A family of bacterial actin homologues forms a 3-stranded tubular structure.

Julien R.C. Bergeron[1*], Justin M. Kollman[2*].

1 Randall Centre, King's College London
2 Department of Biochemistry, University of Washington

* To whom correspondence should be addressed: Julien.bergeron@kcl.ac.uk; jkoll@uw.edu

Abstract:

The cytoskeleton plays a critical role in the organization and movement of cells. In Eukaryotes, actin filaments polymerize into a highly conserved double-stranded linear filamentous structure in the presence of ATP, and disassemble upon ATP hydrolysis. Bacteria also possess actin-like proteins, that drive fundamental cellular function, including cell division, shape maintenance, and DNA segregation. Like eukaryotic actin, bacterial actins assemble dynamic on ATP binding. In general, longitudinal interactions between bacterial actin protomers along each strand are conserved with eukaryotic actin, but variation in interactions between strands gives rise to striking diversity of filament architectures. Here, we report a family of bacterial actins of unknown function, conserved amongst the *Verrucomicrobiota* phylum, which assembles into a unique tubular structure in the presence of nucleotides. A cryo-EM structure of the filaments reveals it is composed of three strands, unlike other described bacterial actin structures. This architecture provides new insights into the organization of actin-like filaments, and has implications for understanding the diversity and evolution of the bacterial cytoskeleton.

Background:

Actin is a small, highly conserved protein that plays a crucial role in the structure and function of cells. Actin monomers (G-actin) polymerize to form filaments (F-actin) in the presence of ATP. Actin also possesses ATPase activity, and the filaments disassemble upon ATP hydrolysis and ADP release. This activity is modulated by numerous proteins, which forms the basis for most cellular processes in Eukaryotic cells, including muscle contraction, cell migration, cell division, intracellular transport, and signal transduction[1].
The bacterial cytoskeleton is a complex network of protein filaments that provides structural integrity and shape to the cell, similar to the cytoskeleton in eukaryotic cells. These filaments are often composed of proteins homologous to actin, although in bacteria each protein performs a specific function: MreB form helical filaments that encircle the cell and help maintain the shape of rod-shaped bacteria; FtsA forms a ring-like structure at the cell's mid-point, known as the Z-ring, and is involved in cell division; ParM transport nascent plasmids to the cell poles; and MamK anchors magnetosome vesicles along a linear track, ensuring their alignment in the cell[2].

Intriguingly, structural studies have revealed that even though the overall fold and ATPase activity is similar across actin-like proteins, they adopt very distinct filamentous architectures, including parallel, antiparallel, staggered and non-staggered arrangement, which have been proposed to be related to their specific function. Nonetheless, they mainly form two-stranded filaments[3], although in some case these have been suggested to be part of a higher-order assembly[4-6].

Here, we report the structure of a previously unreported family of bacterial actin homologues, which forms a rigid, three-stranded, polar filament. This unusual architecture suggests a distinct mechanism of filament assembly, and provides new avenues to our understanding of the evolution of actin-like proteins.

Results and discussion:

Previous studies had demonstrated that each bacterial actin homologue forms a distinct filament architecture[3]. This prompted us to search for previously uncharacterized actin homologues in the bacterial metagenomics database, to identify novel actin-like proteins that could adopt unique filament architectures. This led us to identify a set of proteins, most closely related to MamK[7-10] (25-30 % sequence identity) (**Figure 1a**) but present in non-magnetotacic bacteria, and therefore presumably with a distinct function. Intriguingly, this protein is highly conserved in the *Verrucomicrobiota* phylum, a poorly characterized family of anaerobic bacteria ubiquitously present in soil samples, as well as in the gut microbiome[11]. Sequence analysis of this protein family confirms that it possesses the hallmarks of actin-like homologues, including conserved residues for ATP binding and hydrolysis (**Figure S1**), and structure prediction confirmed that it likely adopts an actin-like fold. Nonetheless, one distinctive feature of this family is the presence of an additional 30-50 amino-acid extension at the N-terminus (NTD) (**Figure S1**), which is predicted to be unstructured and is not conserved in sequence, but not found in other actin-like proteins.

In order to characterize this newly-identified family, we purified one representative orthologue, from the bacterium *Opitutus terrae*[12]. A pelleting assay confirmed that it is soluble in isolation, but polymerizes in the presence of nucleotide (**Figure 1b**), confirming its actin-like property. Intriguingly, under the condition used, we observed a similar propensity for polymerization in the presence of both ATP and ADP. This is distinct to that of most observed actin homologues, where polymerization is vastly more efficient with ATP compared to ADP.
Negative-stain Electron Microscopy (EM) analysis of the obtained polymers revealed that they consist of long, well-ordered filamentous structures, which appeared as twisted rail tracks (**Figure 1c**), very distinct from that of any other actin-like proteins. Notably, these filaments are highly rigid, very long (up to several μm), and visual inspection suggested that it adopts a rail tracks-like architecture, consisting of two strands rotating on their individual axis. This prompted us to name this family the Bacterial elongated entwined Rail-like protein (BeeR).

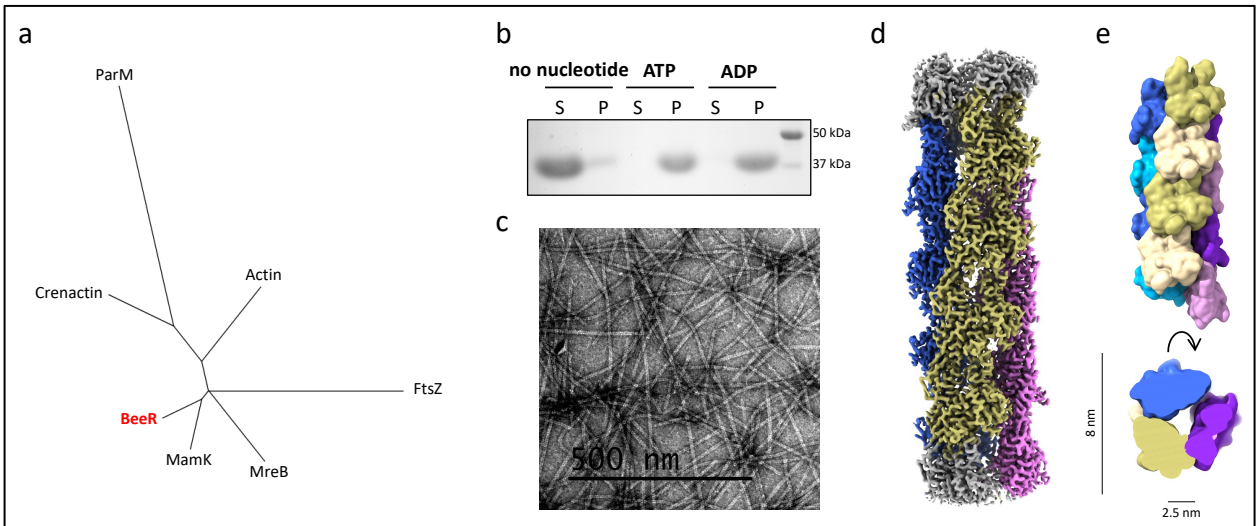

Figure 1: A novel Bacterial actin homologue family forms a three-stranded filament. a) Unrooted phylogenic tree of the main families of actin orthologue. BeeR, which is most closely related to MamK, is in red. b) Pelleting assay for BeeR, Monomeric protein is present in the supernatant (S), whereas polymers are found in the Pellet fraction (P). BeeR is primarily monomeric in the absence of nucleotide, and polymerizes in the presence of ATP, as well as ADP. c) Negative-stain TEM micrograph of BeeR filaments in the presence of ATP. d) Cryo-EM structure of the BeeR filament, to 3.1 Å resolution. The three strands are colored in dark blue, khaki, and magenta, respectively. e) Surface representation of the BeeR filament atomic model, colored as in d), from the side and top. The diameter of the filament, and internal cavity, are indicated.

We next used cryo-EM to gain further insights into the molecular structure of this BeeR filament. As shown on **figure S2a**, these were readily incorporated in ice within cryo-EM grids, presumably due to their length and rigidity. 2D classification of the resulting particles confirmed that they were highly ordered, with readily identifiable secondary structure elements (**figure S2b**). Using particles within the well-resolved 2D classes, we were then able to determine its structure by helical reconstruction, to 3.1 Å resolution (**figures S2** and **S3**, **movie S1**, and **table S1**; see **materials and methods** for details).

Unexpectedly, this unambiguously revealed that BeeR adopts a three-stranded, parallel, right-handed, staggered filament architecture (**figure 1d-e**). The diameter of this filament, at ~ 80 Å, is significantly larger than most other actin-like proteins, and it includes a large

cavity at its core (~ 25 Å-wide), responsible for its rail track-like appearance by negative-stain EM. Accordingly, this helical arrangement can be described in multiple ways: (1) as a 3-start helix, with a rise of one subunit (~ 52 Å) in a right-handed filament with a twist of ~ 19 °; (2) as a 2-start helix in a left-handed filament, with a rise of ~ 35 Å and a twist of ~ -177 °; or (3) as a 1-start helix in a right-handed filament, with a rise of ~ 17 Å and a twist of ~ 126 ° (**figure 2a**). These are equivalent in terms of subunit arrangement, but we note that representation (3) was used as helical parameters for the cryo-EM map reconstruction, as it has the lowest rise and therefore maximizes the number of individual protomers averaged in our reconstruction.

The structure of the BeeR monomer is consistent with that of an actin-like protein, with the canonical domains Ia, Ib, IIa and IIb adopting a U-shaped arrangement (**figures S3c** and **S4a**), and the nucleotide buried between domains Ib and IIb[1]. It is most similar to its closest homologue of known structure, MamK[7,8], with a RMSD of 1.3 Å for aligned atoms (5.1 Å for all atoms). Within the BeeR filament, the longitudinal interface along strands is largely similar to other actin-like filaments (**figure S4b** and **S4c**), with domains Ib and IIb making extensive contacts with domains IIa of the subunit above. Notably, its strand arrangement resembles mostly that of MamK, but also actin, both of which have similar interfaces, but with a slightly more twisted filament in the later. The N-terminal domain, which we identified as a hallmark of the BeeR family (see above and figure S1) is not resolved in our map, supporting its intrinsically disordered nature.

In contrast, the cross-strand contacts in the BeeR filament is strikingly distinct to that of all other actin-like filaments. Each protomer makes contact with two consecutive subunits of the adjacent strand, mainly alongside the 2-start helix interface, as shown on **figure 2b**. Contacts with the top subunit are minimal, with an interface area of ~35 $Å^2$, and consist of a hydrogen bond network between Arg 157 of one subunit, and Thr 307 and Gln 310 in the adjacent subunit. In contrast, the contact with the bottom subunit are extensive, forming a contact surface area of ~ 450 $Å^{2,}$ and consist of multiple hydrogen bonds, between Glu 149/Glu 153 and Asn 246, and between Arg 242/Arg 252 and Asn 380. In addition, a hydrophobic residue at the C-terminus, Leu 385, is buried in a hydrophobic pocket of the adjacent subunit consisting of Ile 277 and Leu 284.

It is noteworthy that most of the aforementioned residues are conserved among BeeR orthologues, but not in MamK or MreB (**figure S1**). This suggests that the three-stranded filament architecture is a feature of this protein family, and not unique to the particular *O. terrae* orthologue characterized here. Most notably, the majority of the cross-strand interaction occurs between helix a3 and the C-terminal ~ 10 residues. In contrast, we note that the loop forming the cross-strand interface in MamK (residues 80-85) is not present in any of the BeeR orthologues, confirming that they cannot adopt a similar 2-stranded architecture.

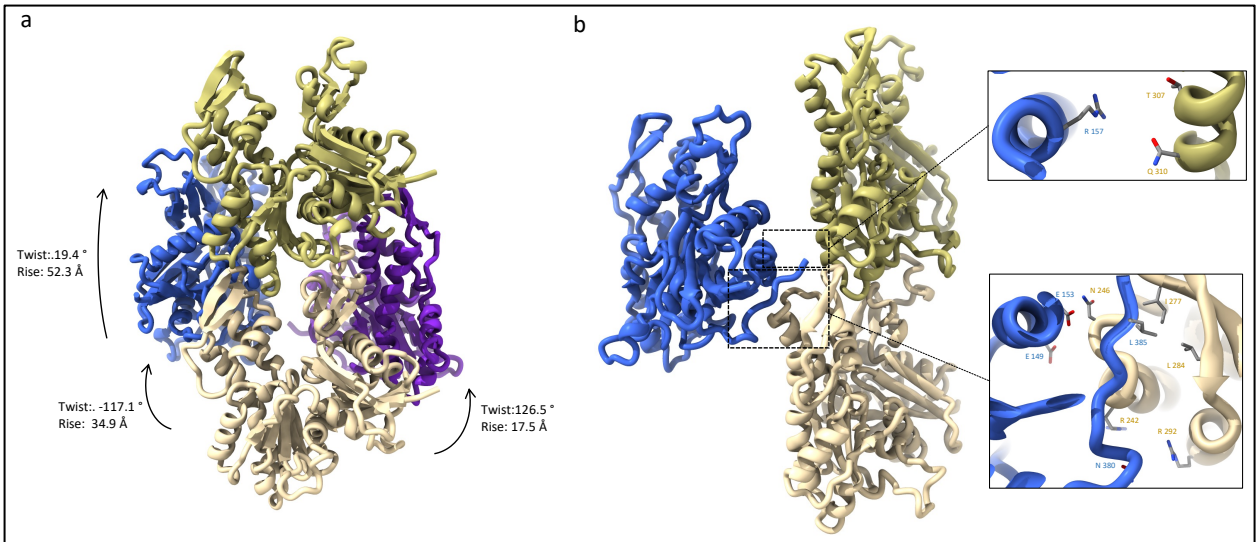

Figure 2: Structural basis for BeeR polymerization. a) Four adjacent BeeR subunits as shown, illustrating the various helical symmetry parameters that can be used to described this helical structure. b) Lateral contact across the strands are shown, with close-up views of the main interacting residues on the right.

As mentioned above, a hallmark of the BeeR family is the presence of a predicted disordered NTD. We sought to assess if this NTD was involved in filament formation; as shown in **figure S5a**, a construct lacking the NTD still retained its capacity to polymerize, and the filaments adopt the same overall rail-track-like morphology (**figure S5b)**. However, we observed that those filaments have a very high propensity to form larger bundles, consisting of multiple filaments sticking together. These were never seen with the WT protein. Based on this, we propose that the NTD of BeeR acts as a “repellant”, preventing the formation of bundles that might alter their cellular function.

We emphasize that the function of this tubular structure formed by BeeR is not known. The diameter of its cavity (~ 2.5 nm), with gaps all along the structure, makes it unlikely that it is involved in solute or macromolecular transport within the tube. Instead, we propose that the three-stranded tubular structure formed by BeeR provides a much more rigid filament than that of other actin homologues, as supported by 2D classes (Fig. S2b), as well as the fact that various Z-lengths used for the reconstruction does not significantly affect the obtained resolution. This likely will have an impact on its capacity to perform its function within the cell.

Nonetheless, the structure of the BeeR filament provides novel insights into the molecular diversity of actin-like proteins, and adds to the diverse structural architectures that can be formed by this family. It uncovers novel paths towards our understanding of the evolution of the actin fold.

Acknowledgements:
We are grateful to Ertan Ozyamak and Arash Komeili for initially identifying BeeR as a MamK homologue. JRCB acknowledges members of the Bergeron lab for occasionally tolerating their PI in the lab. Cryo-EM data was collected at the Imperial College Electron Microscopy Centre, and we acknowledge Paul Simpson for support. This work was

funded by grants from the BBSRC (BB/R009759/2) and HFSP (RGY0080/2021) to JRCB, and the National Institutes of Health (R35149542) to JMK.

Data availability:

The electron potential map of the BeeR filament is available in the EMDB, with accession number EMD-18852. The atomic model is available in the PDB, with accession number 8R2N.

References

1. Dominguez, R. & Holmes, K.C. Actin structure and function. *Annu Rev Biophys* **40**, 169-86 (2011).
2. Izore, T. & van den Ent, F. Bacterial Actins. *Subcell Biochem* **84**, 245-266 (2017).
3. Ozyamak, E., Kollman, J.M. & Komeili, A. Bacterial actins and their diversity. *Biochemistry* **52**, 6928-39 (2013).
4. Szwedziak, P., Wang, Q., Freund, S.M. & Lowe, J. FtsA forms actin-like protofilaments. *EMBO J* **31**, 2249-60 (2012).
5. Koh, F. et al. The structure of a 15-stranded actin-like filament from Clostridium botulinum. *Nat Commun* **10**, 2856 (2019).
6. Jiang, S. et al. Novel actin filaments from Bacillus thuringiensis form nanotubules for plasmid DNA segregation. *Proc Natl Acad Sci U S A* **113**, E1200-5 (2016).
7. Bergeron, J.R. et al. Structure of the magnetosome-associated actin-like MamK filament at subnanometer resolution. *Protein Sci* **26**, 93-102 (2017).
8. Löwe, J., He, S., Scheres, S.H. & Savva, C.G. X-ray and cryo-EM structures of monomeric and filamentous actin-like protein MamK reveal changes associated with polymerization. *Proc Natl Acad Sci U S A* **113**, 13396-13401 (2016).
9. Ozyamak, E., Kollman, J., Agard, D.A. & Komeili, A. The bacterial actin MamK: in vitro assembly behavior and filament architecture. *J Biol Chem* **288**, 4265-77 (2013).
10. Komeili, A., Li, Z., Newman, D.K. & Jensen, G.J. Magnetosomes are cell membrane invaginations organized by the actin-like protein MamK. *Science* **311**, 242-5 (2006).
11. Fuerst, J.A. The PVC superphylum: exceptions to the bacterial definition? *Antonie Van Leeuwenhoek* **104**, 451-66 (2013).
12. Chin, K.J., Liesack, W. & Janssen, P.H. Opitutus terrae gen. nov., sp. nov., to accommodate novel strains of the division 'Verrucomicrobia' isolated from rice paddy soil. *Int J Syst Evol Microbiol* **51**, 1965-1968 (2001).