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Bacterial actins and their diversity

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Abstract

For many years bacteria were considered rather simple organisms, but the dogmatic notion that subcellular organization is a eukaryotic trait has been overthrown for more than a decade. The discovery of homologs of the eukaryotic cytoskeletal proteins actin, tubulin, and intermediate filaments in bacteria has been instrumental in changing this view. Over the recent years we gained an incredible level of insight into the diverse family of bacterial actins and their molecular workings. Here we review the functional, biochemical and structural features of the most well-studied bacterial actins.

Introduction

Bacteria exhibit an incredible level of subcellular organization and rely on fine-tuned processes for their growth and development. Similar to eukaryotic cells, cytoskeletal proteins play a key role in the regulation of many cellular functions in bacteria. Of particular interest is the large and incredibly diverse family of bacterial actin-like proteins that are intimately involved in numerous activities ranging from the coordination of cell wall synthesis to the positioning of subcellular structures. Despite showing limited sequence relatedness, bacterial and eukaryotic actins share a common ancestry and an overall similar tertiary structure. In addition, bacterial actin sequences are highly divergent and can be grouped into distinct protein families based on their phylogenetic and functional relatedness (Fig. 1A, B). It is becoming increasingly evident that different bacterial actin families possess unique biochemical and structural features that distinguish them not only from actin, but also from each other. Here, we discuss a selection of well-studied bacterial actins and their functional, biochemical and structural features. We refer readers to a number of excellent previous reviews on bacterial actins but also on other bacterial cytoskelatal proteins[1-7].

Actin and the discovery of bacterial actins

Actin is a highly conserved protein in eukaryotes with many diverse roles in central processes such as cell shape maintenance, cell motility and cytokinesis[8, 9]. The

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quintessential property of actin is the ability to transition between monomeric and filamentous states. This transition is largely controlled by the binding and hydrolysis of ATP and through the action of a great number of actin modulating proteins[8, 9]. Structurally, actin is composed of two domains with similar folds (domains I and II). A hinge region allowing interdomain movements associated with different functional states of the protein connects domains I and II. ATP, together with Mg^{2+}, bind in this interdomain region (Fig. 1B)[10, 11]. Actin assembles into a two-stranded helical filament with a distinct asymmetry at the filament ends, which is due to the head-to-tail assembly of the asymmetric structure of actin monomers (Fig. 1C). The two filament ends exhibit differential assembly and disassembly kinetics, resulting in a polar dynamic behavior commonly referred to as treadmilling. Here, ATP-bound actin monomers preferentially join the so-called barbed end. Once in a filament, the ATP is hydrolyzed to form actin-ADP subunits that dissociate faster from the so-called pointed end. At steady state this kinetic asymmetry results in the flux of subunits through the filaments, hence the term treadmilling[8-11].

The wealth of knowledge about the function, structure and biochemical activities of eukaryotic actin played a key role in the discovery of bacterial actins. A major step towards the identification, and ultimately the characterization, of bacterial actins was bioinformatics work by Bork et al[12]. Crystallographic evidence that actin, Hsc70 and hexokinase - three functionally distinct proteins with low overall sequence homology - share extensive structural similarity was used by the authors to define “fingerprint” motifs for actin family proteins. Although these motifs are spread throughout the length of the protein sequence, in the three-dimensional structure they are clustered near the ATP binding site. Significantly, Bork et al[12] identified that the bacterial proteins MreB, FtsA and SbtA (now known as ParM) had the same fingerprint and they predicted a common core region with actin. At the time only the broad functions of these bacterial proteins were known and whether they were true actin homologs was questioned. However, several years later a rapid succession of crystal structures from the Löwe group demonstrated that FtsA[13], MreB[14] and ParM[15] all share the actin fold; subsequent structures of two other homologs confirmed the conservation[16, 17] (Fig. 1B). In addition to the actin core structure, its conformational plasticity through domain movements at the hinge[15, 18], thought to play an important role in modulating filament structure, dynamics, and interaction with binding partners, is also conserved. Moreover, all of the bacterial actins that have been examined form ATP-dependent filaments *in vitro*[16, 19-25] and have filamentous localization patterns *in vivo*[26-29], suggesting conservation of core actin-like cytoskeletal functions in this diverse family.

Despite the conservation of tertiary structure and assembly properties among the bacterial actins, the filaments they form show a surprising degree of variation (Fig. 1C). As described below, the variation includes dramatic changes in twist, registration between strands, changes in strand number, and even the possibility of anti-parallel strands. In most of the filaments similar surfaces mediate subunit interactions along each strand, and variation arises primarily from changes to inter-strand contacts. Differences in filament structure correlate with variation in filament dynamics, supporting a causal link between filament architecture and dynamics.

Recent bioinformatic analyses suggest the existence of more than 35 different families of actin-like proteins in bacteria[27]. Whether all of these families are true actin-like proteins, and what cellular functions they might fulfill, is currently unknown. Below, we will highlight the functional, biochemical and structural features of the most-well studied bacterial actins.

MreB, an essential bacterial actin

MreB, one of the first characterized bacterial actins, is fully integrated into the cellular physiology and plays several essential roles. MreB has a major impact on the synthesis of the cell wall, which is the major determinant of cell shape and integrity in bacteria. Due to its central role in the coordination of the cell wall synthesizing machinery, a lack of MreB results in large cell morphological defects in most rod-shaped bacteria where it is found (Fig. 2A)[30-34]. However, MreB also participates in the localization of a number of other proteins, including the gliding motility complexes in *Myxococcus xanthus*[35] or pilus-associated proteins in *Pseudomonas aeruginosa*[36]. Furthermore, MreB appears to be involved in chromosome segregation[37-39].

Subcellular imaging of MreB originally indicated that it forms a continuous, helical structure running along the length the bacteria. These structures were located just below the cytoplasmic membrane and correlated with the organization/localization of several enzymes involved in cell wall synthesis. These observations led to a widely accepted model in which MreB forms a helical scaffold, or track, for cell wall synthesis. However, this helical MreB model has been challenged in recent years[40-43]. Advanced high resolution imaging by electron cryotomography (ECT) of wild type cells failed to detect continuous helical structures either near or along the surface of the inner membrane[42]. A number of recent discoveries suggest instead that MreB exists as discrete “patches” that move perpendicular to the cell axis[40, 41, 43]. This discrepancy can be rationally explained. The early studies on MreB visualization largely relied on fluorescent protein fusions to either its N- or C-terminus. However, the fusion perturbs MreB function since these derivatives do not complement *mreB* deletion strains[33, 44]. Furthermore, this alteration to the protein can also lead to gross localization artifacts. ECT imaging shows that MreB forms helical structures in *E. coli* when fused to yellow fluorescent protein[45]. The molecular reasons promoting the formation of these extensive structures are unclear. However, since some forms of commonly used fluorescent proteins can dimerize, their fusion to a protein that itself oligomerizes could result in the formation of long-ranging structures. It is also worth noting that optical artifacts or misinterpretation of the visual data might have fed the model of a continuous, helical MreB structure in cells[46]. An important step to towards a better understanding of the true ultrastructural organization of MreB in cells, and its dynamics, were so called “sandwich fusions” to MreB. In these functional forms of MreB the fluorescent protein is not fused to the N- or C-terminus, but rather inserted elsewhere between the termini, hence the term “sandwich”. In the case of MreB the suitable location was determined empirically[44]. The visualization of sandwich fusions by fluorescent microscopy showed that MreB patches, presumably composed of several filaments, are highly dynamic and move perpendicular to the cell length. Interestingly, it appears that entire patches move independently of each other, as well as bidirectionally[40, 41, 43]. This

behavior argues against a model from previous *in vivo* studies using the non-functional MreB derivatives, which had suggested that MreB polymers moved by treadmilling[47, 48], like actin. Interestingly, MreB patch movements are dependent on the cell wall synthesis machinery, indicating that the cell wall synthesis itself drives MreB motion and dynamics. This could be in line with a scaffolding role of MreB for the cell wall synthesis machinery. Moreover, MreB organization and movement appear to be influenced by growth conditions[49].

The assembly of MreB filaments *in vitro*

The study of the MreB protein *in vitro* was crucial in revealing its close relationship to actin. To date MreB from *T. maritima* (*Tm*MreB), *B. subtilis* (*Bs*MreB) and *E. coli* (*Ec*MreB) have been investigated. The crystal structure of *Tm*MreB[14] unequivocally showed that this protein adopts an actin-like topology (Fig. 1B). Not only was the overall protein fold between actin and MreB conserved but, remarkably, the MreB crystal packing contacts closely resembled the longitudinal contacts between subunits in an actin filament. Essentially, MreB was incorporated into the crystal lattice as single stranded protofilaments[14]. The conservation of longitudinal interaction surfaces appears to be true for most bacterial actin filaments, which is surprising given the low level of sequence conservation at these sites between different bacterial actins.

In addition to crystallographic evidence, the assembly properties of MreB proteins in solution show some parallels to actin. Actin polymerization requires ATP and Mg^{2+} as cofactors, as mentioned previously. The presence of these cofactors kinetically favors the formation of a nucleation seed onto which additional subunits can assemble to form a filament. Furthermore, seed formation is favored above a certain actin monomer level referred to as the critical concentration[11]. MreB also polymerizes in the presence of ATP and Mg^{2+}, and with critical concentrations that are similar to actin[50-53]. However, the role of these cofactors for MreB assembly has been less clear because of conflicting reports. Early suggestions that Mg^{2+} is not strictly required for assembly of *Tm*MreB[14], or is even inhibitory[54], were contrasted by a study showing that Mg^{2+} was necessary for rapid and extensive *Tm*MreB polymerization[50]. In addition to an unresolved role of Mg^{2+}, the role of ATP was also questioned because *Bs*MreB appeared to form filaments equally well in the presence of ATP, ADP or even the complete absence of any nucleotide[51]. The reason for this discrepancy between *Tm*MreB and *Bs*MreB is unclear. An interesting aspect to consider is that different MreB proteins have species-specific adaptations/properties. *Tm*MreB and *Bs*MreB, for example share approximately 60% sequence identity and 75% similarity but are native to bacteria with presumably different physiologies. *T. maritima* is an aquatic and hyperthermophilic bacterium, whereas *B. subtilis* is a soil bacterium and grows at moderate temperatures. At this time, no study has compared different MreBs side by side. Therefore, one cannot rule out experimental factors and general circumstances as artifactual sources for different MreB behaviors. In contrast to the varying requirements for Mg^{2+} and ATP, the effect of K^+ on MreB assembly is more consistent between different MreB proteins. In most bacteria, K^+ is the major cation and plays important roles, such as maintaining cell turgor pressure and pH homeostasis[55, 56]. Interestingly, MreB assembly is modulated negatively or inhibited by physiological K^+ concentrations[50-52]. One possibility is that MreB proteins may

be tuned in such a way that the K^+ in the cytoplasm prevents excessive polymerization. An "over-assembly" could potentially alter the balance with its interactions partners or interfere with other cellular processes.

Further *in vitro* investigations will likely provide a better framework to understand the molecular mechanisms of MreB function *in vivo*. In fact, recent insights dramatically changed our view of MreB assembly in cells, and challenged the relevance of previous *in vitro* observations. As mentioned earlier, in cells MreB filaments (or patches of filaments) are located close to the inner membrane. This localization was believed to be mediated via MreB's interactions with membrane-bound proteins and the cell wall synthesizing machinery. However, Salje and colleagues[24] demonstrated that a structural feature on MreB itself enables the direct interaction with phospholipid membranes. In the case of *Tm*MreB this is mediated by a small membrane insertion loop, consisting of two hydrophobic residues, close to the N-terminus. For *Ec*MreB, however, direct membrane association involves an N-terminal amphipathic helix[24]. The reason for the different mechanisms of membrane attachment is unknown, but this finding illustrates the existence of species-specific traits within the MreB protein family. In addition to investigations of membrane binding, the authors also studied the MreB filament assembly in more detail. Purified *Tm*MreB assembles on a lipid monolayer into filaments that consist of two protofilaments[24], similar to other actin homologs. The two-stranded MreB filaments are, however, distinct from the filaments of other actin homologs in two ways: binding along the lipid monolayer constrains them to being straight rather than twisted, and the two strands are in register rather than staggered. The orientation of the protofilaments relative to each other is yet unknown[24]. An antiparallel arrangement would place the membrane insertion loops in both protofilaments towards the membrane, maximizing the surface binding area potential. If confirmed, this would be the first case of an antiparallel arrangement in any actin filament. Interestingly, the discovery of MreB binding to membranes also rationalizes a number of previous *in vivo* observations. As discussed earlier, N- or C-terminal fluorescent protein fusions to MreB are not functional in cells. In the MreB monomer both termini are located close to the each other, hence fusions to both ends would interfere with membrane binding. In line with this is the functionality of sandwich fusions to MreB. Here, the fluorescent protein is inserted between helices 6 and 7 of MreB, meaning far away from the membrane interaction site[44].

It is still unknown whether the entire cellular pool of MreB is actually membrane-bound. If so, it raises another important question: Can insights gained from *in vitro* experiments in solution be readily translated to membrane-associated MreB? It is conceivable that at least the assembly kinetics would be influenced as some components of reactions are restricted to only two rather than three dimensions. At the very basic level this could mean that the critical concentration for filament formation *in vivo* may be lower than determined *in vitro*. Overall, although MreB is one of the most extensively studied bacterial actins, it is clear that our understanding of MreB function at the molecular levels is still incomplete.

ParM and other plasmid segregating bacterial actins

A number of bacterial actins are not essential to cell survival but are important for plasmid segregation. The stable propagation of bacterial plasmids can be accomplished by two mechanisms: random or active segregation. Provided the copy number of the plasmid is high enough, stochastic (or diffusive) events will be sufficient to ensure that a large fraction of the cells in the population will inherit the plasmid and traits encoded by it. However, some plasmids are maintained in the cell at a low copy number. For instance, virulence factors of pathogenic bacteria are sometimes encoded on large extrachromosomal plasmids that are kept at a low copy number (as low as 1-5 copies per cell), possibly to minimize the metabolic burden on the carrier cell. Relying on stochastic events for segregation in these instances is a risky strategy. It appears that plasmids evolved systems that govern their active transport into daughter cells[57-59]

The ParMR/*parC* system of the R1 plasmid of enteropathogenic *E. coli* cells is the best studied plasmid segregation system so far. It is composed of three basic components that are all encoded by the plasmid: i) the “motor” protein ParM, ii) the adaptor protein ParR and iii) and a centromere-like region termed *parC*. The ParR protein binds the *parC* sequence and couples the plasmids to ParM which, via its polymerization, pushes the plasmids apart[57-59]. At least two other plasmid segregation systems are driven by actin-like proteins and have been characterized in some detail. The bacterial actin AlfA is encoded on the *B. subtilis* plasmid pLS32 and is found in an operon with AlfB (ParR-like) and a *parC* sequence[20, 26]. The bacterial actin Alp7A, in conjunction with Alp7R (ParR-like) and a*lp7C (parC*-like), is responsible for the segregation of plasmid pLS20 in *B. subtilis*[27, 60]. Although the basic architecture of all these systems is similar there appears to be substantial differences between their bacterial actins, as we will discuss. Additionally, a number of uncharacterized families of bacterial actins are also found on naturally occurring plasmids raising the possibility that the use of these proteins for DNA segregation is a common theme in nature.

One characteristic feature of ParM is its heterogeneous localization pattern in cells across a population. In some cells ParM appears as a filamentous structure spanning the entire cell length (Fig. 2B) while in the majority of cells ParM is either diffuse or appears as foci[29, 61]. This heterogeneity is a reflection of the dynamic nature of ParM filaments in individual cells. ParM filaments elongate for a period of time before switching from elongation to rapid shortening[62]. This dynamic behavior is directly linked to the plasmid segregation process since filaments bound to plasmids are stabilized from disassembly and their elongation actively pushes plasmids apart[61, 62] (Fig. 2B). Once the plasmids reach the cell poles, ParM filaments disassemble essentially completing the segregation event. However, plasmids do not remain at cell poles and can diffuse away, suggesting the lack of a cell pole-anchoring factor. Plasmids that “escape” can re-encounter ParM filaments and again be segregated. In fact, several rounds of segregation occur in a single cell cycle[62].

The behavior of AlfA and Alp7A in cells indicates that some of ParM’s key features are not shared by other plasmid segregating bacterial actins. In contrast to ParM, AlfA-GFP filaments can be visualized in the vast majority of cells of a population[26] (Fig. 2B). Furthermore, these filaments do not undergo rapid disassembly and assembly events.

Nonetheless, AlfA-GFP filaments are dynamic when observed by a Fluorescence Recovery After Photobleaching (FRAP) assay. It appears that AlfA's mode of dynamics is not only different from ParM, but also from actin. For AlfA-GFP, FRAP signal recovery occurs symmetrically from both sides of the bleached area and without polarity[26]. Signal recovery in FRAP experiments is often related to the exchange of bleached subunits by unbleached subunits as a result of assembly and disassembly. For a polar mode dynamics, such as actin treadmilling, one also would expect polar signal recovery. Despite these observations one cannot exclude the possibility of treadmilling events for AlfA. An explanation for the observed signal recovery pattern could be the presence of bundles of filaments with different polarities that could undergo treadmilling. ATP hydrolysis is also important for the dynamics of AlfA filaments and its function, since a mutation expected to abolish nucleotide hydrolysis leads to static filaments and a segregation defect *in vivo*[26]. Dynamics are also important for the function of Alp7A. The behavior of Alp7A-GFP filaments (Fig. 2B) appears more similar to ParM's dynamics, as time-lapse experiments reveal the rapid assembly and disassembly of filaments. But unlike ParM, Alp7A filaments can remain assembled and elongated after segregating plasmids and are still dynamic via a mechanism more consistent with treadmilling[27].

Consistent with their different dynamic properties, ParM and AlfA have very different filament architectures (Fig. 1C), supporting a causal link between structural polymorphisms and variation in functional dynamics. ParM is the best structurally characterized bacterial actin, and the structure of its filaments highlights aspects of both conservation and divergence of bacterial actins from eukaryotic actin. Crystal structures of the ParM monomer clearly revealed a domain organization and conformational flexibility conserved with actin. However, ParM filaments, while two-stranded like actin, have the opposite twist[18, 63], indicating that the inter-strand contacts have changed dramatically over the course of evolution. AlfA is more open and twisted than ParM, with more exposed surface between the strands[20]; understanding the functional impact of AlfA's unique filament architecture awaits higher resolution structural studies.

The different behaviors of ParM, AlfA and Alp7A in cells may suggest that the exact mechanism for plasmid segregation is not conserved between different systems. Investigations into common and variable features of different plasmid segregation systems require both *in vivo* and *in vitro* approaches. To date we have insights into the *in vitro* properties of ParM proteins and AlfA. The bacterial actin Alp12, encoded on the C*lostridium tetani* plasmid pE88, has also been characterized in some detail *in vitro*, but to our best knowledge it is unclear whether Alp12 is actually involved in plasmid segregation. In the following sections we review the behavior of these proteins outside the cell.

ParM polymerization and dynamics *in vitro*

By now, a remarkable level of detail is available about ParM and its behavior *in vitro*. ParM can form filaments in the presence of ATP, GTP or non-hydrolysable ATP analogues provided Mg^{2+} is present. Consistent with their *in vivo* behavior ParM filaments are unstable *in vitro*[18, 29, 63, 64]. Total internal reflection fluorescence (TIRF) microscopy assessing the behavior of single filaments uncovered that ParM filaments suddenly switched between

phases of steady elongation to rapid disassembly. This type of behavior had previously been associated with eukaryotic microtubules and is referred to as dynamic instability[64]. ParM disassembly depends on ATP hydrolysis since either the presence of non-hydrolysable ATP analogs or the mutational inactivation of ATPase activity results in stable filaments[64]. The molecular basis for dynamic instability of ParM filaments appears to be nucleotide-dependent conformational changes within the ParM subunit. The crystal structure of ParM in the nucleotide-bound state is more closed than in the apo state, suggesting that the nucleotide plays a role in stabilizing a closed state[15]. These structural changes are somewhat reminiscent of the hinge-like flexibility seen in actin in various states, but the ParM movement is much greater in magnitude. Cryo-EM structures of ParM filaments have also shown the subunits in open and closed states[18]. The proposed mechanism for dynamic instability is thus a conformational change within the subunits of a filament upon nucleotide release, leading to a disruption of the inter-subunit contacts between subdomains IIa and IIb, and subsequent destabilization of the filament.

TIRF microscopy shows that ParM assembles and disassembles bidirectionally with similar kinetics at both ends[64], in contrast to the kinetic polarity of e.g. actin filaments. However, whether the growth of ParM filaments is truly symmetrical has been a matter of debate, because, similar to actin or MreB, ParM filaments have a structural polarity with distinct barbed and pointed ends. It has been suggested that antiparallel bundles of ParM filament, with asymmetric growth, could lead to an apparent kinetic symmetry[65]. Another question raised by the structural polarity of filaments is about how a single filament with different ends can bind the ParR/*parC* complex[66, 67] equally well at both ends to segregate plasmids. One possibility is that the ParR/*parC* complex binds the end of the ParM filament like a collar, potentially allowing the interaction with similar surfaces on both ends[68]. Another possibility comes from a recent co-crystal of ParM with the ParM-interacting region of ParR (a 17 aa peptide), which suggests that ParR binds to the barbed end of ParM filaments[69]. The binding region of the ParR peptide overlaps with the ParM-ParM interaction surface thus lending support to the idea that ParR can only bind one end of the filament, namely the barbed end[69]. In TIRF experiments, ParR/parC accelerated filament growth and ParM monomers were only added at the ParR/*parC*-bound end of the filament, confirming an insertional ParM polymerization model. An appealing model is that the assembly of at least two filaments in an antiparallel fashion, each with one ParR/*parC* complex at the barbed end, allows for the bipolar segregation of plasmids[69]. Interestingly, the recent study above[69] also presents data that could be consistent with an antiparallel packing of ParM filaments. TIRF microscopy indicates that single filaments can condense into bundles. Within bundles, ParM filaments appear to move by an interfilament sliding mechanism that is not yet fully understood. Molecular modeling of interfilament interaction surfaces favors a model in which ParM filaments are oriented in an antiparallel fashion. Furthermore, mutational analysis and TIRF microscopy of sliding mutant filaments appears to substantiate this model. Introducing negatively charged residues at the proposed antiparallel packing surface leads to splitting of bundles into constituent ParM filaments, because of repulsive electrostatic forces that are generated if antiparallel filaments slide against each other[69]. Building on previous and more recent insights an overall model for ParMR/parC-mediated plasmid segregation is proposed by the Löwe group[69]: a critical concentration of ATP-

bound ParM monomers can form filaments that disassemble due to dynamic instability, unless their barbed ends are stabilized by the ParR/*parC* complex. The ParR/*parC* complex speeds up filament growth at the barbed end. The pointed end remains susceptible to disassembly unless it is paired with another ParR/*parC*-bound filament in an antiparallel manner. Thus, for the bipolar segregation of plasmids at least two antiparallel ParM filaments are needed.

The behavior of divergent members of the ParM family

Most studies to date have concentrated on the properties of the ParM protein from the *E. coli* R1 plasmid (ParM-R1), however, a number of ParM related proteins exist and it is unclear whether ParM-R1's properties are shared by other proposed members of the ParM family. One divergent ParM member is encoded on the *E. coli* plasmid pB171, which shares 41% identity and 52% similarity to ParM-R1[23]. ParM-pB171 shows many of ParM-R1's traits. It polymerizes in the presence of ATP, exhibits dependence on divalent cations and is dynamically unstable. EM reconstruction shows that ParM-pB171 filaments are composed of two protofilaments that are helically wound, similar to ParM-R1 filaments. In fact, modeling of the ParM-R1 crystal structure into the ParM-pB171 filament reconstruction shows that inter- and intra-strand contacts between subunits are nearly identical to those in the ParM-R1 filament reconstruction[23]. Another proposed divergent member of the ParM family is ParM-pSK41 from *Staphylococcus aureus*[16]. ParM-pSK41 filaments also assemble in the presence of ATP and require divalent cations for assembly. However, these filaments exhibit a propensity to form well-ordered bundles. Filtered images of bundles showed that ParM-pSK41 forms filaments that are single-stranded, unlike the double-stranded ones from ParM-R1. Furthermore, the crystal structure of ParM-pSK41 shows a higher correspondence to that of the archaeal actin-like protein Ta0583 from *Thermoplasma acidophilum* (Fig. 1B), with which it shares 22% sequence identity (18% between ParM-R1 and ParM-pSK41). Interestingly, ParM-pSK41 does not exhibit significant dynamic instability but rather displays a form of treadmilling[16]. It remains to be seen whether other ParM proteins are more like ParM-R1 or ParM-pSK41. Considering the presence of other actin-like proteins that are unrelated to ParM but mediate plasmid-segregation, it has been suggested that ParM-pSK41 actually exemplifies a novel family of bacterial actins[23].

The assembly of AlfA and Alp12 filaments

The two unrelated bacterial actins AlfA and Alp12 exhibit properties *in vitro* that are significantly different from ParM. Similar to ParM, both proteins assemble into filaments in the presence ATP or GTP and Mg^{2+}, but the architecture and ultrastructure of these filaments is different[20-22]. AlfA filaments have a strong propensity to form bundles *in vitro*. Bundle formation can be disrupted by very high levels of KCl (e.g. 2 M KCl), indicating that bundling is promoted by electrostatic interactions. Interestingly, EM analysis suggests that these bundles can be composed of filaments with mixed structural polarity. TIRF microscopy reveals AlfA filamentous structures of variable size and fluorescence intensities, consistent with bundles composed of various numbers of AlfA filaments. Bundles appear to grow bidirectionally, but frequent filament annealing events account for most size increases. AlfA filament bundles do not show ParM-like dynamic instability, also consistent with *in vivo* demonstrations of rather stable filamentous structures[20]. Moreover, the capacity to form

mixed-polarity bundles is consistent with observations in FRAP assays carried out using AlfA-GFP in *B. subtilis*[26]. Alp12 forms dynamically unstable filaments and can undergo repeated cycles of assembly and disassembly. Despite this similarity to ParM-R1, Alp12 shows a dramatically different filament architecture. EM reconstructions suggest that Alp12 filament consists of two antiparallel strands that are twisted around each other. The strands themselves consist of two parallel protofilaments in total resulting in an Alp12 filament that is constructed from four protofilaments (Fig. 1C)[22].

ParM and Alp7A behavior - Hints for the existence of nucleation factors in bacteria?

The spontaneous assembly of actin *in vitro* is inefficient because the formation of a nucleus for assembly is kinetically unfavorable. In eukaryotes a number of protein complexes exist that serve as nucleation factors for actin polymerization, such as the Arp2/3 complex[70]. There has been a debate for many years now on whether bacterial actins require nucleation factors, since they assemble quite readily *in vitro*. Several pieces of information hint at the presence of nucleation factors also for bacterial actins.

Early studies reported that ParM-R1 filaments could only be visualized *in vivo* when ParR and *parC* were present. Furthermore, *in vitro*, the presence of ParR and *parC* triggered ParM-R1 polymerization at a low ParM concentration where usually no significant polymerization is seen[29]. These observations could be interpreted in two ways: Either the ParR/*parC* complex stabilizes ParM-R1 filaments from disassembly or it acts as a nucleation point for ParM-R1 filaments. If the nucleation model is true this could imply that ParM-R1 monomer levels *in vivo* are lower than the critical concentration measured *in vitro*. The cellular ParM-R1 concentration was estimated to be ~12 uM[29], considerably higher than the critical concentration *in vitro* (~2 uM)[64], arguing against nucleation as a mode of ParM assembly regulation. However, it is worth pointing out that we do not know whether critical concentration values determined *in vitro* are a true a reflection of the *in vivo* values. Interestingly, recent results on Alp7A also could argue in favor of a nucleation model. Alp7A itself is sufficient to produce filaments *in vivo* as long as it is expressed at sufficiently high levels (5-fold higher than native level). However, at physiological levels no filaments are observed unless Alp7R and *alp7A* are also present, indicating that these might serve as a nucleation factor[60].

The question of whether bacterial actin assembly is nucleated *in vivo* remains largely underexplored. In general we know little about binding partners of bacterial actins and how filament formation is integrated with the general cell physiology. It will be interesting to see if bacteria control filament formation by strategically placing nucleation factors or even disassembly factors in their cytoplasm.

MamK, a bacterial actin involved in magnetosome organization

Whereas MreB and the plasmid segregating actins appear to be widely present in bacteria, the bacterial actins of the MamK family, with a few exceptions, are only found in the phylogenetically diverse group of magnetotactic bacteria. In these bacteria MamK is important for the subcellular organization of organelles termed magnetosomes[71]. Magnetosomes are specialized membrane compartments in which cells synthesize magnetic

nanocrystals, such as magnetite or greigite. A given cell has a number of magnetosomes organized into a chain that runs along the length of the cell (Fig. 2A). The magnetosome crystals are large enough (30 −120 nm in diameter) to hold a permanent dipole moment like small magnets. Their chain-like organization is significant for the cell since in this way a large dipole moment is created, thus maximizing overall magnetism. The cells, with their fixed magnetosome chain, essentially act as a small compass needle and align to geomagnetic field lines. This ability is thought to make their search for low-oxygen concentrations in stratified aquatic environments more efficient[71-74].

The molecular mechanisms of MamK function are not yet understood but high-resolution ECT imaging of magnetosomes and the cytoplasmic space provides some clues. In two magnetotactic bacteria, *Magnetospirillum magneticum* AMB-1 (AMB-1) and *Magnetospirillum gryphiswaldense* MSR-1 (MSR-1), magnetosomes are flanked by a network of actin-like filaments[75, 76]. In cells lacking the *mamK* gene, these filaments disappear, indicating that they are composed of MamK[75, 77]. This is also supported by *in vitro* experimentation showing that the MamK protein is sufficient to form filamentous structures, as we will discuss later. A striking phenotype of cells lacking MamK is the altered magnetosome chain organization. However, depending on the species the deletion results in slightly different phenotypes. In AMB-1 Δ*mamK* cells individual magnetosomes are scattered and are no longer organized into a coherent chain along the length of the cell (Fig. 2A)[75]. In MSR-1 Δ*mamK* cells magnetosome chains are shorter, fragmented and placed ectopically at cell poles[77]. Several possible molecular functions of MamK could be envisaged. One possibility is that MamK filaments act as a scaffold maintaining the magnetosome chain after its formation. Alternatively, MamK filaments might play a more active role and establish the chain by guiding and pushing magnetosomes into place. Recent insights into the cell division process of MSR-1 may indicate that MamK fulfills a more active role[78]. In MSR-1 the magnetosome chain is located at the midcell, which is also the site for cell division. When cells are dividing the magnetosome chain becomes split and the chain halves are essentially positioned at newly-forming cell poles of daughter cells, even at the later stages of cell division[78]. However, in separated wild type MSR-1 cells the magnetosome chains are not located at cell poles which suggests a rapid translocation of magnetosomes during the final stages of cell division[78]. The phenotype of a MSR-1 Δ*mamK* mutant may suggest that MamK filaments are involved in the segregation and midcell positioning of magnetosomes by exerting cytomotive force on magnetosomes[77, 78]. Further research is, however, required to substantiate such a model. Whether MamK's exact molecular function is conserved between different magnetotactic bacteria is also an important question, since different magnetosome chain assembly strategies appear to exist. In MSR-1, empty magnetosome compartments are formed throughout the cells' space but their alignment requires magnetite synthesis and the magnetic interaction of adjacent magnetosomes[76]. In contrast, AMB-1 cells can align empty magnetosome compartments, even without the formation of a magnetite crystal[75, 79] The different phenotypes of *mamK* deletions in AMB-1 and MSR-1 is also another indication for species-specific mechanisms. An additional difference between MSR-1 and AMB-1 is that the latter also encodes another homolog of MamK, named MamK-like[80]. It has been implied that if MamK-like performed

a similar function as MamK, its presence could potentially explain the different phenotypes of AMB-1 and MSR-1 *mamK* deletion strains[77].

The dynamic behavior of MamK filaments *in vivo*

The question of whether MamK forms dynamics filaments has been investigated in AMB-1. FRAP assays using MamK-GFP have shown that, similar to other bacterial actins, MamK filaments are dynamic[28]. As with other bacterial actins, nucleotide hydrolysis is required for MamK dynamics since the mutational inactivation of its ATPase activity renders filaments static *in vivo*[28]. Investigations into the exact mechanism of MamK dynamics are still ongoing. ParM-like dynamic instability, involving cycles of assembly and rapid disassembly events, is not evident for MamK-GFP. Furthermore, unlike ParM, MamK-GFP appears as a continuous straight filament running from cell pole-to cell pole (Fig. 2B) in the vast majority of cells in a population[28, 75]. FRAP assays appear to also exclude an actin-like treadmilling as a mode of dynamics, since the pattern of signal recovery lacks a clear directionality. However, the architecture of the MamK filament network in cells might limit the conclusions that can be reached from FRAP assays alone. ECT imaging of AMB-1 shows that many overlapping MamK filaments of approximately 200 – 250 nm in length run parallel to magnetosomes, but not a continuous filament[75]. This discrepancy to the above mentioned observations of pole-to-pole filaments with MamK-GFP can be explained by the optical limitation of fluorescence microscopy. This limitation gives the impression of a continuous MamK-GFP filament. In fact, ECT imaging of cells with MamK-GFP shows that this fluorescently labeled protein also forms a network of filaments, just as the native protein[75]. An important point is that the polarity of individual filaments in this network cannot be determined. If the network is composed of filaments with mixed orientations, polar treadmilling events of individual filaments might be masked or cancelled out during visualization. Alternatively, the observed fluorescence recovery events could be explained by sliding of entire unbleached filaments into the photobleached areas. The recent evidence of the movement of entire MreB filament patches[40, 41, 43] or even ParM filament sliding[69] make this a plausible model.

Interestingly, the dynamics of MamK filaments in AMB-1 require the presence of other magnetosome proteins[28]. The majority of proteins required for magnetosome formation, including MamK, are encoded by a distinct genomic region called the magnetosome island (MAI)[71, 74]. The MAI varies in length between species, but in AMB-1, it is approximately 100 kb long. The MAI is flanked by two direct repeats and recombination between these two direct repeats can lead to the loss of the entire MAI, thus leaving cells without the ability to form magnetosomes[81]. Significantly, the loss of the MAI also affects MamK filaments dynamics. FRAP assays with ΔMAI cells expressing MamK-GFP from a plasmid show no fluorescence signal recovery, implying that MamK filaments are no longer dynamic[28]. Genetic studies have identified at least two redundant proteins encoded by the MAI, MamJ and LimJ, that are important for MamK dynamics. In a strain lacking both these proteins, FRAP assays with MamK-GFP again do not show fluorescence signal recovery[28]. It appears, however, that MamJ or LimJ are not sufficient, since reconstitution of MamK-GFP and either of these proteins in ΔMAI cells could not restore MamK-GFP dynamics[28]. Interestingly, loss of both MamJ and LimJ also results in the disturbed organization of the

magnetosome chain. In the absence of these proteins the magnetosome chain is disrupted by large gaps to which bundles of MamK filaments localize[28]. Whether this disrupted chain phenotype is directly related to additional functions of MamJ and LimJ, or if it is a consequence of static instead of dynamic MamK filaments is an open question

The *in vitro* behavior of MamK filaments and implications in the cellular context

Compared with MreB or ParM, comprehensive insights into MamK's assembly *in vitro* have only recently been gained. Studies so far have concentrated on MamK from three closely related *Magnetospirillum* species[19, 80, 82, 83] but MamK from AMB-1 has been studied most extensively[19]. In the presence of ATP and Mg^{2+} MamK polymerizes with kinetics and a critical concentration similar to those of other bacterial actins. MamK can also assemble in the presence of GTP but assembly kinetics are generally slower than with ATP, and this is accompanied by a slightly higher critical concentration. MamK assembles into filaments composed of two protofilaments that are twisted around each other[19] (Fig 1C and Fig. 3). The protein can form single, well-separated filaments or bundles of filaments, depending on the experimental conditions[19, 80, 82]. For instance, in the presence of physiological levels of K^+ MamK forms well-structured bundles[19]. The filament structure of the MamK was recently determined by cryo-EM at 12 Å resolution[19]. As with MreB and ParM, the longitudinal contact surfaces in the MamK filament are conserved with actin. Similar to MreB, the two protofilaments of MamK are unstaggered. However, for MamK it is clear that the two strands are parallel and not antiparallel as was suggested for MreB[19]. The unique architecture of MamK further highlights a common theme of variation in bacterial actins: conserved longitudinal contacts along each strand, but strong variation in cross-strand contacts (Fig. 3).

MamK is an ATPase and the mutation of a glutamate residue in the active site of MamK (E143A) abolishes its ATPase activity[19]. This glutamate residue is conserved across most bacterial actins and, in fact, the equivalent mutation also abolishes ParM-R1's ATPase activity[64]. Furthermore, these data are consistent with the ATP hydrolysis model for actin[84]. Similar to ParM, a lack of ATPase activity does not affect MamK's ability to form filaments *in vitro*. However, this leads to filaments that do not disassemble, as bulk measurements by light scattering assays show[19]. It is currently unclear by which mechanism MamK filaments undergo disassembly *in vitro* because single filament measurements are not available. However, the bulk disassembly kinetics appear inconsistent with the rapid disassembly behavior of ParM[19].

In addition to nucleotide hydrolysis, K^+ levels influence MamK filament disassembly *in vitro*. At physiological K^+ concentrations, when MamK filaments bundle, no bulk disassembly is obvious despite ATP hydrolysis[19]. This filament bundling and the lack of disassembly *in vitro* may be related to the static nature of MamK filament in cells lacking MamJ and LimJ. As mentioned earlier, in this mutant MamK filaments appear to bundle, clearly deviating from the behavior in wild type cells. One possibility is that the interaction of MamK filaments with magnetosomes is impaired in cells lacking MamJ and LimJ. In such a model, filaments that are not bound to magnetosomes would be free to associate with each other, resulting in stable bundles. This would fit with the notion that MamJ may

function as a magnetosome-MamK attachment protein[85], although there is no strong evidence for this interaction.

In general, little is known about magnetosome or cellular protein binding partners of MamK. The MAI encoded protein Amb0994 has been shown to co-localize and interact with MamK filaments at cell poles by bimolecular fluorescence complementation assays[86]. Amb0994 exhibits similarities to methyl-accepting chemotaxis proteins (MCP-like) and a possible role in magnetotaxis was suggested[86]. Another study also concluded the interaction with another MCP-like protein with MamK by genetic two-hybrid assays and cross-linking studies with purified proteins[87].In general, it can be expected that a hunt for MamK interaction partners will bring us closer to understanding the true function of MamK and how the function of this protein is integrated with the cell's physiology.

FtsA is an unusual bacterial actin

A common feature of actin and bacterial actins is an overall similar protein fold. However, it appears that not all bacterial actins adhere to this principle. The structure of the essential cell division protein FtsA substantially deviates from the canonical actin fold in that subdomain IB is missing, and a novel domain has been inserted into subdomain IA (Fig. 1B)[13]. Considering this rather large deviation in structure its ability to assemble into actin-like filaments has been debated for many years.

A number of genetic approaches showed that FtsA can interact with itself. In fact, the fusion of two FtsA monomers in a head-to-tail configuration, similar to the configuration in an actin-like filament, is functional in *E. coli* cells. Furthermore, dimerization/oligomerization was shown to be required for the *in vivo* function of FtsA during cell division[88]. Bacterial cell division requires the orchestrated assembly and colocalization of a number of proteins at the site of cell division. Through a C-terminal amphipatic helix, FtsA binds the cytoplasmic membrane and tethers another crucial cell division protein, the tubulin homolog FtsZ, to the cell division site. Moreover, FtsA is involved in the recruitment of a number of other components of the cell division apparatus[89, 90]. When FtsA is depleted cells are unable to divide and grow into long filamentous cells as result (Fig. 2A). In line with its role in cell division FtsA exhibits a rather clear localization to the cell division site at the midcell but, in contrast to other bacterial actins, no filamentous structures are obvious (Fig. 2B). Interestingly, GFP–FtsA lacking the amphipatic helix results in filamentous structures running along the length of *E. coli* cells[91] indicating that membrane binding limits FtsA's ability to form larger polymers *in vivo*.

The formation of filaments *in vitro* was first shown for FtsA from *Streptococcus pneumoniae* (*Sp*FtsA), which forms large corkscrew-like polymers in the presence of ATP and Mg^{2+}[92]. Recently FtsA from *T. maritima* (*Tm*FtsA) was shown to crystallize with longitudinal contacts similar to those of actin and MreB protofilaments[25], further confirming the conservation of longitudinal contacts among the actin family (Fig. 3). In MreB, longitudinal monomer-monomer interactions in the protofilament occur between subdomains IB and IIB on one subunit, and IA and IIA on another. However, strictly speaking, subdomain IB is no longer present in FtsA and is essentially replaced by a domain

with unrelated sequence, termed subdomain IC. Thus in an FtsA protofilament, interactions occur in a somewhat mixed fashion through subdomains IC and IIA on one monomer and subdomains IA and IIB on the other monomer (Fig. 3). Similar to MreB the *Tm*FtsA protein can form filaments on a lipid monolayer *in vitro*[25]. Whether the subunits in these filaments have the exact topology as the subunits in the crystal structure is unknown. However, the longitudinal spacing of subunits in lipid-assembled filaments and crystal protofilaments appear consistent with each other. Interestingly, *Tm*FtsA filaments that form on the lipid share little similarity to the cork-screw-like structure observed for *Sp*FtsA and may hint at differences between FtsA protein from different organisms. In any case, the physiological relevance of extensive filament formation observed *in vitro* remains unclear.

It will be interesting to see if other uncharacterized bacterial actins have undergone similarly drastic alterations in domain architecture.

Conclusions

Bacterial actins constitute a large, diverse family that share the core actin structural fold and are involved in many of the same cellular processes as eukaryotic actin, including cell shape, organelle positioning, and cell division (Fig. 1A,B). Bacterial and eukaryotic actins also share evolutionarily conserved functional properties: they polymerize into dynamic filaments, their assembly dynamics are modulated by regulatory proteins, subunits undergo similar conformational changes, and the filaments can be assembled into larger bundled structures. However, unlike eukaryotic actin, where a single filament form has been adapted to multiple cellular processes through a host of actin binding proteins, bacteria have evolved specialized actins for specific purposes that possibly require fewer interaction partners. This has relaxed evolutionary constraints and allowed bacterial actins to explore a greater range of sequence space. The result is a family of bacterial actins that vary greatly in filament architecture and dynamics (Fig. 1C), while retaining some evolutionarily conserved properties of eukaryotic actin. The functions, architecture and biochemical properties of the vast majority of these bacterial actin families are unknown thus providing an exciting new frontier in the study of bacterial cell organization.

Notes for Figure 1A: Sequences were aligned with ClustalW2 and phylogenetic tree drawn in FigTree v1.4. Eukaryotic actins: *H. sapiens* (4501885), *C. elegans* (17568985), *D. melanogaster* (17530805), *S. cerevisiae* (14318479), *G. lamblia* (159108769). *E. coli*: ParM-R1 (134954), ParM-pB171 (10955418), MreB (486290201), FtsA (446511017). *B. subtilis*: AlfA pLS32 (323651003), Alp7A pLS20 (323651170), MreB (142855), FtsA (221309402). *C. crescentus:* MreB (16125790), FtsA (16126780). *T. maritima:* MreB (15643354), FtsA (15643598). *S. enterica:* ParM-R64 (32470180), ParM-R621a (345134017). *C.tetani:* Alp12 pE88 (28373143, *role in plasmid segregation not confirmed). *M. magneticum* AMB-1: MreB (83312612), FtsA (83312952), MamK (83310064). *M.gryphiswaldense* MSR-1: MamK (33945229), *D. magneticus* RS-1: MamK (239908729), *M.marinus* MC-1: MamK (117925549), *Cd.M.multicellularis:* MamK (317383429).

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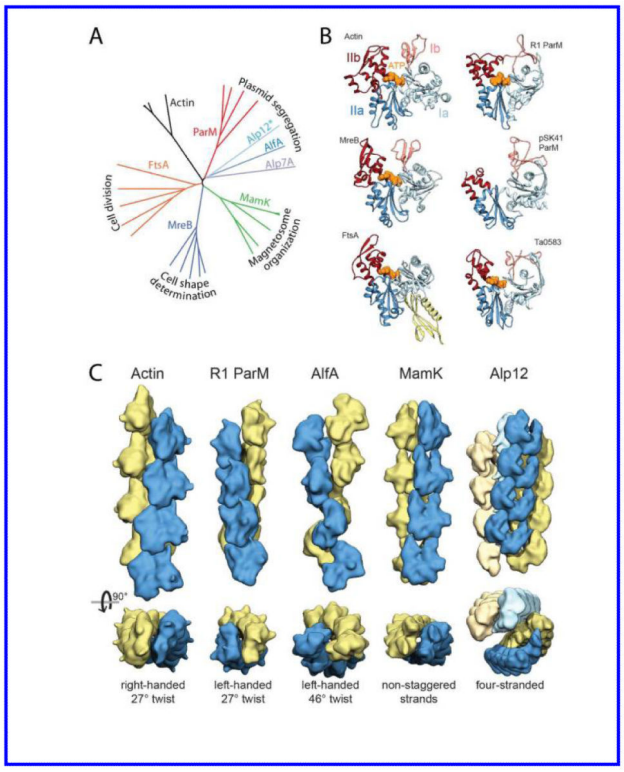

Figure 1. The diversity of bacterial actins

A. Phylogenetic tree of some actin-like proteins indicating functions for characterized members. See Notes section for the identity of proteins used to create phylogenetic tree. *Role in plasmid segregation not confirmed. **B.** The known crystal structures of bacterial actins all share a conserved structural core, with two domains which can each be subdivided into two subdomains: Ia (light blue), Ib (pink), IIa (dark blue), and IIb (red). ATP (orange) binds in cleft between domains. FtsA contains a unique domain insertion (yellow) within subdomain Ia. The PDB ID codes for the crystal structures are: actin – 1ATN, MreB – 1JCG, FtsA – 4A2B, R1 ParM – 1MWM, pSK41 ParM – 3JS6, TA0583 – 2FSN. **C.** Diverse filament architecture of bacterial actins. Although all bacterial actins assemble filaments, these vary dramatically in their quaternary structures. Inter-strand interactions vary extensively, giving rise to changes in filament twist, handedness, filament registration, and filament number. These differences correlate with different dynamic behaviors, suggesting a functional consequence of structural variation. References for structures: actin[93], R1 ParM[18], AlfA[20], MamK[19], Alp12[22]. A short length of filament is shown for each actin, both in side view (top) and down the helical axis (bottom).

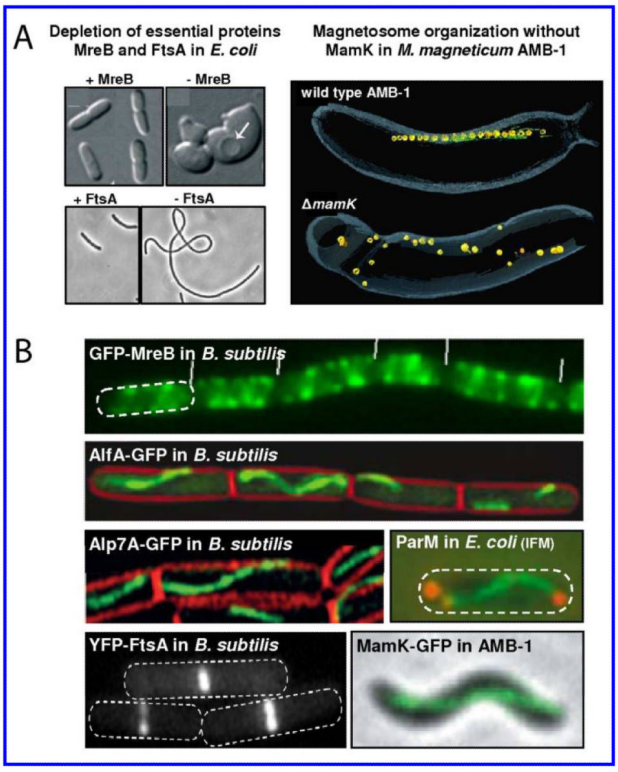

Figure 2. Bacterial actins in cells

A. Left panels: depletion of essential proteins MreB[44] or FtsA[94] results in cell morphological defects in *E. coli.* Right panel: deletion of *mamK* affects magnetosome (yellow) organization in *M. magneticum AMB-1*. Electron cryotomography reconstruction[75]. **B.** Top and left panels: localization of GFP-MreB[95] (dashed lines outline cell), AlfA-GFP[26] (cell membrane in red), Alp7A-GFP[27]and YFP-FtsA[96] in *B. subtilis* cells. Right panels: Immunofluoresce microscopy (IFM) visualizing ParM (green) and segregated plasmid (red) in an *E. coli* cell[29]. MamK-GFP fluorescence in *M. magneticum* AMB-1[28].

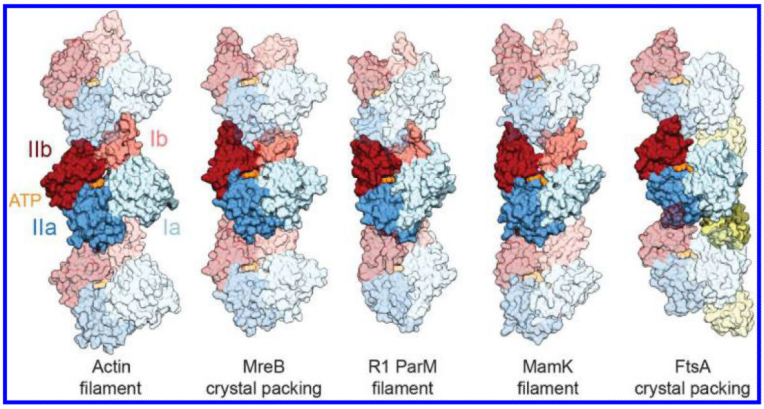

Figure 3. Longitudinal filament interactions are conserved in many bacterial actins

The end-to-end longitudinal interaction surfaces along each protofilament are largely conserved between actin, MreB, ParM and MamK. Here, three subunits from each filament are shown, with the same subdomain coloring used in Fig. 1B. FtsA also forms longitudinal-like contacts in its crystal packing; however, it is missing subdomain Ib, and a large insertion in subdomain 1a (yellow) stabilizes the longitudinal interactions.