The bacterial cytoskeleton: more than twisted filaments

Martin Pilhofer[*] and **Grant J. Jensen**
Howard Hughes Medical Institute and Division of Biology, California Institute of Technology, 1200 E California Blvd, M/C 114-96, Pasadena, CA, USA

Abstract

Far from being simple "bags" of enzymes, bacteria are richly endowed with ultrastructures that challenge and expand standard definitions of the cytoskeleton. Here we review rods, rings, twisted pairs, tubes, sheets, spirals, moving patches, meshes and composites, and suggest defining the term "bacterial cytoskeleton" as all cytoplasmic protein filaments and their superstructures that move or scaffold (stabilize/position/recruit) other cellular materials. The evolution of each superstructure has been driven by specific functional requirements. As a result, while homologous proteins with different functions have evolved to form surprisingly divergent superstructures, those of unrelated proteins with similar functions have converged.

Defining the bacterial cytoskeleton

The word "skeleton" is defined as the basic frame or supporting structure of an object. The term "cytoskeleton" was coined after a network of long, skinny, cell-shape-determining structures was discovered in the cytoplasm of eukaryotic cells. These structures were later found to consist of actin, tubulin and intermediate filament (IF) proteins that move objects through their own growth and disassembly, act as stationary tracks for auxiliary motors, and/ or serve as connectors and scaffolds to position and stabilize other materials. The discovery of bacterial homologs of actins, tubulins and IF proteins then led to a new understanding that bacteria, too, were organized and shaped by a cytoskeleton [reviews: 1,2,3]. Bacterial filaments have also been found to push and pull objects and/or bind them together as connectors and scaffolds, but have not yet been found to act as tracks for other motor proteins. In bacteria (and archaea), the eukaryotic notion of the cytoskeleton is challenged however by both the small size of the cell, which causes filament bundles to sometimes be nearly as wide as they are long (and therefore seem much less obviously "filamentous"), and by diverse new non-actin, non-tubulin, and non-IF superstructures (higher-order assemblies) that exhibit many cytoskeletal characteristics and have already been called "cytoskeletal" in the literature. Thus as one of the purposes of this review, here we explore an expansive definition of the bacterial cytoskeleton that includes all cytoplasmic protein filaments and their superstructures that either move or scaffold (stabilize/position/recruit) materials within the cell. Recent progress is highlighted with emphasis on how specific functions drove the evolution of different superstructures.

[*]martin-pilhofer@web.de.

Superstructures of the bacterial cytoskeleton

Individual rods

Protein polymerization may possibly have first evolved to regulate enzymatic activity, but the resulting filaments might have then proven to be useful as scaffolds as well [review: 4]. The actin homolog MamK may exemplify such simple rods, as it polymerizes into what appear by electron cryotomography (ECT) of intact cells [review: 5] to be simple short rods flanking magnetosome chains (Figure 1A) [6-10]. Despite the apparently simple structure, MamK shows dynamic behavior, promoted by the regulators MamJ and LimJ [9], and plays a role in a pole-to-midcell translocation of the magnetosome chain prior to cell division [10], so there is much complexity to be understood.

Another element of the bacterial cytoskeleton that appears to act as an isolated rod *in vivo* is the tubulin homolog FtsZ, the major cell division protein in almost all bacteria. FtsZ acts as both a scaffold to recruit other proteins to the septum and as a "cytomotive" filament (one that pushes or pulls objects) [reviews: 11,12]. Through ECT of dividing cells, FtsZ filaments were seen to be either straight or curved, isolated filaments typically shorter than the diameter of the cell slightly displaced from the membrane (Figure 1B) [13]. More recent studies showed that fluorescently labeled FtsZ with a short membrane targeting sequence can self-assemble into contractile rings inside liposomes [14]. These *in vitro* studies have shown that FtsZ alone can generate constrictive forces, that FtsZ protofilaments have a preferred direction of bending [15], and that GTP-hydrolysis and filament remodeling are necessary for continuous constriction [16]. Molecular dynamics simulations have supported a hypothesis that FtsZ protofilament bending alone could generate sufficient force to constrict membranes [17]. Super-resolution fluorescence light microscopy (fLM) confirmed the ECT result by showing that FtsZ was unevenly distributed around the ingression "ring" (Figure 1C) [18-20]. Present data therefore favors (though not clearly [11,21]) an "iterative pinching" model in which FtsZ polymerizes into straight filaments in its GTP-bound state, is tethered to the membrane, and then bends upon GTP hydrolysis, pulling the membrane inwards [13,22]. Cell-wall-remodeling enzymes recruited by FtsZ apparently then add new peptidoglcan behind the advancing membrane, preventing it from relaxing when the FtsZ filament depolymerizes.

FtsZ is tethered to the membrane by the actin homolog FtsA [23]. FtsA was recently shown to polymerize into actin-like filaments and 2D sheets *in vitro* [24], but its structure and position with respect to other divisome components *in vivo* remain unknown. Mutants disrupted in polymerization have an elongated cell division phenotype, however, suggesting that polymer formation is important for FtsA function, and truncated FtsA lacking its membrane-binding helix formed straight protofilaments *in vivo*. Overexpression of full-length FtsA resulted in membrane distortions and the formation of polymer-coated lipid tubes.

Rings

Without evolving any additional binding interfaces, but given a special curvature, individual filaments can loop around and bind themselves to form complete rings. SepF may be an example. Thick-walled bacteria such as Gram-positives and cyanobacteria purportedly have special requirements regarding the arrangement of FtsZ filaments [25]. Proper division of these cells requires SepF, which forms ~50-nm diameter rings *in vitro* (Figure 1D) and is thought to bundle FtsZ *in vivo*. If SepF does indeed bundle FtsZ filaments by forming rings around them, it might provide the benefits of filament bundling without the need for intrinsic lateral interactions between FtsZ filaments that might otherwise interfere with FtsZ's conformational changes. While simple linear SepF "crosslinks" might also be able to bundle

FtsZ, the authors point out that a ring superstructure can limit the bundle width, promoting the growth of FtsZ filaments lengthwise.

Twisted filament pairs

A superstructure one step more complex than individual or loosely-bundled filaments is a tightly coupled pair. In fact this appears to be a highly effective superstructure, as a diverse set of cytomotive filaments have evolved to form twisted filament pairs that propel other structures throughout the cell. Filament pairs require an additional lateral binding interface, but offer increased stiffness and the possibility of staying attached to an object even while one of the filaments is growing or shrinking. In contrast to FtsZ's bending mechanism, these cytomotive filaments evolved dynamic instability or treadmilling in order to generate motility.

For plasmid segregation, the cytomotive filament usually acts in concert with a centromere-like region of DNA and a DNA-binding adaptor, as exemplified by the ParMR*C* system [review: 26]. Dynamically instable filaments of the actin homolog ParM cycle between growth and shrinkage until they are stabilized by binding ParR-*parC* complexes at both ends. Plasmids are then pushed to opposite cell poles by bidirectional elongation of the ParM filaments. Like F-actin, ParM assembles into a polar double-helical filament pair *in vitro* (Figure 1E), though with a different handedness [27-31]. Erickson has made an interesting argument that ParM may form an apolar, antiparallel double-helical structure *in vivo*, however, since this could better explain bidirectional filament growth [32]. ECT of cells harboring the ParMR*C* system found that *in vivo*, 3-5 presumably double-stranded filaments aligned in a bundle (Figure 1E)[33]. Bundling provides the stiffness that is likely needed to segregate the large plasmid-adaptor complexes, but it is unclear how many filaments in a bundle are attached to plasmids. Like ParM, AlfA (for actin-like filament) [34-36] and various Alps (for actin-like proteins) [37,38] are also involved in plasmid segregation. AlfA is thought to treadmill *in vitro* and the double-filament has a much higher twist, however, suggesting a different mechanism.

In addition to the above actin homologs, a diverse set of homologs of tubulin (TubZ, phage TubZ and PhuZ) also form DNA-positioning twisted filament pairs. In combination with an adaptor (TubR) and a centromere (*tubC*), TubZ segregates plasmids through treadmilling [39-41]. Electron microscopy (EM) of *in vitro* and *in vivo* TubZ filaments suggests a double-helical, ParM-like structure (Figure 1F) [42]. Well-ordered bundles of presumably double-stranded filaments were observed in cryotomograms of *E. coli* cells overproducing TubZ (Figure 1F). Strikingly, two recent studies showed that tubulin homologs are also involved in bacteriophage DNA positioning. Upon cell entry, the *Clostridium botulinum* c-st phage genome is recircularized into a plasmid-like entity and segregated by a phage TubZ, which again acts together with an adaptor and a centromere, but also an additional modulator [43]. *In vitro*, phage TubZ polymerizes into double-helical filaments that coalesce into bundles. A second phage tubulin homolog, PhuZ from bacteriophage 201! 2-1, is required during the lytic phage cycle [44]. A single-cell assay monitored by fLM revealed that dynamic PhuZ filaments form a spindle that positions phage DNA at the cell center (Figure 1G), enhancing phage production. Like the other DNA positioning filaments, PhuZ forms twisted filament pairs *in vitro*, and the authors speculate that these generate pushing forces *in vivo* to hold the phage DNA at midcell. Surprisingly simple changes in cell length or filament number or dynamics can shift whether filament pairs localize objects at the poles or the center of cells [45].

Another class of proteins involved in DNA and protein positioning are Walker A "cytoskeletal" ATPases (WACA) [reviews: 46,47,48]. Two prominent members are ParA and MinD, which segregate DNA and position the septum, respectively. While WACA

polymerize into filaments *in vitro*; whether they form filaments *in vivo* (and really are therefore "cytoskeletal") remains unclear.

Tubes

Given lateral interactions between filaments, more complex structures like tubes and sheets can form. Compared to simple rods and twisted pairs, tubes offer enhanced stiffness and mechanical strength, allowing them to act as firm tracks for motors, for instance.

Do bacteria contain microtubules? There are indeed scattered reports of bacterial microtubule-like structures [review: 49]; but later sequencing has revealed that tubulin genes are absent from the organisms described. Hence, microtubules were considered exclusively eukaryotic until a recent study on bacterial tubulins BtubA and BtubB in the phylum *Verrucomicrobia* [50]. Early on, it was thought that BtubA/B might have taken over the function of its distant homolog FtsZ, since *Verrucomicrobia* are the closest relatives of FtsZ-lacking *Chlamydiae* and *Planctomycetes*, but FtsZ was later found in the same genomes as BtubA/B [51,52]. The sequences and crystal structures of BtubA/B are more similar to eukaryotic tubulins than to FtsZ [50,51,53-55], but negative stain EM suggested that BtubA/B polymerize into protofilament bundles rather than tubes *in vitro* [54,56]. ECT showed, however, that *in vivo* BtubA/B assembles into slender tubes (Figure 1H) which run the length of the cell close to the cytoplasmic membrane in bundles of up to four [50]. Interestingly and in contrast to eukaryotic tubulins, BtubA/B can fold without chaperones and they can be heterologously expressed in *E. coli*; the recombinant proteins also form tubes *in vitro* and *in vivo*. Because BtubA/B tubes had the same basic architecture as eukaryotic microtubules (but with only five protofilaments), they were termed 'bacterial microtubules (bMTs)' (Figure 1H) [50]. Their 5-protofilament architecture might be ancient, since BtubA/B most probably arose from early tubulin intermediates [50,55]. Interestingly, a gene with low similarities to kinesin light chains is part of the operon encoding bMTs [57], but it remains unclear whether bMTs act as tracks for a motor, cytomotive tubes or scaffolds.

An amazing capability of tubes is illustrated by the bacterial type VI secretion (T6S) system, which injects effector proteins into neighboring cells [review: 58]. A combination of ECT and fLM recently revealed that T6S functions like a spring-loaded molecular dagger (Video 1 and Figure 1I, J) [59]: an inner rod (the dagger) is propelled out of the cell by rapid contraction of a tubular outer sheath (the spring). Because the sheath is a dynamic, proteinaceous, cytoplasmic tube involved in moving material, we include it here as a novel cytomotive element of the bacterial cytoskeleton. Sheath contraction provides the energy needed to move the inner rod [59], and sheath disassembly relies on a AAA+ ATPase [60-62]. T6S genes are highly abundant and widespread among diverse phyla and are involved in competition, defense, pathogenesis and symbiosis [review: 63]. Interestingly, the T6S mechanism and some of its components are homologous to the contractile bacteriophage tail [64]. Phage and bacterium both take advantage of the tube geometry, which is effective to collect the forces needed to transport materials across membranes.

Sheets

In addition to tubes, lateral interactions between filaments can also lead to sheets, which can provide mechanical support, separate compartments, or act as high-surface-area scaffolds. The bactofilins BacA/B in *Caulobacter*, for instance, localize as sheets at the poles, where they assist stalk morphogenesis by recruiting a peptidoglycan synthase (Figure 1K) [65]. Although bactofilins are almost universally conserved among bacteria, they have a variety of functions. A *Myxococcus* strain mutated in one bactofilin paralogue had impaired social motility for instance [65], while mutation of another (BacM) led to altered cell morphology

[66]. Based on fLM and *in vitro* experiments the authors suggested that instead of sheets, BacM forms fibers which bundle into helical cables throughout the cell [66], but such a structure has not yet been directly visualized.

The metabolic enzyme CTP synthase (CtpS) also forms sheets in some species. CtpS is conserved in all domains of life, and some bacterial and eukaryotic homologs have been shown to form filaments [review: 3]. While the function of polymerization might typically be the regulation of enzymatic activity, in *Caulobacter crescentus* CtpS polymers are also involved in cell shape determination [67]. *Caulobacter* CtpS forms stacks of sheets that lie along the inner curvature of the crescent-shaped cells (Figure 1L) [67,68], where it appears to regulate the activity of another shape-determining polymer, crescentin (CreS) [67].

CreS was the first characterized bacterial intermediate-filament-like protein [69]. CreS is thought to induce curvature in *Caulobacter* cells by applying asymmetric tension to the cell wall, inhibiting new cell wall growth on one side [70]. fLM, EM, and biochemical studies suggest that CreS forms a substantial ribbon-like structure with properties similar to eukaryotic intermediate filaments [70-73]. No such ribbon-like structure was seen by ECT [68], however, so the *in vivo* superstructure remains unclear. Like CtpS, CreS localizes to the inner curvature of *Caulobacter* cells where the two have apparently antagonistic effects: CtpS decreases CreS-induced curvature [67]. One of the distinguishing characteristics of intermediate filament proteins are extended coil-coil domains. In addition to CreS, multiple other coiled-coil rich (Ccrp) proteins have been identified in bacteria and found to be involved in cell shape, rigidity and motility [74-77]. Ccrps assemble into various polymerization condition-dependent superstructures *in vitro*, but again their superstructures *in vivo* remain unknown.

Spirals

While the lateral interactions of linear filaments can produce sheets, so can circular coiling. During *Bacillus* endospore formation, the protein SpoIVA localizes to the mother-side of the outer spore membrane and recruits spore coat proteins [78]. SpoIVA was observed to form polymers *in vitro* and contains a Walker A box, so it should be considered a WACA (it has not yet been described as such). In fact, in contrast to other better-known WACAs there is strong evidence that SpoIVA actually forms a cytoskeletal structure *in vivo*: cryotomograms of sporulating *Acetonema longum* cells showed concentric, ring-shaped densities on the outer spore membrane (Figure 1M) which were most likely SpoIVA [79]. As a filamentous cytoplasmic scaffold, these rings/spirals should also be considered part of the bacterial cytoskeleton.

Moving 'Patches'(?)

Perhaps the most well-known supposed element of the bacterial cytoskeleton is the actin homolog MreB, which is present in most non-spherical bacteria and is essential for cell shape determination [80,81]. Early fLM suggested that MreB polymerizes into extended helical filaments that encircle the cell just inside the cytoplasmic membrane [82-85], but extended helices were not found in cryotomograms of diverse rod-shaped cells, even after careful computational searches [86]. In 2011 three groups then reported that instead of forming helices, MreB formed small 'patches' that moved circumferentially around cells (Video 2 and Figure 1N), driven by cell wall synthesis rather than MreB's intrinsic ATPase activity [87-89]. ECT has now shown that at least in *E. coli*, the previously reported helices were an artifact caused by an N-terminal YFP tag (Figure 1O) [90]. This is not surprising given the recent findings that *E. coli* MreB is anchored to the membrane by an N-terminal amphipathic helix (which is most likely blocked by N-terminal tags), and that membrane binding is essential for MreB's role in cell shape determination [91]. Indeed *Thermotoga*

MreB forms double filaments and sheets on a 2D lipid monolayer and it distorts lipid vesicles by the formation of small sheets (Figure 1P). At high protein levels, MreB also distorts membranes *in vivo*. Thus while the structure and function of MreB *in vivo* has yet to be determined, the data available today suggest that MreB forms small sheet-like polymers or 'patches' on the membrane together with cell wall synthetic machinery. Small patches could conceivably be effective in maximizing surface area without overly inhibiting movement along the interface of a complex membrane and a crowded cytoplasm.

Meshes

While lateral binding interactions lead to tubes and sheets, 3-way junctions can lead to 3-D meshes, which allow for cell compartmentalization. Biochemical analyses, *in vitro* polymerization (Figure 1Q), and cellular ECT suggest that the polar organizing protein Z (PopZ) forms such a mesh *in vivo* [92-94]. PopZ provides an anchor for the *parS*/ParB chromosomal centromere at the *Caulobacter* cell pole and later recruits various proteins for stalked pole development. PopZ also establishes a special ribosome-excluding zone (Figure 1Q).

Multi-element composite

Finally, multiple cytoskeletal elements can come together in multi-element composites. Gliding motility, cell division, and attachment of different *Mycoplasma* species, for instance, depend on composite superstructures that have also been described as cytoskeletal. Their composition and structure is species-dependent and can include elements termed jellyfish, cap, oval, bowl, bulge, ankle, thick and thin rods, terminal button, etc. (Figure 1R) [95-97, and review 98].

Conclusion

Function dictates superstructure

Each superstructure of the bacterial cytoskeleton has special advantages that optimize its role in moving objects, arranging materials, or both (Figure 2). Similar functions lead even unrelated proteins to evolve similar superstructures – actins and tubulins segregate plasmids as twisted filaments, CTP synthase and bactofilins (and possibly MreB) recruit proteins to the membrane as sheets. Homologs with different functions, however, form surprisingly different superstructures – tubulins are found as twisted filament pairs (PhuZ/TubZ), bending rods (FtsZ), and tubes (BtubA/B) (and perhaps more - the superstructures of FtsZ-like proteins [99] and archaeal "artubulins" [100] remain unknown).

Boundaries of our definition

Here we have included as bacterial cytoskeletal elements all cytoplasmic filamentous superstructures with either a cytomotive or scaffolding function. As with all definitions, there are ambiguities at the boundaries. Although PhuZ and phage TubZ, for instance, are not truly bacterial proteins, they were included in this review because they act in the bacterial cytoplasm. Phage tails and capsids, however, act mostly outside the cell and were excluded just like flagella and their motors, whose main components are also located outside the cytoplasm. Chemoreceptor arrays and carboxysome shells were not discussed either, since their superstructures are hexagonal or polyhedral, rather than higher-order assemblies of filaments, but these fine distinctions illustrate the growing ambiguities the bacterial "cytoskeleton" is presenting: bactofilin and CtpS sheets may also be hexagonal, some carboxysomes are so long that they could be considered filamentous at the superstructural level [101], and the *in vivo* superstructures of many widely discussed members of the bacterial "cytoskeleton" like MreB, CreS, FtsA, SepF, most WACA, Ccrps, and SepF

remain unknown! Such ambiguities emphasize the importance of molecular-resolution, *in vivo* imaging of wildtype cells [review: 5]: almost all proteins will polymerize under some condition *in vitro* (as proven by the success of X-ray crystallography), and fluorescence signals can be deceiving (as shown by the case of MreB [90] and likely other supposedly "helical" filaments). Thus while the growing diversity of superstructures produces some semantic challenges, it is nevertheless amazing and beautiful (who would have imagined membrane-bending pyramids, as recently seen in archaea [102]!), and there is surely much left to discover.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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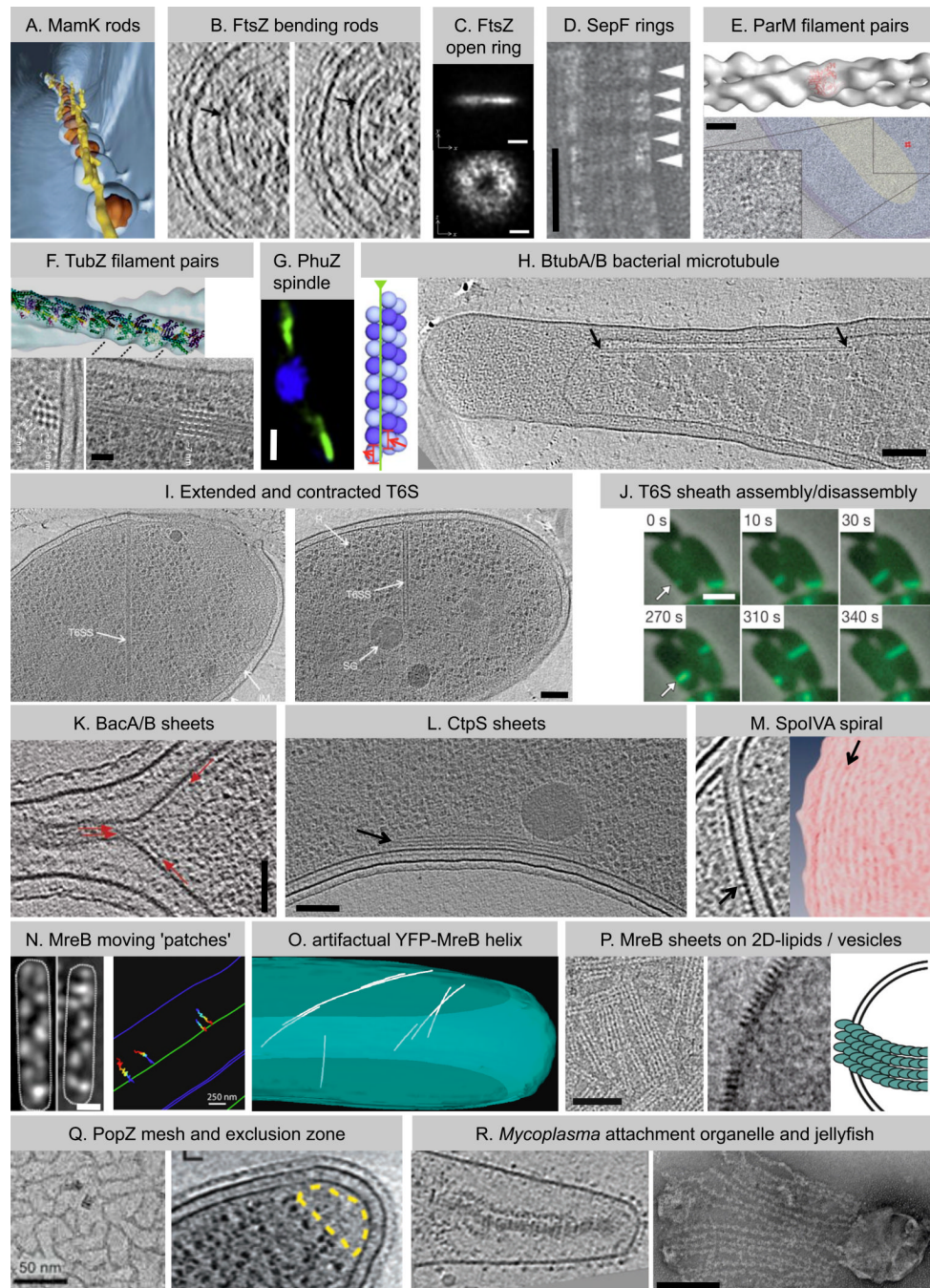
Figure 1. Superstructures of the bacterial cytoskeleton

(A) Individual MamK rods (yellow in shown segmentation) organize the magnetosome chain [7]. (B) ECT of wildtype cells identified the cell division protein FtsZ (arrows) as straight (left) or bent (right) rods [13]. (C) Superresolution fLM observed FtsZ as a ring-shaped signal with uneven density (*y*, cell length axis) [19]. (D) SepF bundles FtsZ filaments (running vertical) *in vitro* by forming rings (arrowheads) around them [25]. (E) Plasmid segregating ParM forms twisted filament pairs *in vitro* (upper [30]) and small bundles of filament pairs *in vivo* (lower: perpendicular view [33]). (F) TubZ also segregates plasmids, assembles twisted filament pairs *in vitro* (upper) and bundles *in vivo* (lower: perpendicular (left) and longitudinal (right) view of overexpressed TubZ) [42]. (G) PhuZ

forms a spindle-like structure (green) that positions phage DNA at midcell (blue, phage/bacterial DNA) [44]. (H) BtubA/B assemble bacterial microtubules (left: model) which localize close to the cytoplasmic membrane *in vivo* (right, arrows) [50]. (I) Type VI secretion (T6S) requires a contractile phage tail-like sheath which is found in extended (left) and contracted (right) confirmation [59]. (J) The tubular T6S sheath is dynamic and cycles between assembly (upper row), quick contraction, and disassembly (lower row) [59]. (K) Bactofilins [65] and (L) CTP synthase [67] both form sheets (arrows) at the cytoplasmic membrane, where they recruit other proteins. (M) Densities thought to represent SpoIVA (left, arrow) form concentric rings (right panel shows density projections, arrow) on the mother side of the outer spore membrane, where SpoIVA recruits spore coat proteins [79]. (N) MreB forms small patches moving circumferentially around the cell driven by the cell wall synthesis machinery (left: fLM images, right: traced patches) [87,88]. (O) Extended MreB helices in *E. coli* (segmentation shown) are an artifact of an N-terminal YFP tag [90]. (P) MreB forms filaments and sheets on 2D-lipids (left) and vesicles (middle and model on right) [91]. (Q) PopZ establishes a ribosome exclusion zone (right, yellow) *in vivo* presumably by the formation of a 3-D mesh (left, *in vitro*) [93,94]. (R) Gliding motility requires multi-element composites such as the attachment organelle in *Mycoplasma pneumoniae* (left, [96]) or the jellyfish-like multi-element complex in *Mycoplasma mobile* (right, [98]). Images are adapted with permissions from references listed in legend. Bars: 200nm in C, R; 50nm in D, K, Q; 100nm in E, H, I, L, P; 25nm in F; 1μm in G, J, N (left); 250nm in N (right).
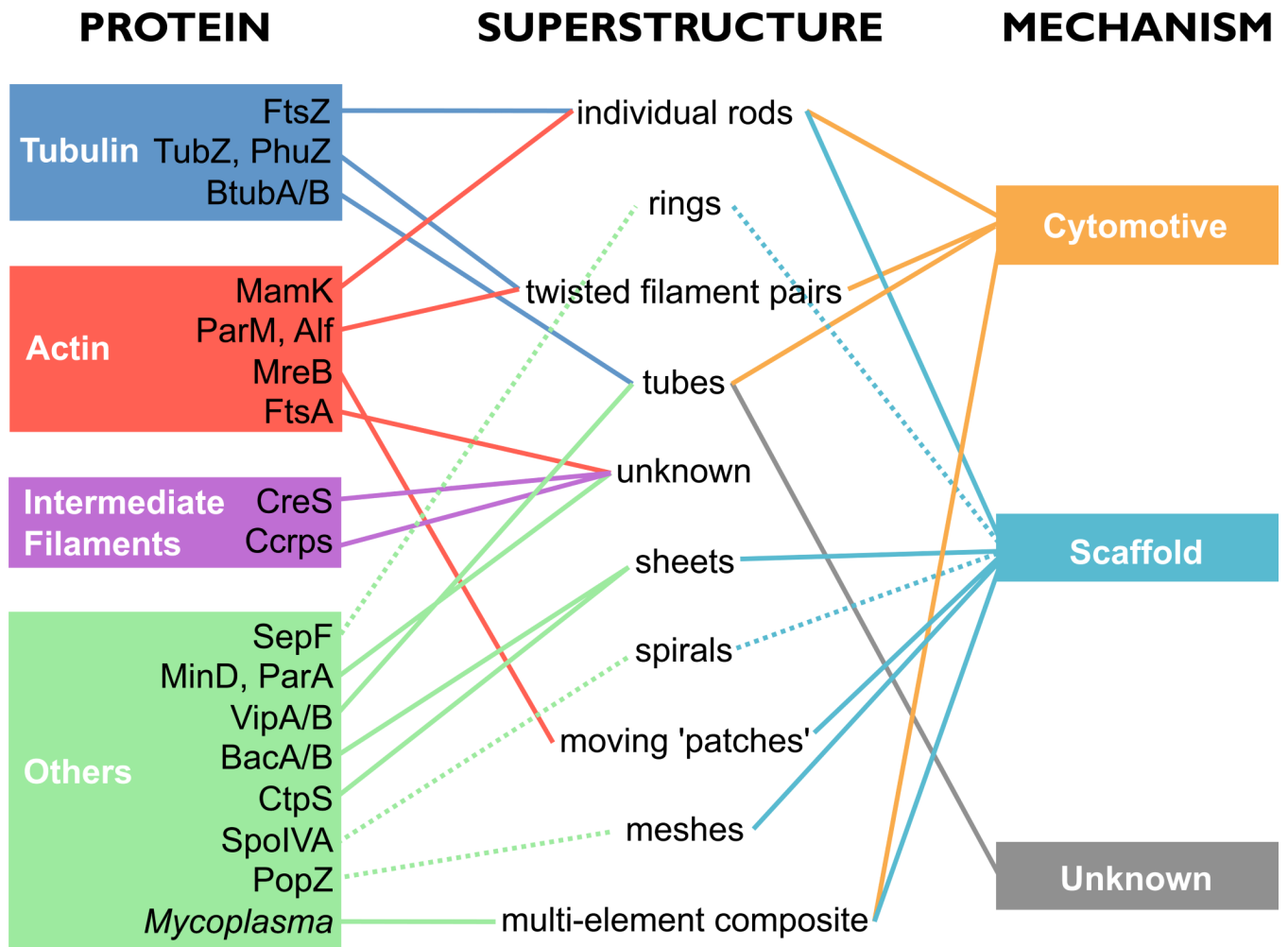
Figure 2. The bacterial cytoskeleton: proteins, superstructures and mechanism of function
Bacterial cytoskeletal elements evolved a variety of superstructures, each adapted to the protein's function in moving (cytomotive) or stabilizing/positioning/recruiting (scaffold) objects within the cell. Connections with weaker evidence are represented as dashed lines.