completion of DNA replication. ParA most likely attaches to a chromosome with bound ParB and then pulls the chromosome across the cell by depolymerizing (Gerdes *et al.* 2010; Banigan *et al.* 2011; Ptacin *et al.* 2010). It may also play a role in the control of chromosome replication, since *B. subtilis* ParA directly affects the function of replication initiator DnaA (Murray and Errington 2008; Scholefield *et al.* 2012). However, the cytoskeletal role of ParA is as yet controversial, and the filaments produced *in vitro* (Hui *et al.* 2010) have not yet been unequivocally established *in vivo*.

FUTURE PERSPECTIVES

In the span of a few years, we have made leaps and bounds toward understanding the mechanisms behind bacterial shape and structure. Twenty years ago, bacterial cytoskeletal elements were unknown; today, actin, tubulin, and intermediate-filament homologs, as well as novel cytoskeletal elements with no apparent eukaryotic counterparts, have all been identified in bacteria. The examples provided in this review demonstrate the vast plasticity and wide variety of roles taken on by prokaryotic cytoskeletal proteins and illustrate how multiscale imaging techniques are leading to new insights and improving our understanding of how bacterial cells function.

To come even closer to an understanding of the complex interactions that occur within the molecular landscape of the cell, static structural information must be coupled with *in vivo* dynamic studies. For this, correlative approaches are necessary. In correlative light and electron microscopy, proteins tagged with a fluorescent reporter, such as enhanced GFP (eGFP), or cell components stained with a selective dye can be directly identified on an EM grid and a tilt series acquired at the location of interest. This should enable mapping of cytoskeletal proteins onto high-resolution images created by electron microscopy, preferably in three dimensions (Plitzko *et al.* 2009; Briegel *et al.* 2010; Sartori *et al.* 2007; van Driel *et al.* 2009; Schwartz *et al.* 2007). In addition, to catch dynamic structural changes, using a rapid-transfer system, samples can be cryoimmobilized once a physiological state has been observed in the cell (Müller-Reichert *et al.* 2007). In this way, the missing links needed to resolve physical models for bacterial growth, division, or propulsion can be determined. Adding the insight provided by correlative methods to the multiscale data of other techniques, we can get even further toward understanding the relationship between the structure of cytoskeletal elements and their position within the cell and function. Given the centrality of the cytoskeleton in regulating and executing key cellular processes, this would mark a great milestone in the field of cell biology.

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