

Characterization of *Spiroplasma citri* Fibril: Insights into polymerization interface and interaction with other cytoskeletal proteins

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उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

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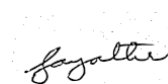


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Dedicated to Aai, Baba, Dadu and Aaji

CERTIFICATE

Certified that the work incorporated in the thesis entitled “Characterization of *Spiroplasma citri* Fibril: Insights into polymerization interface and interaction with other cytoskeletal proteins” submitted by Mrinmayee Anant Bapat was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.



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Abbreviations

GTP	Guanine triphosphate
Cryo-EM	Cryo electron microscopy
hsp70	Heat shock protein 70
nM	nanomolar
μM	micromolar
Å	Angstrom
NMR	Nuclear magnetic resonance
PG	peptidoglycan
LytM	LAS peptidases (lysostaphin-type enzymes, D-Ala-D-Ala metallopeptidases, and sonic hedgehog)
Csd	Cell shape determining
PBP	Penicillin binding protein
IPTG	Isopropyl β -D-1 thiogalactopyranoside
CcmA	Curved cell morphology A
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
GFP	Green fluorescent protein
JCVI-syn3B	J. Craig Venter Institute-synthetic cell 3.0
ATP	Adenosine triphosphate
MTase	Methyltransferase
SAH	S-adenosylhomocysteine
mM	Millimolar
NaH ₂ PO ₄	Sodium dihydrogen phosphate
k_{obs} (min ⁻¹)	Observed rate coefficient per minute
MTA	5'-methylthioadenosine
ADP	Adenosine diphosphate
Pi	Inorganic phosphate
RMSD	Root mean square displacement
μg/mL	Microgram per millilitre
°C	Degree Celsius
LB	Lurie bertini
PCR	Polymerase chain reaction
kDa	kilodalton
OD ₆₀₀	Optical density at 600 nm
PBS	Phosphate buffer saline
λ _{ex}	Excitation wavelength
λ _{em}	Emission wavelength
mL	millilitre
Fib	Fibril
CD spectroscopy	Circular dichroism
ScMreB5 ^{WT}	Spiroplasma citri MreB5 wild type

TEM	Transmission electron microscopy
KCl	Potassium chloride
MgCl ₂	Magnesium chloride
Fibril ^{wt}	Fibril wild type
AMPPNP	Adenylyl-imidodiphosphate
FRET	Forster resonance energy transfer
Ni-NTA	Nickel-nitrilotriacetic acid
NaCl	Sodium chloride
T _m	Melting temperature
pI	Isoelectric point
DTT	dithiothreitol
CHAPS	(3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)
EGFP	Enhanced green fluorescent protein
DIC	Differential interference contrast
PDB	Protein data bank
PGEM	Peptidoglycan elongation machinery
IF proteins	Intermediate filament proteins
Ccrp	Coiled coil rich protein
creS	crescentin
WT	Wild type
TIRFM	Total internal reflection fluorescence microscopy
<i>E. coli</i>	Escherichia coli
<i>B. subtilis</i>	Bacillus subtilis
<i>S. citri</i>	Spiroplasma citri
<i>H. pylori</i>	Helicobacter pylori
μm	micrometre
BLAST	Basic local alignment search tool
NCBI	National centre for biotechnology information
μL	microlitre
NaH ₂ PO ₄	Sodium dihydrogen phosphate
H ₂ SO ₄	Sulphuric acid
NaCl	Sodium chloride
mg/mL	Milligrams per millilitre

Abstract

Characterisation of *Spiroplasma citri* Fibril: Insight into the polymerisation interface and interaction with other cytoskeletal proteins

Spiroplasma is a cell-wall less helical bacterium. Electron microscopy studies show the presence of a flat, monolayered and membrane-bound ribbon-like cytoskeleton structure which consists of Fibril and 5 MreB paralogs. In the absence of any outer appendages, the cytoskeletal ribbon is thought to be responsible for maintaining the cell shape and motility of this organism. My Ph.D. work aims to characterize Fibril and MreBs using structural and biochemical approaches to understand the mechanism by which these proteins contribute to cell shape and motility. For characterization of Fibril, based on the sequence homology of the N-terminal half of the protein to 5'-methylthioadenosine nucleosidase (MTAN), nucleosidase activity was checked for Fibril. We observed that in spite of similar domain architecture, Fibril does not exhibit any nucleosidase activity. Fibril protein forms constitutive filaments. In absence of any information regarding the filament interface, we incorporated EGFP as a fluorescent tag at the loop regions based on the sequence based secondary structure prediction. We found that despite the EGFP insertions at multiple positions, Fibril readily forms filaments and might not be stable as a monomer. Earlier studies have shown that MreB5 interacts with lipids and Fibril. Our results show that Fibril and MreB5 interact in a nucleotide dependent manner similar to MreB5-liposome interaction. Our findings reveal that Fibril does not possess any enzymatic activity or ligand-dependent polymerization and that it acts as a scaffold for cell shape maintenance which is regulated by MreB5 filament dynamics in *Spiroplasma*.

For biochemical characterization like polymerization dynamics, ATP hydrolysis and checking interaction with other MreBs and Fibril, purification of MreB1 was initiated. Purification trialsof MreB1 are also discussed in the thesis.

Synopsis

Characterization of *Spiroplasma citri* Fibril: Insight into the polymerization interface and interaction with other cytoskeletal proteins

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Cytoskeletal assembly plays an important role in important cellular functions like, subcellular organization of organelles, guidance in transportation, maintaining cell morphology etc. Actin, tubulin and intermediate filaments form the quintessential components of cytoskeleton in eukaryotes. Existence of bacterial homologs of actin, tubulin and intermediate filaments have led to the discovery of a number of proteins responsible for essential functions in the prokaryotes. The first cytoskeletal protein identified was a tubulin homolog, FtsZ (Yit et al., 1985). FtsZ is important for cell division in prokaryotes (Adams and Errington, 2009). Subsequently, an actin homolog called MreB was also identified (Jones et al., 2001; van den Ent, 2001). MreB is present in most of the non-spherical bacterial species and helps in maintaining the cell morphology. It does so by interacting with peptidoglycan synthesis machinery and guiding in the insertion of new cell wall material (Domínguez-Escobar et al., 2011; Garner et al., 2011; Jones et al., 2001). In spite of having low sequence similarity, these bacterial homologs show strikingly similar structural properties with their eukaryotic counterparts (Löwe and Amos, 1998; van den Ent et al., 2001). First bacterial homolog of intermediate filament identified was Crescentin from *Caulobacter crescentus* (Ausmees et al., 2003). Crescentin is responsible for the crescent shape of the organism (Ausmees et al., 2003; Charbon et al., 2009). The overall structure of crescentin shows high similarity with its eukaryotic intermediate filaments (Liu et al., 2023). Apart from crescentin, many coiled coil rich proteins have been identified which are unique to the bacterial species. Like intermediate filaments, these proteins tend to polymerize independent of any nucleotide. Bactofilin (BacM) in *Myxococcus xanthus*,

CcmA in *Helicobacter pylori* have been shown to play an important role in cell shape maintenance (Koch et al., 2011; Sycuro et al., 2010).

One such nucleotide independent polymerizing protein called Fibril was identified in a cell wall less bacteria, *Spiroplasma* (Cole et al., 1973; Williamson, 1974). This protein was found to be present only in *Spiroplasma* amongst all the mollicutes (Townsend and Archer, 1983). The pitch of the Fibril protein was found to be similar to that of the cell (Lartigue et al., 2022; Sasajima et al., 2022). This indicates that Fibril might have a structural role in this organism. However, few of the *Spiroplasma* strains lack Fibril and still maintain helicity and motility (Abalain-Colloc et al., 1987; Whitcomb et al., 1997). Hence, conservation and function of Fibril in *Spiroplasma* is still not clear.

Along with Fibril protein, MreB protein was also identified in this organism (Williamson et al., 1979). Most of the *Spiroplasma* species showed the presence of multiple MreB homologs within the cells. As discussed earlier, MreB interacts with peptidoglycan synthesis machinery and guide new cell wall precursors at the longitudinal portion of the cell. Interestingly, *Spiroplasma*, being a cell wall less bacteria show the presence of 5-8 paralogs of MreB protein (Ku et al., 2014). *Spiroplasma* has been shown to be evolved from a regressive evolution through Gram positive bacteria (Razin, 1978; Cole et al., 1973; Weisburg et al., 1989; (Williamson, 1974). In spite of having a reduced genome, conservation of multiple MreB paralogs remains unclear. Structural and biochemical characterization of MreB5 and MreB3 from *Spiroplasma citri* has been done (Pande et al., 2022; Takahashi et al., 2022). MreB5 has been shown to be responsible for helicity and motility in *Spiroplasma citri* (Harne et al., 2020). However, biochemical and structural characterization of other MreB paralogs has not been done. Gene deletion of MreBs from the native organism will help in deciphering the role of Fibril and other MreB paralogs .

Fibril and MreB proteins form a cytoskeletal ribbon underneath the cell membrane (Kürner, 2005). Coordination of these individual proteins for maintaining the cell morphology and motility will require interaction between these proteins. Fibril and MreB5 have been shown to interact in vitro (Harne et al., 2020). As MreB is an ATPase, effect of nucleotide on the Fibril-MreB5 interaction will shed light on the mechanistic details of the interaction. Similar study will be extended for checking the interaction between Fibril and other MreB paralogs.

The thesis talks about some of the questions raised in the previous section. Following is the chapter wise summary of the thesis.

1. Introduction

This chapter talks about discovery and function of different bacterial cytoskeletal proteins. Major cytoskeletal players responsible for cell shape determination in prokaryotes have been explained. Cytoskeletal proteins involved in cell shape determination dependent/independent of MreB, an actin homolog has been summarized.

2. Understanding conservation of biochemical features of *Spiroplasma citri* Fibril

As discussed in the earlier sections, MreB is involved in cell shape maintenance in most of the rod-shaped bacteria and is associated with peptidoglycan elongation machinery (PGEM). However, few coccoid bacteria possess MreB and some rod-shaped bacteria lack the gene and still maintain a well-defined cell shape. Some reports also show that MreB might have different and completely unrelated roles to play in such organisms. On the other hand, Fibril is unique to *Spiroplasma* species and is present as a single copy in most of the species. Its role as a cytoskeletal protein is still not clear. This led us to check the distribution of gene neighborhoods of Fibril. Presence of a separate promoter sequence for Fibril and the downstream gene suggests that Fibril might express independently and is not a part of an operon.

Further, functional characterization of Fibril, for checking for ATPase and nucleosidase activities, was also done based on its partial homology with MTAN. However, no enzymatic activity was seen for Fibril. We propose that Fibril possesses a structural role in *Spiroplasma* for cell shape maintenance.

3. Determination of polymerization interface of Fibril

Fibril is a nucleotide independent polymerizing protein. There are no known ways of its depolymerization. Higher resolution structural data on Fibril will help us in elucidating the mechanism by which it regulates its filament length. For structural studies through X-ray crystallography, we plan to obtain a well folded, non-polymerizing construct of Fibril by introducing a fluorescent tag EGFP at the loop positions. We wished to obtain a fluorescently tagged filaments as well as a fluorescently tagged non-polymerizing construct of Fibril. We observed a gradation of fluorescence for the different Fibril-EGFP constructs.

TEM imaging showed that Fibril-EGFP constructs formed robust filaments irrespective of the position of the EGFP insertion. However, fluorescence levels and CD spectroscopy suggested that the Fibril fold is intact in the tagged constructs, especially those with high fluorescence. Through this fluorescent screen, we were able to identify favorable tag positions for EGFP insertions in Fibril.

4. Interaction studies of Fibril and MreBs

Spiroplasma citri MreB5 have been shown to interact with Fibril as well as liposomes. As MreB belongs to the actin superfamily, it binds and hydrolyses ATP. Based on these observations, we checked the interaction between MreB5 and Fibril in the presence of different nucleotides. We extended this study to check the interaction between ATPase mutants of MreB5 with Fibril as well. Co-sedimentation assay of ScMreB5^{WT} and ATPase mutants with Fibril showed nucleotide dependent interaction.

Liposomes tend to increase the hydrolysis activity of MreB5 (Vani Pande, PhD Thesis, 2023; <http://dr.iiserpune.ac.in:8080/xmlui/handle/123456789/8029>). We also checked if there is any effect of Fibril on the hydrolysis activity of MreB5. However, Fibril interaction with ScMreB5^{WT} did not affect the activity of ScMreB5^{WT}.

5. Preliminary characterization of *S. citri* MreB1 and its interaction with Fibril

Biochemical characterization of MreB proteins will help us in understanding the interfilamentous interaction and dynamics of these filamentous proteins. MreB5 was successfully purified and characterized in the lab previously. I started with purification trials of MreB1. Purification optimization of Fibril helped in separation of bundling of this protein as seen from TEM imaging. This will be useful in future for visualization and structure determination of interacting proteins with Fibril.

6. Conclusion and Future prospects

Major findings from the study have been summarized in this chapter.

List of Publications

1. Harne, S., Duret, S., Pande, V., **Bapat, M.**, Béven, L., Gayathri, P., 2020. MreB5 Is a Determinant of Rod-to-Helical Transition in the Cell-Wall-less Bacterium *Spiroplasma*. *Current Biology* 30, 4753-4762.e7. <https://doi.org/10.1016/j.cub.2020.08.093>

2. **Bapat, M.***, Harne S.*, R, Kashyap., Gayathri, P., 2023. Insights into assembly of constitutive filament-forming proteins of novel fold through domain dissection and EGFP insertion-based screen: A case study of *Spiroplasma* Fibril. 2023. BioRxiv, *Equal contribution
3. **Bapat, M.**, Gayathri, P., 2024. Role of nucleotide on the interaction between MreB5 and Fibril from *Spiroplasma citri* (manuscript under preparation).
4. **Bapat, M.**, Gayathri, P., 2024. Sequence, gene neighbourhood and structure analysis provide insights into evolutionary origins of *Spiroplasma* Fibril (manuscript under preparation)

References

1. Abalain-Colloc, M.L., Chastel, C., Tully, J.G., Bove, J.M., Whitcomb, R.F., Gilot, B., Williamson, D.L., 1987. *Spiroplasma sabaudiense* sp. nov. from Mosquitoes Collected in France. *Int J Syst Bacteriol* 37, 260–265. <https://doi.org/10.1099/00207713-37-3-260>
2. Adams, D.W., Errington, J., 2009. Bacterial cell division: Assembly, maintenance and disassembly of the Z ring. *Nat Rev Microbiol*. <https://doi.org/10.1038/nrmicro2198>
3. Ausmees, N., Kuhn, J.R., Jacobs-Wagner, C., 2003. The Bacterial Cytoskeleton: An Intermediate Filament-Like Function in Cell Shape peptidoglycan (or murein) (Vollmer and Holtje, *Cell*. [https://doi.org/https://doi.org/10.1016/S0092-8674\(03\)00935-8](https://doi.org/https://doi.org/10.1016/S0092-8674(03)00935-8)
4. Razin, S., 1978. The mycoplasmas. *Microbiol Rev* 42, 414–470. <https://doi.org/10.1128/mr.42.2.414-470.1978>
5. Charbon, G., Cabeen, M.T., Jacobs-wagner, C., 2009. Bacterial intermediate filaments : in vivo assembly , organization , and dynamics of crescentin *Genes Dev* 23, 1131–. <https://doi.org/10.1101/gad.1795509>
6. Cole, R.M., Tully, J.G., Popkin, T.J., Joseph, M., Al, C.E.T., Bacteriol, J., 1973. Morphology , Ultrastructure , and Bacteriophage Infection of the Helical Mycoplasma-Like Cultured from " Stubborn " Disease of Citrus 115, 367–386. <https://doi.org/https://doi.org/10.1128/jb.115.1.367-386.1973>
7. Domínguez-Escobar, J., Chastanet, A., Crevenna, A.H., Fromion, V., Wedlich-Söldner, R., Carballido-López, R., 2011. Processive Movement of MreB-Associated Cell Wall Biosynthetic Complexes in Bacteria. *Science* (1979) 333, 225–228. <https://doi.org/10.1126/science.1203466>
8. Garner, E.C., Bernard, R., Wang, W., Zhuang, X., Rudner, D.Z., Mitchison, T., 2011.

- Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in *B. subtilis*. *Science* (1979) 333, 222–225. <https://doi.org/10.1126/science.1203285>
9. Harne, S., Duret, S., Pande, V., Bapat, M., Béven, L., Gayathri, P., 2020. MreB5 Is a Determinant of Rod-to-Helical Transition in the Cell-Wall-less Bacterium *Spiroplasma*. *Current Biology* 30, 4753–4762.e7. <https://doi.org/10.1016/j.cub.2020.08.093>
 10. Jones, L.J.F., Carballido-López, R., Errington, J., 2001. Control of Cell Shape in Bacteria. *Cell* 104, 913–922. [https://doi.org/10.1016/S0092-8674\(01\)00287-2](https://doi.org/10.1016/S0092-8674(01)00287-2)
 11. Koch, M.K., McHugh, C.A., Hoiczyk, E., 2011. BacM, an N-terminally processed bactofilin of *Myxococcus xanthus*, is crucial for proper cell shape. *Mol Microbiol* 80, 1031–1051. <https://doi.org/10.1111/j.1365-2958.2011.07629.x>
 12. Ku, C., Lo, W.S., Kuo, C.H., 2014. Molecular evolution of the actin-like MreB protein gene family in wall-less bacteria. *Biochem Biophys Res Commun* 446, 927–932. <https://doi.org/10.1016/j.bbrc.2014.03.039>
 13. Kürner, J. et al, 2005. Cryo – Electron Tomography Reveals the Cytoskeletal Structure of *Spiroplasma melliferum* 436. <https://doi.org/10.1126/science.1104031>
 14. Lartigue, C., Lambert, B., Rideau, F., Dahan, Y., Decossas, M., Hillion, M., Douliez, J.P., Hardouin, J., Lambert, O., Blanchard, A., Béven, L., 2022. Cytoskeletal components can turn wall-less spherical bacteria into kinking helices. *Nat Commun* 13. <https://doi.org/10.1038/s41467-022-34478-0>
 15. Liu, Y., Van Den Ent, F., Löwe, J., n.d. Structure of crescentin Filament structure and subcellular organization of the bacterial 3 intermediate filament-like protein crescentin. <https://doi.org/10.1101/2023.06.04.543601>
 16. Löwe, J., Amos, L.A., 1998. Crystal structure of the bacterial cell-division protein FtsZ. *Nature* 391, 203–206. <https://doi.org/10.1038/34472>
 17. Pande, V., Mitra, N., Bagde, S.R., Srinivasan, R., Gayathri, P., 2022. Filament organization of the bacterial actin MreB is dependent on the nucleotide state. *Journal of Cell Biology* 221. <https://doi.org/10.1083/jcb.202106092>
 18. R. Townsend and D. B. Archer, 1983. A Fibril Protein Antigen Specific to *Spiroplasma*, *Journal of General Microbiology*. <https://doi.org/http://www.genesdev.org/cgi/doi/10.1101/gad.1795509>
 19. Sasajima, Y., Kato, T., Miyata, T., Kawamoto, A., Miyata, M., 2022. Isolation and structure of the fibril protein, a major component of the internal ribbon for *Spiroplasma* swimming Makoto Miyata Quick freeze replica electron microscopy, *Electron*

- tomography. 20. <https://doi.org/10.1101/2021.02.24.432793>
20. Sycuro, L.K., Pincus, Z., Gutierrez, K.D., Biboy, J., Stern, C.A., Vollmer, W., 2010. Peptidoglycan Crosslinking Relaxation Promotes *Helicobacter pylori* ' s Helical Shape and Stomach Colonization. *Cell* 141, 822–833. <https://doi.org/10.1016/j.cell.2010.03.046>
 21. Takahashi, D., Fujiwara, I., Sasajima, Y., Narita, A., Imada, K., Miyata, M., 2022. ATP-dependent polymerization dynamics of bacterial actin proteins involved in *Spiroplasma* swimming. *Open Biol* 12. <https://doi.org/10.1098/rsob.220083>
 22. van den Ent, F., 2001. Bacterial ancestry of actin and tubulin. *Curr Opin Microbiol* 4, 634–638. [https://doi.org/10.1016/S1369-5274\(01\)00262-4](https://doi.org/10.1016/S1369-5274(01)00262-4)
 23. van den Ent, F., Amos, L.A., Löwe, J., 2001. Prokaryotic origin of the actin cytoskeleton. *Nature* 413, 39–44. <https://doi.org/10.1038/35092500>
 24. Weisburg, W.G., Tully, J.G., Rose, D.L., Petzel, J.P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T.G., Van Etten, J., 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* 171, 6455–6467. <https://doi.org/10.1128/jb.171.12.6455-6467.1989>
 25. Whitcomb, R.F., French, F.E., Tully, J.G., Gasparich, G.E., Rose, D.L., Carle, P., Bove, J.M., Henegar, R.B., Konai, M., Hackett, K.J., Adams, J.R., Clark, T.B., Williamson, D.L., 1997. *Spiroplasma chrysopicola* sp. nov., *Spiroplasma gladiatoris* sp. nov., *Spiroplasma helicoides* sp. nov., and *Spiroplasma tabanidicola* sp. nov., from Tabanid (Diptera: Tabanidae) Flies. *Int J Syst Evol Microbiol* 47, 713–719. <https://doi.org/10.1099/00207713-47-3-713>
 26. Williamson, D.L., 1974. Unusual Fibrils from the Spirochete-Like Sex Ratio Organism, *Journal of Bacteriology*. <https://doi.org/10.1128/jb.117.2.904-906.1974>
 27. Williamson, D.L., Blaustein, D.I., Rhea, Levine, J.C., Elfvin, M.J., 1979. Current Microbiology Anti-Actin-Peroxidase Staining of the Helical Wall-Free Prokaryote *Spiroplasma citri*, *Current Microbiology*. <https://doi.org/10.1007/BF02605871>
 28. Yit, Q.-M., Rockenbach, S., Ward, J.E., Lutkenhaust, J., 1985. Structure and Expression of the Cell Division Genes *ftsQ*, *ftsA* and *ftsZ*, *J. Mol. Biol.* [https://doi.org/10.1016/0022-2836\(85\)90290-6](https://doi.org/10.1016/0022-2836(85)90290-6)

Chapter 1

Introduction

Eukaryotic organisms consist of three major families of cytoskeletal proteins- actin, tubulin and intermediate filaments. These proteins form extensive networks and long-range organization to support various functions like providing mechanical strength to carry out functions like cell division and motility. These large filaments span the entire cell length and provide scaffold for the cytoskeleton-based motor proteins. Till early 1990s, cytoskeleton was thought to exist in eukaryotic cells alone until the discovery of the first tubulin homolog in bacteria - FtsZ (Bi and Lutkenhaus, 1991). Soon after, a bacterial actin homolog called MreB was also identified (Bork et al., 1992). These findings led to further discovery of nucleotide independent filament forming proteins analogous to eukaryotic intermediate filaments. The following section talks about the discovery of some of the filament forming proteins present in prokaryotes, some of which share homology to eukaryotic cytoskeleton whereas few are unique to the prokaryotic system.

1.1 Bacterial Tubulin homologs

Tubulin performs essential functions like intracellular transport of cargo and cell division in eukaryotes. The heterodimer of $\alpha\beta$ tubulin forms the functional and structural subunit of microtubules. Tubulin consists of an N-terminal GTPase domain and a C-terminal activation domain. Polymerization occurs when the N-terminal of GTP bound α subunit interacts with the C-terminal catalytic domain of another dimer (Nogales et al., 1998). The GTP binding and catalytic pocket is highly conserved in FtsZ as well. Similar to tubulin, FtsZ polymerizes and forms filaments only in the presence of GTP (Mukherjee et al., 1993; Mukherjee and Lutkenhaus, 1994; de Boer et al., 1992; RayChaudhuri and Park, 1992). Other than FtsZ, TubZ and RepX form a diverse group yet belong to the tubulin superfamily (Larsen et al., 2007; Tinsley and Khan, 2006). Schematic below shows dimers and filament assembly of bacterial tubulin homologs (Figure 1.1).

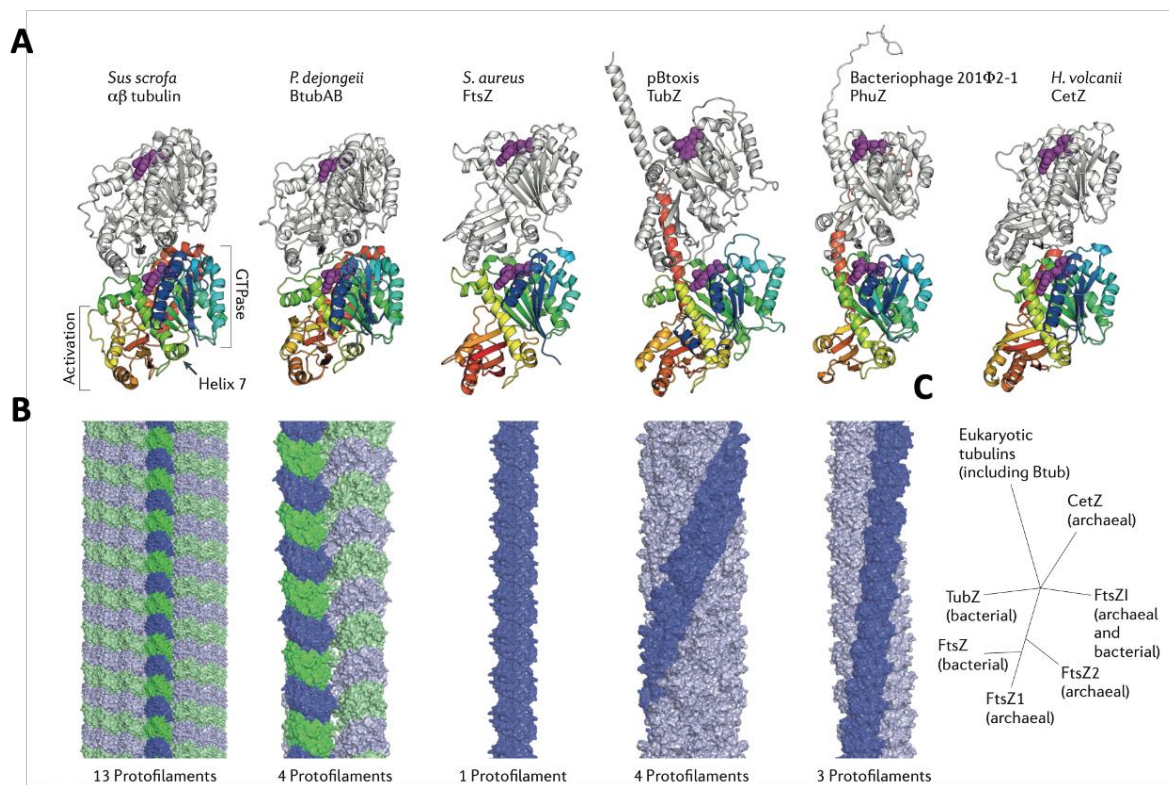


Figure 1.1. Tubulin superfamily. **A.** Dimers of different proteins belonging to tubulin superfamily. The lower subunit is colored as blue to red depicting amino terminal to carboxyl terminal. Purple spheres indicate the bound nucleotide. **B.** Filament assembly of diverse proteins from tubulin superfamily were obtained from high resolution X-ray crystallography or cryo electron microscopy (cryo-EM). Individual protofilaments in each of the filaments has been shown in darker shades. α subunits are colored blue whereas β subunits are shown in green. The number of protofilaments required to form the assembly is mentioned below each filament. **C.** Phylogenetic tree showing conservation among the tubulin family members across different kingdoms. $\alpha\beta$ tubulin (PDB entry 5SYF), BtubAB (PDB entry 5O09), FtsZ (PDB entry 3VOA), TubZ (PDB entry 3J4S), PhuZ (PDB entry 3J5V), CetZ (PDB entry 4B45). BtubAB, bacterial tubulins termed Btubs A and B; FtsZ, cell division protein. Adapted from (Wagstaff and Löwe, 2018).

1.1.1 FtsZ

FtsZ is the most common bacterial tubulin homolog found in many lineages of bacteria. It is one of the major components of the septum formed during the bacterial cell division. Similar to tubulin, FtsZ have been shown to specifically bind and hydrolyse GTP (Mukherjee et al., 1993; de Boer et al., 1992; RayChaudhuri and Park, 1992). The sequence similarity between FtsZ and Tubulin is only ~10% (Erickson, 2007; Vaughan et al., 2004). However, determination of crystal structures of both the proteins revealed that the proteins possess a similar fold (Löwe and Amos, 1998; ; Nogales et al., 1998). FtsZ forms a dynamic Z ring at

the septum and recruits other proteins involved in the divisome machinery. This leads to the constriction of the cell wall leading to the formation of daughter cells (Adams and Errington, 2009). Based on the sequence similarity between various FtsZ sequences, the cell division protein shows the presence of three distinct regions, an N-terminal region, a spacer region of variable length and a conserved C-terminal region. The N-terminal region is required for FtsZ-FtsZ interaction as well as proper localization during the cell division. The conserved C-terminal region of FtsZ has been shown to be essential for its interaction with an actin homolog, FtsA (Bi and Lutkenhaus, 1991; Din et al., 1998; Ma and Margolin, 1999; Yan et al., 2000).

FtsZ is commonly found in most of the bacterial species. However, it is not found in all prokaryotes. For instance, many archaeal species either possess a divergent form of FtsZ protein or completely lack *ftsZ* gene. In the absence of FtsZ, these organisms possess ESCRT III proteins as a part of their cytokinesis machinery (Lindås et al., 2008; Samson et al., 2008).

1.1.2 TubZ and RepX

Other than FtsZ, bacteria show the presence of other tubulin like proteins also, namely, TubZ and RepX. These proteins play an important role in maintaining the stability of plasmids in the cells. TubZ forms dynamic and linear polymers. These filaments are bidirectional with plus and minus ends. Similar to FtsZ, TubZ binds and hydrolyses GTP. The point mutation, D269A, in the conserved catalytic pocket within the T7 loop inhibits treadmilling and leads to the formation of stable filaments. Mutation in its GTPase domain thus, affects the polymerization inhibiting cell septation. Cells expressing wild type TubZ showed 100 % inheritance of the pBtoxin virulence plasmid. However, when TubZ::gfp was expressed in place of wild type TubZ, some cells lost the virulence plasmid. Hence, TubZ is also required for stable inheritance of pBtoxin virulence plasmid (Larsen et al., 2007).

Another distantly related protein to FtsZ is RepX found in *Bacillus anthracis* and related organisms. RepX plays an important role in replication of virulence plasmids in these organisms (Tinsley and Khan, 2006). As these proteins are encoded by the virulence plasmids, they serve as desirable candidates for drug targets.

1.2 Actin homologs in bacteria

Actin, hexokinase and heat shock proteins (hsp70), despite having unrelated functions and a low sequence similarity, share a conserved three-dimensional structure. The structures are divided into two domains, namely, I and II which are divided into Ia, IIa and Ib, IIb subdomains. Superimposition of these structures highlights a very well conserved ATP binding pocket between Ia and IIa domains. Amongst all the structures of these superfamilies, the larger domains, Ia and IIa seem to be highly conserved. However, differences were observed in Ib and IIb domains. This indicated that the unique fold of Ib and IIb domains might be responsible for specialized functions in the different subfamilies. Apart from the sugar kinases, bacterial cytoskeletal proteins like MreB, FtsA and ParM (earlier known as StbA) were also recognized to have similar folds (Bork et al., 1992) (Figure 1.2). Subsequent sections in this chapter will highlight the functions of some of the major bacterial actin homologs.

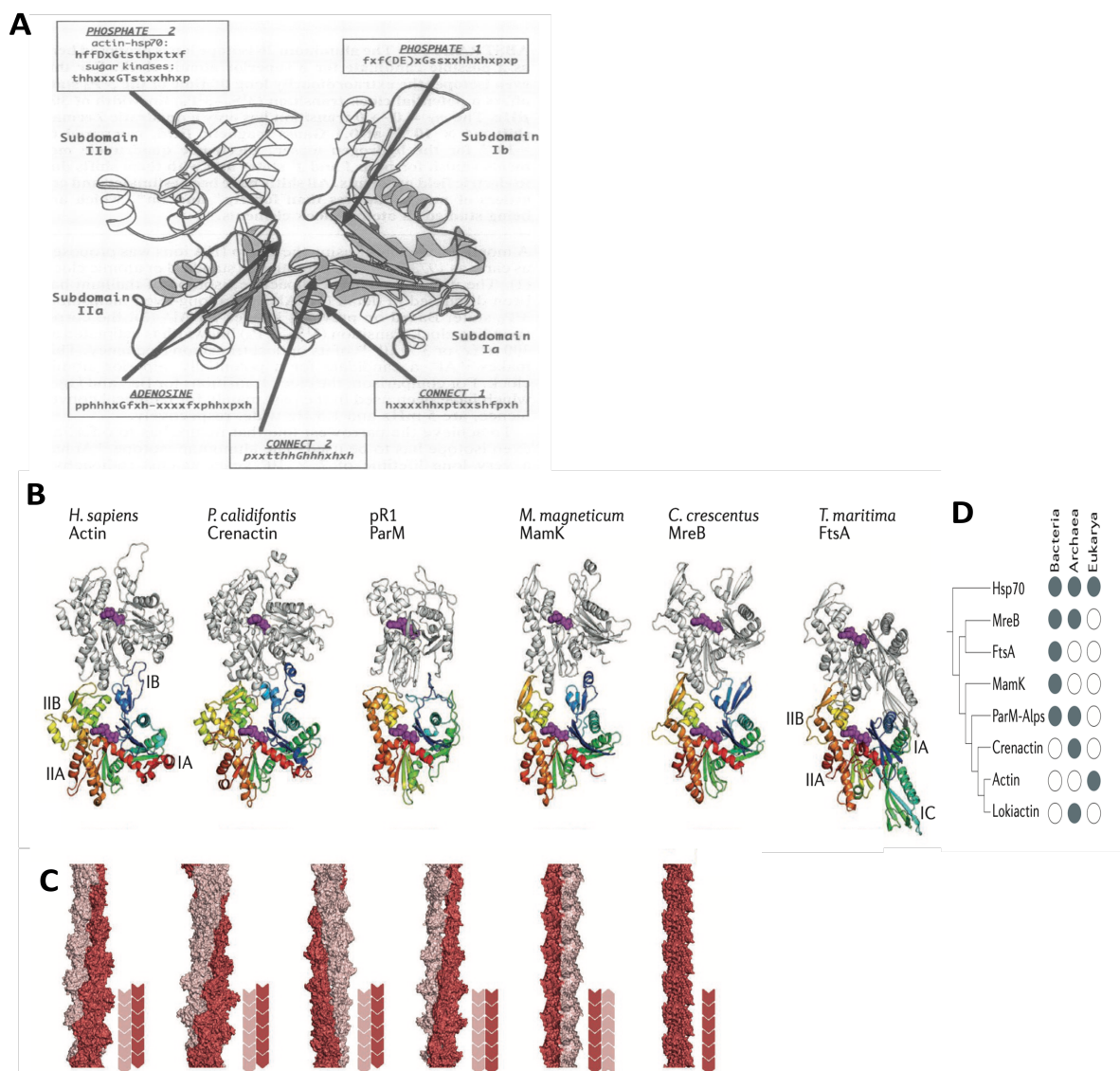


Figure 1.2. Actin superfamily **A.** Diagrammatic representation of actin monomer. The subdomains have been highlighted along with the signature motif sequence for each domain. These 5 regions of the structure are well conserved and characteristic of actin/hexokinase/hsp70 protein families. **B.** Pairs of monomers of various proteins belonging to the Actin superfamily are shown. The lower subunits have been colored blue to red from amino terminus to carboxyl terminus. Purple sphere denotes the bound nucleotide. Subdomains of the lower subunit have been highlighted for actin and FtsA. From the structures, an overall conservation can be seen for IA and IIA domains, but differences could be seen for IB domain. **C.** Individual protofilaments have been shown which give rise to filament assembly. These filament structures were obtained from high resolution X-ray crystallography or Cryo-EM structures. The cartoon represents the polarity of the protofilaments. **D.** Phylogenetic tree showing conservation between the Actin family amongst different kingdoms. Protein Databank (PDB) entries for actin structures: actin (PDB entry 5JLH), FtsA (PDB entry 5 LY4), MreB (PDB entry 5AEY), FtsA (PDB entry 5 LJV), MreB (PDB entry 4CZJ), FtsA (PDB entry 4A2B). Alps, actin-like proteins; Hsp70, heat shock protein 70; MamK, magnetosome protein; MreB, rod shape-determining protein; ParM, plasmid segregation protein. Adapted from (Bork et al., 1992; Wagstaff and Löwe, 2018).

1.2.1 FtsA

As discussed earlier, bacterial tubulin homolog FtsZ forms a cytokinetic ring at the division site. This leads to assembly of other components of the division machinery (Bi and Lutkenhaus, 1991). One of the major components of the divisome is a membrane binding protein called FtsA. Interaction between FtsZ and FtsA was established using yeast hybrid assay. Multiple sequence alignment of FtsZ from several species showed the presence of a conserved motif of 8-10 residues at C-terminus. Deletion of this motif of FtsZ led to disruption of the interaction between the FtsZ and FtsA. However, deletion of this motif had no effect on the interaction between FtsZ-FtsZ interaction. This proved that the C-terminus conserved motif acts as a linker domain and is essential for FtsZ-FtsA interaction (Din et al., 1998; Ma and Margolin, 1999; Yan et al., 2000). FtsA has been shown to bind ATP, however hydrolysis activity of the protein is not well conserved among the bacterial domain (Loose and Mitchison, 2014). Interaction between FtsZ and FtsA requires the presence of ATP, although no hydrolysis is required for this interaction (Beuria et al., 2009; Martos et al., 2012; Szwedziak et al., 2012). FtsA possess two important roles during the later stages of cell division- 1) tethering of FtsZ filaments to the membrane and 2) negative regulation of FtsZ filaments (Loose and Mitchison, 2014).

1.2.2 MreB

Another actin homolog, MreB is most commonly found in most of the non-spherical bacteria and is responsible for rod-shape formation of bacterial cells (Daniel and Errington, 2003; Jones et al., 2001; Wachi et al., 1987). Sequence similarity of MreB and actin from various species shows ~10% sequence identity. However, comparison between the 3-D structures highlight a similar fold (Bork et al., 1992). The filament assembly of MreB is also different from that of actin. As seen from the light scattering data, the critical concentration of *Thermotoga maritima* MreB is ~3 nM whereas actin shows a critical concentration of ~ 0.25 μ M which is 100-fold higher than MreB. When polymerization of MreB was monitored in the presence of ATP, a time lag was observed between the phosphate release and polymerization. This suggests that ATP hydrolysis might occur after MreB assembles into filaments (Esue et al., 2005). MreB and its paralogs play an important role in maintenance of rod shape in many bacterial species. This actin homolog and its paralogs are shown to be responsible for maintaining longitudinal as well as width of the cells (Jones et al., 2001). These proteins do so by interacting with peptidoglycan elongation machinery (PGEM) or with peptidoglycan binding proteins (PBPs) itself. When cells were treated with antibiotics targeting PG synthesis, the MreB filament motion was stalled. This indicates that PG synthesis drives the motion of MreB filaments (Domínguez-Escobar et al., 2011; Garner et al., 2011). The mode of function of MreB in different bacteria will be discussed in detail in subsequent sections.

1.2.3 MamK

Magnetotactic bacteria are motile bacteria which align along the geomagnetic field owing to the presence of its internal component known as magnetosomes. These magnetosomes consist of crystals of the ferrimagnetic iron mineral magnetite (Fe_3O_4) (Bazylinski and Frankel, 2004; Komeili et al., 2006; Scheffel et al., 2006; Schüler, 2004). These magnetosomes form chains of these crystals and act as a compass needle for the proper alignment of the bacteria. These organisms consist of 15-20 magnetite crystals of ~ 50 nm enclosed within a lipid bilayer (Gorby et al., 1988; Grünberg et al., 2001). Magnetosomes align in a straight line across the cell length. A network of long filaments ranging 200-250 nm run parallel to magnetosomes along the cell length. MamK, an actin-like protein, was identified as one of the filaments associated with magnetosomes. When the localization of MamK-GFP was checked in vivo, the filament aligned with the magnetosomes across the cell length. Deletion of this gene led to dispersed magnetosomes. This suggested that bacterial actin homologs may play a role in proper alignment of cell organelles (Komeili et al., 2006). Crystal structure of non-polymerizing mutant of MamK shows high similarity with actin and MreB (Löwe et al., 2016).

However, the helical arrangement of MamK filaments showed high similarity with that of actin as compared to the other bacterial homologs (Bergeron et al., 2017).

1.2.4 ParM and AlfA

Mitotic apparatus has been proved to be essential for the stable inheritance of chromosomes in eukaryotes. However, the existence of such apparatus was not known for the prokaryotic system. Elucidation of crystal structure of ParM revealed the similarity in overall fold with actin and MreB. ParM is a plasmid encoded protein required for segregation of plasmid R1, preventing loss of plasmids (Gerdes et al., 1985). ParM forms double helical protofilaments upon polymerisation whose repeat distance is 49 Å which is similar to 51 Å in MreB and 54 Å in actin (Holmes et al., 1990; van den Ent, 2002, 2001). The critical concentration of ParM is 2.3 µM compared to 0.1 - 0.2 µM of actin (Garner et al., 2007). It forms double filaments which bind to *parC*, a centromere-like region in R1 plasmid through a DNA binding protein called ParR (Salje and Löwe, 2008). ParM filaments polymerise bidirectionally with the plasmids attached to it on both the sides of a spindle formed of paired filaments (Gayathri et al., 2012). As ParM binds to ATP, hydrolysis of ATP leads to disassembly of the filaments once the newly replicated plasmids reach opposite cell poles (Campbell and Mullins, 2007).

AlfA: Actin like filament is another actin homolog responsible for plasmid segregation found in *Bacillus subtilis*. The plasmids were seen to be stably inherited by progeny cells at the exponential phase. To quantify the efficiency of segregation of plasmid pBET131, cells were grown on chloramphenicol and were allowed to sporulate. The resulting colonies were then recovered and grown in the absence of the antibiotic. The plasmid pBET131 was inherited by ~ 58% of the cells. This suggested that AlfA plays an essential role in the segregation of a low copy plasmid pBET131 during growth as well as sporulation events (Becker et al., 2006).

1.3 Intermediate filaments

The domain architecture of intermediate filaments (IF) is fairly conserved across eukaryotes (Herrmann and Aebi, 2004). IF proteins primarily consist of an N-terminal head domain followed by four α-helical coiled coil domains- 1A, 1B, 2A, 2B and a C-terminal tail domain. The coiled-coil domains are connected by short linker sequences (Figure 1.3). The N-terminal and C-terminal domains are highly variable across different IF families. These proteins form dimers through the coiled-coil region which further polymerize to form higher order meshwork. Bacteria shows the presence of IF proteins like crescentin, which appears to be analogous to

eukaryotic IF proteins as well as some IF proteins unique to bacterial species like Ccrps, bactofilins, PopZ and DivIVA (Lin and Thanbichler, 2013). Many of these bacterial coiled-coil rich proteins (CCRPs) form intracellular scaffolds which help in maintaining the cell shape.

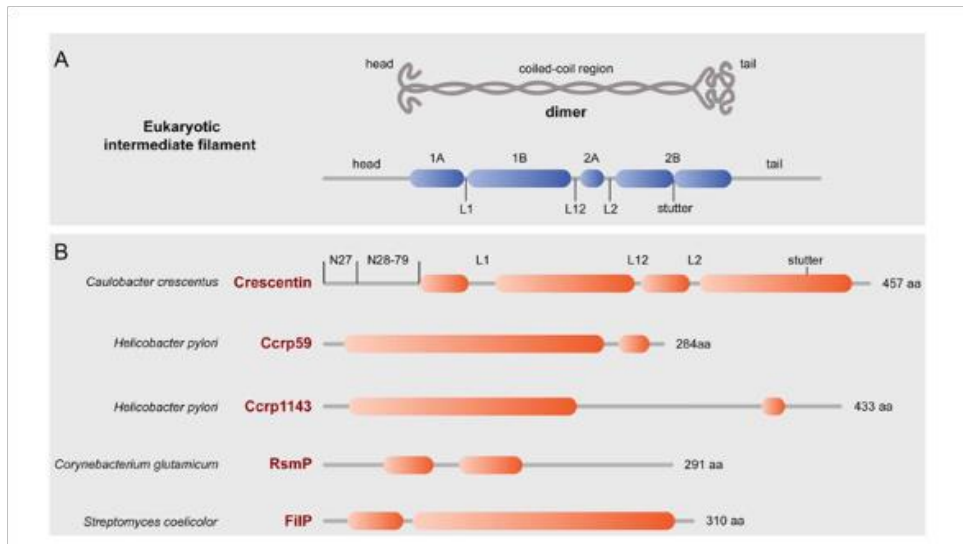


Figure 1.3. Intermediate filaments and bacterial coiled-coil rich proteins. **A.** Domain architecture of eukaryotic IF protein. Typical IF proteins consist of an N-terminal head domain followed by four coiled coil domains with linker regions between them (denoted as L1, L12, L2), a stutter and a C-terminal tail domain. **B.** Domain organization of bacterial coiled coil rich proteins. From the above figure, Crescentin shows presence of domain organization closest to eukaryotic IF protein. Adapted from (Lin and Thanbichler, 2013).

1.3.1 Crescentin

Bacteria exist in a wide range of cell shapes. To name a few, these organisms can attain defined morphologies like rod, curved or helical cells as well as exist as cells of pleomorphic shapes. Cell morphology studies were extensively done on rod shaped bacterial cells. In order to change a rod-shaped bacteria to curved or helical cells, additional proteins must be involved in determining these cell shapes.

Caulobacter crescentus is a Gram-negative bacteria which is widely studied for its asymmetric cell division. The characteristic shape of *Caulobacter crescentus* is due to a cytoskeletal protein called Crescentin (CreS). Earlier studies showed that deletion or mutations that render CreS non-functional, leads to rod shaped cells (Charbon et al., 2009a). *C. crescentus* is usually found in freshwater. The curved shape of *C. crescentus* is functionally important for its survival under hydrodynamic forces (Persat et al., 2014).

Later section of this chapter will talk about the function and crosstalk between crescentin and MreB in maintaining the morphology of *C. crescentus*.

1.3.2 Bactofilin

Caulobacter crescentus undergoes asymmetric cell division to give rise to swarmer and stalked cells. The swarmer cells are mobile whereas the stalked cells are immobile. Stalked cells immediately enter a new cycle of cell division; however, the swarmer cells differentiate into stalked cells to enter a new cell cycle. In order to identify the factors responsible for such differentiation, the genes which showed upregulation during the transition from swarmer to stalked cells were identified (McGrath et al., 2007). These genes were fused to a fluorescent protein to understand the localization pattern. It was found that the bactofilins (BacA and BacB) localised in the stalked pole and base. No fluorescent signal was observed in the swarmer cells. This suggested that bactofilins are required for polar morphogenesis. Bactofilin BacA structure was determined using solid state NMR. The BacA structure consists of a right-handed β helix with six windings and a hydrophobic core. BacA is the only protein consisting of β helical fold (Shi et al., 2015). Cryo-EM structure of non-polar filaments of *Thermus thermophilus* bactofilin was determined using modified nanobodies. These nanobodies prevented the bundling of the filaments making them suitable for structure determination using cryo-EM (Deng et al., 2019). Bactofilins also play a role in recruiting PbpC- a peptidoglycan synthase to the stalked pole. PbpC is predicted to play a role in the insertion of new cell wall material leading to longitudinal elongation of the stalk pole (Kühn et al., 2010). *Myxococcus xanthus*, a Gram-negative soil bacterium, shows the presence of four paralogs of bactofilin. One of the paralogs, BacM has a role in maintaining the wild-type morphology and shows resistance against antibiotics targeting cell wall synthesis machinery (Koch et al., 2011). CcmA protein belonging to the bactofilin family is found in helical bacteria, *Helicobacter pylori*. Deletion of this gene led to loss of characteristic helical morphology of the bacteria (Sycuro et al., 2010).

1.4 Cell morphology

Bacteria exist in several cellular forms and some of them tend to change their shape during their lifetime. The shape of the bacteria also changes depending upon the environmental cues (Young, 2006). Cell shape has also shown to affect translational velocity of the cells (Mitchell, 2002; Young, 2006). So, we can say that maintaining a cell shape is functionally relevant. Cell geometry is one of the important characteristics for the survival of any organism. It is also clinically relevant as cellular components involved in the maintenance of cell shape form a good target for various antibiotics to inhibit the proliferation of bacteria, especially pathogenic ones. In that case, it becomes interesting to understand the mechanism of cell shape maintenance in different bacteria. Following sections talk about the mechanism of cell shape

maintenance by peptidoglycan and about major cytoskeletal proteins in bacteria which help determine its cell morphology.

1.4.1 Peptidoglycan

Cell wall synthesis plays an important role in determining the shape of a bacterial cell. There exists a general scheme of cell wall synthesis which is present in bacteria with cell walls irrespective of the cell shape (Figure 1.1). Penicillin binding proteins (PBPs) form an important constituent of cell wall synthesis. Peptidoglycan consists of glycan chains crosslinked with many peptides. The two important glycans, namely N-acetyl-glucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc) are present in the cytoplasm and are linked by β -1, 4-glycosidic bonds. Various enzymatic reactions in the cytoplasm of the cell yield peptidoglycan precursors namely, UDP-GlcNAc and UDP-MurNAc-L-Ala-Y-D-Glu(L)-meso-diaminopimelic acid (DAP)-D-Ala-D-Ala(UDP-MurNAc-pentapeptide). These precursors are then transferred to a lipid carrier undecaprenyl phosphate to form Lipid I. GlcNAc from UDP-GlcNAc is added to Lipid I to form Lipid II which is then flipped across the inner membrane by an enzyme flippase. This flipping causes the disaccharide pentapeptide moiety to face the periplasm. The periplasmic space contains PG synthases namely transglycosylases and transpeptidases. Transglycosylases catalyse a reaction between the two glycans to form β -1, 4-glycosidic bonds. Transpeptidases help in the crosslinking between two pentapeptide chains. These synthases play a crucial role in activating the polymerization of lipid II into glycan strands and crosslink them to the existing PG (Link, 2017).

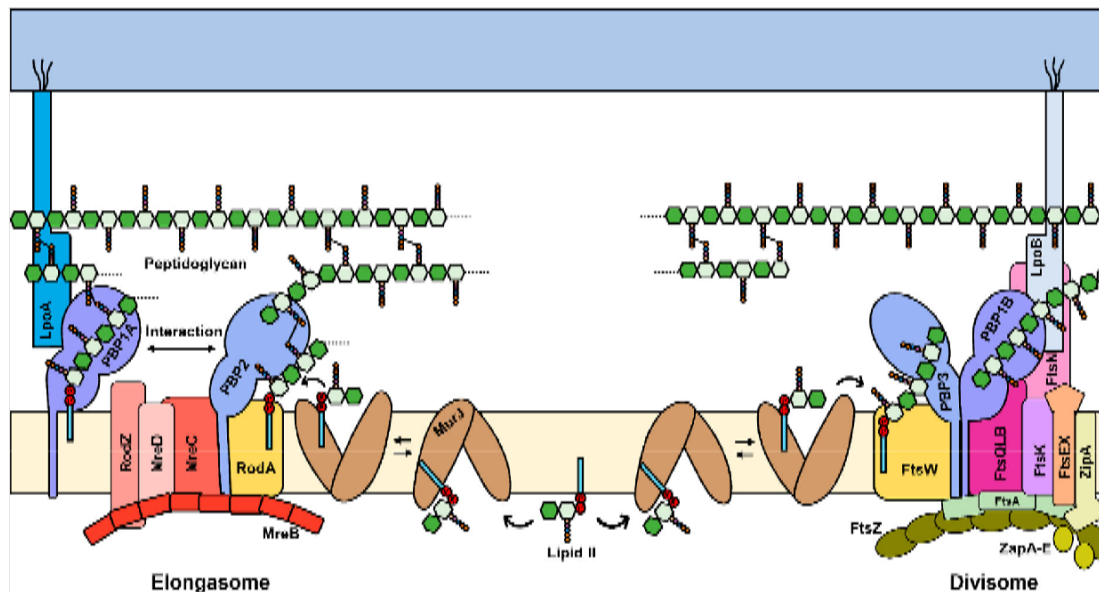


Figure 1.4. Major components of peptidoglycan biogenesis from *E. coli*. Peptidoglycan (PG) building blocks are added to the existing peptidoglycan by a protein complex termed as elongasome. This helps in increasing cell size along the long axis of the cell. During cell division, a new septal wall is formed by addition of new PG. The divisome complex facilitates the daughter cells to separate. The schematic shows two modes of peptidoglycan synthesis, one for elongation and the other for septation, during the cell cycle of a bacteria. Adapted from (Kumar et al., 2022).

In order to attain a particular shape, all bacteria must possess some additional scaffolding proteins that guide this peptidoglycan synthesis machinery. To understand this, the subsequent section will talk about three major cell morphologies namely, spherical, rod and curved shaped bacteria and the cytoskeletal assembly responsible for maintaining such morphology.

1.4.2 MreB dependent cell morphology

1.4.2.1 Rod shaped bacteria

In order to acquire a rod shape, there is a need for additional proteins that stretches the coccoid bacteria. Rod shaped bacteria have two ways of cell wall synthesis - septal and lateral. This section will explain the lateral cell wall synthesis in these bacteria. Most of the rod-shaped bacteria possess MreB, a bacterial actin homolog, that is responsible for cell shape determination. To check the effect of MreB on cell shape, *mreB* gene, from *B. subtilis* was replaced with *mreB* under xylose inducible promoter. The cells lost the rod shape and appeared distorted in the absence of the inducer (Jones et al., 2001) (Figure 1.5).

RodZ acts as an anchor protein for MreB to tether it to the membrane (Henriques et al., 1998). The N-terminal cytoplasmic domain of RodZ interacts with MreB whereas the C- terminal periplasmic domain interacts with RodA, MreC, MreD and other proteins of cell wall synthesis machinery (Berkeley and Christian, 2016; Gerdes, 2009; Morgenstein et al., 2015). This implies that RodZ anchors MreB to peptidoglycan elongation machinery (PGEM) that mediates the dynamics of MreB.

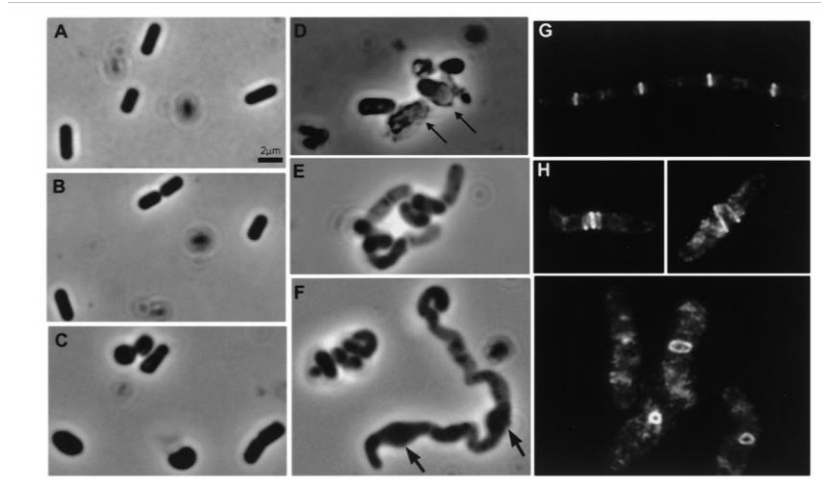


Figure 1.5. Phase contrast images showing effects of mutations in *mreB* and *mbl* in *Bacillus subtilis*. **A.** Wild-type cells showing rod shape. **B-D.** Effect of MreB depletion on cell morphology. *mreB* gene was cloned under xylose inducible promoter. Cells were grown in the absence of an inducer and images were captured at different time points. Cells were recovered for visualization under Phase contrast microscopy after 5 (B and C) and 6 (D) culture doubling times. **E-F.** Effect of disruption of the *mbl* gene led to loss in control over the linear axis of the cells. **G-H.** Cell division protein, FtsZ was immunolabeled. **G.** FtsZ localization in wild-type cells. **H.** Assembly of FtsZ was seen to be disrupted in *mbl* mutant cells. Loss of Mbl was thought to affect proper positioning of cell wall precursors which in turn leads to failure of cell division. Adapted from (Jones et al., 2001).

Earlier it was reported that MreB forms helical filaments and guides peptidoglycan synthesis machinery along the entire length of the cell, resulting in rod shape (Daniel and Errington, 2003). Total internal reflection fluorescence microscopy (TIRFM) studies in *Bacillus subtilis* have shown the relation between MreB and RodA, RodZ, PBPs (Brown et al., 2012; Gerdes, 2009). Deletion of RodA, RodZ and PBPs inhibit MreB dynamics (Figure 1.6).

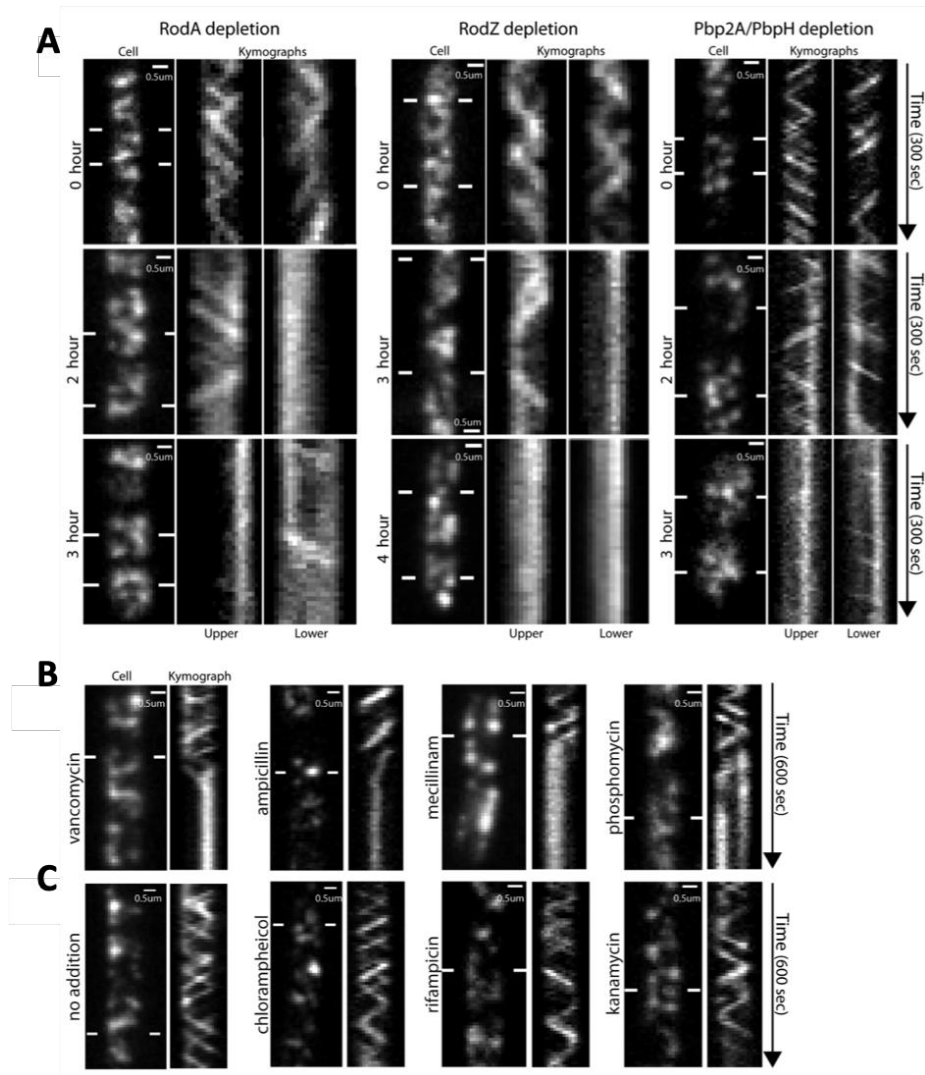


Figure 1.6. Cell wall synthesis machinery drives filament motion. **A.** To test the effect of PGEM (Peptidoglycan elongation machinery) on the filament dynamics of MreB and its paralogs Mbl and MreBH, the motion of GFP-Mbl was monitored in the presence and absence of three components of PGEM. The PGEM genes- *rodA*, *rodZ* and *pbp2A/pbpH* were each cloned under IPTG (isopropyl- β -D- thiogalactopyranoside) inducible promoter. All the strains were grown in a media containing IPTG and then transferred to a media lacking IPTG. Images for the cells were captured at the time intervals specified on the left of each image. Kymographs showing the effect of RodA, RodZ and Pbp2A/PbpH depletion on GFP-Mbl dynamics. As the proteins depleted over time, Mbl filaments appear motionless. **B.** Antibiotics were added in the agar pads used for growing the strains. Antibiotics targeting cell wall synthesis machinery were seen to stop filament dynamics within a time period of 10-30 seconds. **C.** Antibiotics that do not target cell wall synthesis machinery were seen to have no effect on GFP-Mbl dynamics. This suggested that the effect seen was specific to the components of PGEM interacting with MreB and its paralogs. Adapted from (Garner et al., 2011).

MreB has been shown to regulate peptidoglycan synthesis which enables bacterial cells to

elongate bidirectionally. There is a differential localization of MreB in the cells which has been attributed to its oligomeric state and specific interaction with lipids in the cell membrane. Anionic phospholipids have been shown to be enriched at the cell poles of *E. coli*. MreB interacts with anionic phospholipids in monomeric or disassembled state. However, in the presence of ATP, anionic phospholipids exclude MreB from cell poles which leads to its localization to the cylindrical portion of the cell (Kawazura et al., 2017; Shiomi, 2017). This differential localization of MreB helps maintain a rod shape in *E. coli*.

1.5 Maintenance of cell shape -independent of MreB

1.5.1 Coccoid cells

Spherical shape of bacteria is said to be the simplest of all morphologies in bacterial cells in terms of lowest energy state. Rod and curved shaped bacteria show two types of cell wall synthesis- septal and lateral. Similarly, there must be a mechanism which elongates or increases the surface area of spherical cells prior to cell division. In that case there should be a cue that promotes peptidoglycan synthesis over the entire volume of the coccoid bacteria so that the daughter cells retain the parental cell shape and size.

Some reports suggest that coccoid cells lack dedicated elongation machinery and new cell wall synthesis occurs only at the septum which forms at the site of division (Pereira et al., 2016; Pinho and Errington, 2003). To prove this hypothesis, the site for incorporation of new cell wall precursors was studied.

To visualize the site of nascent cell wall, vancomycin labelling was used. Vancomycin is an antibiotic that forms hydrogen bonds with the terminal D-ala-D-ala moieties of NAG-NAM. The position of labelling was similar and undistinguishable to the septum formed by FtsZ during cell division (Figure 1.7A). To validate this result, localization of GFP-PBP2 was studied by immunofluorescence. PBP2 is the only *Staphylococcus aureus* protein that possesses both transpeptidase and transglycosylase activity. The localization pattern of PBP2 was similar to that of FtsZ, indicating that the new cell wall synthesis in these bacteria is FtsZ dependent. The parent cell divides symmetrically to give two hemispherical daughter cells. PG synthesis in each daughter cell then pushes the hemispherical wall to yield spherical cells. However, FtsZ mutants led to the delocalization of FtsZ and the cell shape was also distorted (Figure 1.7 B-D). However, normal peptidoglycan synthesis was seen even in the absence of FtsZ. In this case, the PG precursors were seen to be dispersed over the entire volume of the coccoid cells (Leaver and Errington, 2005; Morgenstein et al., 2015).

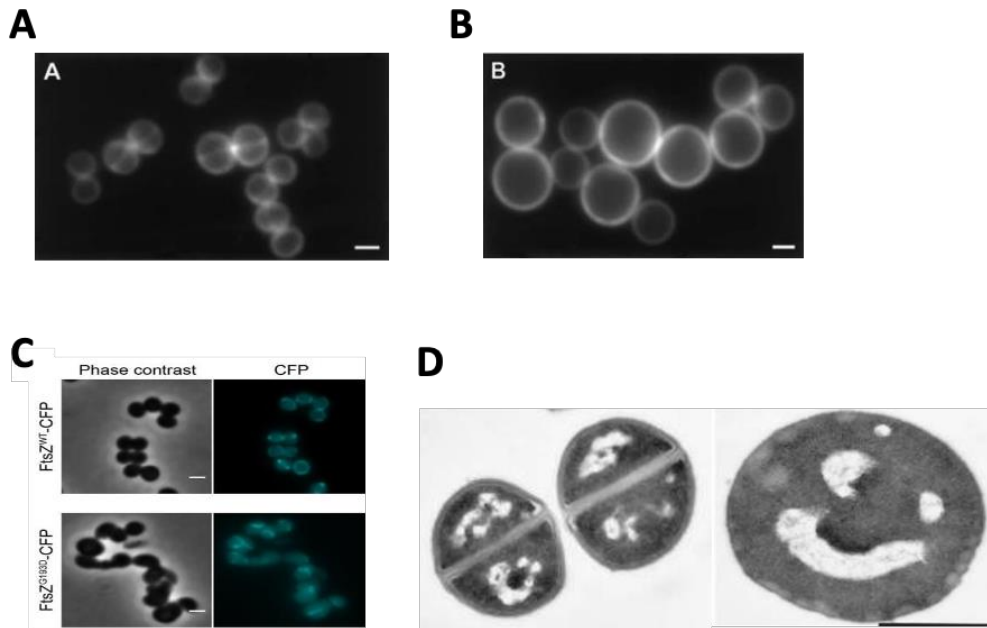


Figure 1.7. FtsZ dependent cell elongation in coccoid bacteria. **A.** Vancomycin-FL labelling was done to identify the site of nascent cell wall in *S. aureus*. New cell wall synthesis is seen at mid-cell. **B.** Vancomycin-FL labelling done in Δ ftsZ mutants of *S. aureus*. Adapted from (Pinho and Errington, 2003). **C.** Left image- Phase contrast microscopy shows the morphology of wild type and cell division mutant FtsZ^{G193D} *S. aureus* cells. FtsZ^{G193D} mutant was isolated during the screening of *S. aureus* strains resistant to the antibiotic PC190723. **D.** Transmission electron microscopy images of wild type and FtsZ^{G193D} cells. Scale bar 1 μ m. Adapted from (Pereira et al., 2016; Pinho and Errington, 2003).

RodA-PBP3 and FtsW-PBP1 have been shown to form cognate pairs which are essential for maintaining the spherical shape of the cells. Depletion of these proteins lead to the formation of multiple divisome sites and incorporation of peptidoglycan at the lateral walls of the cell, leading to the elongated cells (Reichmann et al., 2019).

1.5.2 Coccoid bacteria carrying MreB gene

MreB is known to be present in most of the rod-shaped bacteria and MreB filaments assembled along the cell length and excluded from the cell poles. This ensures incorporation of new peptidoglycan along the longitudinal axis of the cells (Kawazura et al., 2017). However, there exist bacteria which have spherical morphology and still show the presence of *mreB*, *mreC* and *mreD*. *Anabena* sp. PCC 7120 shows the presence of *mreBCD* genes that are transcribed as an operon. Inactivation of *mre* genes led to irregular cell shape and also made the cells more susceptible to lysozyme (Figure 1.8).

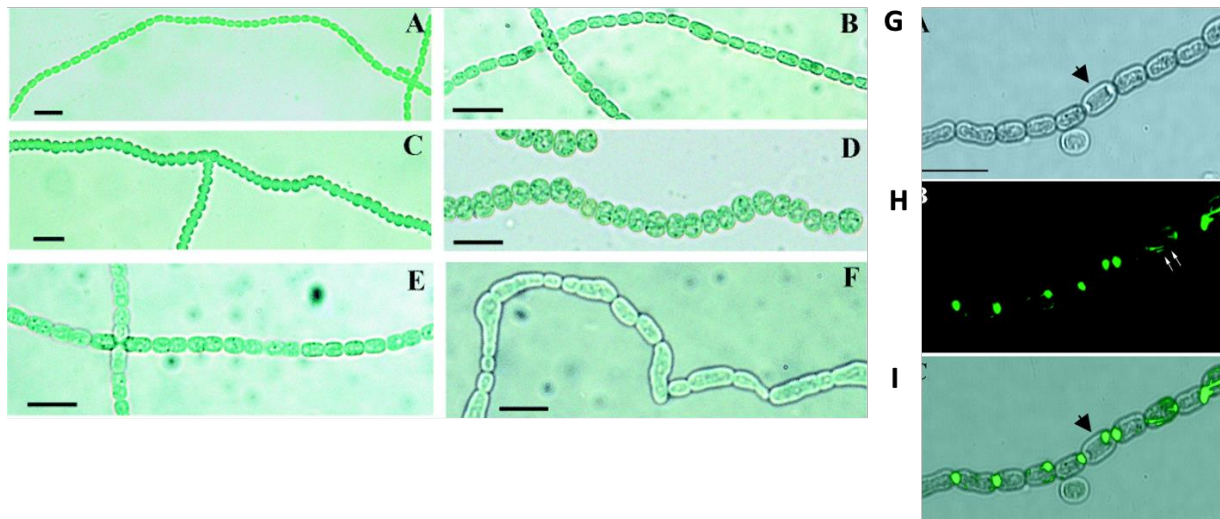


Figure 1.8. Microscopic images of the *Anabaena* sp. PCC 7120. A and B. The wild type cells observed under (40 $\times$) and (100 $\times$) magnifications respectively. C and D. Cells lacking *mreB* were observed at (40 $\times$) and (100 $\times$) magnifications respectively. E and F. Images of the strain complemented with *mreBCD* under control of the *petE* promoter was induced with 0.2 μ M (E) and 2 μ M (F) copper in growth medium respectively. Scale bars: 5 μ m. Localization of GFP tagged MreB in *Anabaena* sp. PCC 7120 using G. Bright field, H. GFP fluorescence and I. merged image of bright field and fluorescence. GFP fluorescence is mostly seen located at the poles. Scale bar 5 μ m. Adapted from (Hu et al., 2007).

Another spherical bacteria *Prochlorococcus* spp. contains *mreB* and *mreC* but lack *mreD*. This suggests that absence of *mreD* prevents formation of rod shape in this organism (Hu et al., 2007; Springstein et al., 2020).

1.5.3 Rod shaped bacteria lacking MreB

Certain alpha -proteobacteria bacteria persist as rod shaped organisms even in the absence of MreB. These include *Rhizobium*, *Agrobacterium* and *Actinobacteria* (Pinho et al., 2013). The proposed model for these bacteria shows unidirectional cell growth (Figure 1.9). *Sinorhizobium melitoli* has been shown to grow in a unipolar elongation manner as well (Krol et al., 2020).

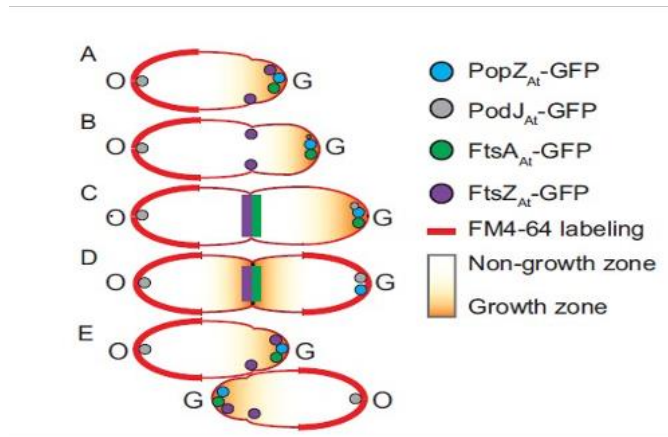


Figure 1.9. Proposed model for cell growth in *Agrobacterium tumefaciens*. Polar localization of PopZ and PopJ tends to decide the site of cell growth. Adapted from (Berkeley and Christian, 2016).

PodJ plays a crucial role in various cellular processes in this organism including maintenance of cell morphology. Δ PodJ mutants in *A. tumefaciens* exhibit ectopic growth and a reduction in cell division. GFP tagged PopZ in *A. tumefaciens* shows localisation at the growing poles and PodJ at the old poles. Hence, these proteins might act as spatial regulators for guiding the PG synthesis machinery (Anderson-Furgeson et al., 2016; Grangeon et al., 2015).

New PG synthesis was observed at the new pole and mid-cell (Figure 1.10A). Spatial localization of FtsZ-eGFP was monitored over a period of 2 hours. A signal was observed at the mid-cell at the junction of regions that formed new and old poles (Brown et al., 2012) (Figure 1.10B).

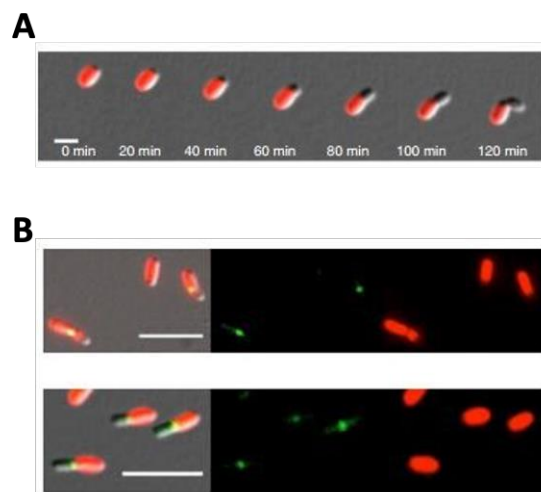


Figure 1.10. *Agrobacterium tumefaciens* growth pattern seen in cells labelled with Texas red succinimidyl ester (TRSE). A. Unlabeled portion indicates the site of new cell wall synthesis. Scale bar 2 μ m. B. 2-hour chase experiment of FtsZ-eGFP localization in TRSE labelled *A. tumefaciens*. The top image shows cell wall synthesis at time zero. Bottom image shows a nascent cell wall at one of the poles and at mid-cell. Adapted from (Brown

et al., 2012).

These observations conclude that, in *A. tumefaciens* new cell wall synthesis takes place at one of the poles and at the mid-cell division site in order to maintain the size of the daughter cell (Brown et al., 2012). However, what drives PopZ and PodJ localization to the poles and how it guides the peptidoglycan machinery to only one of the poles is still unanswered.

1.6 Maintenance of cell shape - Crosstalk between MreB and bacterial intermediate filament-like proteins

1.6.1 Curved shape bacteria

A curved shape cell can be generated with an asymmetric incorporation of peptidoglycan precursors to its walls. Some proteins in such bacteria may twist/bend the cell wall to give rise to a curvature. They may provide mechanical force or spatially prevent the incorporation of PG precursors. To validate the actual mechanism, we can look at two curved shaped bacteria.

The following schematic figure shows the cell cycle of *Caulobacter crescentus* (Figure 1.11A). To identify the cell shape determining factor in curved shaped bacteria like *C. crescentus*, transposon insertion was used as a method to isolate the mutants that gave altered cell shapes (Figure 1.11B). Such cells were isolated and sequenced. The gene was designated as *creS* encoding crescentin protein which is a coiled coil rich protein. When sequence similarity of crescentin was compared with the eukaryotic IF proteins, a considerable similarity was observed. The most common characteristic of IF proteins is formation of filaments measuring 10 nm diameter without the presence of any nucleotide or a cofactor. Crescentin too has been shown to form higher order oligomers in vitro without the need of any nucleotide or a cofactor (Ausmees, 2006). The domain organization of crescentin and its eukaryotic counterparts show conservation of domain organization (Figure 1.11C).

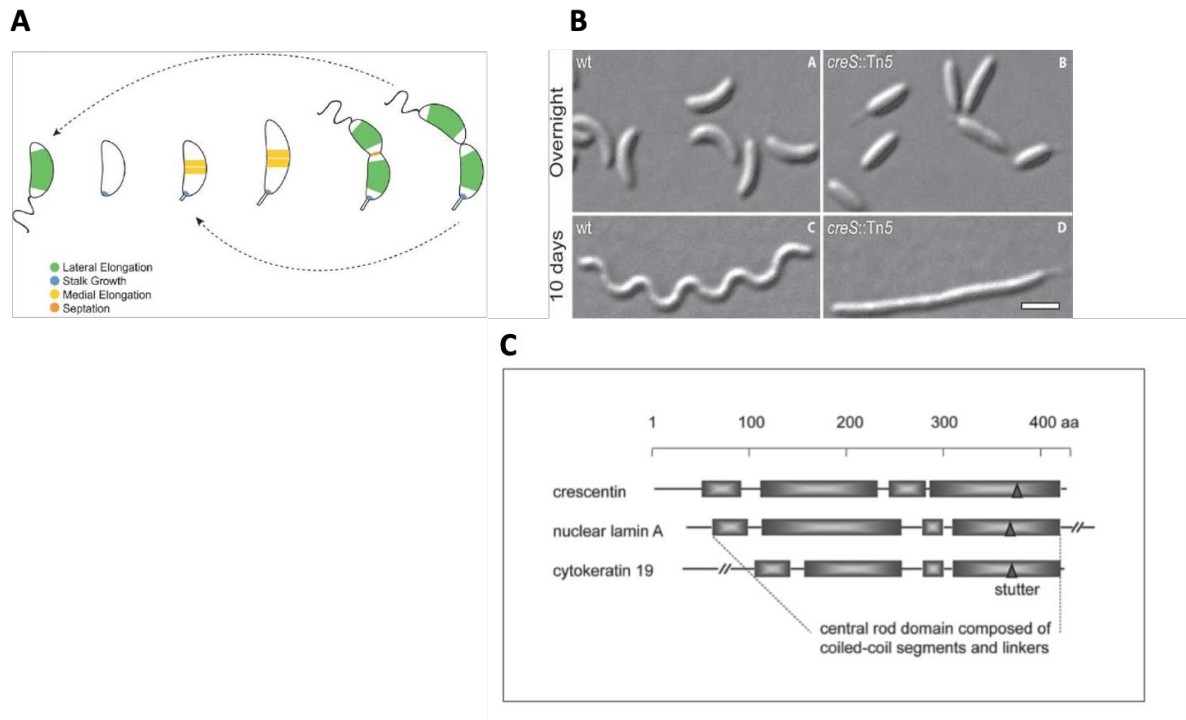


Figure 1.11. Crescentin is responsible for curved morphology in *Caulobacter crescentus*.
A. Schematic diagram showing the cell cycle of *C. crescentus*. Swarmer cell is shown to divide and give rise to a stalked cell. Adapted from (Randich and Brun, 2015). **B.** Phase contrast images of $\Delta creS$ phenotypic effect in *C. crescentus*. Adapted from (Ausmees, 2006).

Recent structure of crescentin validates this observation. Determination of crescentin structure using cryo-EM shows the presence of two intertwined strands consisting of four CreS dimers each. These coiled coil structures form polar filaments. Both these protein structures tend to show presence of a single secondary structure. The structure further proves that crescentin shows similar coiled coil dimer formation and possesses no overall polarity (Liu et al., 2023).

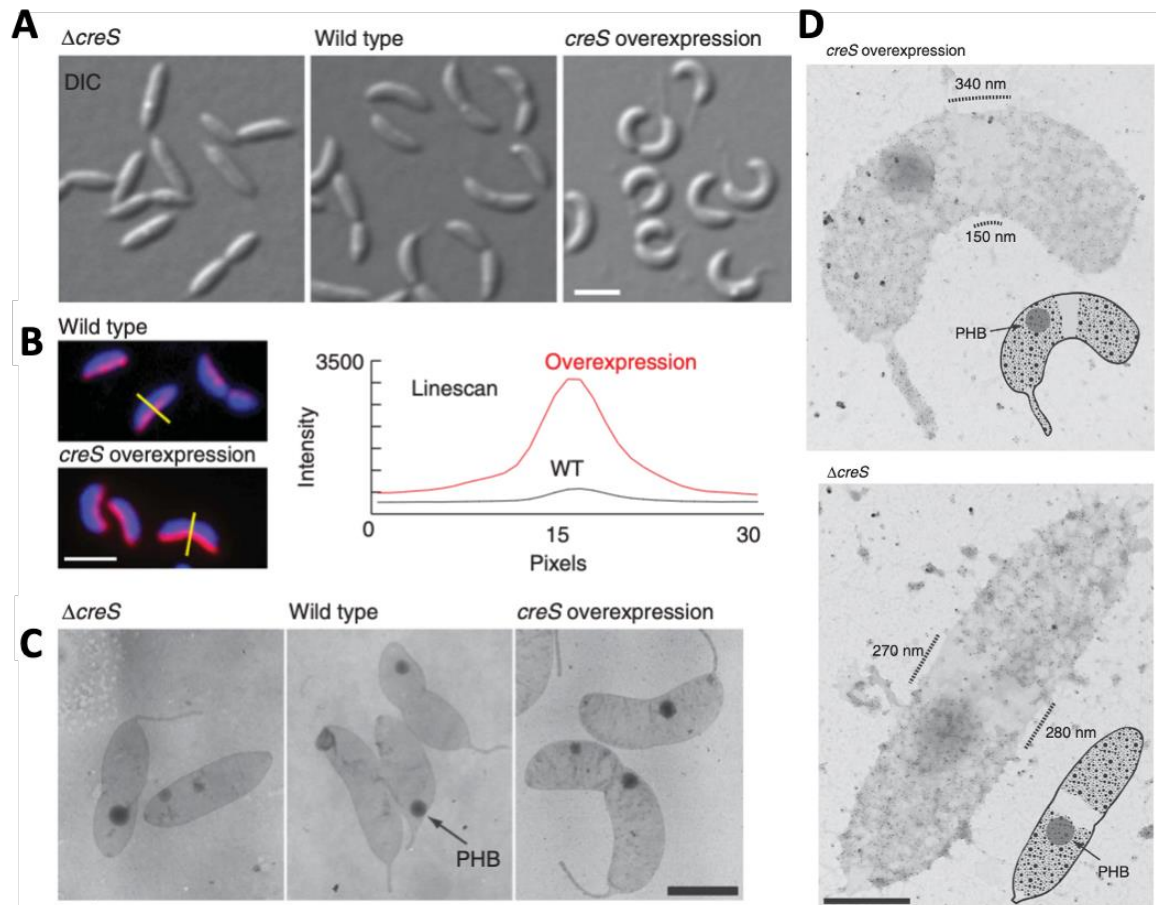


Figure 1.12. Differential PG insertion at inner and outer surface of *C. crescentus*. **A.** Cells lacking the *creS* gene, wild-type levels of crescentin, overexpression of crescentin. Scale bar 2 μm . **B.** Cells stained with 4',6-diamidino-2-phenylindole (DAPI) (blue) and crescentin were stained with anti-crescentin immunolabel (red) shows localization of crescentin. The micrograph shows fluorescence values across the yellow line. Scale bar 2 μm . **C.** Transmission electron microscope images of negatively stained cells lacking crescentin, wild-type levels and overexpressing crescentin. Scale bar 1 μm . **D.** Transmission EM images of peptidoglycan sacculi from a *creS*-overexpressing strain and a $\Delta creS$ strain carrying an empty vector. The cells were grown in D-Cys for 90 minutes and then imaged to visualize the regions of new peptidoglycan insertion. D-Cys residues were biotinylated, the sacculi were labelled with anti-biotin and gold-coupled anti-rabbit antibodies. The central part of the cells lacking the gold label indicates the regions of new peptidoglycan insertion. Measured lengths of the cleared region on each side of the sacculus are shown with broken lines. The sacculi of the cells showed presence of polyhydroxybutyrate granules (PHB). Scale bar 0.5 μm . Adapted from (Charbon et al., 2009).

To visualize the localization of crescentin, a merodiploid strain of *C. crescentus* (CJW815) was made carrying a chromosomal copy of each *creS* and *creS-gfp*. Fluorescence microscopy revealed that crescentin shows asymmetric localization (Ausmees et al., 2003). Transmission

electron microscopy images showed that there is a differential gradient of PG incorporation in the outer and inner curvature of the cells. More of the PG incorporation was seen at the outer layer (Figure 1.12). This leads to the curved morphology of the cells (Charbon et al., 2009). MreB plays a critical role in the adherence of crescentin to the inner cell wall. The *mreB* null mutants showed distorted morphology. The detached crescentin converted cells from stretched form to relaxed helical form. Also, during the course of cell growth, this coiled coil structure was inherited by only one of the daughter cells (Ausmees, 2006).

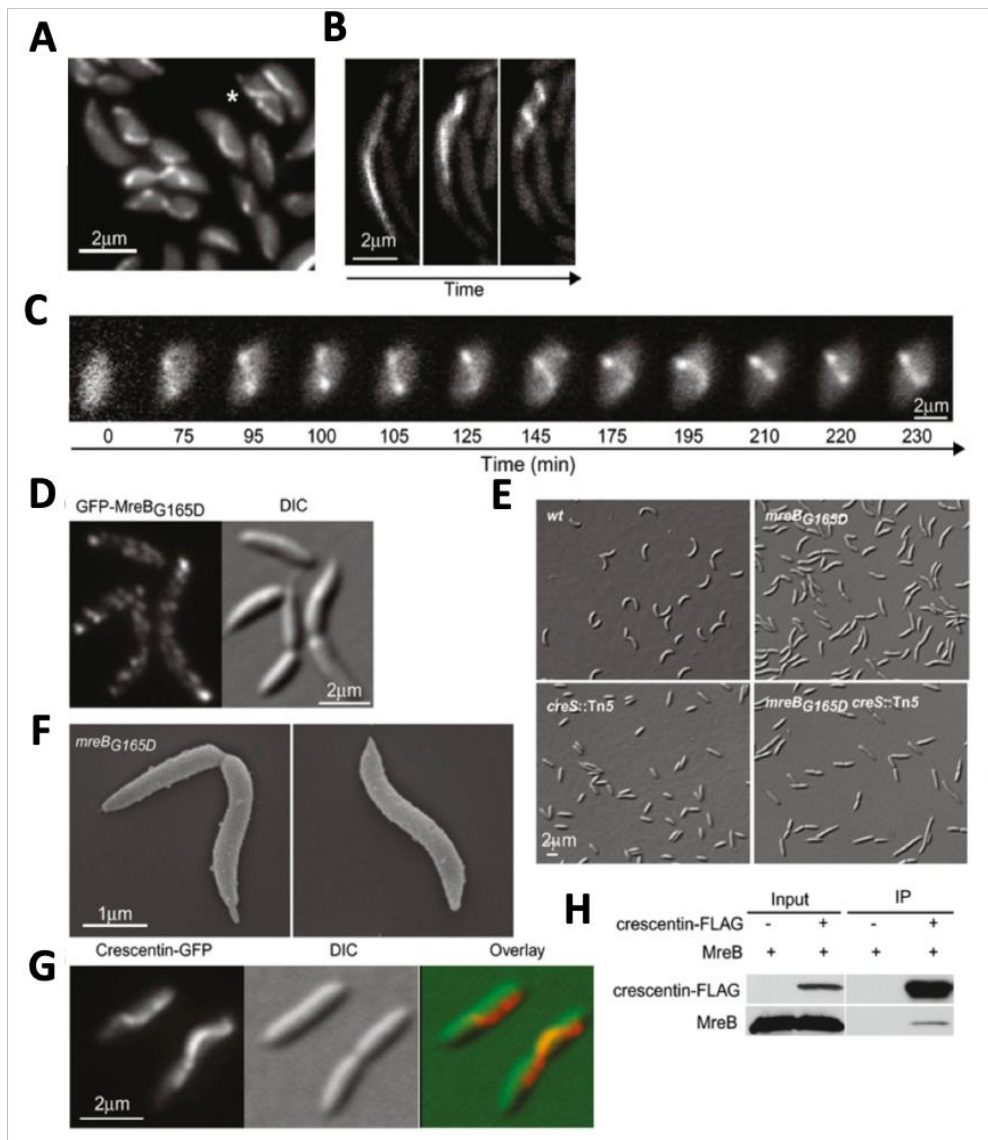


Figure 1.13. Proper localization of crescentin requires MreB. **A.** Cells expressing crescentin and crescentin-GFP were treated with A22. A22 has been shown to disrupt MreB filament assembly by binding near the nucleotide binding site of MreB. **B.** Time lapse imaging of cells expressing crescentin and crescentin-GFP when *ftsZ* was depleted. *FtsZ* was expressed under xylose- inducible promoter. The expression of *FtsZ* was restored after 3 hours and the cells were treated with A22 to disrupt MreB localization. $\Delta creS$ strain of *C. crescentus* was grown in a medium containing A22. The expression of crescentin-GFP was slowly

induced to visualize the localization of crescentin. **D.** Fluorescence and DIC micrographs of cells expressing GFP- MreB^{G165D}, an MreB mutant that is unable to assemble as rings at the mid-cell during cell division. **E.** DIC images of cells expressing wild-type, GFP-MreB^{G165D}, *crec*::Tn5 and GFP- MreB^{G165D} *crec*::Tn5. **F.** Scanning electron microscopy images of GFP-MreB^{G165D}. **G.** Mutant cells of *C. crescentus* expressing crescentin-GFP. **H.** Crescentin-Flag coimmunoprecipitation with MreB using anti-FLAG antibodies. Adapted from (Charbon et al., 2009).

Crescentin forms a stretched coiled coil structure at the concave side of the cell and prevents the PG precursors from getting incorporated at the site of its attachment to the cell wall. *C. crescentus* cells when treated with A22 drug disrupted MreB filaments. It was observed that disruption of MreB filaments led to detachment of crescentin from the cell membrane of the cells (Figure 1.13). Crescentin could polymerize when the cells were pretreated with A22. However, the filaments could not attach to the cell membrane (Charbon et al., 2009). This indicates that MreB may act as an anchor that helps crescentin to interact with penicillin binding proteins.

CreS binds to one of the walls of the cell and provides a curvature. This side of the cell surface then becomes the concave surface. It is unclear as to how crescentin decides which side of the cell to bind. Also, there must be a regulation for the extent of curvature on both the sides of the cell.

1.6.2 Helical bacteria

Helicobacter pylori is a Gram-negative pathogenic bacteria. The helical shape of this bacterium is important for its pathogenicity. The helical shape is required to penetrate the lining of the stomach. The proteins which give *Helicobacter* its helical shape include three LytM peptidoglycan endopeptidases (Csd 1-3) and CcmA. Deletion of LytM peptidoglycan endopeptidase homologs (Csd 1-3) *csd1*, *csd2* and *ccmA* led to curved rod cells (Sycuro et al., 2010). *csd3* deletion mutants resulted in varying degrees of cell curvature (Figure 1.14).

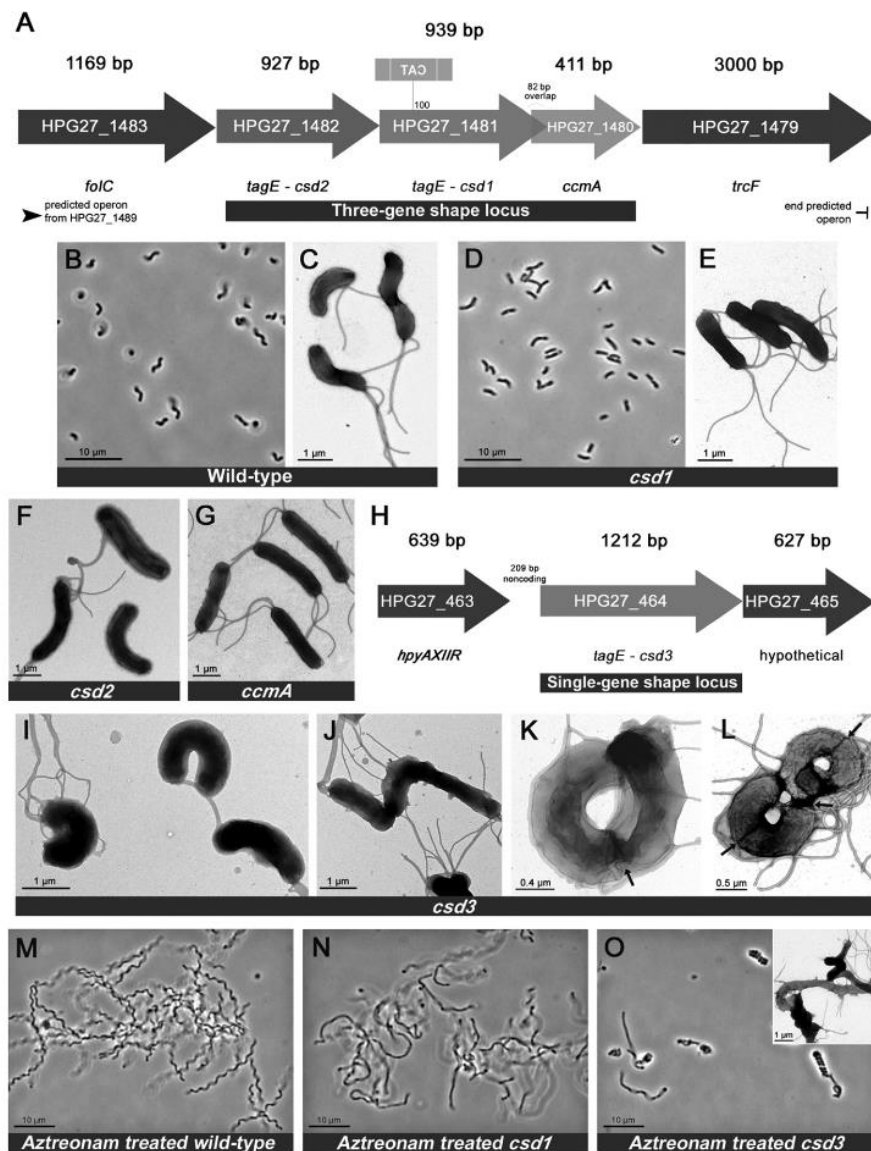


Figure 1.14. Csd and CcmA deletion mutants show defects in the morphology of *Helicobacter pylori*. **A.** Gene arrangement of *csd1*, *csd2* and *ccmA*. Insertion of the transposon encoding chloramphenicol acetyltransferase (*cat*) caused rod shaped mutants. Insertion site of the transposon occurred approximately 100 bp into the *csd1* gene. **B-G.** Phase contrast and transmission electron microscopy (TEM) images of wild-type and curved shape mutant cells. **H)** locus containing *csd3* as a single gene. **(I-L)** TEM images of cells expressing single mutant *csd3*. **I and J.** *csd3* mutant cells were elongated and preparing to divide **K and L.** Arrow indicates the site of septal formation. **M-O.** Phase contrast and TEM images (inset) of wild-type and mutant cells treated with the filamentation inducing drug aztreonam. Adapted from (Sycuro et al., 2010).

Helicobacter elongasome machinery shows three PBPs- Class A PBP1, Class B-PBP2 and PBP3. Yeast two hybrid assay shows that PBP2 interacts with MreC but not with PBP1 and

PBP3 (Sycuro et al., 2010). To visualise the phenotypic effect of MreC depletion in cells, the wild type gene was replaced by MreC under IPTG inducible promoter. In the absence of IPTG, the cells turned coccoid. This shows that MreC has a role to play in cell shape determination (Figure 1.15).

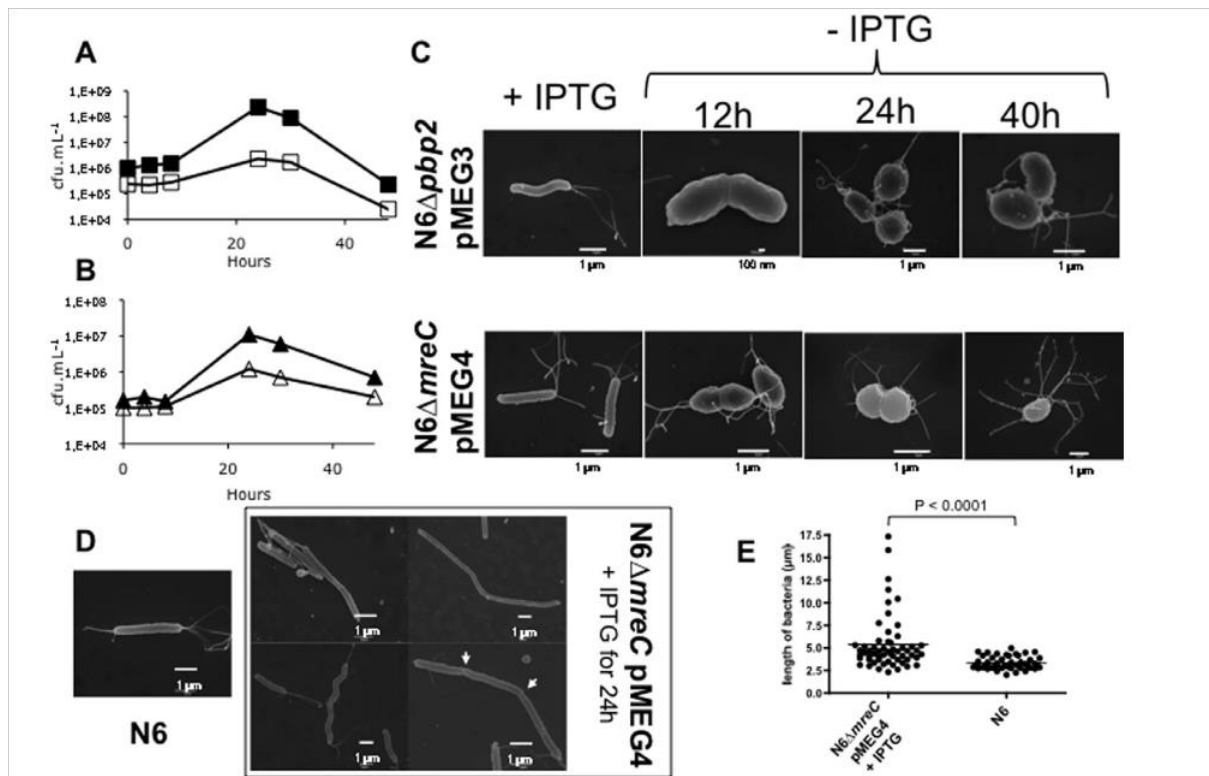


Figure 1.15. Effect of depletion of Pbp2 and MreC on the cell morphology. Viability of the cell was estimated for cells expressing **A.** *mreC* and **B.** *pbp2* in the presence (filled) and absence (open) symbols of IPTG. **C.** SEM images of the cells at different time intervals of IPTG depletion. Both the mutants formed coccoid cells post 12h of depletion and failed to divide post 24 hours. **D.** Induction of MreC for 24 hours led to filamentous cells. White arrows show the probable site of incomplete cell division. **E.** The length of individual bacteria was measured using ImageJ software. The average size of *mreC* mutant was 5.35 ± 0.4 mm compared to wild-type strain, which had an average size of 3.33 ± 0.1 mm ($n = 50$). Adapted from (Ghachi et al., 2011).

Earlier reports stated that MreB does not have a role in maintaining cell shape in *H. pylori* cells. Although it was observed to have an effect on chromosome segregation leading to cell division delay. MreB mutant cells showed elongated cells with extended non-segregated nuclei (Waidner et al., 2009). In the absence of RodZ, whether MreB takes up a different function remains elusive. However, a recent study has shown that MreB is enriched in negative Gaussian

curvature of *H. pylori* cells. MreB associated peptidoglycan synthesis is enriched at negative curvature while CcmA based cell wall synthesis is enriched at the positive Gaussian curvature (Figure 1.16). This preferential localization of the two cytoskeletal proteins helps maintain the curvature of the cells (Taylor et al., 2020).

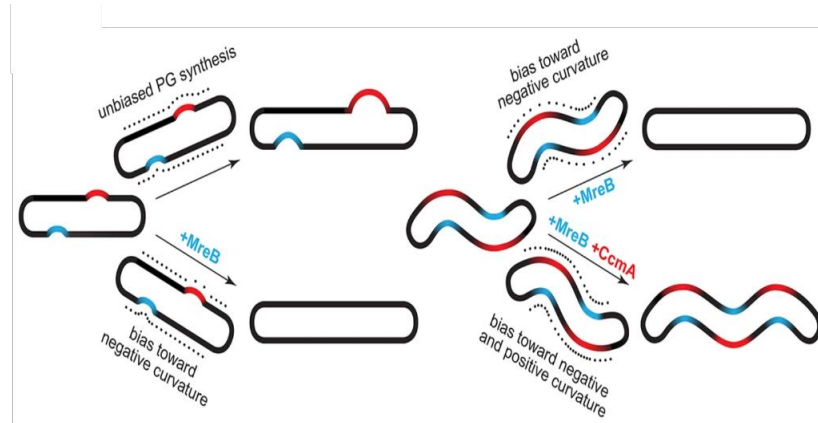


Figure 1.16. MreB and CcmA lead to differential cell wall synthesis patterns. Model showing pattern of cell wall synthesis in *Helicobacter pylori* cells. The dotted points indicate insertion of new cell wall precursors giving rise to non-zero Gaussian curves. Red indicates a region of positive Gaussian curvature and blue indicates a region of negative Gaussian curvature. Adapted from (Taylor et al., 2020).

Multiple factors are responsible for determining the helical nature of *H. pylori*. However, the exact role of each factor is not yet clear. Also, in order to cause differential curvature along the cell length, the factors involved in this system must coordinate with each other. No evidence for the regulation of these proteins have been suggested so far.

1.6.3 Cell wall less bacteria

The phylum mycoplasmatota, earlier known as “Tenericutes” contains the class Mollicutes. This class contains *Mycoplasma*, *Ureaplasma*, *Spiroplasma*, *Acholeplasma* and *Candidatus phytoblasma*. These bacteria have evolved through a regressive evolution and genome reduction from a Gram-positive bacteria *Clostridia* (Razin, 1978; Cole et al., 1973; Weisburg et al., 1989; Williamson, 1974). *Spiroplasma* genera of this phylum shows a well-defined helical cell geometry and consists of a flat monolayered membrane bound cytoskeleton (Figure 1.17 A). It is approximately 150 nm in width and 3 - 10 micrometers in length (Shaevitz et al., 2005). The first evidence about the existence of such species came from the study of infected *Drosophila* which showed the presence of spirochaeta like organisms. They called these organisms “sex ratio organisms” (SRO) as infection of these organisms led to lethality of male progenies in *Drosophila*. Electron microscopic studies of SROs showed that

the organism lacks a cell wall and any outer appendages for its movement (Williamson, 1974; Chen, 1971; Chen and Granados, 1970; Doi et al., 1967; Shafy A. Fudl-Allah, 1972). Further studies on the metabolic pathways of the organism showed that the absence of cell wall is due to the lack of enzymatic pathways for cell wall synthesis (Dybvig and Voelker, 1996; Melcher and Fletcher, 1999; Pollack et al., 1997). Despite the absence of the cell wall and obvious motility appendages such as flagella and pili, the organism maintains a helical shape and is motile. *Spiroplasma* cells show a tapered end and are said to swim in the direction of this tip (Ammar et al., 2004; Garnier et al., 1981; Liu et al., 2017; Wang et al., 2004). Studies on the helical, motile strains of *Spiroplasma* hypothesized that a linear motor constituted from intracellular filaments was responsible for motility (Davis et al., 1972). Fresh cultures of *S. citri* showed the presence of helical filaments ranging from 2 - 4 μm . Prolonged incubation of the culture led to fragmentation of these helical filaments (Cole et al., 1973). Sodium deoxycholate treatment of SROs helped in the visualization of these filaments (Williamson, 1974). Electron microscopic studies have shown that *Spiroplasma* possesses a flat, monolayered and membrane-bound ribbon which comprises six or seven pairs of Fibrils that is a part of the linear motor (Figure 1.17 B and C). Based on the repeat distance observed in the filaments, the outer two ribbons were postulated to be composed of Fibril protein whereas the inner ribbon could comprise MreB filaments (Kürner et al., 2005).

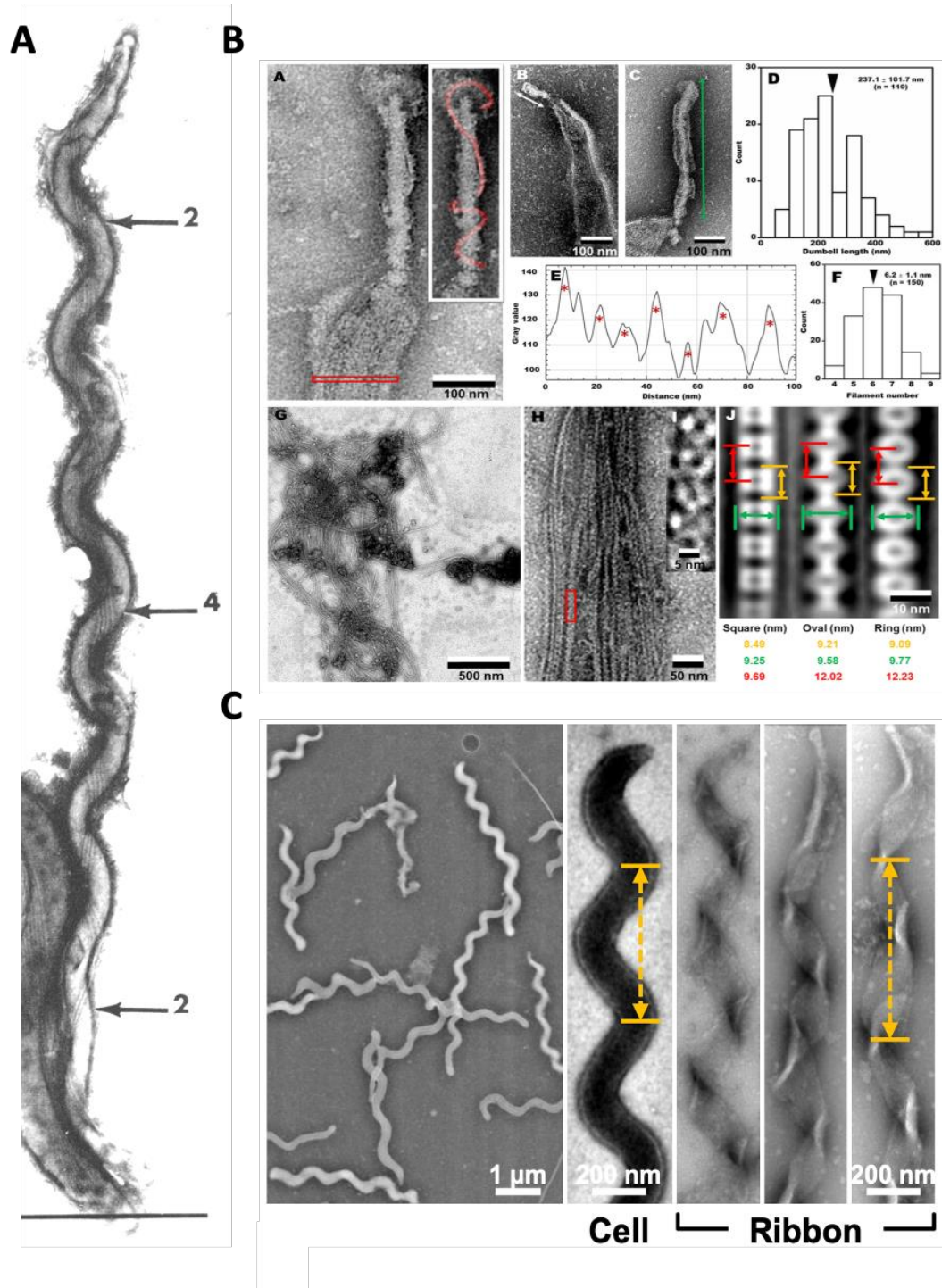


Figure 1.17. Visualization and analyses of cytoskeletal ribbon. A. Negative staining image of *Spiroplasma* cells showing filaments running along the shortest path of cell length. B. (A) Tip of the cell surrounded by helical ribbon. The inset shows the ribbon in red. (B) and (C) short and long tip of the cell exposed after TritonX-100 treatment. (D) length distribution of the dumbbell structure at the tip of the cells. (E) density profile of ribbon highlighted in red box in (A). seven filaments were detected and are marked by red asterisks. (F) Number of filaments detected in a cell. Average number of filaments denoted by black arrow. (G) Negatively stained filaments isolated after treating cells with 0.8 % Triton X-100. (H) Magnified image of isolated filaments. (I) Inset of filament in red box in (H). (J) Three kinds

of filaments were observed. Dimensions and shape of the subunits are highlighted below each filament. C. Negative staining images of *Spiroplasma eriocheiris* and isolated ribbon. Helical pitch of the cell was comparable to that of the isolated ribbon. Adapted from (Liu et al., 2017; Listgarten and Socransky, 1964; Sasajima et al., 2022).

Fibril is a 59-kDa core molecule of the ribbon and the full-length protein has no homologue in any of the currently available protein or DNA sequence databases. Sequence identity-based search suggests that nearly half of the sequence (residues 1 – 234) at the N-terminal domain of Fibril shares homology with MTAN (5'-methylthioadenosine nucleosidase).

Fibril is known to form very stable filaments for which there are no known ways of depolymerization (Cohen-Krausz et al., 2011). Conformational changes in Fibril have been proposed to be responsible for structural changes in Fibril ribbon, which in turn results in motility (Figure 1.17B). It was suggested that Fibril might be responsible for the cork-screw movement of the helical organism because of the force generated by attaching to the cell membrane (Townsend et al., 1980).

Most of the rod-shaped bacteria show the presence of a single copy of MreB gene (Ku et al., 2014). Interestingly, *Spiroplasma citri* which is a cell wall less bacteria consist of 5 copies of MreB (Cohen-Krausz et al., 2011; Townsend and Archer, 1983; Trachtenberg, 2004). Isolation of phenotypically different serotypes of *S. citri* resulted in characterization of SP-A, GII3 strains that were helical and motile whereas the ASP1 strain that was non-helical, non- motile (Figure 1.18A). Pathogeny and serology tests have proved that the non-helical, non- motile is a naturally occurring pathogenic strain of *S. citri*. DNA hybridization and SDS-PAGE studies also suggested that both the phenotypic variants of *Spiroplasma* are pathogenic (Townsend et al., 1977). Comparison of membrane protein profile of the helical (GII3) and non-helical (ASP1) strains of the organism suggested that there is one protein band of ~39 kDa missing in the non-helical strain (Figure 1.18B) (Townsend et al., 1977). MreB is another cytoskeletal element of *Spiroplasma*. Genome sequencing of ASPI strain revealed that *mreB5* gene shows the presence of a stop codon at 400th nucleotide position (Figure 1.18C) (Harne et al., 2020). This leads to non-functional protein in ASPI strain. Complementation of *mreB5* from GII3 strain led to gain of helicity and motility in ASPI strain (Figure 1.18D) (Harne et al., 2020).

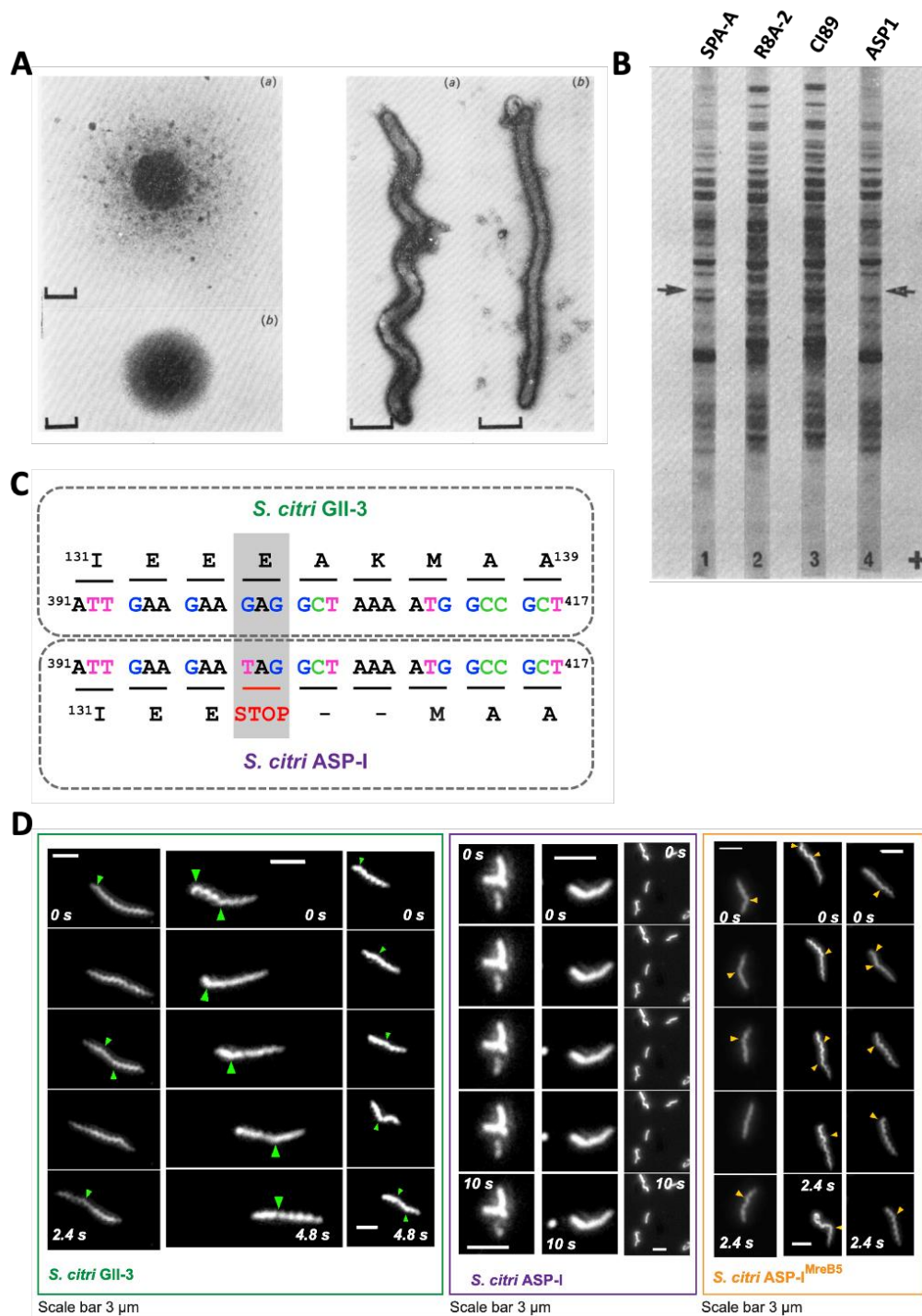


Figure 1.18. MreB5 determinant of helicity and motility in *Spiroplasma citri*. **A.** Stained colonies of *S. citri* strains (a)SP-A and (b) ASP-I on a medium solidified with 0.75% agar. Scale bar represents 0.01 mm. Negatively stained organisms from exponential phase cultures of *S. citri* strains (a)SP-A and (b) ASP-I. Bar markers represent 0.5 μ m. **B.** Profile of membrane proteins from different strains of *S. citri*. The arrow in ASP-I indicates absence of a 39 kilodalton band. **C.** Comparison of DNA sequences of MreB5 from GII-3 and ASP-I shows mutation in ASP-I at the 400th nucleotide position leading to a stop codon. The numbers highlighted in the box represent the amino acid position (top) and nucleotide position (bottom) of each sequence. **D.** Images of *S. citri* strains GII-3 (green box), ASP-I (purple box) and ASP-

I^{MreB5} (orange box) captured at different time intervals. Adapted from (Harne et al., 2020; Townsend et al., 1977).

Sequence alignment of various MreBs have shown that the polymerization interface (inter and intrafilament) and the ATPase catalytic pocket is fairly conserved (Takahashi et al., 2020). Based on these observations, polymerization dynamics and assembly of *Spiroplasma poulsonii* MreB proteins was studied. As MreB1-MreB4 and MreB2-MreB5 share 96% and 86.4 % sequence similarity respectively, polymerization patterns were studied for the fluorescently tagged MreB1, 2 and 3. These MreBs tagged with GFP were overexpressed in *E.coli* and the following observations were made-

- i. SpMreB1^{GFP} formed long filaments that spanned the entire cell
- ii. SpMreB2^{GFP} formed thick filaments connected to large puncta
- iii. SpMreB3^{GFP} formed ring like filaments across the cell

To understand the interaction network of these filaments inside the cells, a different combination of GFP tagged isoforms was coexpressed with the untagged isoforms. MreB1^{GFP}23 and MreB123^{GFP} formed longitudinal filaments attached to large puncta whereas MreB12^{GFP}3 showed diffuse phenotype. These observations suggest that the filament formation of different isoforms of MreBs are not independent events. Instead, these isoforms form an elaborate network which in turn regulate the filament formation (Masson et al., 2021).

Recent study did reconstitution of 5 MreBs and Fibril in *Mycoplasma capricolum* through a transposon vector. A large population of pleomorphic cells gained helicity and motility after the introduction of the 6 proteins (Figure 1.19A). Proteomics analyses of *Spiroplasma citri* showed that there is a variation in the abundance of these cytoskeletal proteins, the order being, MreB5 > MreB4 > Fibril > MreB3 > MreB1 > MreB2. After several passages of *M.cap*^{mre1-5-fib}, MreB2, MreB3, MreB4 and Fibril were seen to be absent in some clones. This suggested that these proteins might be dispensable for helicity and motility in *S. citri*. When the Mycoplasma cells were transformed with MreB1, no membrane deformations were seen. However, expression of MreB5 alone rendered the cells helical with a kinking ability. MreB1, MreB5 and Fibril led to helical and motile cells with a helical pitch similar to *S. citri* cells (Figure 1.19 B). This indicates that MreB1, MreB5 or Fibril each form the minimal requirement for conferring helicity and motility in a cell wall less bacteria (Lartigue et al., 2022).

Another recent study showed the effect of individual cytoskeletal proteins on the cell shape and motility when expressed in synthetic cells of JCVI-syn3B. All the MreB and Fibril proteins were reconstituted in the pleomorphic, non-motile cells individually and in different combinations. Upon the reconstitution of all MreB proteins along with Fibril, syn3B cells became helical as well as motile. Cells expressing Fibril and five MreB paralogs and a lipoprotein between MreB3 and MreB4 were named syn3Bsw. The helical pitch and motility rates of these cells were comparable to *Spiroplasma eriocheiris* (Figure 1.19 C). Deletion of one gene at a time did not show significant difference when compared to syn3Bsw cells. However, in the absence of MreB5, the cells were unable to swim (Figure 1.19 D). It was observed that expression of MreB2 alone makes the cells filamentous. However, expression of MreB1, MreB3, MreB4 and MreB5 did not change the phenotype of syn3B cells (Figure 1.19 E). When different MreBs were expressed in pairs, MreB5 and MreB1, MreB5 and MreB4 pairs showed movement and helicity. Fibril was also expressed along with MreB5 and MreB4 (Figure 1.19 F-I). However, the phenotypic change observed was very subtle and hence the role of Fibril is still not clear (Kiyama et al., 2022).

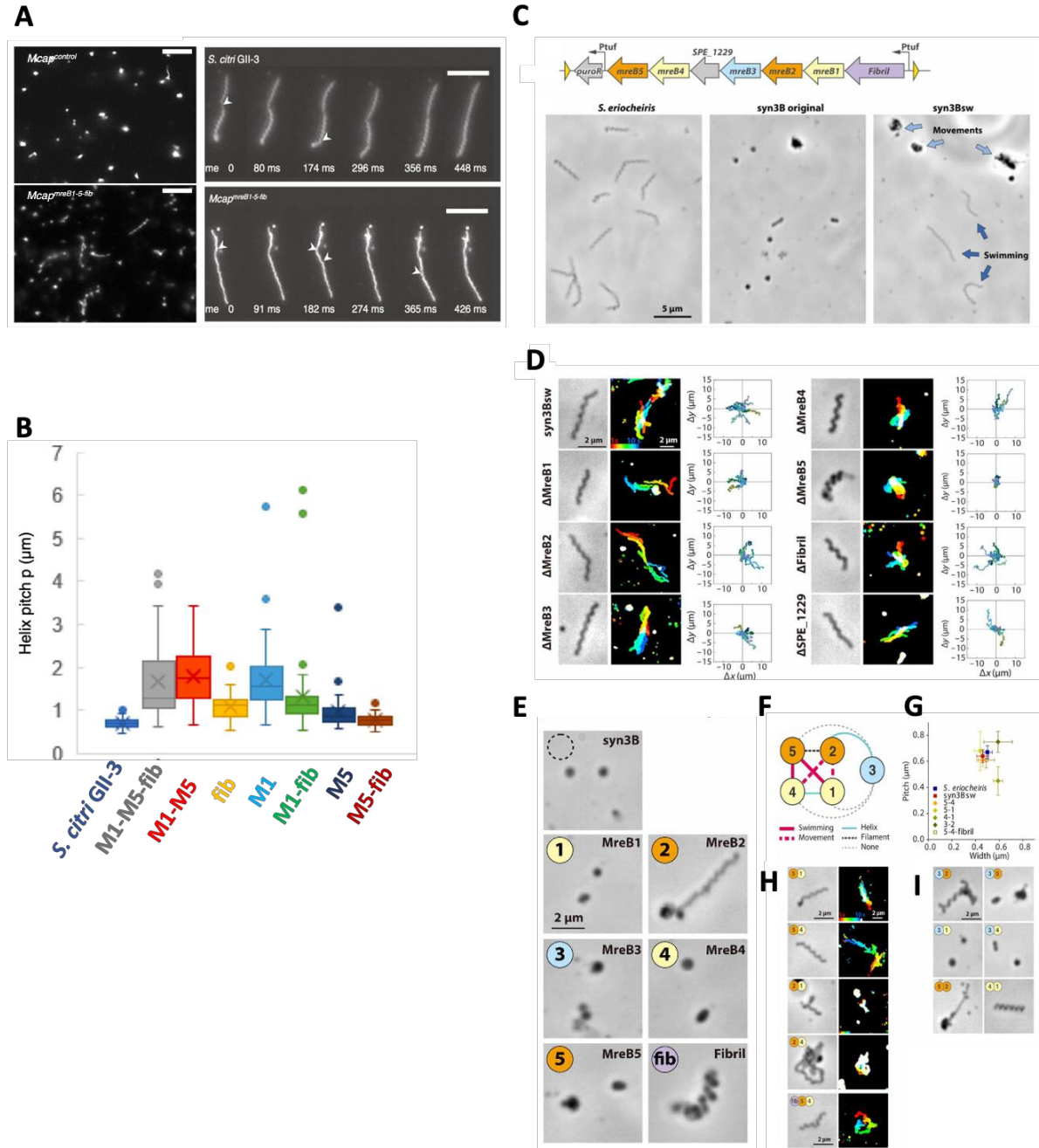


Figure 1.19. Reconstitution of cytoskeletal assembly in mycoplasma cells. **A.** Darkfield microscopy images of *Mycoplasma capricolum*- $Mcap^{control}$ and $Mcap^{mreB1-5-fib}$. Kink based motility displayed in *S. citri* cells (top) and $Mcap^{mreB1-5-fib}$ cells. **B.** DNA fragment highlighting seven genes from *Spiroplasma* along with puromycin resistant genes. This fragment present between two loxP sites was introduced into syn3B cells. Images showing wild-type *S. eriocheiris* cells, syn3B and syn3Bsw (expressing seven genes from *Spiroplasma*). **C.** The box plot represents the pitch of $Mcap$ transformants. The central line in each box represents the median. **D.** Phase contrast images of syn3sw cells lacking one of the seven genes (5 MreBs, Fibril and lipoprotein between MreB3 and MreB4). The cells were tracked for 10 seconds (red to blue). **E.** Phase contrast images of syn3B and cells expressing a single gene. **F.** Schematic

showing the effect of different combinations of gene expression on the cell morphology and movement. Line formats represent the characteristic of each combination. **G.** distribution of cell helicity across transformants. **H-I.** Phase contrast images of cells expressing different pairs of proteins. Image (left) and motility behavior (right) in **H.** Adapted from (Kiyama et al., 2022; Lartigue et al., 2022).

1.6.3 Models proposed for *Spiroplasma* cytoskeletal ribbon.

Based on tomography studies of *Spiroplasma melliferum*, two models have been proposed so far, namely, three ribbon models (Kürner et al., 2005) and one ribbon model (Trachtenberg et al., 2008).

- I. **Three ribbon model:-** Two types of filaments (thick and thin) were observed under the cell membrane. The outer 5 thick filaments had a spacing of ~ 11 nm whereas the inner 9 thinner filaments had a spacing of ~ 4 nm. Both these filaments were placed parallel to one another. The total width of the ribbon comes to 146 nm. Based on the repeat distance of the isolated and purified proteins, the outer thick filaments were identified as Fibril and the inner thin filaments were identified as MreB.
- II. **One ribbon model:-** The ribbon from *S. melliferum* cells was isolated as a single entity. The ribbon appears to be ~70 nm wide possessing 7 Fibrils with a lateral spacing of ~ 10 nm spanning the entire cell length (Liu et al., 2017; (Trachtenberg et al., 2008; Trachtenberg and Gilad, 2002).

1.7 Objectives

Spiroplasma belongs to the class mollicutes. In the absence of any outer appendages like cilia or flagella, an intracellular cytoskeletal ribbon has been proposed to provide helicity and motility to the organism (Williamson, 1974; Davis et al., 1972). This ribbon consists of 5 MreB paralogs and a unique protein called Fibril (Kürner et al., 2005). Fibril protein is found only in *Spiroplasma* species and hence shows no homology to any known protein.

The N-terminal domain of the protein possesses ~24% sequence similarity to 5'-methylthioadenosine nucleosidase (MTAN). Also, different conformational changes in the cytoskeletal ribbon have been proposed for the helicity of the organism. Owing to the fact that the major component of the ribbon is Fibril (Sasajima et al., 2022), it is likely that the energy source of these conformational changes comes from ATP. However, no biochemical assays

have been done to check if Fibril retains the enzymatic activity. Structural details of the protein were also not known until recently.

The main goal of my thesis is to characterize Fibril, a novel cytoskeletal protein form *Spiroplasma citri*. This involved identifying the polymerization interface of Fibril using the EGFP based method. I also carried out biochemical assays for functional characterization of Fibril proteins. Interaction between two of the cytoskeletal proteins, Fibril and MreB5 were also carried out in the presence of different nucleotide conditions. Following section talks about the specific goals of my thesis.

1. Understanding conservation of Fibril and its gene neighborhood amongst *Spiroplasma* species. This involves construction of a phylogenetic tree based on 16srRNA of *Spiroplasma* species. This result compared with phylogeny based on *fibril* genes will help understand presence or absence of the gene in a clade-based manner.
2. Biochemical characterization of Fibril from *Spiroplasma citri*. Hydrolysis of adenosine-based metabolites has been checked to see if Fibril retains any enzymatic activity. This study also involves checking ATP hydrolysis activity for Fibril.
3. Understanding the polymerization interface of Fibril. This study involves introducing EGFP tag to block the polymerization interface. This will provide insights into the probable regions of the protein present at the interfilament/intrafilament interface. This will also result in the identification of residue positions suitable for the addition of EGFP tag.
4. Interaction studies of Fibril-EGFP and MreB5. This study involves checking interaction between Fibril-EGFP and MreB5 using co-pelleting assay. Interaction between Fibril-EGFP constructs and MreB5 will help us identify the effect of EGFP on the interaction, if any. This will also help in the identification of functional fluorescently tagged Fibril proteins. Interaction between MreB5^{WT} and the ATPase mutants, MreB5^{E134A} and MreB5^{T161A} with Fibril has been done in the presence of different nucleotides. Effect of Fibril interaction on the ATP hydrolysis activity of MreB5 has been checked using Malachite green assay.
5. Purification optimization of Fibril and MreB1. Further optimization of Fibril purification has been tried to get well separated filaments. This protein is being used for negative staining to observe the filaments. Parallelly, purification optimization of MreB1 has also been tried.

1.8 References

1. Adams, D.W., Errington, J., 2009. Bacterial cell division: assembly, maintenance and disassembly of the Z ring. *Nat Rev Microbiol* 7, 642–653. <https://doi.org/10.1038/nrmicro2198>
2. Ammar, E.-D., Fulton, D., Bai, X., Meulia, T., Hogenhout, S.A., 2004. An attachment tip and pili-like structures in insect- and plant-pathogenic spiroplasmas of the class Mollicutes. *Arch Microbiol* 181, 97–105. <https://doi.org/10.1007/s00203-003-0630-8>
3. Anderson-Furgeson, J.C., Zupan, J.R., Grangeon, R., Zambryski, P.C., 2016. Loss of PodJ in *Agrobacterium tumefaciens* Leads to Ectopic Polar Growth, Branching, and Reduced Cell Division. *J Bacteriol* 198, 1883–1891. <https://doi.org/10.1128/JB.00198-16>
4. Ausmees, N., 2006. Intermediate Filament-Like Cytoskeleton of *Caulobacter crescentus* 152–158. <https://doi.org/10.1159/000094051>
5. Ausmees, N., Kuhn, J.R., Jacobs-Wagner, C., 2003. The Bacterial Cytoskeleton. *Cell* 115, 705–713. [https://doi.org/10.1016/S0092-8674\(03\)00935-8](https://doi.org/10.1016/S0092-8674(03)00935-8)
6. Bapat, M., Harne, S., Kashyap, R., Gayathri, P., 2023. Insights into assembly of constitutive filament-forming proteins of novel fold through domain dissection and EGFP insertion-based screen: A case study of *Spiroplasma Fibril*. <https://doi.org/10.1101/2023.10.26.564286>
7. Bazylinski, D.A., Frankel, R.B., 2004. Magnetosome formation in prokaryotes. *Nat Rev Microbiol* 2, 217–230. <https://doi.org/10.1038/nrmicro842>
8. Becker, E., Herrera, N.C., Gunderson, F.Q., Derman, A.I., Dance, A.L., Sims, J., Larsen, R.A., Pogliano, J., 2006. DNA segregation by the bacterial actin AlfA during *Bacillus subtilis* growth and development. *EMBO J* 25, 5919–5931. <https://doi.org/10.1038/sj.emboj.7601443>
9. Bergeron, J.R.C., Hutto, R., Ozyamak, E., Hom, N., Hansen, J., Draper, O., Byrne, M.E., Keyhani, S., Komeili, A., Kollman, J.M., 2017. Structure of the magnetosome-associated actin-like MamK filament at subnanometer resolution. *Protein Science* 26, 93–102. <https://doi.org/10.1002/pro.2979>
10. Berkeley, U.C., Christian, J., 2016. Electronic Theses and Dissertations Polar growth and cell division in *Agrobacterium tumefaciens* By James Christian Anderson- - Furgeson A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy In Microb.
11. Beuria, T.K., Mullapudi, S., Mileykovskaya, E., Sadasivam, M., Dowhan, W.,

- Margolin, W., 2009. Adenine nucleotide-dependent regulation of assembly of bacterial tubulin-like FtsZ by a hypermorph of bacterial actin-like FtsA. *Journal of Biological Chemistry* 284, 14079–14086. <https://doi.org/10.1074/jbc.M808872200>
12. Bi, E., Lutkenhaus, J., 1991. FtsZ ring structure associated with division in *Escherichia coli*. *Nature* 354, 161–164. <https://doi.org/10.1038/354161a0>
 13. Bork, P., Sander, C., Valencia, A., 1992. An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proceedings of the National Academy of Sciences* 89, 7290–7294. <https://doi.org/10.1073/pnas.89.16.7290>
 14. Brown, P.J.B., Pedro, M.A. De, Kysela, T., Henst, C. Van Der, Kim, J., Bolle, X. De, Fuqua, C., Brun, Y. V, 2012. Polar growth in the Alphaproteobacterial order Rhizobiales 109. <https://doi.org/10.1073/pnas.1200309109>
 15. Campbell, C.S., Mullins, R.D., 2007. In vivo visualization of type II plasmid segregation: bacterial actin filaments pushing plasmids. *J Cell Biol* 179, 1059–1066. <https://doi.org/10.1083/jcb.200708206>
 16. Charbon, G., Cabeen, M.T., Jacobs-Wagner, C., 2009a. Bacterial intermediate filaments: in vivo assembly, organization, and dynamics of crescentin. *Genes Dev* 23, 1131–1144. <https://doi.org/10.1101/gad.1795509>
 17. Charbon, G., Cabeen, M.T., Jacobs-Wagner, C., 2009b. Bacterial intermediate filaments: in vivo assembly, organization, and dynamics of crescentin. *Genes Dev* 23, 1131–1144. <https://doi.org/10.1101/gad.1795509>
 18. Chen, M., 1971. Mycoplasma-like Bodies Associated with Likubin-Diseased Ponkan Citrus. *Phytopathology* 61, 598. <https://doi.org/10.1094/Phyto-61-598>
 19. Chen, T.-A., Granados, R.R., 1970. Plant-Pathogenic Mycoplasma-Like Organism: Maintenance in vitro and Transmission to *Zea mays* L. *Science* (1979) 167, 1633–1636. <https://doi.org/10.1126/science.167.3925.1633>
 20. Cohen-Krausz, S., Cabahug, P.C., Trachtenberg, S., 2011. The Monomeric, Tetrameric, and Fibrillar Organization of Fib: The Dynamic Building Block of the Bacterial Linear Motor of *Spiroplasma melliferum* BC3. *J Mol Biol* 410, 194–213. <https://doi.org/10.1016/j.jmb.2011.04.067>
 21. Cole, R.M., Tully, J.G., Popkin, T.J., Bové, J.M., 1973. Morphology, Ultrastructure, and Bacteriophage Infection of the Helical Mycoplasma-Like Organism (*Spiroplasma citri* gen. nov., sp. nov.) Cultured from “Stubborn” Disease of Citrus. *J Bacteriol* 115, 367–386. <https://doi.org/10.1128/jb.115.1.367-386.1973>

22. Daniel, R.A., Errington, J., 2003. Control of Cell Morphogenesis in Bacteria. *Cell* 113, 767–776. [https://doi.org/10.1016/S0092-8674\(03\)00421-5](https://doi.org/10.1016/S0092-8674(03)00421-5)
23. Davis, R.E., Worley, J.F., Whitcomb, R.F., Ishijima, T., Steere, R.L., 1972. Helical Filaments Produced by a Mycoplasma-Like Organism Associated with Corn Stunt Disease. *Science* (1979) 176, 521–523. <https://doi.org/10.1126/science.176.4034.521>
24. de Boer, P., Crossley, R., Rothfield, L., 1992. The essential bacterial cell-division protein FtsZ is a GTPase. *Nature* 359, 254–256. <https://doi.org/10.1038/359254a0>
25. Deng, X., Gonzalez Llamazares, A., Wagstaff, J.M., Hale, V.L., Cannone, G., McLaughlin, S.H., Kureisaite-Ciziene, D., Löwe, J., 2019. The structure of bactofilin filaments reveals their mode of membrane binding and lack of polarity. *Nat Microbiol* 4, 2357–2368. <https://doi.org/10.1038/s41564-019-0544-0>
26. Din, N., Quardokus, E.M., Sackett, M.J., Brun, Y. V., 1998. Dominant C-terminal deletions of FtsZ that affect its ability to localize in *Caulobacter* and its interaction with FtsA. *Mol Microbiol* 27, 1051–1063. <https://doi.org/10.1046/j.1365-2958.1998.00752.x>
27. DOI, Y., TERANAKA, M., YORA, K., ASUYAMA, H., 1967. Mycoplasma- or PLT Group-like Microorganisms Found in the Phloem Elements of Plants Infected with Mulberry Dwarf, Potato Witches' Broom, Aster Yellows, or Paulownia Witches' Broom. *Japanese Journal of Phytopathology* 33, 259–266. <https://doi.org/10.3186/jjphytopath.33.259>
28. Domínguez-Escobar, J., Chastanet, A., Crevenna, A.H., Fromion, V., Wedlich-Söldner, R., Carballido-López, R., 2011. Processive Movement of MreB-Associated Cell Wall Biosynthetic Complexes in Bacteria. *Science* (1979) 333, 225–228. <https://doi.org/10.1126/science.1203466>
29. Dybvig, K., Voelker, L.L., 1996. MOLECULAR BIOLOGY OF MYCOPLASMAS. *Annu Rev Microbiol* 50, 25–57. <https://doi.org/10.1146/annurev.micro.50.1.25>
30. Erickson, H.P., 2007. Evolution of the cytoskeleton. *BioEssays* 29, 668–677. <https://doi.org/10.1002/bies.20601>
31. Esue, O., Cordero, M., Wirtz, D., Tseng, Y., 2005. The Assembly of MreB, a Prokaryotic Homolog of Actin. *Journal of Biological Chemistry* 280, 2628–2635. <https://doi.org/10.1074/jbc.M410298200>
32. Garner, E.C., Bernard, R., Wang, W., Zhuang, X., Rudner, D.Z., Mitchison, T., 2011. Coupled, Circumferential Motions of the Cell Wall Synthesis Machinery and MreB Filaments in *B. subtilis*. *Science* (1979) 333, 222–225.

<https://doi.org/10.1126/science.1203285>

33. Garner, E.C., Campbell, C.S., Weibel, D.B., Mullins, R.D., 2007. Reconstitution of DNA Segregation Driven by Assembly of a Prokaryotic Actin Homolog. *Science* (1979) 315, 1270–1274. <https://doi.org/10.1126/science.1138527>
34. Garnier, M., Clerc, M., Bove, J.M., 1981. Growth and division of spiroplasmas: morphology of *Spiroplasma citri* during growth in liquid medium. *J Bacteriol* 147, 642–652. <https://doi.org/10.1128/jb.147.2.642-652.1981>
35. Gayathri, P., Fujii, T., Møller-Jensen, J., van den Ent, F., Namba, K., Löwe, J., 2012. A Bipolar Spindle of Antiparallel ParM Filaments Drives Bacterial Plasmid Segregation. *Science* (1979) 338, 1334–1337. <https://doi.org/10.1126/science.1229091>
36. Gerdes, K., 2009. RodZ, a new player in bacterial cell morphogenesis. *EMBO J* 28, 171–172. <https://doi.org/10.1038/emboj.2008.287>
37. Gerdes, K., Larsen, J.E., Molin, S., 1985. Stable inheritance of plasmid R1 requires two different loci. *J Bacteriol* 161, 292–298. <https://doi.org/10.1128/jb.161.1.292-298.1985>
38. Ghachi, M. El, Matteï, P., Ecobichon, C., Martins, A., Hoos, S., Schmitt, C., Colland, F., Ebel, C., Prévost, M., Gabel, F., England, P., Dessen, A., Boneca, I.G., 2011. Characterization of the elongasome core PBP2 : MreC complex of *Helicobacter pylori* 82, 68–86. <https://doi.org/10.1111/j.1365-2958.2011.07791.x>
39. Gorby, Y.A., Beveridge, T.J., Blakemore, R.P., 1988. Characterization of the bacterial magnetosome membrane. *J Bacteriol* 170, 834–841. <https://doi.org/10.1128/jb.170.2.834-841.1988>
40. Grangeon, R., Zupan, J.R., Anderson-Furgeson, J., Zambryski, P.C., 2015. PopZ identifies the new pole, and PodJ identifies the old pole during polar growth in *Agrobacterium tumefaciens*. *Proceedings of the National Academy of Sciences* 112, 11666–11671. <https://doi.org/10.1073/pnas.1515544112>
41. Grünberg, K., Wawer, C., Tebo, B.M., Schüler, D., 2001. A Large Gene Cluster Encoding Several Magnetosome Proteins Is Conserved in Different Species of Magnetotactic Bacteria. *Appl Environ Microbiol* 67, 4573–4582. <https://doi.org/10.1128/AEM.67.10.4573-4582.2001>
42. Harne, S., Duret, S., Pande, V., Bapat, M., Béven, L., Gayathri, P., 2020. MreB5 Is a Determinant of Rod-to-Helical Transition in the Cell-Wall-less Bacterium *Spiroplasma*. *Current Biology* 30, 4753–4762.e7. <https://doi.org/10.1016/j.cub.2020.08.093>

43. Henriques, A.O., Glaser, P., Piggot, P.J., Moran Jr, C.P., 1998. Control of cell shape and elongation by the *rodA* gene in *Bacillus subtilis*. *Mol Microbiol* 28, 235–247. <https://doi.org/10.1046/j.1365-2958.1998.00766.x>
44. Herrmann, H., Aebi, U., 2004. Intermediate Filaments: Molecular Structure, Assembly Mechanism, and Integration Into Functionally Distinct Intracellular Scaffolds. *Annu Rev Biochem* 73, 749–789. <https://doi.org/10.1146/annurev.biochem.73.011303.073823>
45. Holmes, K.C., Popp, D., Gebhard, W., Kabsch, W., 1990. Atomic model of the actin filament. *Nature* 347, 44–49. <https://doi.org/10.1038/347044a0>
46. Hu, B., Yang, G., Zhao, W., Zhang, Y., Zhao, J., 2007. MreB is important for cell shape but not for chromosome segregation of the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Mol Microbiol* 63, 1640–1652. <https://doi.org/10.1111/j.1365-2958.2007.05618.x>
47. Jones, L.J.F., Carballido-López, R., Errington, J., 2001. Control of Cell Shape in Bacteria. *Cell* 104, 913–922. [https://doi.org/10.1016/S0092-8674\(01\)00287-2](https://doi.org/10.1016/S0092-8674(01)00287-2)
48. Kawazura, T., Matsumoto, K., Kojima, K., Kato, F., Kanai, T., Niki, H., Shiomi, D., 2017. Exclusion of assembled MreB by anionic phospholipids at cell poles confers cell polarity for bidirectional growth. *Cell* 104, 472–486. <https://doi.org/10.1111/mmi.13639>
49. Kiyama, H., Kakizawa, S., Sasajima, Y., Tahara, Y.O., Miyata, M., 2022. Reconstitution of a minimal motility system based on *Spiroplasma* swimming by two bacterial actins in a synthetic minimal bacterium. *Sci Adv* 8. <https://doi.org/10.1126/sciadv.abo7490>
50. Koch, M.K., McHugh, C.A., Hoiczky, E., 2011. BacM, an N-terminally processed bactofilin of *Myxococcus xanthus*, is crucial for proper cell shape. *Mol Microbiol* 80, 1031–1051. <https://doi.org/10.1111/j.1365-2958.2011.07629.x>
51. Komeili, A., Li, Z., Newman, D.K., Jensen, G.J., 2006. Magnetosomes Are Cell Membrane Invaginations Organized by the Actin-Like Protein MamK. *Science* (1979) 311, 242–245. <https://doi.org/10.1126/science.1123231>
52. Krol, E., Yau, H.C.L., Lechner, M., Schäper, S., Bange, G., Vollmer, W., Becker, A., 2020. Tol-pal system and rgs proteins interact to promote unipolar growth and cell division in *Sinorhizobium meliloti*. *mBio* 11, 1–21. <https://doi.org/10.1128/MBIO.00306-20>
53. Ku, C., Lo, W.S., Kuo, C.H., 2014. Molecular evolution of the actin-like MreB protein

- gene family in wall-less bacteria. *Biochem Biophys Res Commun* 446, 927–932. <https://doi.org/10.1016/j.bbrc.2014.03.039>
54. Kühn, J., Briegel, A., Mörschel, E., Kahnt, J., Leser, K., Wick, S., Jensen, G.J., Thanbichler, M., 2010. Bactofilins, a ubiquitous class of cytoskeletal proteins mediating polar localization of a cell wall synthase in *Caulobacter crescentus*. *EMBO J* 29, 327–339. <https://doi.org/10.1038/emboj.2009.358>
 55. Kumar, S., Mollo, A., Kahne, D., Ruiz, N., 2022. The Bacterial Cell Wall: From Lipid II Flipping to Polymerization. *Chem Rev* 122, 8884–8910. <https://doi.org/10.1021/acs.chemrev.1c00773>
 56. Kürner, J., Frangakis, A.S., Baumeister, W., 2005a. Cryo-Electron Tomography Reveals the Cytoskeletal Structure of *Spiroplasma melliferum*. *Science* (1979) 307, 436–438. <https://doi.org/10.1126/science.1104031>
 57. Kürner, J., Frangakis, A.S., Baumeister, W., 2005b. Cryo-Electron Tomography Reveals the Cytoskeletal Structure of *Spiroplasma melliferum*. *Science* (1979) 307, 436–438. <https://doi.org/10.1126/science.1104031>
 58. Larsen, R.A., Cusumano, C., Fujioka, A., Lim-Fong, G., Patterson, P., Pogliano, J., 2007. Treadmilling of a prokaryotic tubulin-like protein, TubZ, required for plasmid stability in *Bacillus thuringiensis*. *Genes Dev* 21, 1340–1352. <https://doi.org/10.1101/gad.1546107>
 59. Lartigue, C., Lambert, B., Rideau, F., Dahan, Y., Decossas, M., Hillion, M., Douliez, J.P., Hardouin, J., Lambert, O., Blanchard, A., Béven, L., 2022. Cytoskeletal components can turn wall-less spherical bacteria into kinking helices. *Nat Commun* 13. <https://doi.org/10.1038/s41467-022-34478-0>
 60. Leaver, M., Errington, J., 2005. Roles for MreC and MreD proteins in helical growth of the cylindrical cell wall in *Bacillus subtilis* 57, 1196–1209. <https://doi.org/10.1111/j.1365-2958.2005.04736.x>
 61. Lin, L., Thanbichler, M., 2013. Nucleotide-independent cytoskeletal scaffolds in bacteria. *Cytoskeleton* 70, 409–423. <https://doi.org/10.1002/cm.21126>
 62. Lindås, A.-C., Karlsson, E.A., Lindgren, M.T., Ettema, T.J.G., Bernander, R., 2008. A unique cell division machinery in the Archaea. *Proceedings of the National Academy of Sciences* 105, 18942–18946. <https://doi.org/10.1073/pnas.0809467105>
 63. Link, C., 2017. The ABC 's of Cell Division : Regulation of Peptidoglycan Amidase Activity during Cytokinesis in *Escherichia coli* The Harvard community has made this article openly available . Please share how this access benefits you . Your story

matters .

64. Listgarten, M.A., Socransky, S.S., 1964. ELECTRON MICROSCOPY OF AXIAL FIBRILS, OUTER ENVELOPE, AND CELL DIVISION OF CERTAIN ORAL SPIROCHETES. *J Bacteriol* 88, 1087–1103. <https://doi.org/10.1128/jb.88.4.1087-1103.1964>
65. Liu, P., Zheng, H., Meng, Q., Terahara, N., Gu, W., Wang, S., Zhao, G., Nakane, D., Wang, W., Miyata, M., 2017. Chemotaxis without Conventional Two-Component System, Based on Cell Polarity and Aerobic Conditions in Helicity-Switching Swimming of *Spiroplasma eriocheiris*. *Front Microbiol* 8. <https://doi.org/10.3389/fmicb.2017.00058>
66. Liu, Y., Van Den Ent, F., Löwe, J., 2023. Structure of crescentin Filament structure and subcellular organization of the bacterial 3 intermediate filament-like protein crescentin. <https://doi.org/10.1101/2023.06.04.543601>
67. Loose, M., Mitchison, T.J., 2014. The bacterial cell division proteins ftsA and ftsZ self-organize into dynamic cytoskeletal patterns. *Nat Cell Biol* 16, 38–46. <https://doi.org/10.1038/ncb2885>
68. Löwe, J., Amos, L.A., 1998. Crystal structure of the bacterial cell-division protein FtsZ. *Nature* 391, 203–206. <https://doi.org/10.1038/34472>
69. Löwe, J., He, S., Scheres, S.H.W., Savva, C.G., 2016. X-ray and cryo-EM structures of monomeric and filamentous actin-like protein MamK reveal changes associated with polymerization. *Proceedings of the National Academy of Sciences* 113, 13396–13401. <https://doi.org/10.1073/pnas.1612034113>
70. Ma, X., Margolin, W., 1999. Genetic and Functional Analyses of the Conserved C-Terminal Core Domain of *Escherichia coli* FtsZ. *J Bacteriol* 181, 7531–7544. <https://doi.org/10.1128/JB.181.24.7531-7544.1999>
71. Martos, A., Monterroso, B., Zorrilla, S., Reija, B., Alfonso, C., Mingorance, J., Rivas, G., Jiménez, M., 2012. Isolation, characterization and lipid-binding properties of the recalcitrant FtsA division protein from *Escherichia coli*. *PLoS One* 7. <https://doi.org/10.1371/journal.pone.0039829>
72. Masaaki, W., Masaki, D., Taro, U., Michiko, U., Katsuki, T., Kazuo, N., Michio, M., 1991. Sequence of the downstream flanking region of the shape-determining genes mreBCD of *Escherichia coli*. *Gene* 106, 135–136. [https://doi.org/10.1016/0378-1119\(91\)90578-Y](https://doi.org/10.1016/0378-1119(91)90578-Y)
73. Masson, F., Pierrat, X., Lemaitre, B., Persat, A., 2021. The wall-less bacterium

- Spiroplasma poulsonii* builds a polymeric cytoskeleton composed of interacting MreB isoforms. *iScience* 24. <https://doi.org/10.1016/j.isci.2021.103458>
74. McGrath, P.T., Lee, H., Zhang, L., Iniesta, A.A., Hottes, A.K., Tan, M.H., Hillson, N.J., Hu, P., Shapiro, L., McAdams, H.H., 2007. High-throughput identification of transcription start sites, conserved promoter motifs and predicted regulons. *Nat Biotechnol* 25, 584–592. <https://doi.org/10.1038/nbt1294>
 75. Melcher, U., Fletcher, J., 1999. Genetic Variation in *Spiroplasma citri*. *Eur J Plant Pathol* 105, 519–533. <https://doi.org/10.1023/A:1008757716803>
 76. Mitchell, J.G., 2002. The Energetics and Scaling of Search Strategies in Bacteria. *Am Nat* 160, 727–740. <https://doi.org/10.1086/343874>
 77. Morgenstein, R.M., Bratton, B.P., Nguyen, J.P., Ouzounov, N., Shaevitz, J.W., 2015. RodZ links MreB to cell wall synthesis to mediate MreB rotation and robust morphogenesis. <https://doi.org/10.1073/pnas.1509610112>
 78. Mukherjee, A., Dai, K., Lutkenhaus, J., 1993. *Escherichia coli* cell division protein FtsZ is a guanine nucleotide binding protein. *Proceedings of the National Academy of Sciences* 90, 1053–1057. <https://doi.org/10.1073/pnas.90.3.1053>
 79. Mukherjee, A., Lutkenhaus, J., 1994. Guanine nucleotide-dependent assembly of FtsZ into filaments. *J Bacteriol* 176, 2754–2758. <https://doi.org/10.1128/jb.176.9.2754-2758.1994>
 80. Nogales, E., Wolf, S.G., Downing, K.H., 1998. Erratum: Structure of the $\alpha\beta$ tubulin dimer by electron crystallography. *Nature* 393, 191–191. <https://doi.org/10.1038/30288>
 81. Pereira, A.R., Hsin, J., Król, E., Tavares, A.C., Flores, P., Hoiczky, E., Ng, N., Dajkovic, A., 2016. FtsZ-Dependent Elongation of a Coccoid Bacterium 7, 1–9. <https://doi.org/10.1128/mBio.00908-16>.Editor
 82. Persat, A., Stone, H.A., Gitai, Z., 2014. enhances surface colonization in flow. *Nat Commun* 5, 1–9. <https://doi.org/10.1038/ncomms4824>
 83. Pinho, M.G., Errington, J., 2003. Dispersed mode of *Staphylococcus aureus* cell wall synthesis in the absence of the division machinery 50, 871–881. <https://doi.org/10.1046/j.1365-2958.2003.03719.x>
 84. Pinho, M.G., Kjos, M., Veening, J.W., 2013. How to get (a)round: Mechanisms controlling growth and division of coccoid bacteria. *Nat Rev Microbiol*. <https://doi.org/10.1038/nrmicro3088>
 85. Pollack, J.D., Williams, M. V., McElhaney, R.N., 1997. The Comparative Metabolism

- of the Mollicutes (*Mycoplasmas*): The Utility for Taxonomic Classification and the Relationship of Putative Gene Annotation and Phylogeny to Enzymatic Function in the Smallest Free-Living Cells. *Crit Rev Microbiol* 23, 269–354. <https://doi.org/10.3109/10408419709115140>
86. Randich, A.M., Brun, Y. V, 2015. Molecular mechanisms for the evolution of bacterial morphologies and growth modes 6, 1–13. <https://doi.org/10.3389/fmicb.2015.00580>
 87. RayChaudhuri, D., Park, J.T., 1992. Escherichia coli cell-division gene ftsZ encodes a novel GTP-binding protein. *Nature* 359, 251–254. <https://doi.org/10.1038/359251a0>
 88. Razin, S., 1978. The mycoplasmas. *Microbiol Rev* 42, 414–470. <https://doi.org/10.1128/mr.42.2.414-470.1978>
 89. Reichmann, N.T., Tavares, A.C., Saraiva, B.M., Jouselin, A., Reed, P., Pereira, A.R., Monteiro, J.M., Sobral, R.G., VanNieuwenhze, M.S., Fernandes, F., Pinho, M.G., 2019. SEDS–bPBP pairs direct lateral and septal peptidoglycan synthesis in Staphylococcus aureus. *Nat Microbiol* 4, 1368–1377. <https://doi.org/10.1038/s41564-019-0437-2>
 90. Salje, J., Löwe, J., 2008. Bacterial actin: architecture of the ParMRC plasmid DNA partitioning complex. *EMBO J* 27, 2230–2238. <https://doi.org/10.1038/emboj.2008.152>
 91. Samson, R.Y., Obita, T., Freund, S.M., Williams, R.L., Bell, S.D., 2008. A Role for the ESCRT System in Cell Division in Archaea. *Science* (1979) 322, 1710–1713. <https://doi.org/10.1126/science.1165322>
 92. Sasajima, Y., Kato, T., Miyata, T., Kawamoto, A., Miyata, M., 2022. Isolation and structure of the fibril protein, a major component of the internal ribbon for Spiroplasma swimming Makoto Miyata Quick freeze replica electron microscopy, Electron tomography. 20. <https://doi.org/10.1101/2021.02.24.432793>
 93. Scheffel, A., Gruska, M., Faivre, D., Linaroudis, A., Plitzko, J.M., Schüler, D., 2006. An acidic protein aligns magnetosomes along a filamentous structure in magnetotactic bacteria. *Nature* 440, 110–114. <https://doi.org/10.1038/nature04382>
 94. Schüler, D., 2004. Molecular analysis of a subcellular compartment: The magnetosome membrane in Magnetospirillum gryphiswaldense. *Arch Microbiol*. <https://doi.org/10.1007/s00203-003-0631-7>
 95. Shaevitz, J.W., Lee, J.Y., Fletcher, D.A., 2005. Spiroplasma Swim by a Processive Change in Body Helicity. *Cell* 122, 941–945.

<https://doi.org/10.1016/j.cell.2005.07.004>

- 96.** -Shafy A. Fudl-Allah, A. El, 1972. Culture of a Mycoplasma-like Organism Associated with Stubborn Disease of Citrus. *Phytopathology* 62, 729. <https://doi.org/10.1094/Phyto-62-729>
- 97.** Shi, C., Fricke, P., Lin, L., Chevelkov, V., Wegstroth, M., Giller, K., Becker, S., Thanbichler, M., Lange, A., 2015. Atomic-resolution structure of cytoskeletal bactofilin by solid-state NMR. *Sci Adv* 1. <https://doi.org/10.1126/sciadv.1501087>
- 98.** Shiomi, D., 2017. Polar localization of MreB actin is inhibited by anionic phospholipids in the rod-shaped bacterium *Escherichia coli*. *Curr Genet*. <https://doi.org/10.1007/s00294-017-0696-5>
- 99.** Springstein, B.L., Weissenbach, J., Koch, R., Stücker, F., Stucken, K., 2020. The role of the cytoskeletal proteins MreB and FtsZ in multicellular cyanobacteria. *FEBS Open Bio* 10, 2510–2531. <https://doi.org/10.1002/2211-5463.13016>
- 100.** Sycuro, L.K., Pincus, Z., Gutierrez, K.D., Biboy, J., Stern, C.A., Vollmer, W., 2010. Peptidoglycan Crosslinking Relaxation Promotes *Helicobacter pylori* 's Helical Shape and Stomach Colonization. *Cell* 141, 822–833. <https://doi.org/10.1016/j.cell.2010.03.046>
- 101.** Szwedziak, P., Wang, Q., Freund, S.M., Löwe, J., 2012. FtsA forms actin-like protofilaments. *EMBO Journal* 31, 2249–2260. <https://doi.org/10.1038/emboj.2012.76>
- 102.** Takahashi, D., Fujiwara, I., Miyata, M., 2020. Phylogenetic origin and sequence features of MreB from the wall-less swimming bacteria *Spiroplasma*. *Biochem Biophys Res Commun* 533, 638–644. <https://doi.org/10.1016/j.bbrc.2020.09.060>
- 103.** Taylor, J.A., Bratton, B.P., Sichel, S.R., Blair, K.M., Jacobs, H.M., Demeester, K.E., Kuru, E., Gray, J., Biboy, J., Vannieuwenhze, M.S., Vollmer, W., Grimes, C.L., Shaevitz, J.W., Salama, N.R., 2020. Distinct cytoskeletal proteins define zones of enhanced cell wall synthesis in *Helicobacter pylori*. *Elife* 9. <https://doi.org/10.7554/eLife.52482>
- 104.** Tinsley, E., Khan, S.A., 2006. A novel FtsZ-like protein is involved in replication of the anthrax toxin-encoding pXo1 plasmid in *Bacillus anthracis*. *J Bacteriol* 188, 2829–2835. <https://doi.org/10.1128/JB.188.8.2829-2835.2006>
- 105.** Townsend, R., Archer, D.B., 1983. A Fibril Protein Antigen Specific to *Spiroplasma*. *Microbiology (N Y)* 129, 199–206. <https://doi.org/10.1099/00221287-129-1-199>

106. Townsend, R., Burgess, J., Plaskitt, K.A., 1980. Morphology and ultrastructure of helical and nonhelical strains of *Spiroplasma citri*. *J Bacteriol* 142, 973–981. <https://doi.org/10.1128/jb.142.3.973-981.1980>
107. TOWNSEND, R., MARKHAM, P.G., PLASKITT, K.A., DANIELS, M.J., 1977. Isolation and Characterization of a Non-Helical Strain of *Spiroplasma citri*. *J Gen Microbiol* 100, 15–21. <https://doi.org/10.1099/00221287-100-1-15>
108. Trachtenberg, S., 2004. Shaping and Moving a *Spiroplasma*. *Microb Physiol* 7, 78–87. <https://doi.org/10.1159/000077872>
109. Trachtenberg, S., Dorward, L.M., Speransky, V. V., Jaffe, H., Andrews, S.B., Leapman, R.D., 2008. Structure of the Cytoskeleton of *Spiroplasma melliferum* BC3 and Its Interactions with the Cell Membrane. *J Mol Biol* 378, 778–789. <https://doi.org/10.1016/j.jmb.2008.02.020>
110. Trachtenberg, S., Gilad, R., 2002. A bacterial linear motor: cellular and molecular organization of the contractile cytoskeleton of the helical bacterium *Spiroplasma melliferum* BC3. *Mol Microbiol* 41, 827–848. <https://doi.org/10.1046/j.1365-2958.2001.02527.x>
111. van den Ent, F., 2002. F-actin-like filaments formed by plasmid segregation protein ParM. *EMBO J* 21, 6935–6943. <https://doi.org/10.1093/emboj/cdf672>
112. van den Ent, F., 2001. Bacterial ancestry of actin and tubulin. *Curr Opin Microbiol* 4, 634–638. [https://doi.org/10.1016/S1369-5274\(01\)00262-4](https://doi.org/10.1016/S1369-5274(01)00262-4)
113. Vaughan, S., Wickstead, B., Gull, K., Addinall, S.G., 2004. Molecular Evolution of FtsZ Protein Sequences Encoded Within the Genomes of Archaea, Bacteria, and Eukaryota. *J Mol Evol* 58, 19–39. <https://doi.org/10.1007/s00239-003-2523-5>
114. Wachi, M., Doi, M., Tamaki, S., Park, W., Nakajima-Iijima, S., Matsushashi, M., 1987. Mutant isolation and molecular cloning of mre genes, which determine cell shape, sensitivity to mecillinam, and amount of penicillin-binding proteins in *Escherichia coli*. *J Bacteriol* 169, 4935–4940. <https://doi.org/10.1128/jb.169.11.4935-4940.1987>
115. Wagstaff, J., Löwe, J., 2018. Prokaryotic cytoskeletons: Protein filaments organizing small cells. *Nat Rev Microbiol* 16, 187–201. <https://doi.org/10.1038/nrmicro.2017.153>
116. Waidner, B., Specht, M., Dempwolff, F., Haebeler, K., Schaetzle, S., Kist, M., Graumann, P.L., 2009. A Novel System of Cytoskeletal Elements in the Human

Pathogen *Helicobacter pylori* 5. <https://doi.org/10.1371/journal.ppat.1000669>

117. Wang, W., Chen, J., Du, K., Xu, Z., 2004. Morphology of spiroplasmas in the Chinese mitten crab *Eriocheir sinensis* associated with tremor disease. *Res Microbiol* 155, 630–635. <https://doi.org/10.1016/j.resmic.2004.04.010>
118. Weisburg, W.G., Tully, J.G., Rose, D.L., Petzel, J.P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T.G., Van Etten, J., 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* 171, 6455–6467. <https://doi.org/10.1128/jb.171.12.6455-6467.1989>
119. Williamson, D.L., 1974a. Unusual Fibrils from the Spirochete-Like Sex Ratio Organism. *J Bacteriol* 117, 904–906. <https://doi.org/10.1128/jb.117.2.904-906.1974>
120. Williamson, D.L., 1974b. Unusual Fibrils from the Spirochete-Like Sex Ratio Organism. *J Bacteriol* 117, 904–906. <https://doi.org/10.1128/jb.117.2.904-906.1974>
121. Williamson, D.L., 1974c. Unusual Fibrils from the Spirochete-Like Sex Ratio Organism. *J Bacteriol* 117, 904–906. <https://doi.org/10.1128/jb.117.2.904-906.1974>
122. Yan, K., Pearce, K.H., Payne, D.J., 2000. A Conserved Residue at the Extreme C-Terminus of FtsZ Is Critical for the FtsA-FtsZ Interaction in *Staphylococcus aureus*. *Biochem Biophys Res Commun* 270, 387–392. <https://doi.org/10.1006/bbrc.2000.2439>
123. Young, K.D., 2006. The Selective Value of Bacterial Shape. *Microbiology and Molecular Biology Reviews* 70, 660–703. <https://doi.org/10.1128/MMBR.00001-06>

Chapter 2

Understanding conservation of biochemical features of *Spiroplasma citri* Fibril

2.1 Introduction

Electron microscopy of *Spiroplasma*, previously referred to as spirochete-like organisms/sex ratio organisms, showed Fibril filaments running along the cell length (Listgarten and Socransky, 1964). Fibril was found out to be the most abundant protein in the cytoskeletal ribbon of *Spiroplasma* followed by MreB paralogs (Sasajima et al., 2022). When the antiserum against Fibril was checked, it was found to be conserved in most of the *Spiroplasma* species. However, it was not detected in other classes of mollicutes like *Mycoplasma* and *Acholeplasma*. The amount of cellular Fibril was suggested to be correlated with the motility rates (Townsend and Archer, 1983). Isolated Fibril filaments from *Spiroplasma eriocheiris* revealed that the length of the filaments was equivalent to the cell length. This suggests that Fibril forms stable filaments and lacks dynamic nature (Sasajima et al., 2022). When overexpressed in *Mycoplasma capricolum*, Fibril was able to induce membrane curvature but with a higher pitch than the cells overexpressing MreB5 and Fibril (Lartigue et al., 2022). This supports the fact that Fibril might be one of the major determinants for helicity in *Spiroplasma*. As discussed earlier, *fibril* gene is only present in *Spiroplasma*. To investigate the conservation of the *fibril* gene across the species, we sampled the genome sequences available in the current database. This will help us in understanding the conservation and evolutionary relation of the protein amongst the species. Cytoskeletal proteins like *ftsZ* and *ftsA* are a part of an operon containing *ftsQ*, *ftsA* and *ftsZ* which are essential for cell division (Yit et al., 1985). Similarly, *mreBCD* forms an operon and these proteins are involved in cell shape maintenance in *E.coli* (Masaaki et al., 1991). However, in the absence of a cell wall, the cell wall synthesis genes are absent in *Spiroplasma*. MreB1 is upstream of other MreB paralogs in *Spiroplasma*. However, MreB2 - 5 are closely placed in the genome and are predicted to be a part of the same operon (Bové et al., 2003; Harne et al., 2020). We were interested to understand if *fibril* is also a part of a functional gene cluster and whether its gene expression is dependent on the neighboring genes. To achieve this, an available genome database was checked to identify the gene neighborhood of all *Spiroplasma* species.

It was speculated that the switch in helicity of the ribbon might be arising due to change in the length of protofilaments of Fibril. This in turn leads to an overall change in helicity of the bacteria (Cohen-Krausz et al., 2011). The energy source for these conformational changes is not known. In order to find whether Fibril uses ATP as the energy source for these conformational changes, we monitored ATP hydrolysis activity of Fibril using malachite green assay.

Sequence based homology search of Fibril shows 24% identity with 5'-methylthioadenosine nucleosidase (MTAN) (Cohen-Krausz et al., 2011) based on alignment with 1-236 amino acids of Fibril. The sequence from 237-512 amino acids does not show homology to any protein in the available database. MTAN is a multifunctional enzyme and catalyzes the hydrolysis of N-ribosidic bond of adenosine-based metabolites. The 4 substrates of the enzyme are *S*-adenosylhomocysteine (SAH), 5'-methylthioadenosine (MTA), 5'-deoxyadenosine (5'-DOA) and 6-amino-6-deoxyfutasolose (Lee et al., 2001; Mishra and Ronning, 2012). As Fibril shows partial sequence similarity with MTAN, we checked the hydrolysis activity of Fibril using SAH as the substrate.

This chapter describes the sequence analysis, gene neighborhood analysis of Fibril sequences in *Spiroplasma*, and checking for the presence of biochemical activities of ATPase and nucleosidase in Fibril.

2.2 Materials and Methods

2.2.1 Phylogenetic tree of *Spiroplasma* species

For the phylogenetic analysis of *Spiroplasma* species, 16srRNA sequences were procured from 16S RefSeq records (https://www.ncbi.nlm.nih.gov/refseq/targetedloci/16S_process/). The sequences for *fibril* for each *Spiroplasma* strain were selected from NCBI. (<https://www.ncbi.nlm.nih.gov/>). The sequences were aligned using the MUSCLE program and the phylogenetic tree was prepared using MEGA 11 (Tamura et al., 2021). The evolutionary trees were generated using the Maximum likelihood method and inferred from 200 replicates. *Escherichia coli*, *Bacillus subtilis* and *Clostridium botulinum* were selected as outgroups and as the root of the tree. BOOTSTRAP value of 70% was used for dividing the strains and Fibril sequences into clades (Scott Muerhoff et al., 1997). *S. helicoides*, *S. sabaudiense*, *S. ixodetis*

and *S. platyhelix* were removed from the evolutionary analysis for *fibril* gene as either *fibril* was a pseudogene/absent or the genomic data was unavailable for the strain.

2.2.2 Model based structural analysis of Fibril using I-Tasser and AlphaFold

3D model building for Fibril was done using I-TASSER (Zhang, 2008) and AlphaFold (Jumper et al., 2021). I-TASSER uses homology-based modelling whereas AlphaFold builds models based on the nature of primary amino acid sequence of the protein using AI-based approaches including sequence and structure databases. Protein sequence of Fibril (Accession number-Q14M84) from *Spiroplasma citri* was selected as the query sequence for generating a model. Models were also generated for Fibril Δ (1-236) and Fibril Δ (237-512). The AlphaFold model for Fibril Δ (1-236) was used as the query structure for DALI search (Holm and Rosenström, 2010). Models were viewed and analyzed using UCSF Chimera (Version 1.16).

2.2.3 Comparison of gene neighborhood of Fibril

Genes adjacent to *fibril* were identified from the genome database of the NCBI (<https://www.ncbi.nlm.nih.gov/>). 5 genes upstream and downstream of *fibril* were selected to check conservation of the adjacent genes. The directionality of the genes was decided based on whether *fibril* was present on the plus strand or minus strand of the genome. The unannotated genes were identified using NCBI BLAST (McGinnis and Madden, 2004). Hits obtained from BLAST search which were above 70% identical to the query sequences were selected for the identification of unannotated genes. The genes which gave hits below 70% identity were not used for identification and are termed as hypothetical genes.

2.2.4 Identification of promoter and terminator sequence of Fibril

To predict the promoter sequences for Fibril in each strain of *Spiroplasma*, sequences which were 100 base pairs upstream and downstream of *fibril* gene were analysed. The -10 and -35 promoter sequences were identified using Softberry (<http://www.softberry.com/>). The BPROM program under Softberry was used to predict the promoter sequences. To obtain consensus sequences for -10 and -35 promoters, the sequences obtained for each *Spiroplasma* strain were then put into Weblogo (Crooks et al., 2004).

2.2.5 Sequence alignment with MTAN

25 sequences of Fibril used for generating phylogenetic trees were selected for sequence alignment with MTAN. 3 adenine bound MTAN structures were selected from PDB (PDB ID:-

1JYS, 3NM6, 6K2Q). Sequences from these 3 structures were aligned with Fibril using the MUSCLE program in Jalview Version 2. Adenine binding residues in the MTAN sequences were identified for *E. coli* and compared with the Fibril sequences. The identification of residues from the *E. coli* active site was based on the information available for the crystal structure of *E. coli* MTAN in complex with adenine (Lee et al., 2001).

2.2.6 MTase assay

Enzymatic activity of Fibril was checked using MTase-Glo™ Methyltransferase Assay (Promega - V7601). Activity was checked using 3 different concentrations of Fibril - 1 μ M, 10 μ M and 25 μ M. SAH standards were used in the range of 0.1 μ M to 1 μ M. For the reactions, 1 μ M of SAH and 1X methyltransferase buffer was used. A reaction containing Fibril, SAH, methyltransferase buffer and MilliQ were mixed in appropriate quantities and incubated at 25 °C for 30 minutes. 5 μ L of 5X Glo reagent was added into the reactions as well as the standards. The reactions were incubated again at 25 °C for 30 minutes. 10 μ L of detection reagent was added and the reactions were incubated in similar conditions as mentioned above. All the reactions were transferred in a Costar black 96 well plate (Corning - CLS3915). Luminescence readings were recorded as a spectra from 555-570 nm. The readings obtained were plotted against the protein concentrations using GraphPad Prism 9.2.

2.2.7 ATP hydrolysis using malachite green assay

ATP hydrolysis activity was checked for Fibril using Malachite green assay (Feng et al., 2011). The amount of inorganic phosphate released by the protein upon hydrolysis of ATP was monitored using the colorimetric assay. Fibril was resuspended in 100 - 200 μ L MilliQ depending on the turbidity of the solution. A fraction of the protein was solubilized by adding 0.5 μ L 20 % SDS to 9.5 μ L of Fibril. The SDS containing protein sample was spun at 21000 g for 1 minute at room temperature. The solubilized protein was used for protein concentration estimation using Nanodrop. A solution of 0.5 μ L 20 % SDS and 9.5 μ L MilliQ was used as a blank. The extinction coefficient was set as 13.4 M⁻¹ cm⁻¹ (<https://web.expasy.org/protparam/>) and absorption at 280 nm was measured.

For the malachite green assay, 10 μ M of Fibril and 2 mM ATP was used for the reactions. All the reactions were maintained in 300 mM KCl, 50 mM Tris pH-8.0. 100 μ L of reaction was prepared and incubated at 25 °C. As Fibril protein is insoluble and can interfere with the absorption at 630 nm, reactions were spun at 21000 g for 2 minutes before taking sample for each timepoint. 20 μ L of the reaction mixture was taken out after the interval of 30 minutes.

ATP hydrolysis for Fibril reactions was monitored for 30 and 60 minutes whereas the control reaction of MreB^{WT} was monitored for 30 minutes. To stop the reactions, 5 μ L of 0.5 M EDTA was added in the 96 well plate.

Phosphate standards (0, 25, 50, 75, 150, 200 μ M) were prepared from a stock of 400 μ M NaH₂PO₄. For the preparation of 800 μ L of malachite green solution 3.5 mM Malachite green (Sigma Aldrich - 38800) was dissolved in 3N H₂SO₄. 16 μ L 11 % Tween 20 (Sigma Aldrich - P-1379) and 200 μ L 7.5 % ammonium molybdate (Sigma Aldrich - 277908) were added to the solution. 50 μ L malachite green solution was added to the 25 μ L reactions and phosphate standards. Readings were taken 5 to 8 minutes post addition of malachite green reagent.

The absorbance was measured at 630 nm in EnSight Multimode plate reader (Perkin Elmer). Standard curve was plotted for phosphate standards to obtain the slope. The absorbance obtained for protein reactions were subtracted with respective blanks (buffer + ATP). Amount of phosphate release was calculated by dividing the above value by the slope obtained from the standard curve. The rate of phosphate release was calculated by dividing the time of incubation. The rate of phosphate release was further divided by protein concentration to get k_{obs} (min⁻¹). GraphPad Prism 9.2 was used for plotting the graphs. Statistical significance was estimated using 2-way ANOVA. The data is expressed as mean $\pm$ SEM.

2.3 Results

2.3.1 Sequence similarity between Fibril correlates with evolution of *Spiroplasma*

Genomes of all *Spiroplasma* strains were checked from the NCBI database. *S. helicoides fibril* gene showed the presence of a frameshift and *S. sabaudiense* had an internal stop codon (Chang et al., 2014; Shen et al., 2016) (Figure 2.1 A and B). Sequence alignment of *S. citri* and *S. helicoides* show frameshift leading to a premature stop codon (Figure 2.1 C and D). Genome of *S. platyhelix* (Accession no:- NZ_CP051215) does not show the presence of *fibril* as well. Due to the presence of a non-functional gene of *fibril*, the three strains were removed from the phylogenetic analysis for Fibril. As there is no genomic data available for *S. ixodetis*, the strain was also removed from further analysis.

A

<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	10 20 30 40 50 60	ATGATAGGAATTTATCTCAACAGCATCTTCACATTGAAAGACCGTCACGACATCAAGCATAT
<i>S. citri</i>	NZ_CP013197.1/1-1539		ATGATCGGGCTAATTTCAACAGCATATTTACGATGAAAGATAGCACAGTATTAAGACAG
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	70 80 90 100 110 120	TAAAAAGTACTGATGAAGAAATATGGTTATTTCAATCTTGAAGTATAGAGGAAAAACCTTTA
<i>S. citri</i>	NZ_CP013197.1/1-1539		AAAAAATATTTGGTGAAGAACCTGTGTAATTCACATGTTAAATACCATGGAAAAACCTTTA
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	130 140 150 160 170 180	TCATACCTACAAATGCTTATGGTAAAGCAAACCCCGCAATGGCAATACATATTTAATGGAA
<i>S. citri</i>	NZ_CP013197.1/1-1539		TTATAGCAACAGTAGGATATGGTAAAGCAAATGCTGCAATGACTATTACTTACTTATTAGAA
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	190 200 210 220 230 240	GAGTACAAAACTTAGAAACTGTTTTAAACATTGACTTAGCTCTATCAACAAATGATAAATT
<i>S. citri</i>	NZ_CP013197.1/1-1539		AAATATCCTGGTTTACAAACAAATTTTAAATGTAGACTTAGCAATGTCAACAAACGATAAGCA
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	250 260 270 280 290 300	TGATACACAGATCTGTTATGTCAACTTAAATTCATTTACAGAGATGCAGATTTGACTGTTT
<i>S. citri</i>	NZ_CP013197.1/1-1539		TGATACAGGAGATACCACTATCTCAACAAATTTTATTTATCTGATGCTGACTTTAACAGTTT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	320 330 340 350 360 370	TTAAAGATATTAATATGGACAAATTTGTTCCGAACCCAGAACATTCCAATTCGATACCTGAA
<i>S. citri</i>	NZ_CP013197.1/1-1539		TTAAAGATATTAATATGGACAAATTTGTTATGAACCTGAATCATTCCAATTCGATGCTGAA
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	380 390 400 410 420 430	TTAGTTCAAAAGTTTAAACCTTTAAATTTAGGAGTTTCAAGTGAATTTGTTGGAACAGCTGA
<i>S. citri</i>	NZ_CP013197.1/1-1539		TTGCTTAAAGTTTAAAGACTTTAAATTTGGGTTTAAACAGAGAGTTTACTGGAAACAGCAGA
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	440 450 460 470 480 490	CATGTTAATCTACAACCTCAAAACAAATTTAAAGAAATGGTTGACAAATACGCAACAACTTG
<i>S. citri</i>	NZ_CP013197.1/1-1539		TATGTTGATTTTAACTCAAAACAAATTTAAAGAAATGGTTGATAAATATGGGCATACATTG
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	500 510 520 530 540 550	ACGTAATTTGATCTGAAGCAGGAGCAATTAGCTCAAGTTGCAAAAAAATCAACAGTAAATTTTC
<i>S. citri</i>	NZ_CP013197.1/1-1539		ATGTAATTTGATCTGAAGCAGGGCAATTCGCAAGTTGCTAAAAAATCAAGTATTAATTTAT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	560 570 580 590 600 610	ATTGCAATGAAATTTATGTACACAAATGCATTAACTCTTGAGACAAATGATCCATTACATAA
<i>S. citri</i>	NZ_CP013197.1/1-1539		ATTGCTTTAAAAATTTATTAATAATGCATTATCACCATGAGATAATGATCCAATCCATAA
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	630 640 650 660 670 680	ATTTAAAAATTTATGAACAACTAATCTTTAAAAATCTTACTTGTAAAGATTATTAATTTAT
<i>S. citri</i>	NZ_CP013197.1/1-1539		ATTTAAAAATTTATGAACAACTAATCTTTAAAAATCTTATTAAAGAGATTATTTAACTTAT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	690 700 710 720 730 740	TATCTTCAAACTAGTTCTTGATTTTCAAGTAAACCTCACTTGATGAATTAGATATCATCAAT
<i>S. citri</i>	NZ_CP013197.1/1-1539		TAAGTTCAAAATATATTTATTTATTTATCACAATGTTTCACAAGATGATTTTAGATTCATAAT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	750 760 770 780 790 800	GACTTATTTGAAATCGAACATGATTCATGAGTGAAACCGTTTCAGCCAGCTGCAACTCAATT
<i>S. citri</i>	NZ_CP013197.1/1-1539		GAAATATTTGAAATTAACATGACCAATGAATTAATTTTAAACCAATACATCAATAGT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	810 820 830 840 850 860	ACTTTCAAGTGCAGGACCATCTCTAATGCTCTTGACGCTAAAGGTTTAAAAACGAAAGCAA
<i>S. citri</i>	NZ_CP013197.1/1-1539		ATTATCAGGATTTGGGCTTCATTAATGTTTATGATAAACAAAGAAAAAATCCTGTTGCAT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	870 880 890 900 910 920	TTGACTTGTGTAAGTTTATGAACAAAAAGTTGAAGAA--AAGGACCAAGTAAAAATTTAT
<i>S. citri</i>	NZ_CP013197.1/1-1539		TAGATATTTCAAGTAATCCGTTCAAAAAACAAAGAAAGCAGAGGACCAAGTAAAGTAAAT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	940 950 960 970 980 990	TTAGGGAGGATGAATGAAAAACATGCTCCTAAAAAATGATTGAAAAAGTTATGTTCTTGA
<i>S. citri</i>	NZ_CP013197.1/1-1539		TTAGGAGAGGATGAATGAAAAAATGCTCCTAAAAAATGATTACGTAAATTTATTTTITAGA
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	1000 1010 1020 1030 1040 1050	AAATGTTTCAATGTAATTAAGATGAATTTATTTATGAAACCGTTTCAACAAATACGATGTTAAAT
<i>S. citri</i>	NZ_CP013197.1/1-1539		ACAAGTTTCTGTTAATGATGATGAATTTATTTATGAAATAATCAACAAATATGAT-TTAAAT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	1060 1070 1080 1090 1100 1110	CAGAAAAAATCTT-CACAAATTGAAGAACTGCTAAAGCAATCGCTAAAAAATGCTGACCGG
<i>S. citri</i>	NZ_CP013197.1/1-1539		AATGAAAAATTTATATAAATTTGAACAGTTGCAAAATGAATTTGCTGCAGCCATTGCTGAAAA
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	1120 1130 1140 1150 1160 1170	TTCAAGAGCAAAATCATCATATCGATACGATGGAAACACAGCTTAAACAAACACTTAAATGG
<i>S. citri</i>	NZ_CP013197.1/1-1539		ATGTCAGATAAATCATCATATACATATAATGGTGCACAGTACCAGAAAAATACTTATTAG
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	1180 1190 1200 1210 1220 1230	TAACTTGTGATCCTTTTCTTTCTACATTACAAATAAACCAACTCACGAATTCGTTGAG
<i>S. citri</i>	NZ_CP013197.1/1-1539		TAAATTTGTGATGCAAGAAATTCATTTTACATTACCCATAACCAATCACATGAATTTTGA
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	1250 1260 1270 1280 1290 1300	GAAAGAAAAATTTGGTAGTGAAATAGTCGTTAATGAATTTATTAATTTCTTAAACACGAATT
<i>S. citri</i>	NZ_CP013197.1/1-1539		GATAAAAAATTTGGAAACACAAATAGTAAGTAATGAATTTGTAATATTTAAATGAAGCAT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	1310 1320 1330 1340 1350 1360	GAAAGAACTGGAATCACCAATTTACTAAAATTTGCTATTTTATTAATAATCCAGCAATCGGAA
<i>S. citri</i>	NZ_CP013197.1/1-1539		GAAAGATGTTGACTCACCATATCAACAAATTTGTTATTTACATGACAAATCCAGCATTAGATT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	1370 1380 1390 1400 1410 1420	ACAGAAAGAAATCCAGTATTTATTTGAACTGCTAAAAAAGCAACCAACCAATTAATTTCTTG
<i>S. citri</i>	NZ_CP013197.1/1-1539		ATCGAAAAATTTCAAGTGTATTCTTTCAACAAAGGAGCAACAGAGGTGTTAAATTTCTTG
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	1430 1440 1450 1460 1470 1480	GGTT--ACTCAGAAATCAACCAACAAAAATACACAGTTGTAGATATTTCAAGAAATGACTAC
<i>S. citri</i>	NZ_CP013197.1/1-1539		GCTTTAAATCAAAAACTA--CAACGAGATATACTGTTGTTGATATTACAGAAATGATTAT
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545	1490 1500 1510 1520 1530 1540	CACCAATTAAGTTTGGATCATTTCAAAGTAACTGTTTGAATTAATAAAGCAAGTAACTAAATA
<i>S. citri</i>	NZ_CP013197.1/1-1539		GATCCAATTAAGTTTGGTTCATTCAAAGTTACAAATTCGTTTAAAAAGTGAA-TAA-----
<i>S. sabaudiense</i>	NZ_CP006934.1/1-1545		G
<i>S. citri</i>	NZ_CP013197.1/1-1539		-

B

<i>S. sabaudiense</i>	NZ_CP006934.1/	970 980 990 1000 1010 1020 1030 1040 1050 1060	ATGATGAAAAAGTATGTTCTTCAAAAAATGTCATGTAAGTAAATGAATGAAATATATGAAGCCGTTCAAGCAAAATACGATGTTAAATCAGAAAAAT
<i>S. citri</i>	NZ_CP013197.1/		ATGATGCGTAAATATATTTTAAAGCAAGTGTGCTGTAAGTAAATGAATGAAATATATGAAGCAAAATCAGCAAAATATGAT-TTAAATTAAGAAAAAT
<i>S. sabaudiense</i>	NZ_CP006934.1/	330 340 350	K K W L K V M F L N V H V N D D E L L W N R S A S Y D V - K -
<i>S. citri</i>	NZ_CP013197.1/		K K W L R K L L F L F Q V R V N D D E L L W N K S A S Y D - L - N

C

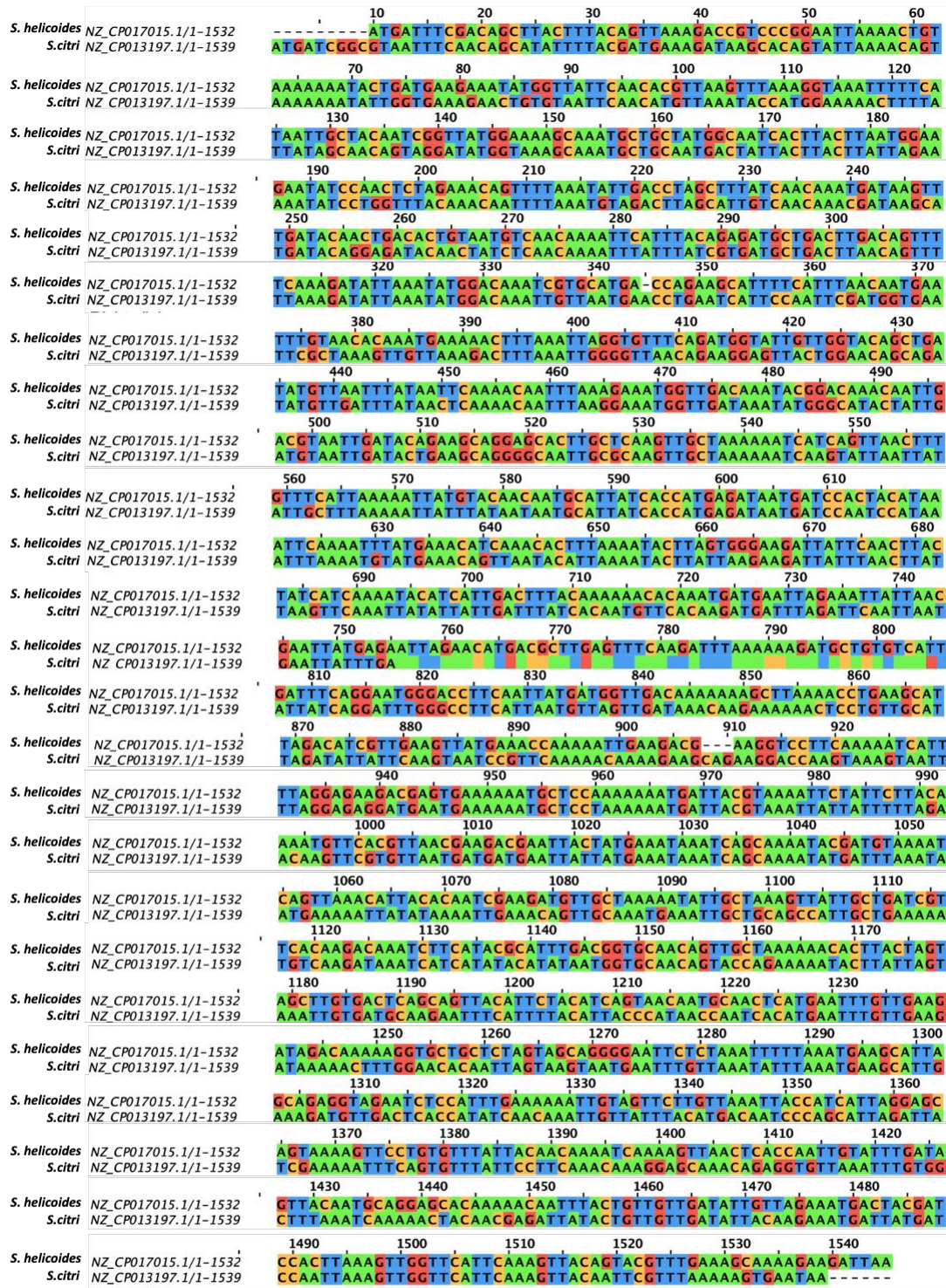


Figure 2.1. Sequence alignment of *S. citri* with *S. sabaudiense* and *S. helicoides*. **A.** Nucleotide sequence alignment of *S. citri* and *S. sabaudiense*. **B.** Zoomed image of the alignment highlighting mutation at the nucleotide and protein sequence. **C.** Nucleotide sequence alignment of *S. citri* and *S. helicoides* Fibril sequences. **D.** Zoomed image of the alignment highlighting frameshift at the nucleotide and protein sequence. The region of mutation in nucleotide and the corresponding protein sequence is highlighted within a black box. The red box in **D** denotes the premature stop codon in *S. helicoides* caused by the frameshift.

We compared the 16srRNA of the 29 *Spiroplasma* strains, for which genome sequence is available. The phylogenetic tree shows division into 2 clades, namely, Apis and Citri (Figure 2.2 A). This division was in agreement to the previous studies where the conservation of MreB was monitored amongst the strains (Ku et al., 2014; Rattner et al., 2021). *E. coli*, *B. subtilis*, *C. botulinum* were identified as outgroups and the clade was selected as the root for the tree. The evolution study for Fibril was carried out with 25 *Spiroplasma* strains. The phylogenetic tree for Fibril showed a similar trend as observed for the 16srRNA except for *S. alleghenense* (Figure 2.2 B). In spite of lacking a functional Fibril, *S. helicoides* and *S. sabaudiense* show similar morphology as other *Spiroplasma* strains (Abalain-Colloc et al., 1987; Whitcomb, et al., 1997). This indicates that Fibril alone might not be responsible for conferring helicity and motility and there might be additional proteins that contribute to maintaining this morphology.

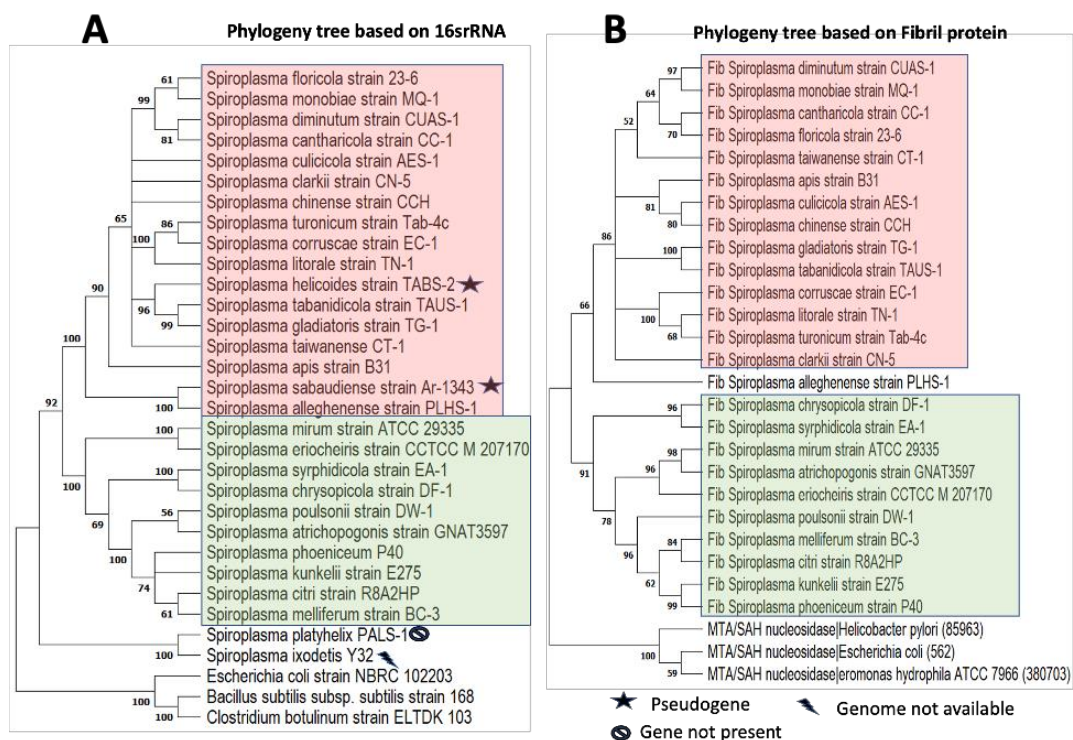


Figure 2.2. Phylogenetic and evolution of *Spiroplasma* strains and Fibril. **A.** Phylogenetic tree of *Spiroplasma* strains with cell-walled organisms like *E. coli*, *B. subtilis* and *C. botulinum* as an outgroup. **B.** Phylogenetic tree of Fibril protein from *Spiroplasma* strains and its distribution in different clades.

Interestingly, two of the strains *S. helicoides* and *S. sabaudiense* lacking a functional Fibril protein belong to the Apis clade. *S. platyhelix* has no *fibril* gene and shows the presence of two *mreB* genes unlike other *Spiroplasma* strains which shows the presence of 5 to 8 *mreB* genes. *S. platyhelix* morphology shows almost straight rods with little or no rotary movement (Williamson et al., 1997). This might be due to the loss of most of the cytoskeletal proteins in *S. platyhelix*. It will be interesting to check the cellular concentrations of MreB and compare within the strains in order to understand if absence of a cytoskeletal protein is compensated by overexpression of other cytoskeletal proteins. Estimation of helical pitch and motility rates can help in correlating the cellular levels of the MreBs and Fibril with the morphology of all the strains.

2.3.3 Conservation of *fibril* gene neighborhood

From the above study, we observed that Fibril is present in most of the *Spiroplasma* strains sequenced so far. However, as discussed in the previous section, Fibril alone might not be enough to configure the helical and motile phenotype to *Spiroplasma* species. In order to address this, we checked the gene neighborhood of *fibril* to identify the presence of any accessory proteins that might have a role in cell shape maintenance (Figure 2.3A). The gene neighborhood of the *fibril* looks fairly conserved. However, except a lipoprotein identified in the neighborhood, other genes present upstream and downstream of *fibril* have no known role in either membrane binding or conferring shape phenotype of any organism (Figure 2.3 B).

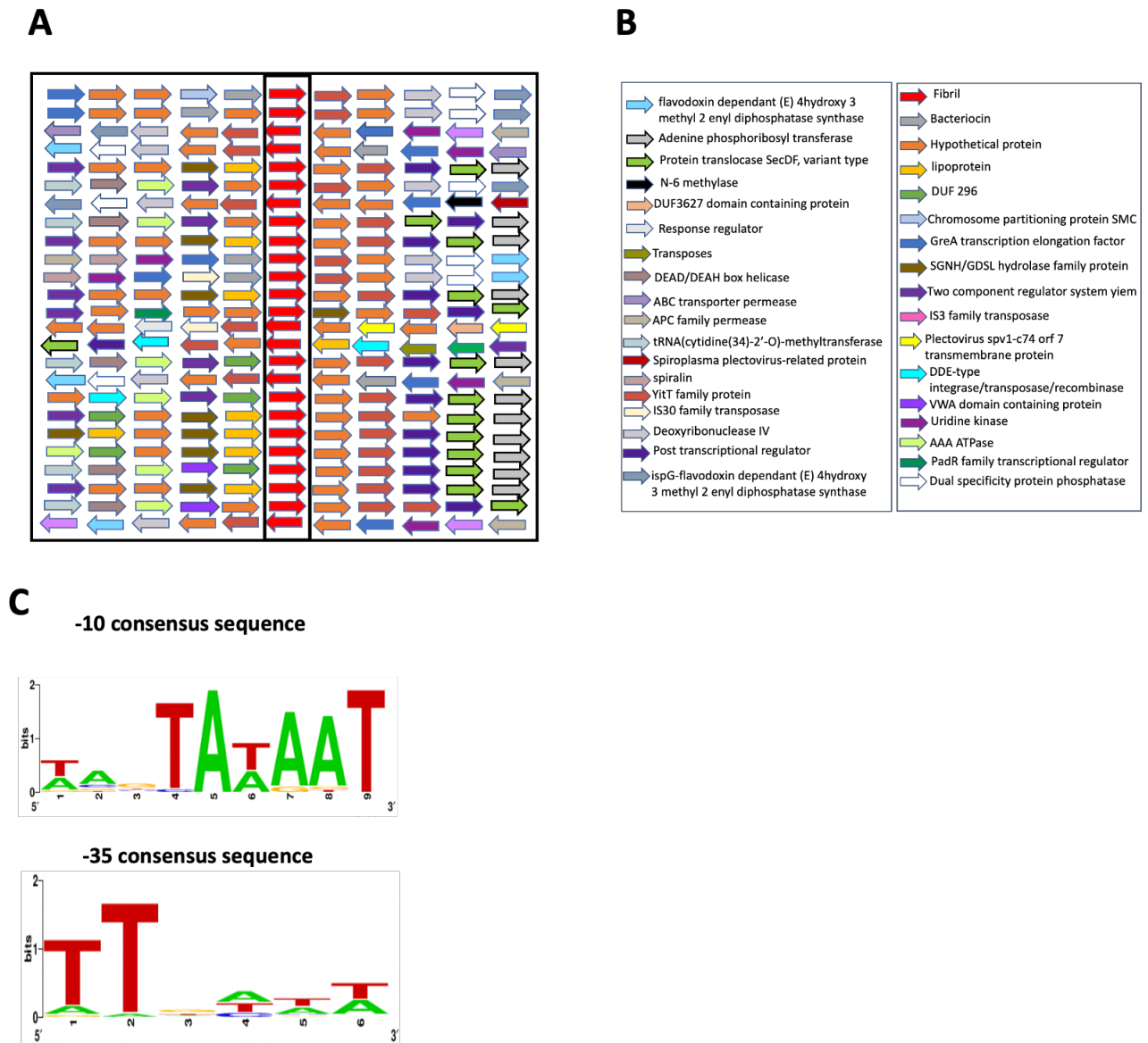


Figure 2.3. Gene neighborhood and promoter sequences of Fibril. **A.** 5 genes upstream and downstream of Fibril were identified and are denoted with different colors. Conservation of a protein can be monitored by color scheme amongst the strains. **B.** Genes present in the neighborhood of Fibril. Some of the genes which were not annotated in the genome were identified using NCBI BLAST. The genes which showed 70% and above sequence identity were selected for gene identification. **C.** -10 and -30 consensus promoter sequences for 25 *Spiroplasma* strains.

Presence of -10 and -35 consensus sequences showed that Fibril might have its own promoter (Figure 2.3 B). To confirm whether *fibril* is a part of an operon, we checked the promoter sequence of the downstream gene (Figure 2.4).

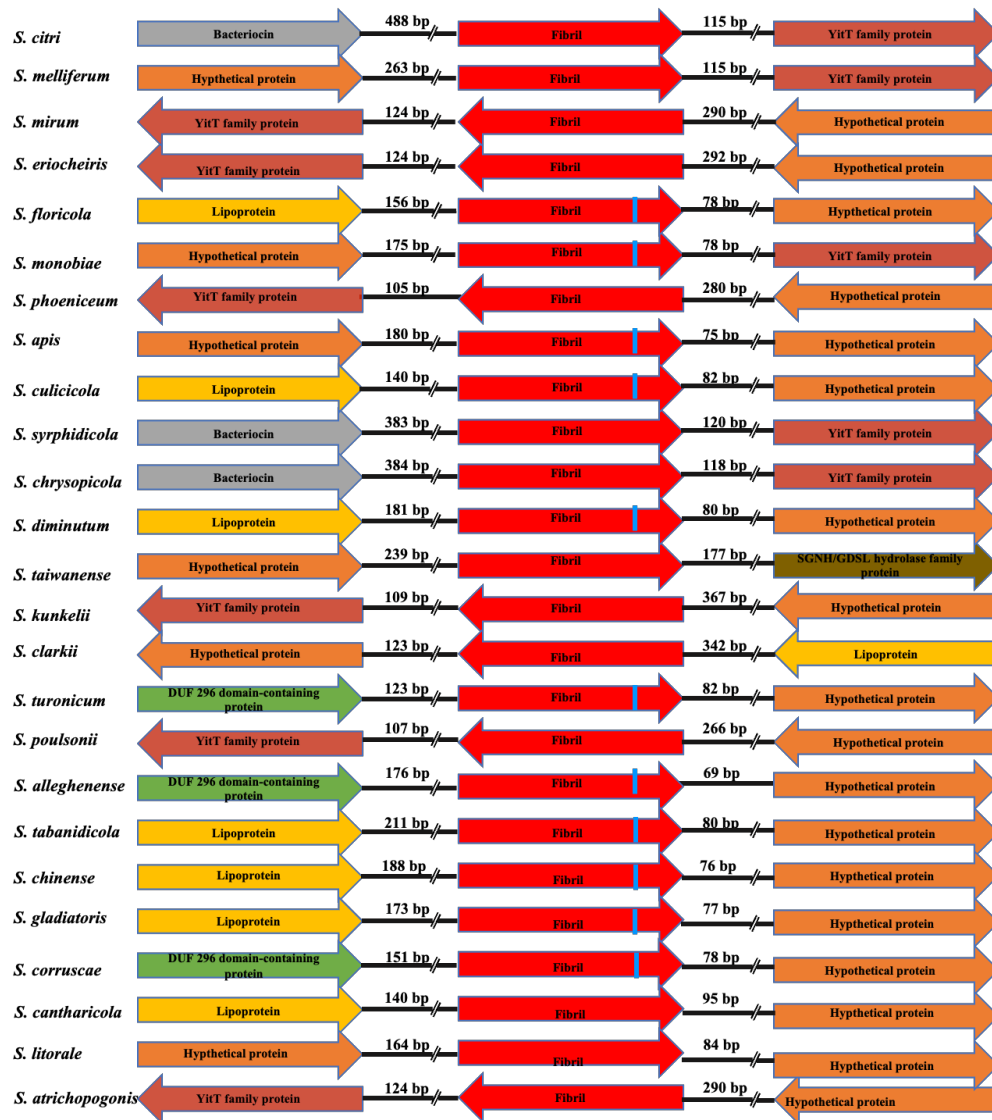


Figure 2.4. Intergenic distance of -1, Fibril and +1 indicates position of promoter for each gene. 25 strains of *Spiroplasma* possessing *fibril* genes were analyzed to check the presence of any operon. Intergenic distances are highlighted above the black lines connecting each gene. Fibril and the downstream gene having intergenic distance below ~ 84 base pairs showed the presence of a promoter within *fibril* gene. Promoter sequences predicted for downstream genes which were present within the *fibril* gene are denoted by the blue line. (Break in the line denotes a nucleotide sequence higher than 105 base pairs for -1 gene and 69 base pairs for +1 gene).

When the distance between *fibril* and the upstream gene was calculated, it was found that for some strains of *Spiroplasma*, the two genes lie close to each other (intergenic distance <85 base pairs). For the strains, *S. floricola*, *S. monobiae*, *S. apis*, *S. diminutum*, *S. turonicum*, *S. alleghenense*, *S. tabanidicola*, *S. chinense*, *S. gladiatoris* and *S. corruscae*, no promoter sequence was predicted between the two genes. However, when a 50-nucleotide long sequence from *fibril* was added for the analysis, a promoter was predicted for the downstream gene

within the *fibril* gene. This indicates that the promoter sequence of the downstream gene might be a part of the *fibril* sequence.

In order to study the effect of *fibril* deletion on the morphology of the *Spiroplasma* strains, it will be useful to check the promoter region of the adjacent genes so that there is no downstream effect. Amplification of this region and sequencing of the transcript will help in experimental validation of this observation.

2.3.2 Model building and structural analysis of Fibril and its domains

In the absence of any homologous structures and prior to obtaining experimental structures, we built models of Fibril for getting structural insights. Structural analysis will help us to identify the domain boundaries, and in identifying surface residues which might be interacting with other cytoskeletal proteins like MreBs. We can also predict the residues or domains which might interact with the membrane. Two model prediction softwares were used for model building of Fibril, namely, I-TASSER and AlphaFold. The N-terminus domain of Fibril, ranging from 1 - 236 amino acids highlighted in pink, were aligned to the MTAN structure (PDB :- 1JYS) (Figure 2.5). When the AlphaFold model was superimposed with 1JYS, the root mean square displacement (RMSD) value was 0.92 Å between 130 pruned pairs out of 214 pairs. The overall RMSD observed was 4.29 Å. When the I-TASSER model was superimposed with 1JYS, the RMSD value was 0.96 Å between 142 pruned pairs out of 214 pairs. The overall RMSD observed was 3.47 Å. However, the overlap between the structures was only seen for the Fibril N-terminal domain (1-236).

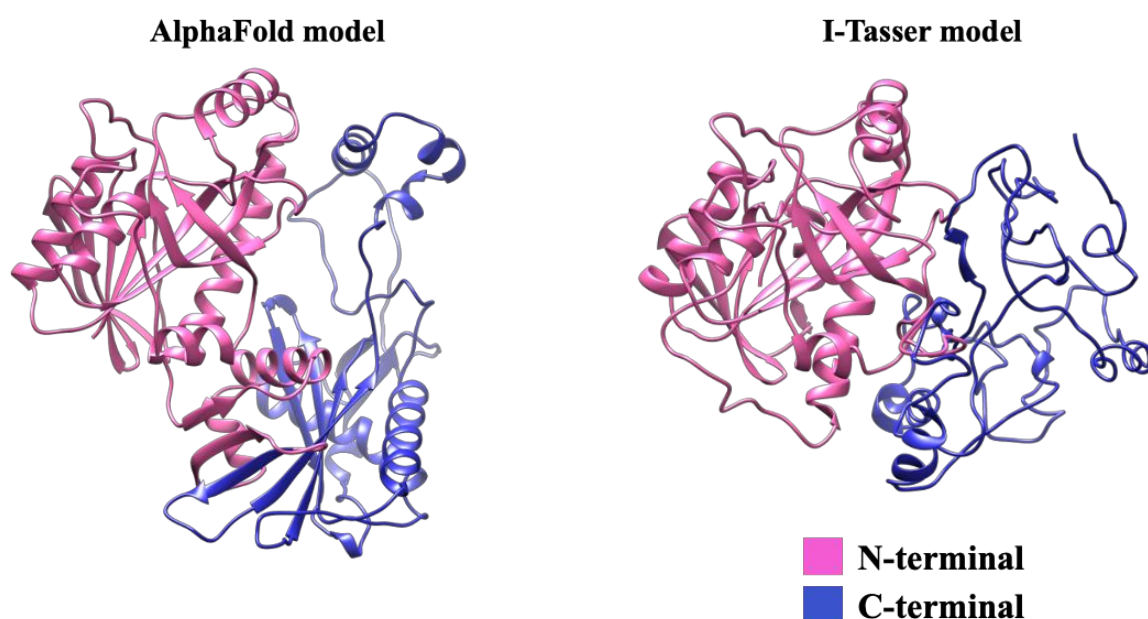


Figure 2.5. I-TASSER and AlphaFold structures of Fibril. 3D models were generated using I-TASSER and AlphaFold. N terminal domain (1-236) is highlighted in pink whereas C terminal domain (236-512) is highlighted in blue.

The C-terminal domain obtained from both the models showed high variation. A model was generated for the C-terminal domain (236-512) by AlphaFold and checked for homologous structures in DALI (Figure 2.6). Figure 2.6 A and B show the AlphaFold models for Fibril wild type and C-terminal domain (236 - 512) respectively. The homology search for the C-terminal domain gave Acetyl CoA carboxylase (PDB :- 5CSK) as a hit (Figure 2.6 C). However, the percent identity was only 10% and the RMSD value obtained was 9.8 (Figure 2.6 D). The information obtained from the homology-based search for C-terminal domain was not reliable as the root mean square displacement value was very high and the sequence identity was very low. Based on these observations, we concluded that the C-terminal half of the protein Fibril has a novel fold.

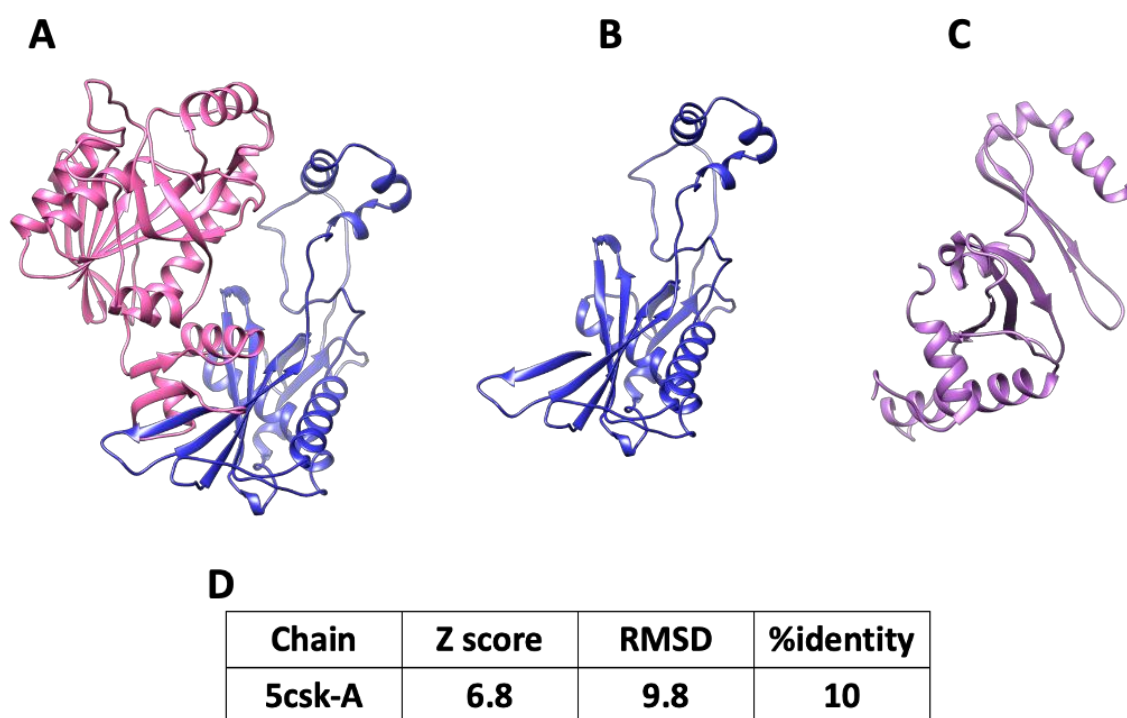


Figure 2.6. Structural analyses of C terminal domain. **A.** AlphaFold structure of Fibril with the N terminal and C terminal domains highlighted in pink and blue respectively. **B.** AlphaFold model of C terminal domain (237-512). **C.** Superimposed model obtained from DALI search when AlphaFold model of C terminal domain (237-512) was given as query structure. **D.** Acetyl CoA carboxylase was identified having very low homology to the C terminal domain of Fibril. The structural alignment parameters are mentioned in the box.

2.3.4 Adenine binding residues of Fibril and MTAN are not conserved

As discussed earlier, Fibril shares 24% sequence identity with 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN). Metabolism of one of the substrates, SAH by MTAN, gives rise to adenine and S-ribosyl homocysteine as products. Based on this information, we searched for MTAN structures bound to either of the products in PDB. Three structures were selected which were bound to adenine. The residues interacting with adenine were selected from the *E.coli* structure (PDB ID :- 1JYS). The available information from PDB structures 3NM6, 1JYS and 6K2Q was taken to identify corresponding residues in Fibril. Multiple sequence alignment of different MTAN and Fibril sequences showed that there is no conservation in the adenine binding residues (Figure 2.7). This indicated that Fibril might not hydrolyze SAH.

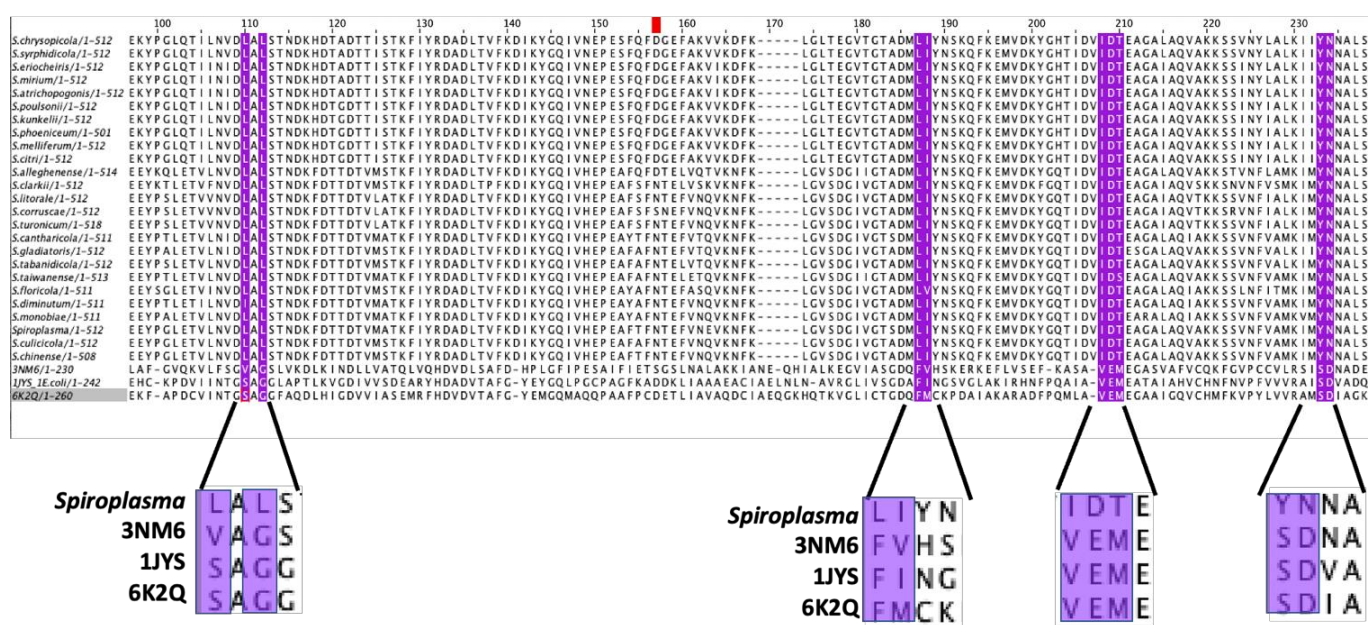


Figure 2.7. Sequence alignment of Fibril and MTAN. *Spiroplasma* Fibril sequences from 25 strains were aligned with MTAN from *E. coli*, *H. pylori* and *A. hydrophila*. Sequences from adenine bound MTAN structures were selected for this alignment (PDB ID :- 1JYS, 3NM6, 6K2Q). Adenine binding residues were identified for 1JYS and corresponding residues highlighted in other sequences.

Bacterial MTAN shows similar affinity for MTA and SAH (Siu et al., 2008). *Arabidopsis thaliana* MTAN1 and MTAN2 shows similar catalytic activity and substrate specificity for MTA and SAH (Park et al., 2009; Siu et al., 2011, 2008). However, MTAN2 shows high catalytic activity and substrate specificity for SAH.

In case of differential binding and catalytic activity of substrate, Fibril sequence was compared with AtMTAN1 and AtMTAN2 (Table 2.1). However, active site residues showed no conservation when Fibril sequence was compared to the bacterial MTAN and plant MTANs.

Table 1. Comparison of the residues of the substrate binding pocket of *Escherichia coli*, *Arabidopsis thaliana* MTAN with Fibril.

EcMTAN	Fibril	AtMTAN1	AtMTAN2
Ser 196	Tyr 193	Thr 224	Thr 211
Asp 197	Asn 194	Asp 225	Asp 212
Phe 151	Leu 147	Leu 181	Met 168
Ile 152	Ile 148	Asp 182	Asp 169

To prove this further, we checked if Fibril shows enzymatic activity similar to MTAN. We used an enzyme-based bioluminescence assay to monitor the change in SAH concentrations. The readout for the assay monitors formation of ATP upon hydrolysis of adenosine substrates. Hence, the assay plays a useful tool to monitor change in the concentration of any adenosine-based substrate in spite of the differential substrate specificity of MTANs.

2.3.5 Fibril does not show nucleosidase activity

Owing to the fact that Fibril shares similarity with MTAN, we performed enzymatic reactions to check if Fibril can catalyze the hydrolysis of SAH (Figure 2.8A). MTase assay showed that there is no formation of adenine as the luminescence values for Fibril-SAH reactions are similar to only SAH control (Figure 2.8B). This further proves that in spite of having similar domain structure of Fibril N terminus 1-236 with MTAN, Fibril does not possess enzymatic activity as MTAN.

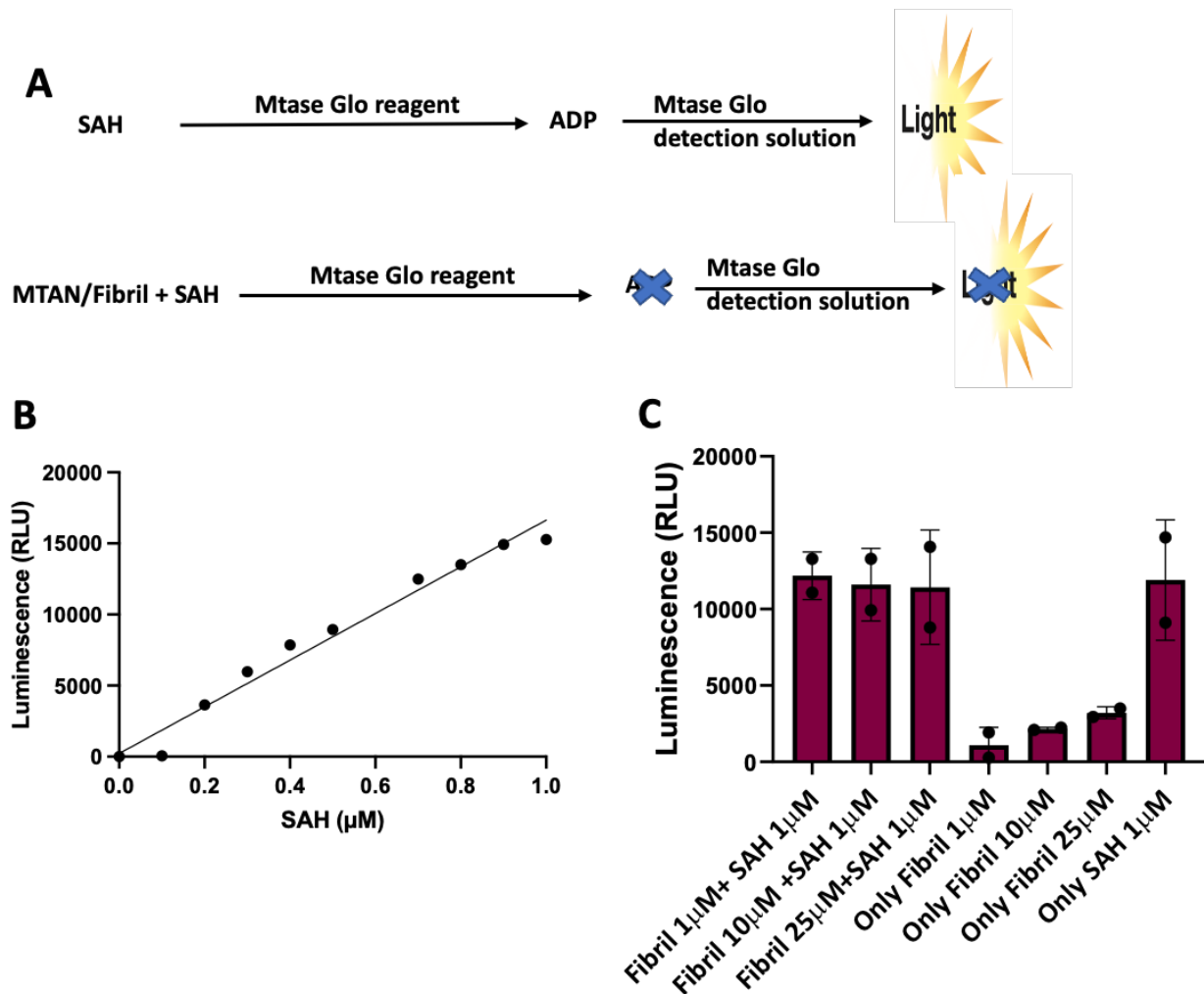


Figure 2.8. MTase assay for Fibril. **A.** Hydrolysis of SAH was monitored using the MTase Glo kit. SAH conversion to adenosine/adenine was monitored by the enzyme linked bioluminescence reaction. **B.** SAH standards from 0.1-1 μM were plotted against bioluminescence signals. **C.** SAH hydrolysis was checked by monitoring the luminescence signal (relative light units) for Fibril + SAH, only Fibril and only SAH reactions. Concentrations of Fibril taken were - 1 μM , 10 μM and 25 μM . Concentration of SAH used - 1 μM .

MTase assay proves that Fibril lacks enzymatic activity. Despite the similar domain architecture of Fibril N-terminal domain (1-236) to MTAN, Fibril might have lost the enzymatic activity.

2.3.6 Fibril does not possess ATPase activity

Unlike MreB, Fibril does not show the presence of a phosphate binding loop. It was suspected that conformational changes in Fibril might be a result of energy transfer from ATP. However, malachite green assay showed that Fibril does not show ATP hydrolysis (Figure 2.9).

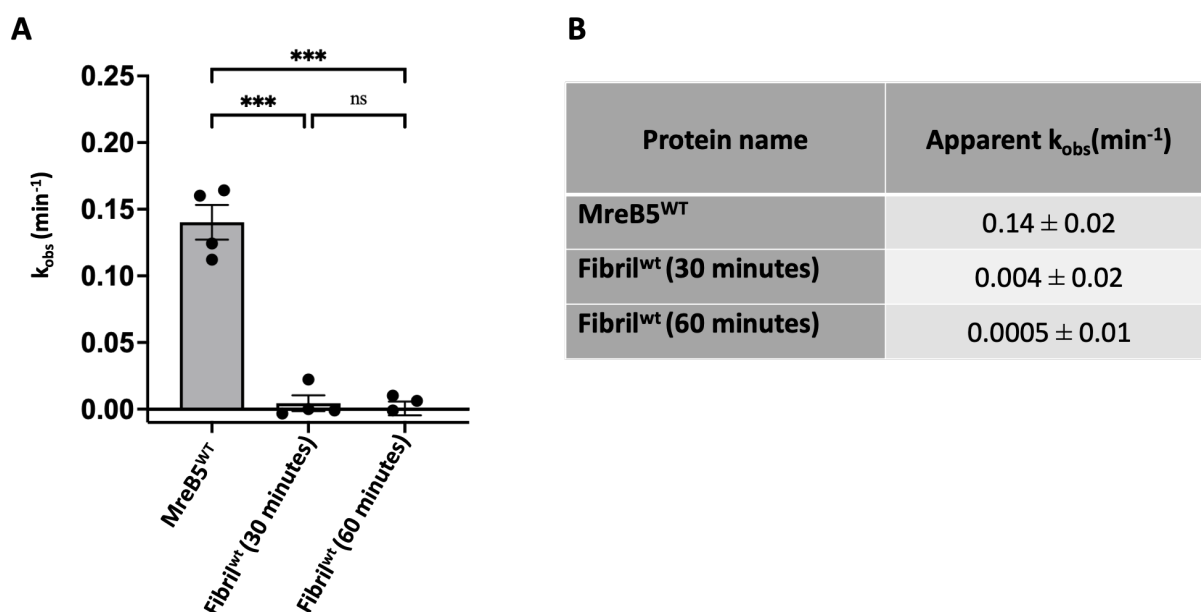


Figure 2.9. Fibril does not possess ATPase activity. **A.** ATP hydrolysis of Fibril was checked using malachite green assay. **B.** 10 μM of each protein was used to check the hydrolysis activity of the protein at two different time points- 30 minutes and 60 minutes. Above graph shows that Fibril does not show ATP hydrolysis.

MTase assay and malachite green assay proves that Fibril does not possess any enzymatic activity. Fibril might act as a scaffold for MreB proteins to interact with its paralogs as well as the membrane. The conformational changes in Fibril filament might be a result of the energy released from MreB proteins interacting with it in the cytoskeletal ribbon.

2.4 Discussion

In this chapter, we tried to compare the evolution of *Spiroplasma* strain with that of Fibril. From this analysis we observed that phylogeny based on 16srRNA of *Spiroplasma* and sequence-based identity of Fibril shows direct correlation. Presence of promoter sequences for each *fibril* indicates that the gene might be transcribed independently and is not a part of an operon. This observation is consistent with the requirement of high expression levels of *Spiroplasma* Fibril, since it forms one of the major components of the cytoplasmic ribbon. Hence, it might be essential to express the protein individually from an independent promoter in high amounts. For most of the species, the promoter sequences were identified for the downstream gene of *fibril* as well. This further supports our hypothesis.

As discussed earlier, Fibril is the major component of the cytoskeletal ribbon present beneath the cell membrane of *Spiroplasma*. The helical pitch of the ribbon was comparable to that of

the cell (Sasajima et al., 2022). Interestingly, Fibril was found in non-helical strains of *Spiroplasma citri* as well (Townsend et al., 1980). Comparison of morphological features like helical pitch and motility rate with the strains possessing Fibril and comparing it with the strains lacking a functional Fibril might shed light on the function of this protein. Absence of Fibril from strains of *Spiroplasma* also raises a question whether Fibril is an essential gene and required for the viability of this species. Many *Spiroplasma* strains especially, those belonging to the Citri clade, show the presence of many plectoviral genes leading to bigger genome size as compared to Apis clade. As seen in the previous section, two of the strains lacking *fibril* genes belong to the Apis clade. As Fibril is present in *Spiroplasma* species alone, it will be interesting to study whether the gene was acquired by horizontal gene transfer from its host or emerged during speciation.

In order to elucidate the function of Fibril, we used enzyme linked bioluminescence assay to monitor hydrolysis of SAH substrate. Model structure of Fibril N- terminal domain (1-236) and MTAN (PDB ID:- 1JYS) shares high structural similarity with RMSD value 0.92. Despite that, we found that Fibril does not possess the SAH hydrolysis activity. Adenine is the common product for all the substrates catalyzed by MTAN. As the bioluminescence levels depend on the amount of adenine formed and getting converted to ATP, this assay can be used to check enzymatic activity with other substrates of MTAN as well.

We also monitored ATP hydrolysis activity of Fibril. However, no enzymatic activity was seen for this protein. These results prove that unlike bacterial actin or tubulin homologs, Fibril function is independent of any nucleotide or cofactor. Fibril might have a role in providing structural support to the other cytoskeletal proteins such as MreB in conferring helicity and motility.

2.5 References

1. Abalain-Colloc, M.L., Chastel, C., Tully, J.G., Bove, J.M., Whitcomb, R.F., Gilot, B., Williamson, D.L., 1987. *Spiroplasma sabaudiense* sp. nov. from Mosquitoes Collected in France. *Int J Syst Bacteriol* 37, 260–265. <https://doi.org/10.1099/00207713-37-3-260>
2. Bové, J.M., Renaudin, J., Saillard, C., Foissac, X., Garnier, M., 2003. *Spiroplasma citri*, A Plant Pathogenic Mollicute: Relationships with Its Two Hosts, the Plant and the Leafhopper Vector. *Annu Rev Phytopathol.* <https://doi.org/10.1146/annurev.phyto.41.052102.104034>
3. Chang, T.-H., Lo, W.-S., Ku, C., Chen, L.-L., Kuo, C.-H., 2014. Molecular Evolution of the Substrate Utilization Strategies and Putative Virulence Factors in Mosquito-Associated *Spiroplasma* Species. *Genome Biol Evol* 6, 500–509. <https://doi.org/10.1093/gbe/evu033>
4. Cohen-krausz, S., Cabahug, P.C., Trachtenberg, S., 2011. The Monomeric , Tetrameric , and Fibrillar Organization of Fib : The Dynamic Building Block of the Bacterial Linear Motor of *Spiroplasma melliferum* BC3. *J Mol Biol* 410, 194–213. <https://doi.org/10.1016/j.jmb.2011.04.067>
5. Crooks, G.E., Hon, G., Chandonia, J.M., Brenner, S.E., 2004. WebLogo: A sequence logo generator. *Genome Res* 14, 1188–1190. <https://doi.org/10.1101/gr.849004>
6. Feng, J., Chen, Y., Pu, J., Yang, X., Zhang, C., Zhu, S., Zhao, Y., Yuan, Y., Yuan, H., Liao, F., 2011. An improved malachite green assay of phosphate: Mechanism and application. *Anal Biochem* 409, 144–149. <https://doi.org/10.1016/j.ab.2010.10.025>
7. Harne, S., Duret, S., Pande, V., Bapat, M., Béven, L., Gayathri, P., 2020. MreB5 Is a Determinant of Rod-to-Helical Transition in the Cell-Wall-less Bacterium *Spiroplasma*. *Current Biology* 30, 4753–4762.e7. <https://doi.org/10.1016/j.cub.2020.08.093>
8. Holm, L., Rosenström, P., 2010. Dali server: Conservation mapping in 3D. *Nucleic Acids Res* 38. <https://doi.org/10.1093/nar/gkq366>
9. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Židek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S.A.A., Ballard, A.J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A.W., Kavukcuoglu, K., Kohli, P., Hassabis, D., 2021. Highly accurate protein structure prediction with AlphaFold. *Nature* 596, 583–589. <https://doi.org/10.1038/s41586-021->

10. Ku, C., Lo, W.S., Kuo, C.H., 2014. Molecular evolution of the actin-like MreB protein gene family in wall-less bacteria. *Biochem Biophys Res Commun* 446, 927–932. <https://doi.org/10.1016/j.bbrc.2014.03.039>
11. Lartigue, C., Lambert, B., Rideau, F., Dahan, Y., Decossas, M., Hillion, M., Douliez, J.P., Hardouin, J., Lambert, O., Blanchard, A., Béven, L., 2022. Cytoskeletal components can turn wall-less spherical bacteria into kinking helices. *Nat Commun* 13. <https://doi.org/10.1038/s41467-022-34478-0>
12. Lee, J.E., Cornell, K.A., Riscoe, M.K., Howell, P.L., 2001. Structure of *E. coli* 5'-methylthioadenosine/S-adenosylhomocysteine Nucleosidase Reveals Similarity to the Purine Nucleoside Phosphorylases. *Structure* 9, 941–953. [https://doi.org/10.1016/S0969-2126\(01\)00656-6](https://doi.org/10.1016/S0969-2126(01)00656-6)
13. Listgarten, M.A., Socransky, S.S., 1964. ELECTRON MICROSCOPY OF AXIAL FIBRILS, OUTER ENVELOPE, AND CELL DIVISION OF CERTAIN ORAL SPIROCHETES. *J Bacteriol* 88, 1087–1103. <https://doi.org/10.1128/jb.88.4.1087-1103.1964>
14. McGinnis, S., Madden, T.L., 2004. BLAST: At the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Res* 32. <https://doi.org/10.1093/nar/gkh435>
15. Mishra, V., Ronning, D.R., 2012. Crystal structures of the *Helicobacter pylori* MTAN enzyme reveal specific interactions between S-adenosylhomocysteine and the 5'-alkylthio binding subsite. *Biochemistry* 51, 9763–9772. <https://doi.org/10.1021/bi301221k>
16. Park, E.Y., Choi, W.S., Oh, S.-I., Kim, K.-N., Shin, J.S., Song, H.K., 2009. Biochemical and structural characterization of 5'-methylthioadenosine nucleosidases from *Arabidopsis thaliana*. *Biochem Biophys Res Commun* 381, 619–624. <https://doi.org/10.1016/j.bbrc.2009.02.106>
17. R. Townsend and D. B. Archer, 1983. A Fibril Protein Antigen Specific to Spiroplasma, *Journal of General Microbiology*. <https://doi.org/http://www.genesdev.org/cgi/doi/10.1101/gad.1795509>
18. Rattner, R., Thapa, S.P., Dang, T., Osman, F., Selvaraj, V., Maheshwari, Y., Pagliaccia, D., Espindola, A.S., Hajeri, S., Chen, J., Coaker, G., Vidalakis, G., Yokomi, R., 2021. Genome analysis of *Spiroplasma citri* strains from different host plants and its leafhopper vectors. *BMC Genomics* 22. <https://doi.org/10.1186/s12864-021-07637-8>
19. Sasajima, Y., Kato, T., Miyata, T., Kawamoto, A., Miyata, M., 2022. Isolation and

- structure of the fibril protein, a major component of the internal ribbon for *Spiroplasma* swimming. Makoto Miyata. Quick freeze replica electron microscopy, Electron tomography. 20. <https://doi.org/10.1101/2021.02.24.432793>
20. Muerhoff, A.S., Smith, D.B., Leary, T.P., Erker, J.C., Desai, S.M., Mushahwar, I.K., 1997. Identification of GB virus C variants by phylogenetic analysis of 5'-untranslated and coding region sequences. *J Virol* 71, 6501–6508. <https://doi.org/10.1128/jvi.71.9.6501-6508.1997>.
 21. Shen, W.-Y., Lo, W.-S., Lai, Y.-C., Kuo, C.-H., 2016. Complete Genome Sequence of *Spiroplasma helicoides* TABS-2^T (DSM 22551), a Bacterium Isolated from a Horsefly (*Tabanus abactor*). *Genome Announc* 4. <https://doi.org/10.1128/genomeA.01201-16>
 22. Siu, K.K.W., Asmus, K., Zhang, A.N., Horvatin, C., Li, S., Liu, T., Moffatt, B., Woods, V.L., Howell, P.L., 2011. Mechanism of substrate specificity in 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidases. *J Struct Biol* 173, 86–98. <https://doi.org/10.1016/j.jsb.2010.06.006>
 23. Siu, K.K.W., Lee, J.E., Sufrin, J.R., Moffatt, B.A., McMillan, M., Cornell, K.A., Isom, C., Howell, P.L., 2008. Molecular Determinants of Substrate Specificity in Plant 5'-Methylthioadenosine Nucleosidases. *J Mol Biol* 378, 112–128. <https://doi.org/10.1016/j.jmb.2008.01.088>
 24. Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol Biol Evol* 38, 3022–3027. <https://doi.org/10.1093/molbev/msab120>
 25. Townsend, R., Burgess, J., Plaskitt, K.A., 1980. Morphology and ultrastructure of helical and nonhelical strains of *Spiroplasma citri*. *J Bacteriol* 142, 973–981. <https://doi.org/10.1128/jb.142.3.973-981.1980>
 26. Masaaki, W., Masaki, D., Taro, U., Michiko, U., Katsuki, T., Kazuo, N., Michio, M., 1991. Sequence of the downstream flanking region of the shape-determining genes mreBCD of *Escherichia coli*. *Gene* 106, 135–136. [https://doi.org/10.1016/0378-1119\(91\)90578-Y](https://doi.org/10.1016/0378-1119(91)90578-Y)
 27. Whitcomb, R.F., French, F.E., Tully, J.G., Gasparich, G.E., Rose, D.L., Carle, P., Bove, J.M., Henegar, R.B., Konai, M., Hackett, K.J., Adams, J.R., Clark, T.B., Williamson, D.L., 1997. *Spiroplasma chrysopicola* sp. nov., *Spiroplasma gladiatoris* sp. nov., *Spiroplasma helicoides* sp. nov., and *Spiroplasma tabanidicola* sp. nov., from Tabanid (Diptera: Tabanidae) Flies. *Int J Syst Evol Microbiol* 47, 713–719. <https://doi.org/10.1099/00207713-47-3-713>

28. Williamson, D.L., 1974. Unusual Fibrils from the Spirochete-Like Sex Ratio Organism. *J Bacteriol* 117, 904–906. <https://doi.org/10.1128/jb.117.2.904-906.1974>

Chapter 3

Determination of polymerization interface of Fibril

3.1 Introduction

Spiroplasma is a cell wall less helical and motile bacteria (Saglio et al., 1973). In the absence of cilia or flagella, the helicity and motility are predicted to be maintained by cytoskeletal proteins present underneath the cell membrane. The cytoskeletal ribbon contains Fibril and MreB paralogs (Williamson, 1974; Williamson et al., 1979; Kürner et al., 2005). Fibril is a unique protein which is found only in *Spiroplasma* species (Townsend and Archer, 1983). It forms nucleotide independent filaments and the biochemical function remains unclear (Kürner et al., 2005a). In spite of polymerizing independently of any nucleotide or cofactor similar to intermediate filaments, AlphaFold model of Fibril shows that the fold consists of beta sheets as well as alpha helices and forms a globular fold (also seen in Harne and Gayathri, 2022). As Fibril forms constitutive filaments, there is a possibility that Fibril interacts with MreB paralogs only in filament form. Polymerized Fibril might be the functional form inside the cell. In absence of a homologue, information regarding the polymerization interface is not available. Sequence based homology search for Fibril shows 24% sequence similarity to 5'-methylthioadenosine nucleosidase (MTAN) (Cohen-Krausz et al., 2011). The sequence similarity shown is for the region amino acid position 1-236 of Fibril. The sequence 237-512 does not show sequence identity to proteins in any of the present databases. MTAN is known to dimerize through a loop region (Lee et al., 2001). Taking this fact into consideration, two approaches were taken to identify the minimum domain required for the polymerization of Fibril. Based on the secondary structure prediction of Fibril, one secondary structure at a time was deleted from the C-terminus of the protein sequentially. These constructs were then purified and checked for the oligomeric state (Bapat et al., 2023). Another approach, described in this thesis, was to introduce monomeric EGFP (enhanced green fluorescence protein) (Zacharias et al., 2002), a 26 kDa protein in loop regions of Fibril. EGFP being a bulky protein will sterically block the next monomer to interact with the other monomers, if EGFP is present at the interface. There can be a possibility of EGFP being away from the interface as well. In that case, we expect to obtain fluorescently tagged Fibril filaments. Fluorescently tagging the protein gives us an advantage of visualizing the protein *in vivo* as well. GFP and its variants have been used routinely as a folding reporter

to monitor misfolding and formation of aggregate of the protein fused to it (Gregoire et al., 2012, Waldo et al., 1999). This approach has been used to identify the folding state of membrane proteins bound to it as well (Kawate and Gouaux, 2006). As Fibril does not possess any biochemical function such as an enzymatic activity, EGFP was used as a folding reporter to identify folded polymerizing as well as non- polymerizing constructs. The second approach to obtain a well-folded construct of Fibril has been discussed in this chapter.

3.2 Materials and Methods

In order to identify the loop positions for EGFP insertion in Fibril, a secondary structure prediction approach of the protein was used. The secondary structure prediction was done using the Psipred tool (<http://bioinf.cs.ucl.ac.uk/psipred/>).

3.2.1 Amplification of *egfp* with *fibril* overhangs and Restriction free cloning

The cloning *fibril-egfp* was done using restriction free cloning method (van den Ent and Löwe, 2006). *egfp* was amplified using *egfp* in pET15b vector as a template. Primers used for the amplification had position specific *fibril* sequence as overhangs. Primers used for *fibril-egfp* cloning are listed in **Table 3.1**. The amplified product had overhang sequences complementary to the *fibril* gene. The overhang sequences varied depending on the position of insertion in *fibril* genes. The ~1.5 kb amplified PCR product was purified using Qiagen PCR purification kit. Concentration was estimated for the purified product using Nanodrop at 260 nm. This PCR product was then used as a megaprimer and *fibril* in the pHis17 vector was used as the template. Control reaction was also kept containing a reaction with template and without any megaprimer. To remove parent plasmid from the restriction free reactions, 0.5 μ L of DpnI was added to 9.5 μ L of PCR products and incubated at 37 °C for 2 hours. DpnI digested product was transformed into NEB electrocompetent cells using electroporation method. The cells were plated on a luria bertani (LB) agar plate containing ampicillin (final concentration – 100 μ g/mL). The colonies obtained were grown in LB media at 37 °C for 12 hours. Plasmids were isolated using the Qiagen miniprep kit (Qiagen - 27104). The expected size of the gene was checked using NdeI and BamHI digestion. The clones were further confirmed through sequencing.

Table 3.1. List of primers for preparing Fibril-EGFP constructs

Serial Number	Name of the primer	Sequence of the primer (5' - 3')
1	Fibril 1 EGFP f	GCTGTTGAAATTACGCCGATCTTGTACAGCTCGTCC
2	Fibril 1 EGFP r	GTTTAACTTTAAGAAGGAGATATACATATGCTCGAA GATCCGGTG
3	Fibril 27 EGFP f	CAGTAAAAAAATATTGGTGAAGCCGCGCGGCAG CCACATG
4	Fibril 27 EGFP r	GGTATTTAACATGTTGAATTACACAGTTCTTGTACA GCTCGTCC
5	Fibril 37 EGFP f	GTGTAATTCAACATGTAAATACCATCCGCGCGGCA GCCACATG
6	Fibril 37 EGFP r	CTGTTGCTATAATAAAAGTTTTTCCCTTGTACAGCT CGTCC
7	Fibril 47 EGFP f	CTTTTATTATAGCAACAGTAGGACCGCGCGGCAGC CACATG
8	Fibril 47 EGFP r	CATTGCAGCATTTGCTTTACCATACTTGTACAGCTC GTCC
9	Fibril 65 EGFP f	CTTACTTATTAGAAAAATATCCTCCGCGCGGCAGCC ACATG
10	Fibril 65 EGFP r	CATTTAAAATTGTTTGTAACCCTTGTACAGCTCGT CC
11	Fibril 80 EGFP f	GTAGACTTAGCATTGTCAACAAACCCGCGCGGCAG CCACATG
12	Fibril 80 EGFP r	GTTGTATCTCCTGTATCATGCTTATCCTTGTACAGCT CGTCC
13	Fibril 109 EGFP f	GACTTAACAGTTTTTAAAGATATTAAATATCCGCGC GGCAGCCACATG
14	Fibril 109 EGFP r	CAGGTTCATTAACAATTTGTCCCTTGTACAGCTCGT CC
15	Fibril 134 EGFP f	GCTAAAGTTGTAAAGACTTTAAATTGCCGCGCGG CAGCCACATG

16	Fibril 134 EGFP r	CCAGTAACTCCTTCTGTTAACCCCTTGTACAGCTCG TCC
17	Fibril 149 EGFP f	GGAACAGCAGATATGTTGATTTATCCGCGCGGCAG CCACATGCTCGAAGATCCGGTGAGC
18	Fibril 149 EGFP r	CCATTTCTTAAATTGTTTTGAGTTCTTGTACAGCT CGTCCATGCCG
19	Fibril 161 EGFP f	CAATTTAAGGAAATGGTTGATAAATATCCGCGCGGC AGCCACATG
20	Fibril 161 EGFP r	GTATCAATTACATCAATAGTATGCCCTTGTACAGC TCGTCC
21	Fibril 183 EGFP f	GCGCAAGTTGCTAAAAAATCAAGTCCGCGCGGCA GCCACATG
22	Fibril 183 EGFP r	GCATTATTATAAATAATTTTAAAGCAATATAATTAA TCTTGTACAGCTCGTCC
23	Fibril 198 EGFP f	GCTTTAAAAATTATTTATAATAATGCATTATCACCGC GCGGCAGCCACATG
24	Fibril 198 EGFP r	GGATTGGATCATTATCCCATGGCTTGTACAGCTCGT CC
25	Fibril 237 EGFP f	GTTCAAATTATATTATTGATTTATCACCGCGCGGCAG CCACATG
26	Fibril 237 EGFP r	CTAAATCATCTTGTGAACATTGCTTGTACAGCTCGT CC
27	Fibril 271 EGFP f	CCAAATACACATAAAGTATTATCACCGCGCGGCAG CCACATG
28	Fibril 271 EGFP r	CTAACATTAATGAAGGCCCAAATCCCTTGTACAGCT CGTCC
29	Fibril 304 EGFP f	CCGTTCAAAAACAAAAGAAGCAGAACCGCGCGGC AGCCACATG
30	Fibril 304 EGFP r	CCTAAAATTACTTTACTTGGTCCCTTGTACAGCTCG TCC
31	Fibril 414 EGFP f	CCAATCACATGAATTTGTTGAAGATCCGCGCGGCA GCCACATG

32	Fibril 414 EGFP r	CTTACTAATTGTGTTCCAAAGTTTTTCTTGTACAGC TCGTCC
33	Fibril 479 EGFP f	GGTGTTAAATTTGTGGCTTTAAATCAACCGCGCGG CAGCCACATG
34	Fibril 479 EGFP r	CAGTATAATCTCGTTGTAGTTTCTTGTACAGCTCGT CC
35	Fibril 493 EGFP f	CTGTTGTTGATATTACAAGAAATCCGCGCGGCAGC CACATG
36	Fibril 493 EGFP r	CCAACCTTAATTGGATCATAATCCTTGTACAGCTCG TCC
37	Fibril 512 EGFP f	GTTACAATTCGTTTAAAAAGTGAACCGCGCGGCAG CCACATGC
38	Fibril 512 EGFP r	GCTTTTAATGATGATGATGATGATGGGATCCTTACTT GTACAGCTCGTCC

3.2.2 Expression check of *fibril- egfp* constructs

The *fibril-egfp* constructs were transformed in BL21AI strains using heat shock method. The cells were grown on 1X LB media with ampicillin at 37 °C. The cultures were induced at OD₆₀₀ – 0.6 with 0.2 % L-arabinose and further grown at 18 °C for 12 - 16 hours. 3 – 5 mL cultures were pelleted at 21000 g and resuspended using a lysis buffer – 200 mM NaCl, 50 mM Tris pH - 8.0. The buffer used was the same buffer used for Fibril wild type protein (Harne and Gayathri, 2022). The cells were lysed using probe sonicator with a cycle of 1s ON and 3s OFF, 60 % amplitude for 3 minutes. 10 µl of this cell lysate was taken as total fraction and added to 10 µl of 1X SDS dye. The lysate was then spun at 21000 g at 4 °C for 10 minutes. 10 µL supernatant was added to 10 µL 1X SDS dye to check the amount of soluble protein in the buffer. The total and supernatant fractions were heated at 99 °C for 10 minutes. 10 µL of this sample was then loaded on 12 % SDS-PAGE gel. BioRad protein ladder (BioRad - 161-0374) was used as a marker to check the molecular weight of the recombinant protein. The molecular weight of Fibril wild type is 59 kDa whereas the expected molecular weight of Fibril-EGFP is 85 kDa.

3.2.3 Sample preparation for whole cell fluorescence

fibril-egfp constructs were transformed in BL21AI strain and plated on LB agar plates containing 100 µg/mL of ampicillin. The plate was incubated at 37 °C for 12 hours. 8 - 10

colonies were inoculated in 1X LB media and grown at 37 °C. The cultures were induced at OD₆₀₀ – of 0.6 with 0.2 % L-arabinose. Post induction, the cultures were kept at 18 °C for 12 - 16 hours. The cell density at the time of induction and post induction was maintained to 4.8*10⁸ (corresponding to OD₆₀₀- 0.6) and 2*10⁹ (corresponding to OD₆₀₀ - 2.5) respectively. The cell density was estimated by measuring optical density of the cultures and calculating the values of corresponding cell densities using Agilent *E. coli* cell culture bio calculator (<https://www.agilent.com/store/biocalculators/calcODBacterial.jsp>). Post induction all the cultures were normalized to cell density 2*10⁹. 3 mL of the culture was pelleted and washed with 1X PBS to get rid of residual LB media. The pellet was resuspended in 300 µL 1X PBS and added to the Costar® 96 well black plate (Corning® - CLS3915-100EA Corning-3915). Fluorescence was measured at λ_{ex}- 488 nm and λ_{em}- 509 nm.

3.2.4 Sample preparation for confocal microscopy

The cultures were grown in a similar manner as described in section 3.2.3. The cell density post induction was normalized to 1.6*10⁹ (corresponding to OD₆₀₀ - 2). 1 mL of induced cultures were harvested by spinning at 15800 g at 4 °C for 1 minute. The pellets were washed 2 - 3 times with 200 µL 1X PBS and the supernatant was discarded. 100 µL of 4% paraformaldehyde was added to the cells and incubated at room temperature for 30 minutes. The vials were covered to prevent any quenching. The cells were pelleted at 15800 g, 4 °C for 1 minute. Washing step was repeated twice and cells were spun down. The cells were finally resuspended in 100 µL of 1X PBS. 2 µL of this suspension was used to make smear on a slide cleaned with 70 % ethanol. Once the slide dried, 5 µL of 80 % glycerol was added as a mountant. Coverslip was carefully placed on the drop to avoid trapping any bubbles. The coverslip was sealed using nail polish. The images were captured using GFP filter and DIC mode under Leica SP8 confocal microscope with argon laser (20 % laser power) with gain - 874.9 and offset - 0.1 %, pinhole - 1AU, line average - 2.

3.2.5 Quantification of fluorescence of *E. coli* cells

The fluorescent and differential interference contrast (DIC) images were analyzed using ImageJ (version: 2.9.0/1.53t) (Schneider et al., 2012). The cell boundaries of the cells were selected from the DIC images using a freehand tool and the fluorescence and area of the cells was estimated. For the background noise estimation, the same area selected for fluorescence estimation of each cell was used in the regions surrounding where no cells were present. The

background intensity was subtracted from the fluorescence intensity of each cell. The background subtracted integrated density/area of each cell was plotted against each construct to compare the difference in the fluorescence intensity.

3.2.6 Purification of *fibril-egfp* constructs

The protocol published in (Harne and Gayathri, 2022) was followed for the purification of all the fluorescent constructs. The separation of protein in the urografin gradient was similar to that observed for Fibril wild type.

3.2.7 Quantification of fluorescence and scattering of purified protein

The Fibril-EGFP gradient was fractionated in a Corning 96 well plate (Sigma - CLS3635-50EA) to measure the scattering. Every plate contained 300 μ L of samples. Each sample was then transferred to a Costar 96 well black plate to estimate the fluorescence signal. Fluorescence was measured using EnSight plate reader (Perkin Elmer). The fluorescence and scattering values were plotted separately using Graphpad Prism (Version 9.4.1) .

3.2.8 Sample preparation for CD spectroscopy

500 μ L of Fibril 27 EGFP, Fibril 183 EGFP and Fibril 493 EGFP were prepared for CD spectroscopy. The proteins were treated with DNase at 37°C for 1 hour. The samples were prepared by using 1 % SDS to dissolve 5 μ M of protein. MilliQ was added to make up the volume to 500 μ L. The samples were mixed properly by inverting the tube several times. Samples were spun at 21000 g for 1 minute to remove any suspended particles. The solution was then transferred to a fresh 1.5 mL microcentrifuge tube. Same volume of control was taken containing MilliQ and 1% SDS. The samples were transferred to a cuvette and readings were taken from 200 - 250 nm.

3.2.9 Visualization of fluorescent constructs under transmission electron microscope

Different concentrations of Fibril-EGFP samples were prepared ranging from 1 mg/mL to 50 mg/mL. Samples with and without 1% SDS were used to prepare samples for negative staining. 10 μ L of protein was loaded on Carbon/Formvar Cu grids (TED-PELLA, INC) for 3 minutes. The solution was back blotted using Whatman paper. 10 μ L of MilliQ was used to wash the grid surface and again back blotted. 4 μ L of 0.5 % uranyl acetate was added on the grid and blotted after 30 s. The grid was air dried and imaged under TEM (JEM- 2200FS Joel Ltd.).

3.3 Results

3.3.1 Fluorescence level shows difference amongst different constructs

In the absence of any information about the polymerization interface, a secondary structure-based approach was used to identify the loop regions for EGFP insertion (Figure 3.1). 19 *fibril-egfp* constructs were cloned for the fluorescence screen.

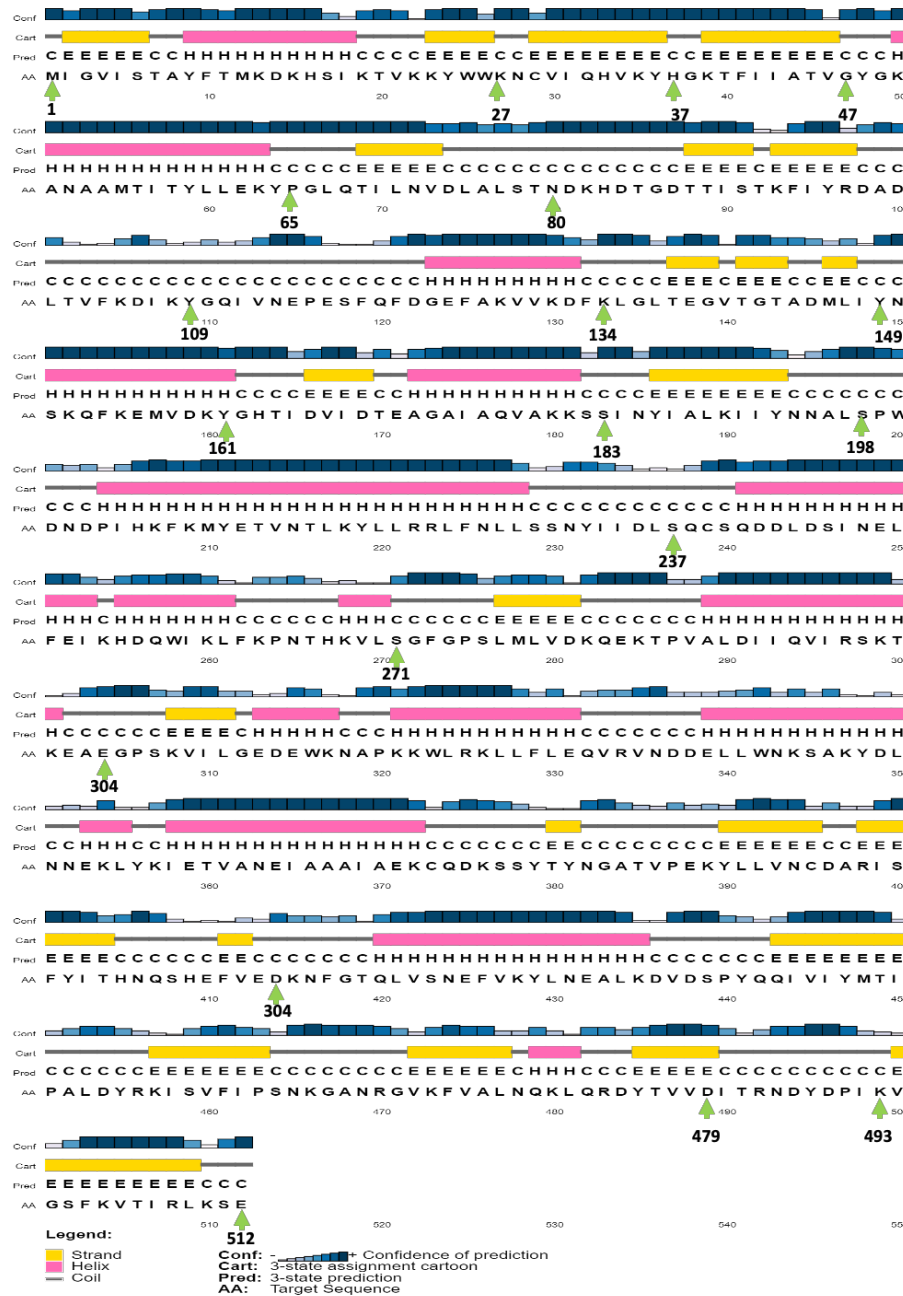


Figure 3.1. Secondary structure prediction of Fibril. Loop regions of Fibril identified by secondary structure prediction were used to introduce EGFP. Green arrow signifies amino acid position for EGFP insertion.

Expression check of all the *fibril-egfp* constructs was done to check the presence of 86 kDa fusion protein. All the constructs expressed the desired band, however, similar to Fibril wild type protein, there was no solubility seen. Whole cell fluorescence was used to screen all the fluorescent constructs and identify the well folded proteins (Figure 3.2). Fluorescence of the overexpressed proteins in *E. coli* was estimated for the quantification of whole cell fluorescence.

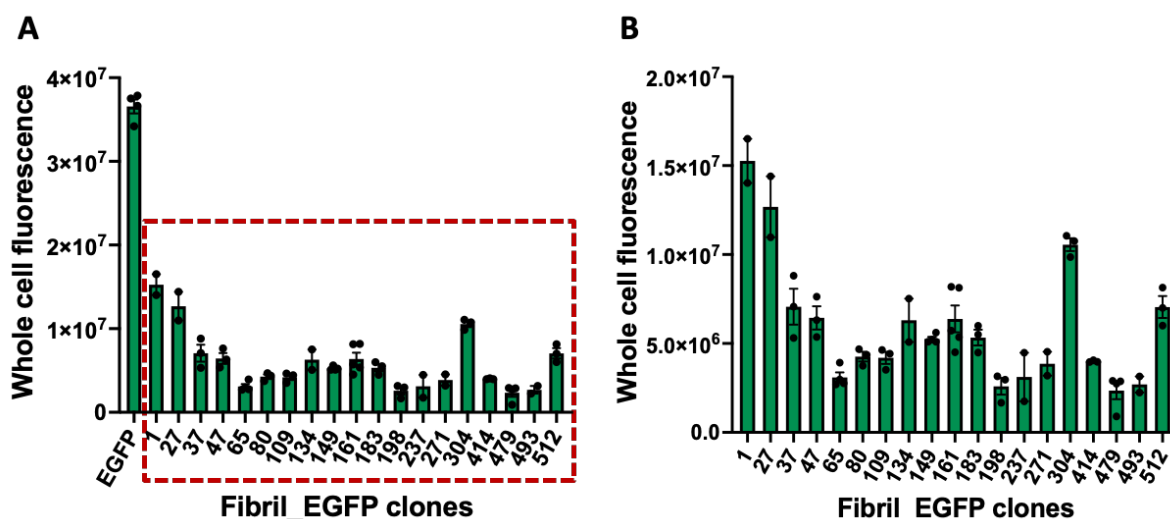


Figure 3.2. Whole cell fluorescence across Fibril-EGFP constructs. **A.** Whole cell fluorescence of all Fibril-EGFP constructs at same cell density with EGFP as a control. **B.** Plot **B** shows fluorescence of Fibril-EGFP constructs for a zoomed version of panel A, excluding EGFP.

The fluorescence of all overexpressed Fibril-EGFP was lower than only EGFP. We observed a gradation of fluorescence amongst the overexpressed proteins (Figure 3.2). There might be a possibility of EGFP experiencing steric hindrance near the Fibril surfaces which led to partial misfolding of the protein. The protein might contain some portion of misfolded protein that led to a decrease in fluorescence. To confirm whether this variation is present at the individual cells, fluorescence signal was checked by visualizing individual cells expressing Fibril-EGFP (Figure 3.3).

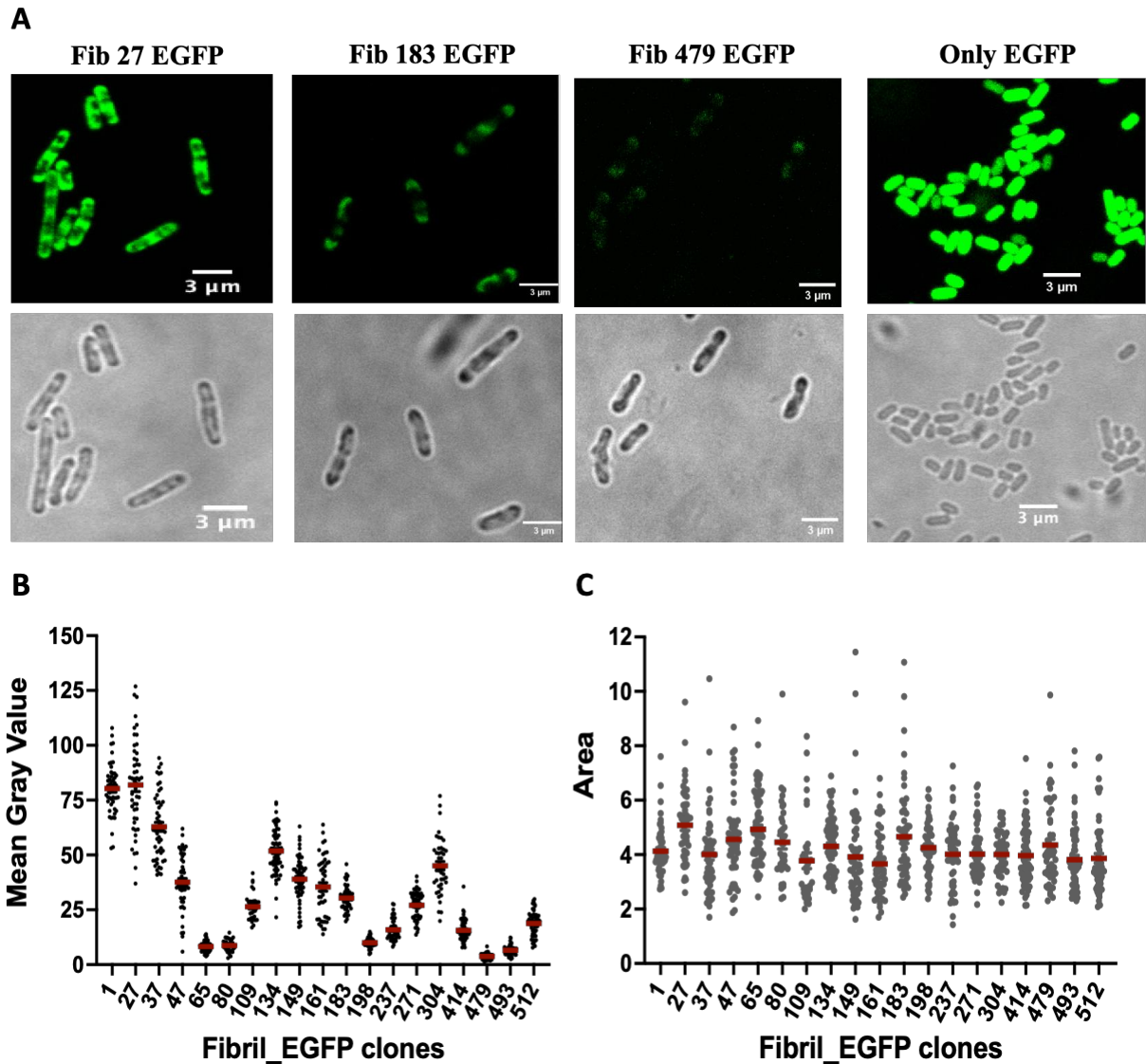


Figure 3.3. Quantification of fluorescence and cell size from individual cells. A. Confocal and DIC images of three Fibril-EGFP constructs and only EGFP overexpressed in *E. coli*. **B.** Fluorescence level estimated for 50 cells overexpressing different Fibril-EGFP constructs. **C.** Cell size measurement of individual cells overexpressing different Fibril-EGFP constructs.

The signal from individual cells followed a similar pattern as seen in whole cell fluorescence. Cells expressing only EGFP showed a diffused localization whereas the cells expressing Fibril-EGFP showed non-diffused localization (Figure 3.3A). This might indicate the protein is either in inclusion bodies or due to the formation of filaments. The fluorescence signal and the area of the cell was quantified to check if the variation is seen due to difference in cell size. The area measured for around 50 cells for each construct showed that the constructs showed variation on fluorescence signals in spite of having similar cell size (Figure 3.3B). Variation in the fluorescence signal seen amongst the constructs in whole cell fluorescence and microscopy

can be a result of Fibril and EGFP getting folded independently of each other. There is also a possibility of differential expression of *fibril-egfp* constructs as well. To compare the total expression with the amount of folded fluorescent protein, we purified all the fusion constructs.

3.3.2 Purified Fibril-EGFP shows similar trend of fluorescence

To check the correlation between the amount of protein produced and fluorescence signal, we purified all the constructs using urografin density gradient centrifugation (Figure 3.4A).

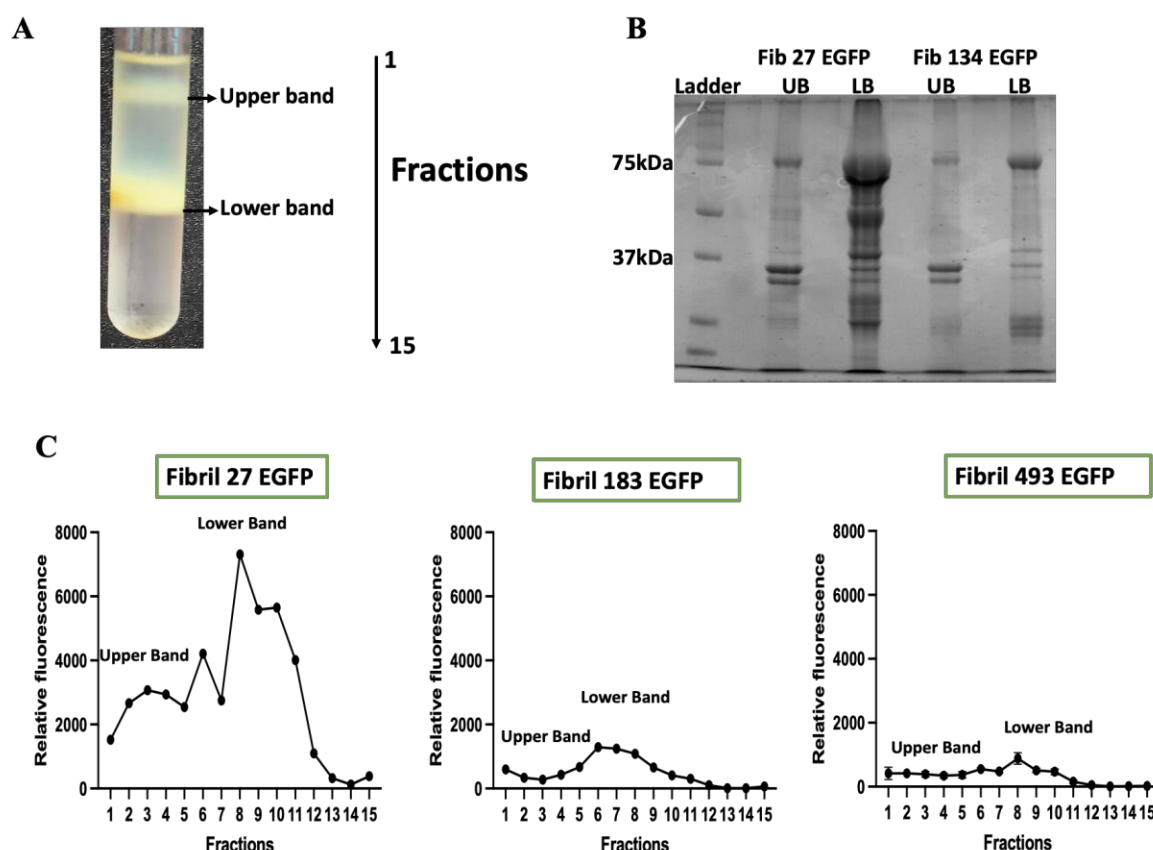


Figure 3.4. Purification of Fibril-EGFP. **A.** Fibril-EGFP construct purified using urografin density gradient. **B.** 10% SDS showing profile of purified Fibril-EGFP proteins. **C.** Fluorescence profile of purified protein from urografin density gradient tube fractionated in 96 well plate.

Fibril-EGFP forms two distinct bands in the urografin gradient as seen for Fibril wild type in (Harne and Gayathri, 2022). The lower bands for all constructs were more fluorescent as a result of a higher amount of protein present in the lower band as compared to upper band (Figure 3.4B and C). In order to check the correlation between expression and the fluorescence levels, scattering and fluorescence signals of the purified proteins were checked.

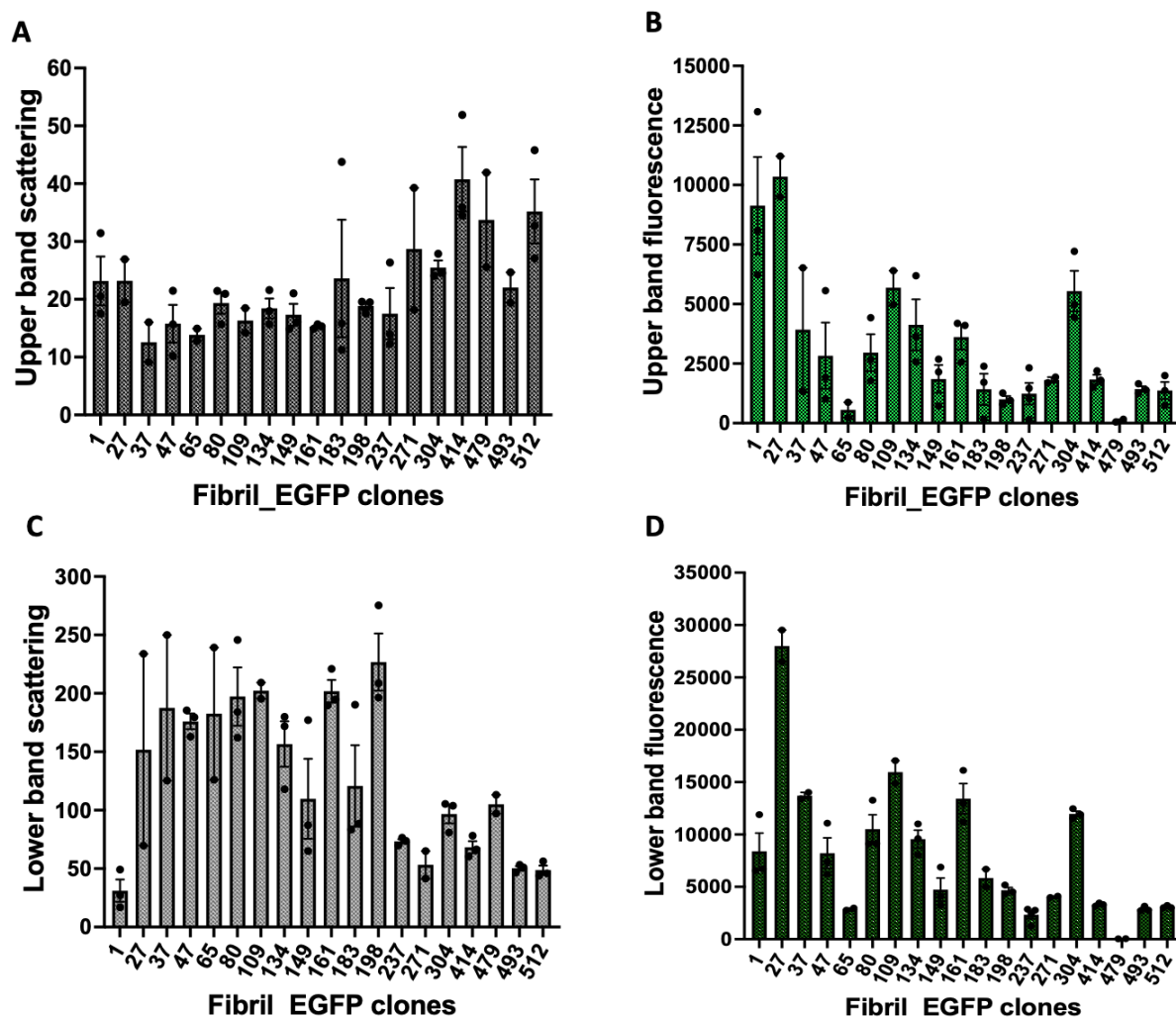


Figure 3.5. Quantification of fluorescence signal of purified protein. A. Fluorescence levels of upper band and lower bands of purified Fibril-EGFP constructs. B. Scattering levels indicate the amount of total protein expressed.

Scattering of the purified proteins indicates the total amount of fluorescent protein expressed by the cells. Comparison of fluorescence from the purified protein and scattering showed that there is poor correlation which indicates that difference in the expression does not lead to variation in fluorescence levels (Figure 3.5). For instance, Fibril 65 EGFP shows scattering similar to other constructs. However, the fluorescence of the upper as well lower band is at the lower side.

To understand the effect of EGFP insertion on the folded state of Fibril, CD spectroscopy was performed. Effect of EGFP on the polymerization and filament assembly was analyzed by visualizing the proteins under transmission electron microscope.

3.3.3 CD spectroscopy reveals presence of secondary structures in the fluorescent proteins

In absence of any functional assays to confirm the folding state of the protein, CD spectroscopy and visualization of the protein might help in the identification of the well folded fusion proteins. Equimolar concentrations of three proteins with varying fluorescent signals were taken as representatives. Fibril wild type protein was taken as a control. EGFP structure contains β -barrel structure containing 11 β sheets. If only EGFP is folded, the expected signal from CD spectroscopy should correspond to β only signal. However, if Fibril/ Fibril-EGFP is folded, we expect a combined signal from α helices as well β sheets.

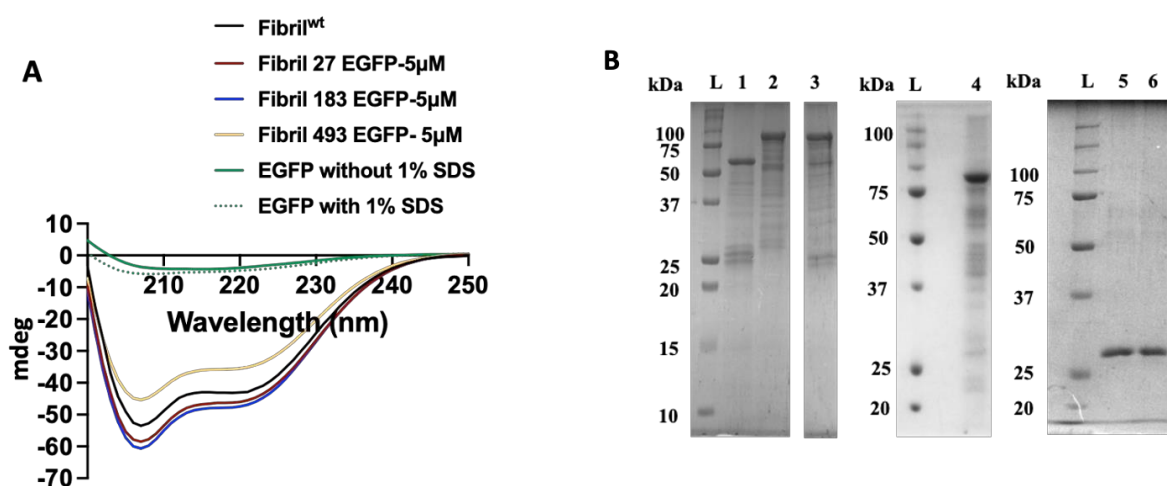


Figure 3.6. Circular dichroism of Fibril-EGFP constructs. **A.** Folded state of three Fibril-EGFP constructs were compared with Fibril wild type. **B.** Corresponding gel of the samples used for CD spectroscopy. L- ladder, 1- Fibril^{WT}, 2- Fibril 27 EGFP, 3- Fibril 493 EGFP, 4- Fibril 183 EGFP, 5- EGFP with 1% SDS and 6- EGFP without 1% SDS.

The CD signal showed presence of both the secondary structures in all Fibril EGFP proteins (Figure 3.6 A). The minima of the proteins Fibril 27 EGFP and Fibril 183 EGFP was similar to the Fibril wild type. However, in spite of equal concentration of protein used (Figure 3.6 B), the signal of Fibril 493 was lesser compared to the other constructs. Signals for α helices and β sheets for Fibril 493 EGFP support that the protein is not completely misfolded. However, there is a possibility of the presence of a heterogeneous population consisting of misfolded and folded protein. This might lead to a decrease in the signal from EGFP. and explains the low fluorescence of this construct.

As discussed earlier, fluorescence level does not provide any information about the oligomeric state of the protein. The fluorescent proteins might be in different filament states. To check if EGFP positions have any effect on the filament state of the protein, negatively stained samples were prepared and observed under transmission electron microscope.

3.3.4 Fibril-EGFP forms filaments irrespective of the position of EGFP insertion

The fluorescently tagged proteins were visualized under transmission electron microscope. Representative images of Fibril 27 EGFP and Fibril 493 EGFP are shown below (Figure 3.7). Big bundles of filaments were observed for all the Fibril-EGFP proteins irrespective of the position of EGFP insertion.

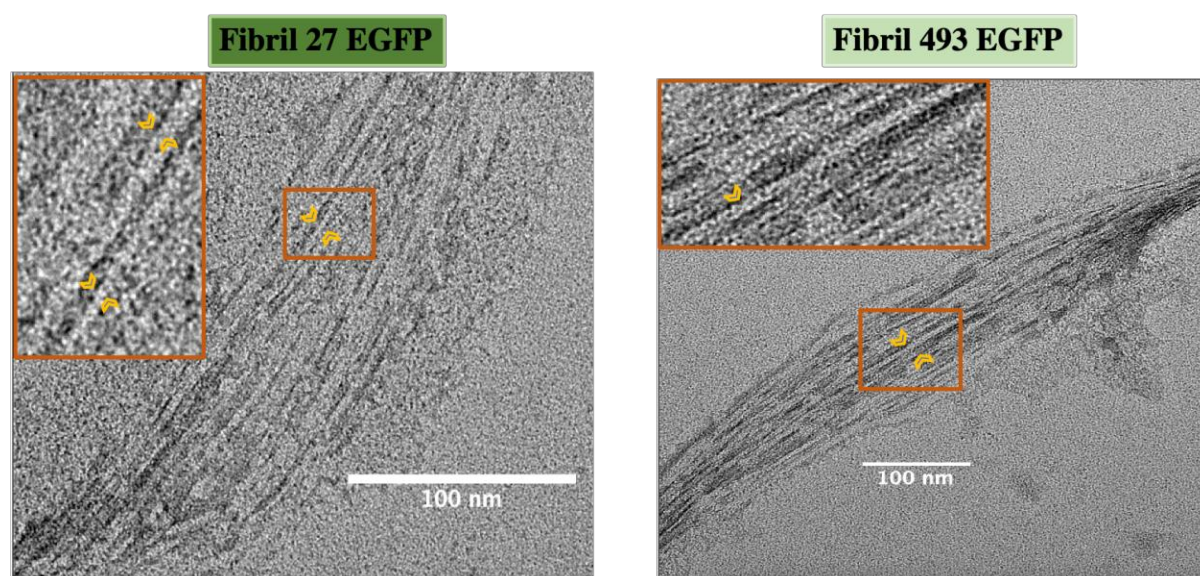


Figure 3.7. Transmission electron microscopy images of purified Fibril-EGFP. Negative staining images of Fibril 27 EGFP and Fibril 493 EGFP. Inset shows zoomed image of Fibril-EGFP bundles. A single protofilament is highlighted using arrows.

Filaments were seen even at lower concentrations. When the concentrations were increased, protein formed multi-layered bundles which led to decrease in the resolution of the filaments. The filaments formed with/without 1 % SDS looked similar. All these observations indicate that Fibril once expressed forms robust filaments which polymerizes readily and does not show depolymerization under any condition. Introducing EGFP at different loop positions did not hamper the polymerization of Fibril.

However, the fluorescent filaments expressed from a heterologous system were similar in morphology as compared to the filaments extracted from the native organisms. This also suggests that the proteins are in native folded form.

3.3.5 Fibril-EGFP filaments are folded and interact with ScMreB5

Fibril and ScMreB5^{WT} have been shown to interact (Harne et al., 2020). To further confirm that the Fibril-EGFP filaments possess a similar fold as the wild-type protein, co-sedimentation assay between fluorescent constructs and ScMreB5^{WT} was performed. Co-sedimentation of the two proteins will also help in identification of the position of EGFP insertion affecting interaction between the proteins, if any.

For the assay, Fibril 27 EGFP, Fibril 183 EGFP and Fibril 493 EGFP were selected for checking interaction with ScMreB5^{WT} (Figure 3.8). When pelleting assay was performed with the individual proteins only, Fibril-EGFP protein was seen in pellet fraction whereas ScMreB5^{WT} was seen in supernatant (Figure 3.8 A). For Fibril 27 EGFP, Fibril 183 EGFP and Fibril 493 EGFP, the amount of ScMreB5^{WT} band (~ 38 kDa) increased in the pellet fraction with increasing concentration of Fibril (Figure 3.8 B-E).

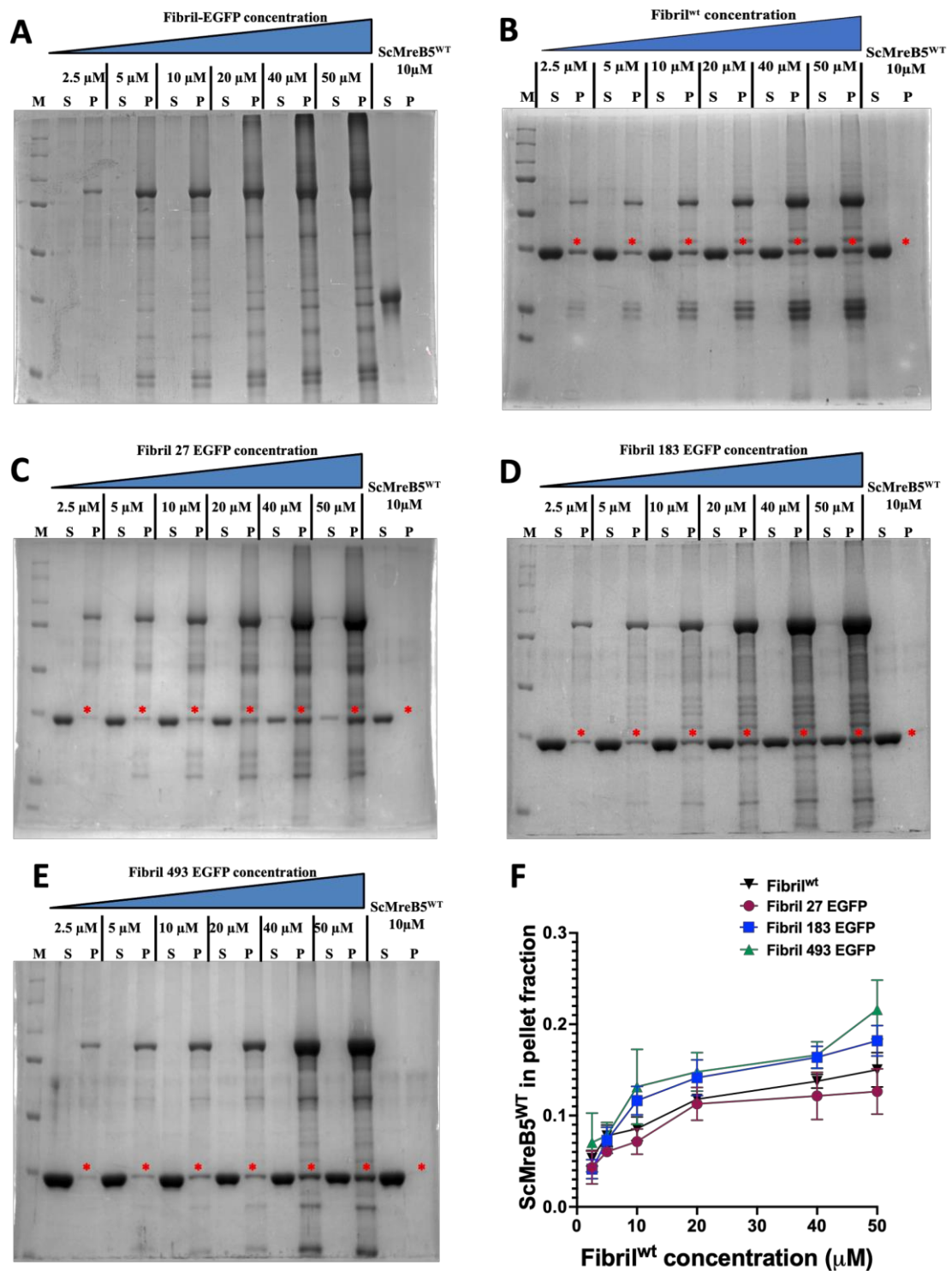


Figure 3.8. Fibril-EGFP interaction with MreB5^{WT} was unaffected by the EGFP tag position for three of the constructs. **A.** Control gel with only Fibril-EGFP and ScMreB5^{WT}. **B.** Fibril^{wt} with ScMreB5^{WT}. **C.** Fibril 27 EGFP with ScMreB5^{WT}, **D.** Fibril 183 EGFP with ScMreB5^{WT}, **E.** Fibril 493 EGFP with ScMreB5^{WT}. **F.** Pellet fraction was quantified as a fraction of total ScMreB5^{WT} loaded. The gel images were analyzed using ImageJ. MreB5^{WT} protein in the pellet fraction used for quantification of interaction is denoted by the red star.

Pellet fraction was quantified as a fraction of total ScMreB5^{WT} protein loaded (Figure 3.8 F). The fluorescent proteins showed similar interaction with ScMreB5^{WT} irrespective of the position of EGFP insertion. The interaction pattern was also indistinguishable than the wild type. This proves that the Fibril-EGFP proteins are well folded despite having varying fluorescence. These observations further support our hypothesis of EGFP being misfolded rather than fibril.

3.4 Discussion

We introduced EGFP tag to Fibril in order to obtain a fluorescently tagged monomer or filament. We expected fluorescence signal for folded filaments equivalent to only EGFP and no signal for misfolded filaments. However, we observed that insertion of EGFP at different positions of Fibril gave rise to variation in fluorescence of filaments. Whole cell fluorescence and signals from individual cells expressing each fusion protein suggested that there might be a mixture of different folded states of Fibril-EGFP filaments. To differentiate the amount of fluorescent protein from cytoplasm from that of inclusion bodies, we purified all fluorescently tagged proteins to quantify the amount of the total protein expressed. Scattering of Fibril-EGFP helped in the quantification of total protein expressed in the cells. Fluorescence of these purified proteins signify the amount of folded protein. Comparison of the scattering data with that of the fluorescence suggested that difference in fluorescence signal amongst the constructs is not due to difference in expression of the *fibril-egfp* genes.

In absence of any functional assay for Fibril, we performed CD spectroscopy and visualized the fusion proteins under TEM to identify the folded state of the fusion proteins. CD spectroscopy showed the presence of secondary structure for different Fibril-EGFP proteins irrespective of the tag position. However, Fibril 493 EGFP showed lower signal than other constructs indicating presence of a heterogeneous population of folded/misfolded protein or partially misfolded protein. This is consistent with the low fluorescence of the construct as seen from whole cell fluorescence and microscopy. TEM of various fluorescent proteins also suggested that these proteins formed filaments irrespective of the fluorescence levels and tag position.

Fibril forms constitutive filaments and has a very high propensity to form bundles. EGFP is linked to Fibril with a flexible linker. The fluorescent tag can move freely and hence might

have various orientations in a particular construct. Orientations in which EGFP faces steric hindrance with Fibril might give rise to misfolded EGFP and hence less fluorescence.

The tag position in this study has been limited to loop regions of the protein. It is also possible that the dimerization of Fibril happens through the α -helical and/or β -sheet regions and not only through loop regions. The fluorescent filaments when visualized under TEM were indistinguishable from the wild type protein. This also supports that Fibril is folded irrespective of the fluorescence levels of the different constructs.

From this study, we were able to identify the position which is favorable for EGFP insertion. The highly fluorescent filaments can be used to study localization and assembly in *Spiroplasma citri*. These constructs can also be used for colocalization studies with MreB paralogs tagged with another fluorescent protein to identify interacting partners in this organism. Earlier studies have shown that position of tag insertions plays a role in the function and localization of the protein of interest (Daniel and Errington, 2003; Jones et al., 2001). In such cases, it becomes essential to identify regions in the protein where a tag can be introduced without affecting the folded state and localization of the protein. Incorrect position of tag may lead to suboptimal fluorescence or misfolding of the protein of interest. This strategy can be used as a tool for the identification of favorable positions to introduce a tag in the gene of interest.

3.5 References

1. Bapat, M., Harne, S., Kashyap, R., Gayathri, P., 2023. Insights into assembly of constitutive filament-forming proteins of novel fold through domain dissection and EGFP insertion-based screen: A case study of *Spiroplasma Fibril*. <https://doi.org/10.1101/2023.10.26.564286>
2. Cohen-krausz, S., Cabahug, P.C., Trachtenberg, S., 2011. The Monomeric , Tetrameric , and Fibrillar Organization of Fib : The Dynamic Building Block of the Bacterial Linear Motor of *Spiroplasma melliferum* BC3. *J Mol Biol* 410, 194–213. <https://doi.org/10.1016/j.jmb.2011.04.067>
3. Daniel, R.A., Errington, J., 2003. Control of Cell Morphogenesis in Bacteria. *Cell* 113, 767–776. [https://doi.org/10.1016/S0092-8674\(03\)00421-5](https://doi.org/10.1016/S0092-8674(03)00421-5)
4. Gregoire, S., Irwin, J., Kwon, I., 2012. Techniques for monitoring protein misfolding and aggregation in vitro and in living cells. *Korean Journal of Chemical Engineering*. <https://doi.org/10.1007/s11814-012-0060-x>
5. Harne, S., Duret, S., Pande, V., Bapat, M., Béven, L., Gayathri, P., 2020. MreB5 Is a Determinant of Rod-to-Helical Transition in the Cell-Wall-less Bacterium *Spiroplasma*. *Current Biology* 30, 4753-4762.e7. <https://doi.org/10.1016/j.cub.2020.08.093>
6. Harne, S., Gayathri, P., 2022. Characterization of heterologously expressed Fibril, a shape and motility determining cytoskeletal protein of the helical bacterium *Spiroplasma*. *iScience* 25. <https://doi.org/10.1016/j.isci.2022.105055>
7. Jones, L.J.F., Carballido-López, R., Errington, J., 2001. Control of Cell Shape in Bacteria. *Cell* 104, 913–922. [https://doi.org/10.1016/S0092-8674\(01\)00287-2](https://doi.org/10.1016/S0092-8674(01)00287-2)
8. Kawate, T., Gouaux, E., 2006. Fluorescence-Detection Size-Exclusion Chromatography for Precrystallization Screening of Integral Membrane Proteins. *Structure* 14, 673–681. <https://doi.org/10.1016/j.str.2006.01.013>
9. Kürner, J. et al, 2005. Cryo – Electron Tomography Reveals the Cytoskeletal Structure of *Spiroplasma melliferum* 436. <https://doi.org/10.1126/science.1104031>
10. Lee, J.E., Cornell, K.A., Riscoe, M.K., Howell, P.L., 2001. Structure of *E. coli* 5'-methylthioadenosine/S-adenosylhomocysteine Nucleosidase Reveals Similarity to the Purine Nucleoside Phosphorylases. *Structure* 9, 941–953. [https://doi.org/10.1016/S0969-2126\(01\)00656-6](https://doi.org/10.1016/S0969-2126(01)00656-6)
11. Saglio, P., Lhospital, M., Lafleche, D., Dupont, G., Bove, J.M., Tully, J.G., Freundt, E.A., 1973. *Spiroplasma citri* gen. and sp. n.: A Mycoplasma-Like Organism Associated with “Stubborn” Disease of Citrus. *Int J Syst Bacteriol* 23, 191–204. <https://doi.org/10.1099/00207713-23-3-191>

12. Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. <https://doi.org/10.1038/nmeth.2089>
13. R. Townsend and D. B. Archer, 1983. A Fibril Protein Antigen Specific to Spiroplasma, *Journal of General Microbiology*.
<https://doi.org/http://www.genesdev.org/cgi/doi/10.1101/gad.1795509>
14. van den Ent, F., Löwe, J., 2006. RF cloning: A restriction-free method for inserting target genes into plasmids. *J Biochem Biophys Methods* 67, 67–74.
<https://doi.org/10.1016/j.jbbm.2005.12.008>
15. Waldo, G.S., Standish, B.M., Berendzen, J., Terwilliger, T.C., 1999. Rapid protein-folding assay using green fluorescent protein 691–695.
16. Williamson, D.L., 1974. Unusual Fibrils from the Spirochete-Like Sex Ratio Organism, *JOURNAL OF BACTERIOLOGY*.
17. Williamson, D.L., Blaustein, D.I., Levine, R.J.C., Elfvin, M.J., 1979. Anti-actin-peroxidase staining of the helical wall-free prokaryote *Spiroplasma citri*. *Curr Microbiol* 2, 143–145. <https://doi.org/10.1007/BF02605871>
18. Zacharias, D.A., Violin, J.D., Newton, A.C., Tsien, R.Y., 2002. Partitioning of Lipid-Modified Monomeric GFPs into Membrane Microdomains of Live Cells. *Science* (1979) 296, 913–916. <https://doi.org/10.1126/science.1068539>

Chapter 4

Interaction studies of Fibril and MreBs

4.1 Introduction

In eukaryotic organisms, intermediate filaments interact with actin filaments to carry out important biological functions (Herrmann and Aebi, 2004). Bacterial actin homologs like MreB, have also been shown to interact with intermediate filaments. *Caulobacter crescentus* MreB mediates attachment of crescentin to the cell wall. Disruption of MreB leads to loss of curved shape in *C. crescentus* (Charbon et al., 2009). MreB and CcmA contribute to cell wall synthesis patterning in *Helicobacter pylori*. MreB senses negative cell curvature whereas CcmA senses positive cell curvature (Taylor et al., 2020). This differential affinity in turn gives rise to helical morphology. Similarly, *Spiroplasma citri* MreB5 has been biochemically and structurally characterized and like all other MreBs, shows ATP hydrolysis activity (Figure 4.1 A-C). It has been shown to interact with liposomes as well as Fibril of *S. citri* (Figure 4.1 D and E) (Harne et al., 2020; Pande et al., 2022). Pelleting assays for MreB5 wild type and its mutants with liposomes showed that the interaction is nucleotide dependent (Figure 4.1 D) (Pande et al., 2022).

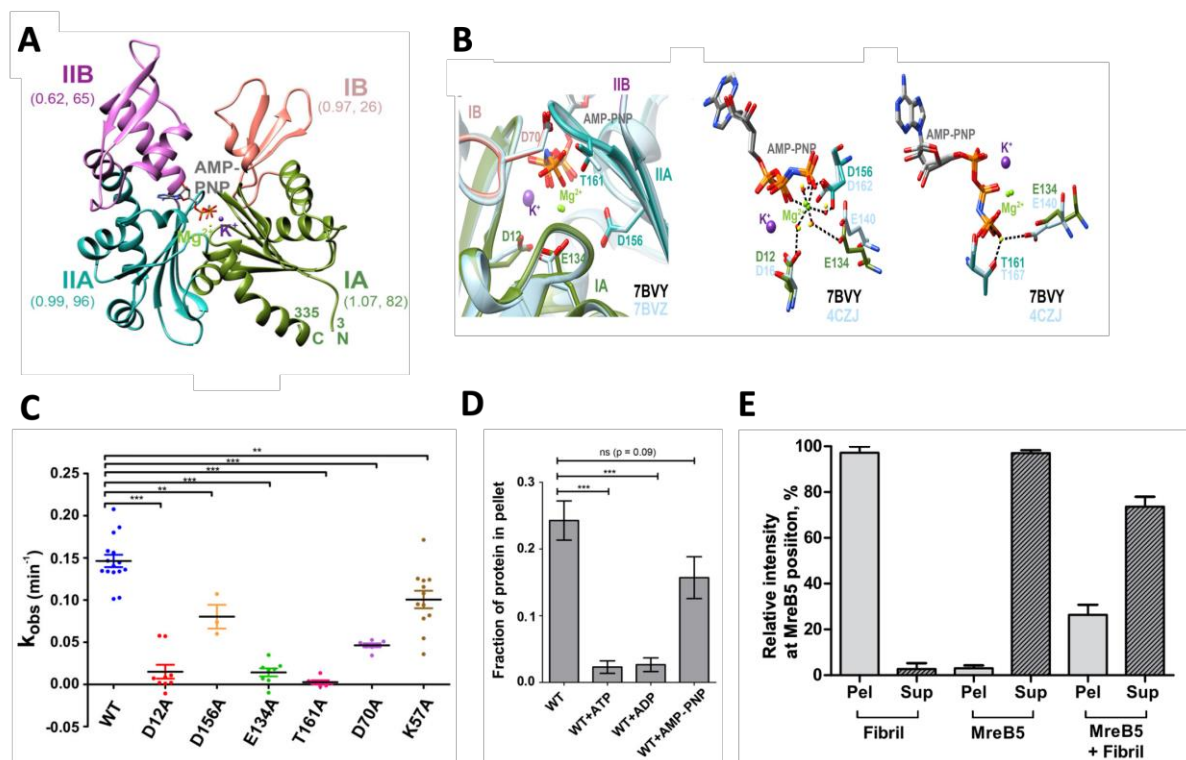


Figure 4.1. ScMreB5^{WT} is an ATPase and interacts with liposomes as well as Fibril **A.** Crystal structure of ScMreB5^{WT} (7BVY). **B.** Active site residues of ScMreB5^{WT} interacting with AMPPNP and involved in Mg²⁺ coordination. **C.** ATP hydrolysis activity of ScMreB5^{WT} and active site mutants has been characterized and shown as k_{obs} (min⁻¹). **D.** Co-sedimentation assay showed that ScMreB5^{WT} interacts with liposomes in a nucleotide *dependent* manner. **E.** ScMreB5^{WT} shows interaction with Fibril as well. Adapted from (Harne et al., 2020; Pande et al., 2022).

With this information in hand, we were interested to know whether MreB5 interaction with Fibril is also nucleotide dependent. In this chapter, we describe interaction of Fibril with MreB5 wild type in presence of different nucleotide states using co-sedimentation assay. We also performed pelleting assay for Fibril wild type with MreB5 ATPase mutants, MreB5^{E134A} and MreB5^{T161A}. The mutants were selected based on the previous characterization of MreB5 and its mutants (Pande et al., 2022).

4.2 Materials and Methods

4.2.1 Protein concentration estimation

Fibril concentration was estimated in a similar manner as described in section 2.5.3. Fibril solubilized in 1 % SDS was used for concentration estimation. MilliQ with 1% SDS was used as a blank. MreB5 was purified using the protocol mentioned in (Harne et al., 2020). The purified protein was spun at 21000 g at 4 °C for 10 minutes before estimation of the concentration. 300 mM KCl, 50 mM Tris pH - 8.0 buffer used for MreB5 purification was used as blank. Fibril and MreB5 concentration were estimated by checking absorption at 280 nm using Nanodrop. Post concentration estimation, Fibril was spun at 21000 g for 10 minutes and resuspended in 300 mM KCl, 50 mM Tris pH - 8.0 for assay.

4.2.2 Pelleting assay reaction conditions

For the pelleting assay, MreB5 concentration was kept constant at 10 μ M and Fibril concentration was taken in a range of 2.5 μ M - 50 μ M. For control runs, only MreB5 and Fibril were added to 300 mM KCl, 50 mM Tris pH - 8.0. For ATP/ADP/AMPPNP control runs, 1 mM of the nucleotide and 1 mM MgCl₂ was added with the individual proteins. For the co-pelleting reactions, MreB5 and Fibril were mixed together with 300 mM KCl, 50 mM Tris pH - 8.0 with or without the nucleotide. The reactions were incubated at 25 °C for 30 minutes. The reactions were spun at 100000 g at 4 °C for 30 minutes. Supernatant and pellet fractions were run on 12 % SDS polyacrylamide gel.

Quantification of the gels was done by using ImageJ (Version 2.9.0/1.53t). Supernatant and pellet bands of ScMreB5^{WT} and ATPase mutants were quantified. Intensities obtained from areas under the curve for both the bands were noted. The intensity of pellets was divided by the total amount of protein to get pellet fraction. These pellet fractions were plotted against Fibril^{WT} concentrations using GraphPad Prism 9.2.

4.2.3 ATPase activity of MreB5 with Fibril

Effect of ATP hydrolysis of ScMreB5^{WT} in the presence of Fibril^{WT} was checked using malachite green assay. The protein concentrations of MreB5 and Fibril used for the assay were 10 μ M each. The protocol used for the assay is the same as mentioned in Chapter 2 section 2.5.3. ScMreB5^{WT} and Fibril^{WT} reactions were monitored for 30 minutes only. However, Fibril^{WT} only reactions were monitored for 30 and 60 minutes as mentioned in the earlier section.

4.3 Results

4.3.1 Interaction of Fibril with MreB5 is nucleotide dependent

ScMreB5-ADP (PDB ID:- 7BVZ) and ScMreB5-AMPPNP (PDB ID:- 7BYY) structures show high similarity with an overall root mean square displacement (RMSD) of 0.87 Å (Pande et al., 2022). Cryo-electron microscopy images of ScMreB5^{WT} in the presence of ATP and AMPPNP showed laterally associated double protofilaments. However, less bundles were seen in the presence of ADP and for ATPase mutant E134A in the presence of AMPPNP (Pande et al., 2022). These observations highlight the fact that in spite of having negligible difference in crystal structures of monomeric proteins, nucleotide bound state may have an effect on the

overall filament assembly of MreB.

As shown in the previous study, Fibril and MreB5 interact when no nucleotide is added externally (Harne et al., 2020). Taking all these observations into account, we were interested to see the effect of different nucleotide bound states of ScMreB5^{WT} and the ATPase mutants on the interaction with Fibril. We performed a pelleting assay to study the interaction between the proteins. Varying concentrations of Fibril were used to check interaction with ScMreB5^{WT} (Figure 4.2).

For the pelleting assay, a control gel containing only Fibril^{WT} and ScMreB5^{WT} were spun after 159000 g spin (Figure 4.2 A). Fibril^{WT} can be seen in pellet fraction (59 kDa) whereas ScMreB5^{WT} is present in supernatant fraction (~39 kDa). No 39 kDa band is seen in Fibril^{WT} only lanes. MreB5 band was seen in pellet fraction upon interaction with Fibril^{WT} (Figure 4.2 B-E). The interaction was seen to increase with increasing Fibril^{WT} concentration.

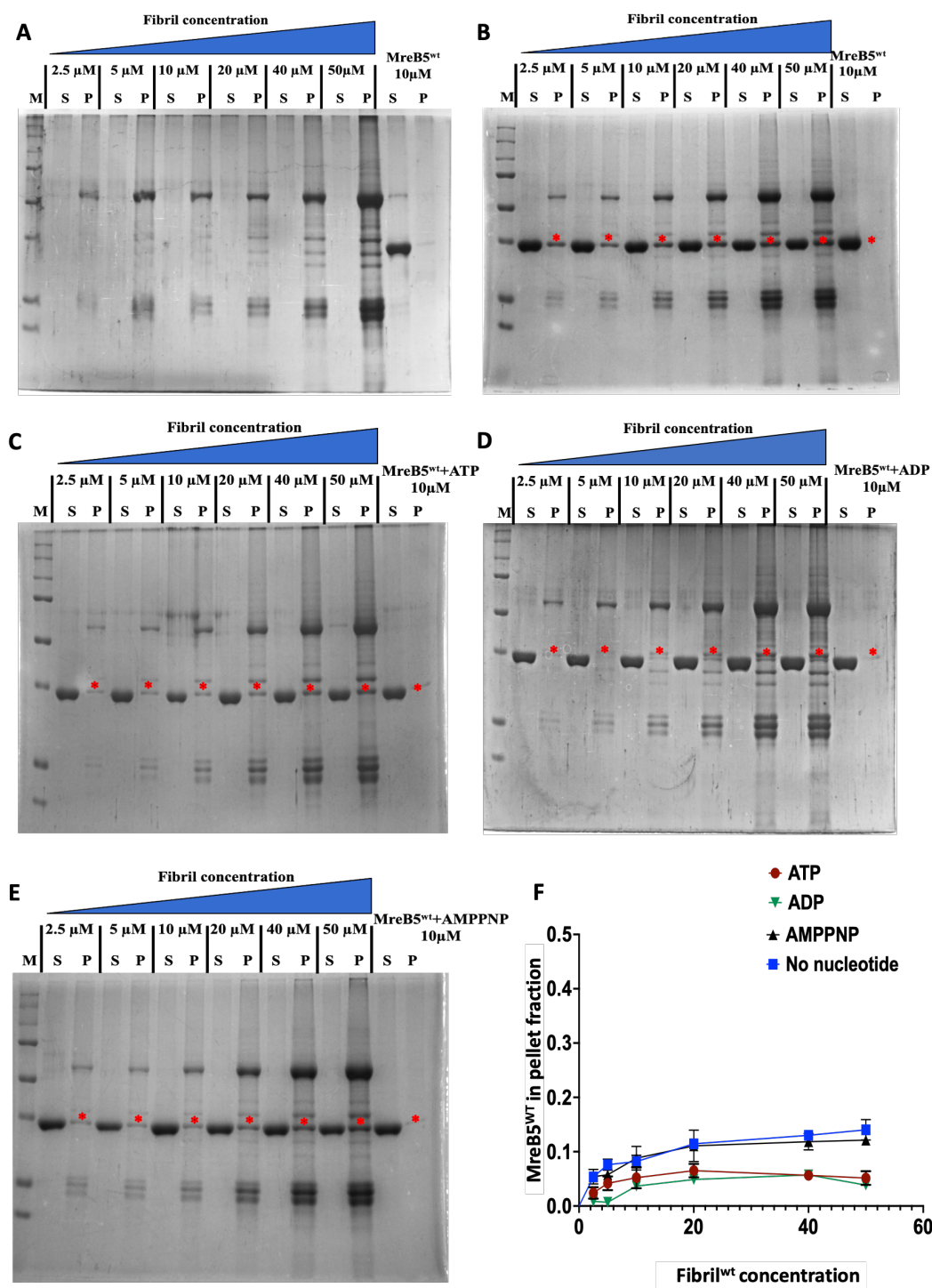


Figure 4.2. Interaction between Fibril^{wt} and MreB5^{WT} is nucleotide dependent. Pelleting assay gels of Fibril and MreB5 control (A), no nucleotide (B), in the presence of ATP (C), ADP (D), AMPPNP (E). Fibril concentration was varied from 2.5 μM to 50 μM. MreB5 concentration used for the assay was 10 μM. Interaction between the two proteins was quantified by selecting supernatant and pellet band MreB5. Intensity of the pellet fraction of MreB5 control was subtracted from all the pellet intensity fraction.

F. Intensities of pellet fraction was plotted for all nucleotide conditions. Binding between no nucleotide and AMPPNP was similar. Decrease in binding was seen in the presence of ATP and ADP. MreB5^{WT} protein in the pellet fraction used for quantification of interaction is denoted by the red star.

ScMreB5^{WT} and Fibril showed higher interaction in the presence of AMPPNP and when no nucleotide was added externally. However, ADP and ATP conditions showed lower interaction (Figure 4.2 F). Interestingly, this interaction pattern was similar to the interactions seen between ScMreB5^{WT} and liposomes in different nucleotide states.

To further decipher the mechanism of nucleotide dependency on Fibril and MreB5 interaction, we carried out pelleting assays of Fibril with MreB5 ATPase mutants with Fibril.

4.3.2 Fibril interaction with MreB5 is dependent on the ATP hydrolysis efficiency

MreB5^{E134A} and MreB5^{T161A} ATPase mutants were used for the assay. MreB5^{E134A} ATP hydrolysis has been shown to be less efficient as compared to the wild type protein. However, MreB5^{T161A} is an ATPase dead mutant (Pande et al., 2022).

MreB5^{T161A} showed a very strong interaction with Fibril when no nucleotide was added externally (Figure 4.3 A and E). This interaction was seen to be higher than the interaction between MreB5 wild type and Fibril. Binding between MreB5^{T161A} and Fibril in the presence of AMPPNP was less efficient as compared to no nucleotide condition (Figure 4.3 D and E). However, very weak interaction was seen in the presence of ADP and ATP (Figure 4.3 B,C and E).

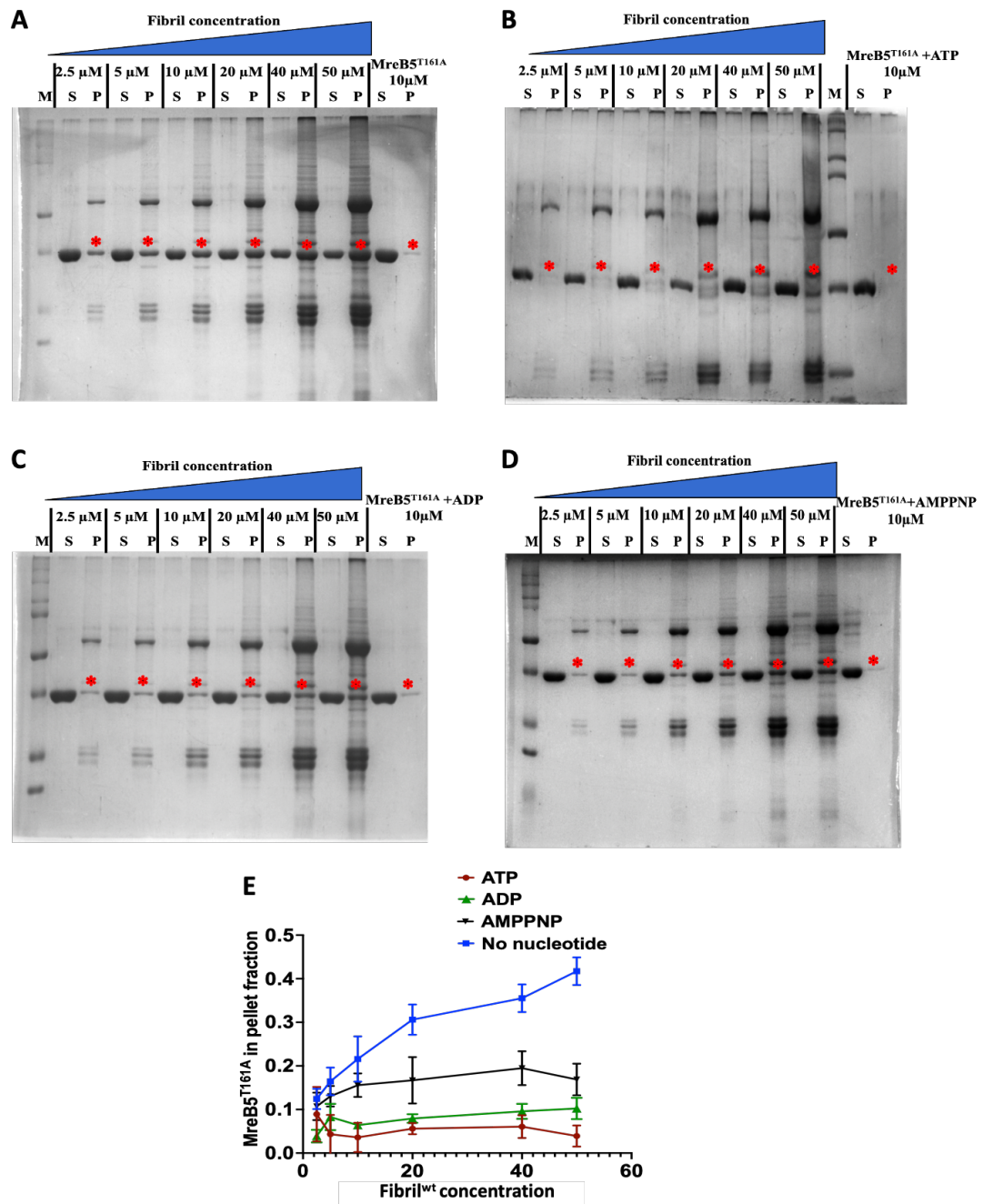


Figure 4.3. Interaction between Fibril^{wt} and ScMreB5^{T161A} is nucleotide dependent. Pelleting assay gels of Fibril and MreB5^{T161A} in the presence of no nucleotide (A), ATP (B), ADP (C), AMPPNP (D). Fibril concentration was varied from 2.5 μM to 50 μM. MreB5^{T161A} concentration used for the assay was 10 μM. Interaction between the two proteins was quantified by selecting supernatant and pellet band MreB5^{T161A}. Intensity of the pellet fraction of MreB5^{T161A} control was subtracted from all the pellet intensity fraction. E. Intensities of pellet fraction were plotted for all nucleotide conditions. The decreasing order in binding

between Fibril and MreB5^{T161A} is no nucleotide>AMPPNP>ADP>ATP. The MreB5^{T161A} protein in the pellet fraction used for quantification of interaction is denoted by red star.

ScMreB5^{T161A} shows very high affinity towards Fibril protein when no nucleotide is added externally. Surprisingly, despite being an ATPase dead mutant, ScMreB5^{T161A} dependency on the interaction with Fibril is similar to that observed for ScMreB5^{wt}. These two results highlight that hydrolysis of ATP might not have any role in binding of MreB5 filaments with Fibril filaments.

Similar conditions were repeated for checking interaction between MreB5^{E134A} and Fibril. Interestingly, binding between the proteins was seen in all nucleotide states unlike MreB5^{wt} and MreB5^{T161A} (Figure 4.4).

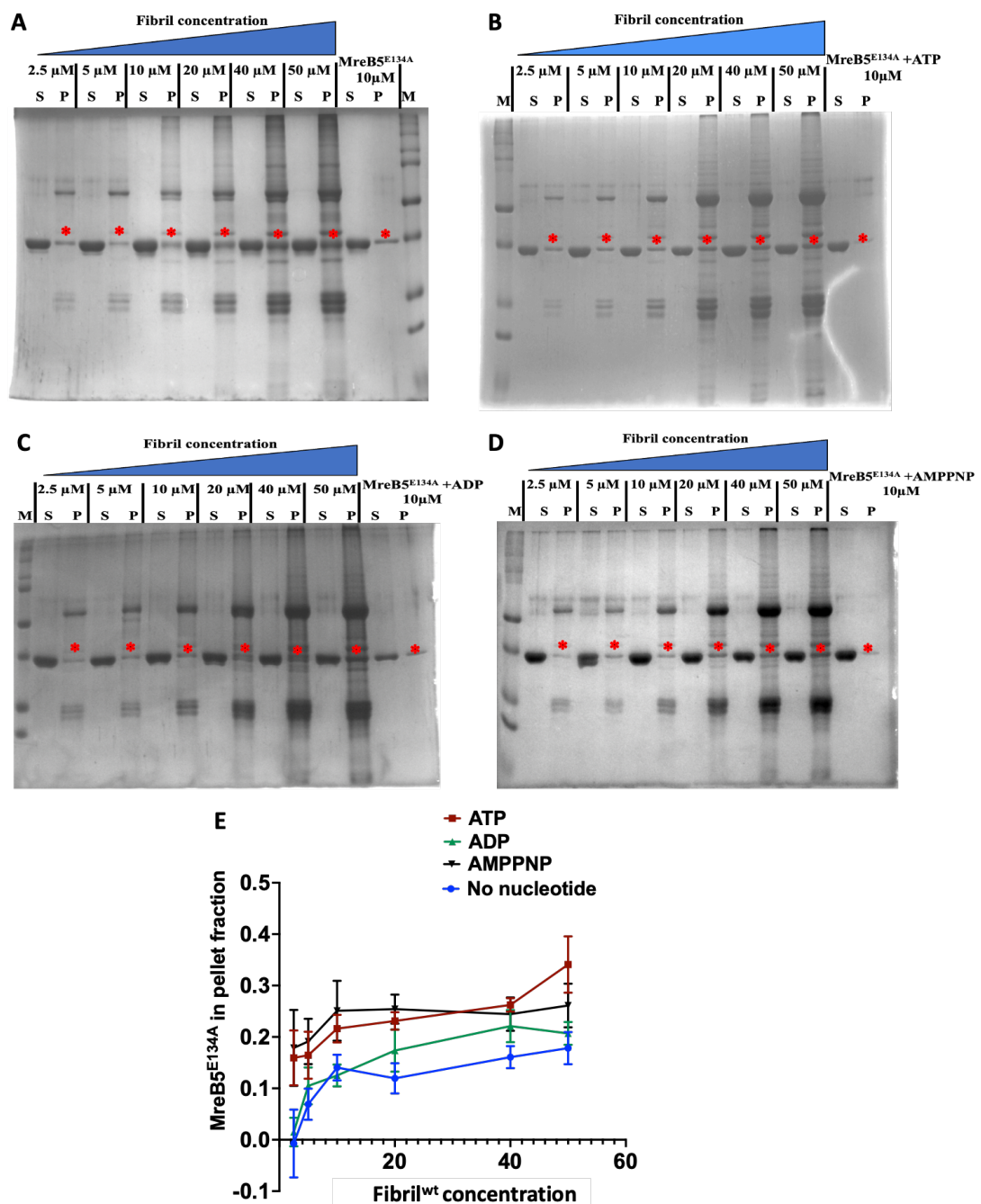


Figure 4.4. Interaction between Fibril^{wt} and MreB5^{E134A} is nucleotide dependent. Pelleting assay gels of Fibril and MreB5^{E134A} in the presence of no nucleotide (A), ATP (B), ADP (C), AMPPNP (D). Fibril concentration was varied from 2.5 μ M to 50 μ M. MreB5^{E134A} concentration used for the assay was 10 μ M. Interaction between the two proteins was quantified by selecting supernatant and pellet band MreB5^{E134A}. Intensity of the pellet fraction of MreB5^{E134A} control was subtracted from all the pellet intensity fraction. E. Intensities of pellet fraction were plotted for all nucleotide conditions. Unlike MreB5^{T161A} and wild type

MreB5 which shows higher interaction with Fibril^{WT} in the presence of no nucleotide and AMPPNP condition, the binding between MreB5^{E134A} and Fibril showed higher binding in all nucleotide conditions. The MreB5^{E134A} protein in the pellet fraction used for quantification of interaction is denoted by red star.

As MreB5^{E134A} is a less efficient ATPase, it is also possible that because MreB5^{E134A} is not able to hydrolyze all the ATP in the solution, MreB5 filaments are bound to ATP as well as ADP. This nucleotide state might favor the interaction between the two proteins. Interaction between Fibril and ScMreB5^{WT}, ScMreB5^{E134A} and ScMreB5^{T161A} in different nucleotide states indicate that hydrolysis of ATP is not crucial for the interaction between the two proteins. Changes in filament assembly of ScMreB5 owing to the nucleotide bound state seem to have an effect on its interaction with Fibril.

4.3.3 Fibril does not enhance ATP hydrolysis of MreB5

Liposomes have been shown to stimulate the ATPase activity of MreB5 (<http://dr.iiserpune.ac.in:8080/xmlui/handle/123456789/8029>). From previous results, we saw that interaction conditions between Fibril^{WT}-MreB5 wild type and Fibril^{WT}-MreB5^{T161A} are similar to those of liposome-MreB5. Based on these results, we were interested to check if Fibril too stimulates the ATPase activity of MreB5, similar to liposomes. We monitored the activity of MreB5 with and without Fibril in the reaction conditions. The k_{obs} for MreB5 wild type was 0.14 ± 0.02 and is consistent with the previous study (Pande et al., 2022). However, upon addition of Fibril in the reaction mixture, the k_{obs} was 0.152 ± 0.06 , which was comparable to MreB5^{WT} without Fibril (Figure 4.5).

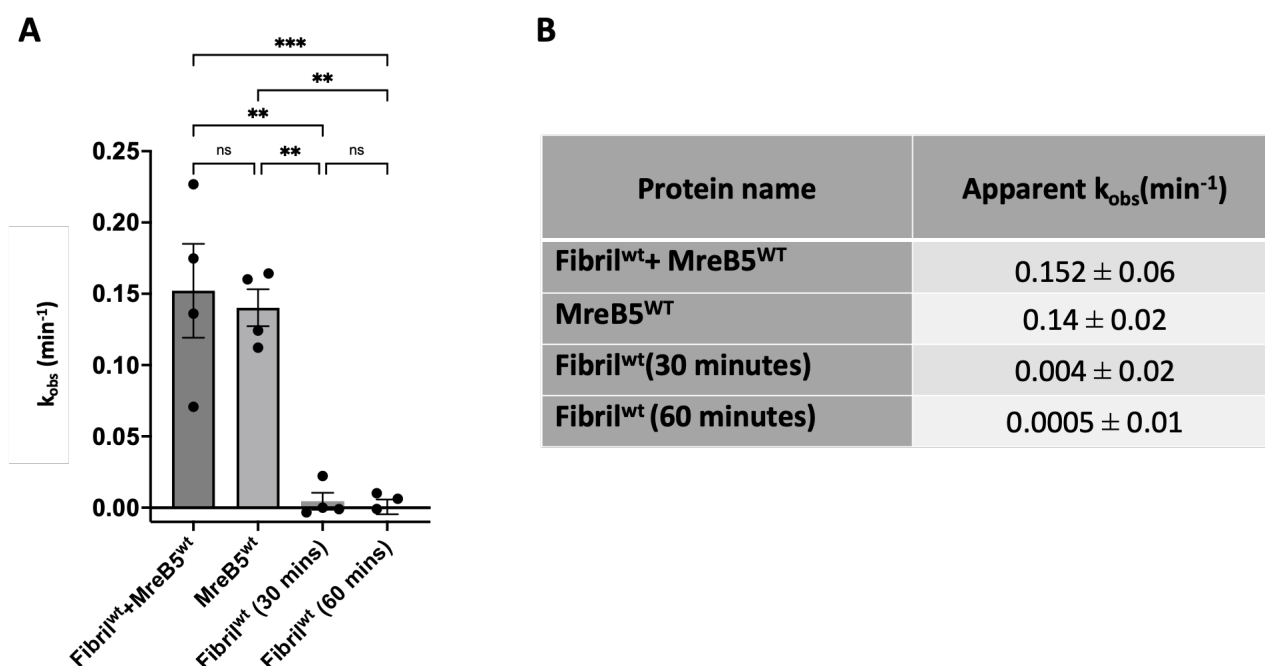


Figure 4.5. Effect of Fibril on the ATPase activity of MreB5. A. ATP hydrolysis of MreB5 and Fibril with MreB5 was checked using malachite green assay. 10 μ M of each protein was used for the assay. Neither stimulation nor inhibition was seen on ATP hydrolysis by MreB5 upon the addition of Fibril. **B.** k_{obs} values of the reactions

This showed that unlike liposomes, Fibril neither possess ATPase activity nor affects the ATP hydrolysis activity of MreB5. As discussed in Chapter 2, these observations suggest that the conformational changes in Fibril might arise from the energy transfer from the ATP hydrolysis of MreBs.

4.4 Discussion

Cytoskeletal proteins of *Spiroplasma eriocheiris* were shown to be placed close to one another just beneath the cell membrane (Kürner, 2005). It is highly likely that these proteins either interact with each other or with the membrane. Recent studies have shown that MreB5 interacts with Fibril independent of any nucleotide (Harne et al., 2020). MreB5 has been shown to interact with liposomes as well (Pande et al., 2022). We were interested in understanding the molecular mechanism of these interactions. To understand these, we checked the interaction between MreB5 and Fibril in different nucleotide conditions through pelleting assays. Interaction between ATPase mutants of MreB5 and Fibril was also checked. We found out that MreB5^{WT} and MreB5^{T161A} show high affinity to Fibril when no nucleotide is added externally. The binding is least efficient in the presence of AMPPNP and the affinity further decreases in

the presence of ATP and ADP condition. However, there is an overall increase in binding of MreB5^{E134A} with Fibril in all nucleotide conditions. MreB5^{E134A} and MreB5^{T161A} ATPase mutants characterized earlier show that MreB5^{T161A} is ATPase dead whereas MreB5^{E134A} is less efficient as compared to MreB5^{WT} (Pande et al., 2022). This might indicate that MreB5 filaments show different conformations depending on the nucleotide bound which in turn changes its affinity towards Fibril. Pelleting assay of MreB5^{WT}, MreB5^{E134A} and MreB5^{T161A} with Fibril proves that MreB5 interaction with Fibril is nucleotide dependent. As discussed earlier, the double protofilament assembly seems to differ in the presence of different nucleotides. In future, it will be interesting to understand the filament assembly of the MreB5^{E134A} and MreB5^{T161A} and compare it with MreB5^{WT}. This might help us in understanding if the filament assembly of MreB5 in different nucleotide states has any effect in Fibril binding.

The fact that MreB5 shows ATPase activity and interacts with Fibril, led us to check the ATP hydrolysis activity of MreB5 in the presence of Fibril. Malachite green assay shows that Fibril shows neither inhibition or stimulation in ATPase activity of MreB5. As discussed earlier, MreB5 interacts with Fibril as well as liposomes, it will be interesting to check the effect of liposomes on the interaction of MreB5-Fibril. Effect of liposome on the ATPase activity of MreB5-Fibril can also be checked. This can help us understand whether Fibril and liposomes compete with each other for MreB5 binding.

Caulobacter MreB has been shown to form stable straight, double protofilaments in the presence of ATP (Nurse and Marians, 2013; Popp et al., 2010; van den Ent et al., 2014, 2001). Disruption of the double protofilaments leads to irregular cell shape. It also affects the membrane binding ability of the filaments (Salje et al., 2011). In the case of *Spiroplasma*, MreB5 has been shown to interact with liposomes (Pande et al., 2022) and Fibril in a nucleotide dependent manner (this study). We propose that MreB5 induces conformational changes in Fibril filaments depending on the bound nucleotide state. This in turn leads to change in helicity and handedness of the cells.

4.5 References

1. Charbon, G., Cabeen, M.T., Jacobs-wagner, C., 2009. Bacterial intermediate filaments : in vivo assembly , organization , and dynamics of crescentin 1131–1144. <https://doi.org/10.1101/gad.1795509.et>
2. Harne, S., Duret, S., Pande, V., Bapat, M., Béven, L., Gayathri, P., 2020. MreB5 Is a Determinant of Rod-to-Helical Transition in the Cell-Wall-less Bacterium *Spiroplasma*. *Current Biology* 30, 4753–4762.e7. <https://doi.org/10.1016/j.cub.2020.08.093>
3. Herrmann, H., Aebi, U., 2004. Intermediate Filaments: Molecular Structure, Assembly Mechanism, and Integration Into Functionally Distinct Intracellular Scaffolds. *Annu Rev Biochem* 73, 749–789. <https://doi.org/10.1146/annurev.biochem.73.011303.073823>
4. Kürner, J. et al, 2005. Cryo – Electron Tomography Reveals the Cytoskeletal Structure of *Spiroplasma melliferum* 436. <https://doi.org/10.1126/science.1104031>
5. Nurse, P., Marians, K.J., 2013. Purification and Characterization of *Escherichia coli* MreB Protein. *Journal of Biological Chemistry* 288, 3469–3475. <https://doi.org/10.1074/jbc.M112.413708>
6. Pande, V., Mitra, N., Bagde, S.R., Srinivasan, R., Gayathri, P., 2022. Filament organization of the bacterial actin MreB is dependent on the nucleotide state. *Journal of Cell Biology* 221. <https://doi.org/10.1083/jcb.202106092>
7. Popp, D., Narita, A., Maeda, K., Fujisawa, T., Ghoshdastider, U., Iwasa, M., Maéda, Y., Robinson, R.C., 2010. Filament Structure, Organization, and Dynamics in MreB Sheets. *Journal of Biological Chemistry* 285, 15858–15865. <https://doi.org/10.1074/jbc.M109.095901>
8. Salje, J., van den Ent, F., de Boer, P., Löwe, J., 2011. Direct Membrane Binding by Bacterial Actin MreB. *Mol Cell* 43, 478–487. <https://doi.org/10.1016/j.molcel.2011.07.008>
9. Taylor, J.A., Bratton, B.P., Sichel, S.R., Blair, K.M., Jacobs, H.M., Demeester, K.E., Kuru, E., Gray, J., Biboy, J., Vannieuwenhze, M.S., Vollmer, W., Grimes, C.L., Shaevitz, J.W., Salama, N.R., 2020. Distinct cytoskeletal proteins define zones of enhanced cell wall synthesis in *helicobacter pylori*. *Elife* 9. <https://doi.org/10.7554/eLife.52482>
10. van den Ent, F., Amos, L.A., Löwe, J., 2001. Prokaryotic origin of the actin cytoskeleton. *Nature* 413, 39–44. <https://doi.org/10.1038/35092500>
11. van den Ent, F., Izoré, T., Bharat, T.A., Johnson, C.M., Löwe, J., 2014. Bacterial actin MreB forms antiparallel double filaments. *Elife* 3. <https://doi.org/10.7554/eLife.02634>

Chapter 5

Preliminary characterization of *S. citri* MreB1 and its interaction with Fibril

5.1 Biochemical characterization of MreB1 from *S. citri*

5.1.1 Introduction

Most of the cell walled rod shaped bacteria possess 1-3 paralogs of MreB (Ku et al., 2014). MreB, Mbl and MreBH are essential for maintaining cell morphology in *Bacillus subtilis* (Jones et al., 2001; Soufo et al., 2003). Mutational studies have shown that MreB plays a role in controlling width of the cells whereas Mbl is required for proper positioning of FtsZ for maintaining the linear axis of the cell (Jones et al., 2001). *B. subtilis mreB* and *mbl* shows ~ 53% sequence similarity. These observations indicate that in spite of having high sequence similarity, different paralogs of MreB show non-redundant functions in the cell. Similarly, in spite of having a reduced genome, most of the strains of *Spiroplasma* show the presence of 5-8 MreB paralogs (Ku et al., 2014). *Spiroplasma citri* possesses five paralogs of MreB. MreB5 has been shown to be essential for conferring helicity and motility to the organism. Sequence alignment shows high similarity between the five paralogs of MreB proteins in *S. citri* (Harne et al., 2020). As Fibril and MreB proteins form the cytoskeletal ribbon of *Spiroplasma*, it is desirable to decipher the role of each of the components. In order to understand the biochemical properties of the protein and check the interaction between different MreBs, we plan to purify all the paralogs of MreBs from *S. citri*. This section discusses purification trials carried out for the biochemical characterization of MreB1, and attempts to improve sample preparation of Fibril to structurally characterize the interaction between MreB1 and Fibril.

5.1.2 Materials and Methods

5.1.2.1 Sequence conservation of MreBs and Actin

The amino acid sequences of MreB protein for *Spiroplasma citri*, *Bacillus subtilis* (Bs-MreB), *Escherichia coli* (Ec-MreB), *Thermotoga maritima* (Tm-MreB) and the actin sequence from *Saccharomyces cerevisiae* (Sc-Actin) were obtained from Uniprot (uniprot.org). Sequence alignment was done using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Table 5.1 lists the accession numbers of the protein sequences.

Table 5 1. Accession number of MreB and Actin sequences

MreB sequence	Uniprot Accession number
MreB1	Q8VP64
MreB2	Q8VQG4
MreB3	Q8VQG3
MreB4	Q8VQG2
MreB5	Q8VQG1
Bs MreB	Q01465
Ec-MreB	P0A9X4
Tm-MreB	R4NQM0
Sc-Actin	P60010

5.1.2.2 Cloning of *mreb1-H₆* and ATP hydrolysis mutant *mreb1^{E137A,T323I}-H₆*

pHis17 mreB1 untagged construct was available in the lab and the insert was amplified from *Spiroplasma citri* GII3 strain. For the purification of MreB1 using affinity-based chromatography, we cloned a construct which contained a 6X histidine tag at C terminal with GS as a linker of two amino acids. *pHis17 mreB1* plasmid was taken as template for the gene amplification and the 6X histidine tag with the linker sequence was added in the primer sequence. This linear product was then used as the mega primer to amplify using *pHis17 mreB1* untagged clone as the template. MreB1^{T323I}-H₆ mutant (T323I was a non-specific mutation introduced during cloning) was available in the lab. ATP hydrolysis mutant MreB1^{E137A,T323I}-H₆ was cloned using MreB1^{T323I}-H₆ as the template.

Restriction free (RF) cloning method was used to clone MreB1-H₆ (Ent and Lo, 2006; van den Ent and Löwe, 2006) . A control was kept consisting of a template without the mega primer. The RF products were digested with DpnI to get rid of plasmid used as the template. The digested products were transformed in the NEB electrocompetent cells using BioRad Gene Pulser Xcell. Plasmids were extracted using Qiagen mini prep kit (Qiagen - 27104). The clones were confirmed by sequencing.

5.1.2.3 Expression check of MreB1-H₆

Expression check of MreB1-H₆ was checked by transforming the plasmid in BL21AI chemically competent cells (Stratagene). The gene encoding T7 polymerase in BL21AI is under

the *araBAD* operon. L-arabinose is used as an inducer for the protein expression. The cells were plated on LB agar plate consisting of 100 µg/mL ampicillin and incubated at 37 °C for 12 hours. 3 - 4 colonies were grown in 1X Luria Bertani broth containing 100 µg/mL of ampicillin at 37 °C till OD₆₀₀ reached 0.6 - 0.8. The culture was induced with 0.2% arabinose and shifted to 18 °C for further growth. After an incubation of 12 hours, 3 mL of the culture was pelleted down and resuspended in a 500 µL of lysis buffer consisting of 300 mM KCl, 50 mM Tris pH-8 and 10 % glycerol. The lysate was subjected to sonication using the SONICS Vibra cell. The settings used for sonication were 1 second ON, 3 seconds OFF, 60% amplitude for 3 minutes. 10 µL of this sample was added to the 2X Laemmli buffer. The lysate was then spun at 21000 g for 10 minutes at 4 °C. 10 µL of supernatant was added to the 2X Laemmli buffer. The two samples were heated at 99 °C for 10 minutes. 10 µL of the samples were loaded on 12 % SDS PAGE gel. BioRad precision plus ladder (BioRad - 1610374) was loaded as a reference for the molecular weight.

5.1.2.4 Purification of MreB1-H₆ , MreB1^{E137A} -H₆, MreB1^{T323I} -H₆, MreB1^{E137A,T323I} -H₆

For purification of MreB1^{T323I} -H₆ and MreB1^{E137A,T323I} -H₆, 1 L culture was processed whereas for MreB1-H₆ and MreB1^{E137A} -H₆, 2 L culture was used for purification. Transformation and culture growth conditions were similar as mentioned in Section 5.1.1.2 unless specifically mentioned. Two sonication cycles were used for cell lysis with a gap of 5 minutes. The supernatant after sonication was spun at 27,216 g for 20 minutes at 4 °C. The buffer conditions used for the purification of MreB1 constructs was similar to *S. citri* MreB5 mentioned in (Harne et al., 2020). The lysate was loaded on a Ni-NTA column (HisTrap, GE Healthcare) pre-equilibrated with Buffer A containing 300 mM KCl, 50 mM Tris pH -8.0. Protein was eluted with Buffer B containing 300 mM KCl, 50 mM Tris pH-8, 500 mM imidazole in a step gradient manner. The protein was eluted in 100 mM and 250 mM imidazole. The protein was spun at 27,216 g for 10 minutes at 4 °C. The protein was then concentrated till 800 µL using Sartorius 10 kDa centricons (catalogue number VS15T01). The protein was then injected in gel filtration column Superdex 200 (10/300 GE Healthcare). The column was pre-equilibrated with 300 mM KCl, 50 mM Tris pH - 8.0. 10 µL of the protein fractions eluted were added to 2X Laemmli buffer and heated at 99 °C for 10 minutes. Samples were checked on 10 % SDS PAGE gel. Protein containing fractions were concentrated and aliquots of 10 - 20 µL were made and flash frozen in liquid nitrogen. The purified protein was stored in -80 °C till further use.

5.1.2.5 Thermal shift assay

Thermal shift assay was performed to check the folding state of proteins (Huynh and Partch, 2015). Thermal shift assay monitors the change in thermal stability of proteins in varying conditions like ligand, salt, pH etc. 2.5 μ M - 5 μ M of MreB1-H₆ was used for the assay. A reaction of 25 μ L was set up for every condition. Dilution of Sypro orange dye (S5692) was made in fresh MilliQ water. 2.5 μ L of 5X Sypro Orange was added in all the reactions. Protein sample with the desired concentration was added to the buffer and additives to be checked and mixed well. These samples were then added to multiplate PCR 96 well plate (MLL9651). After adding the samples in the 96 well plate, the wells were sealed with BioRad microseal 'B' seal (MSB1001). The assay was carried out in a Bio-Rad CFX96 Real-Time System. The change in the fluorescence of Sypro Orange dye as it binds to hydrophobic regions of the protein is measured using FRET channel (λ_{ex} - 450 - 490 nm) and (λ_{em} - 560 - 580 nm). A temperature gradient of 4 - 90 °C was set up with initial incubation at 4 °C for 10 minutes followed by a rise of 0.4 °C every 20 seconds. The first derivative of the melting curve was plotted against temperature.

5.1.2.6 Malachite Green assay

Bradford method was used to estimate the concentration of the protein. BSA standards were used to plot a standard curve and determine the protein concentration. 5 μ M and 10 μ M of protein was used for checking ATP hydrolysis activity using malachite green assay (Feng et al., 2011). A reaction of 70 μ L was set up consisting of protein, 1 mM of ATP and 1 mM MgCl₂ buffer (300 mM KCl, 50 mM Tris pH - 8.0) was added to make up the volume. The reactions were incubated at 25 °C and the activity was monitored for 1 hour. The protocol followed for the assay is mentioned in section 2.5.3.

5.1.2.7 Purification of Fibril wild type

The Fibril wild type was overexpressed in *E. coli*. For the purification, we used urografin density gradient as discussed in (Harne and Gayathri, 2022). The lower band obtained from the gradient was carefully separated from the rest of the gradient and washed with 50 mM Tris pH - 8.0. This sample was spun at 159000 g in order to get rid of urografin. The protein obtained was resuspended in 2 % cholic acid (sodium deoxycholate), 20 mM Tris-Cl pH - 8.0, 150 mM NaCl and kept for stirring at 4°C for 8 hours. The partially purified protein sample was then layered on the urografin density gradient and spun at 159000 g for 2 hours at 4 °C. The protein

obtained was again washed with 50 mM Tris pH - 8.0. The pellet was stored in -80 °C until further use.

5.1.2.7 Sample preparation of Fibril wild type for negative staining

The protein obtained from the two rounds of density gradient was resuspended in 1 mL of 20 mM Tris-Cl pH - 8.0, 150 mM NaCl. The concentration was adjusted to 1 mg/mL. The protein sample was then subjected to sonication of 5 s ON and 5 s OFF cycle and 60 % amplitude. Protein sample was sonicated for a total of 1 hour. 10 µL of sample was taken at different timepoints for preparing grids. 10 µL of protein was loaded on Carbon/Formvar Cu grids (TED-PELLA, INC) for 3 minutes. The solution was back blotted using Whatman paper. 10 µL of MilliQ was used to wash the grid surface and again back blotted. 4 µL of 0.5% uranyl acetate was added on the grid and blotted after 30 s. The grid was air dried and imaged under TEM (JEM - 2200FS Joel Ltd.).

5.1.3 Results

5.1.3.1 MreB1 shows conserved motifs of Actin family

Sequence alignment of *Spiroplasma citri* MreB paralogs with *B. subtilis*, *E. coli* and *T. maritima* MreB and Yeast actin shows conservation of motifs. Table 5.2 summarizes the pairwise sequence alignment of MreB1 with actin and MreB sequences from other bacterial species.

Table 5.2. Pairwise sequence alignment of MreB sequences

Sequence pairs	Percentage identity
MreB1 - MreB2	54.49
MreB1 - MreB3	40.35
MreB1 - MreB4	86.99
MreB1 - MreB5	53.64
MreB1 - Bs MreB	38.81
MreB1 - Ec MreB	36.95
MreB1 - Tm MreB	38.62
MreB1 - Yeast actin	19.8

In spite of having low sequence identity with actin, MreBs show conservation of motifs (Figure 5.1). The residues involved in ATP hydrolysis for MreB1 were identified using multiple sequence alignment. Residue E137 was selected as an ATPase mutant for biochemical characterization of the ATPase mutant activity. The yellow asterisk shows the E137 residue of MreB1 and the corresponding residues in actin and other MreB sequences. The sequence alignment shows conservation of the active site glutamate residue in all *Spiroplasma* MreB sequences.

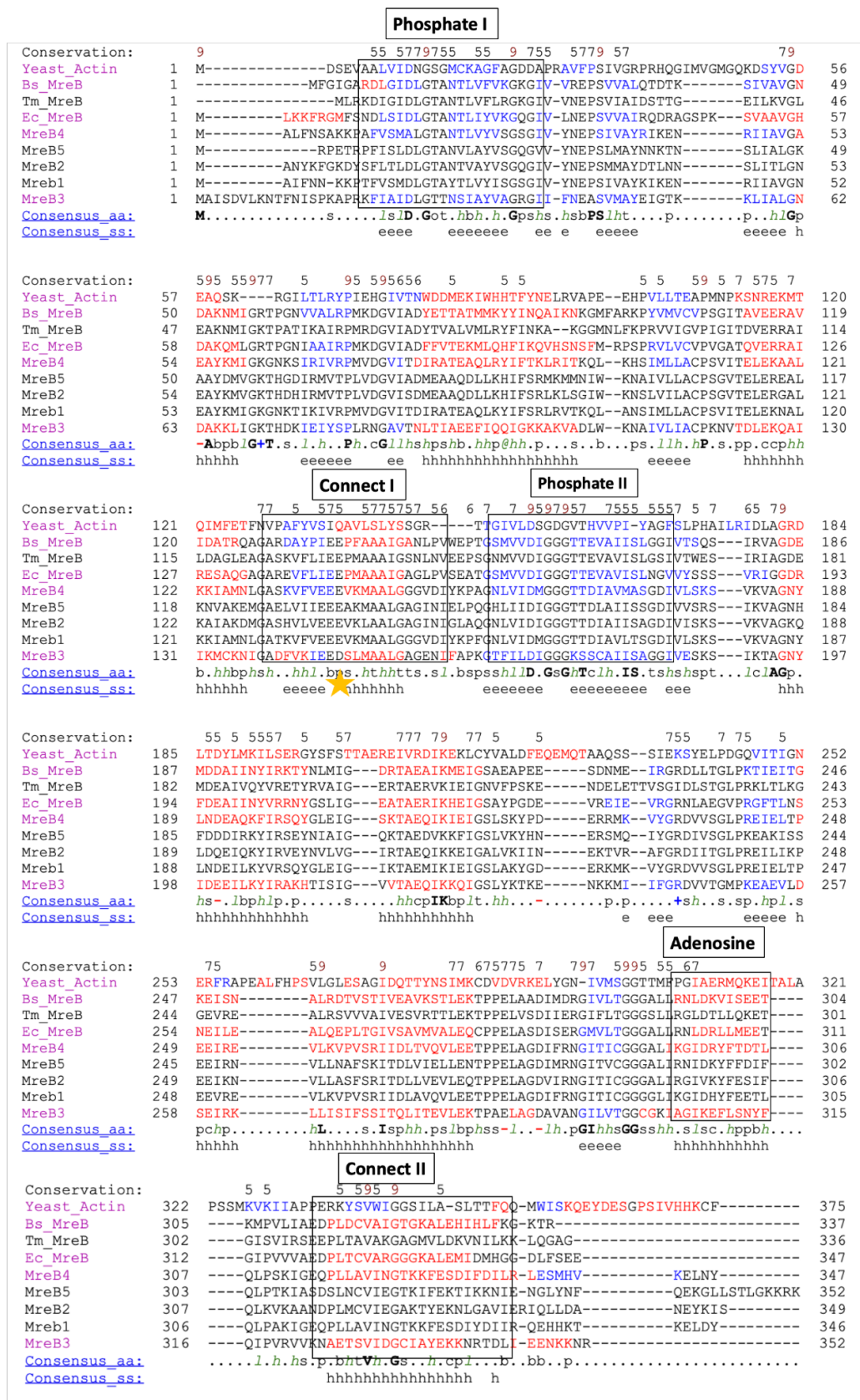


Figure 5.1. All the 5 motifs are conserved in *Spiroplasma citri* MreBs. Conservation of Phosphate 1, Connect 1, Connect 2, Phosphate 2 and Adenosine motifs show that all five paralogs of MreBs in *S. citri* can bind and potentially hydrolyse ATP.

5.1.3.2 Stability and folded state of MreB1-H₆^{T323I}

MreB1^{T323I}-H₆ construct was available in the lab. The construct was purified using Ni-NTA affinity chromatography followed by size exclusion chromatography (Figure 5.2 A and B). The buffer conditions for the purification used were similar to MreB5 wild type (Harne et al., 2020; Pande et al., 2022). However, the protein showed heavy precipitation and the protein yield was very low. In order to increase the stability and yield of the protein, buffer optimization for the purification of MreB1-H₆^{T323I} was done using Thermal shift assay. Purified protein showed highly unstable protein (Figure 5.2 C). When 2 mM ADP was added along with the protein, the thermostability showed an increase from 50 mM to 500 mM KCl (Figure 5.2 D). The T_m at 300 mM KCl and 500 mM KCl was similar and hence 300 mM KCl was chosen for the further purifications.

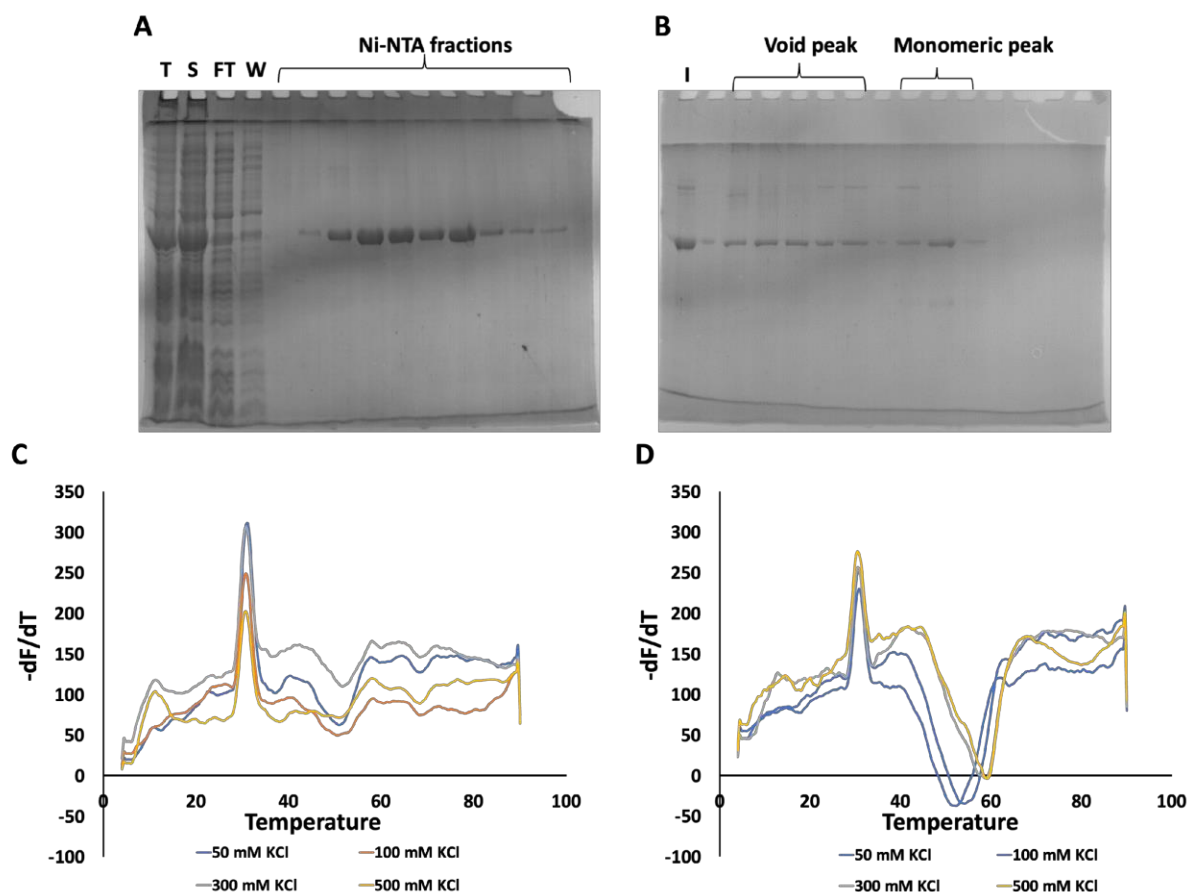


Figure 5.2. Thermal shift assay of MreB1^{T323I}-H₆ shows stability in the presence of nucleotide. A. Ni-NTA gel of MreB1^{T323I}-H₆. B. Superdex 200 size exclusion gel profile of MreB1^{T323I}-H₆. C. The first derivative melting curve of MreB1^{T323I}-H₆ indicates that protein might be misfolded/unstable in the buffer conditions tried. Salt concentrations of 50 mM, 100 mM, 300 mM and 500 mM KCl were tried to check stability of the protein. D. Melting curve

of purified MreB1^{T323I}-H₆ after adding 2 mM ADP. Stability of the protein was checked with similar salt concentrations after adding ADP in all the reactions. Highest T_m was observed for the salt concentration of 300 mM and 500 mM KCl.

Further batches for MreB1^{T323I}-H₆ were purified by adding 2 mM ADP in the fraction collection tubes as well as during the protein concentration step. The protein purified by adding ADP was used to check stability in the presence of different salts, namely, KCl and NaCl (Figure 5.3). No significant change was observed between the two different salt conditions.

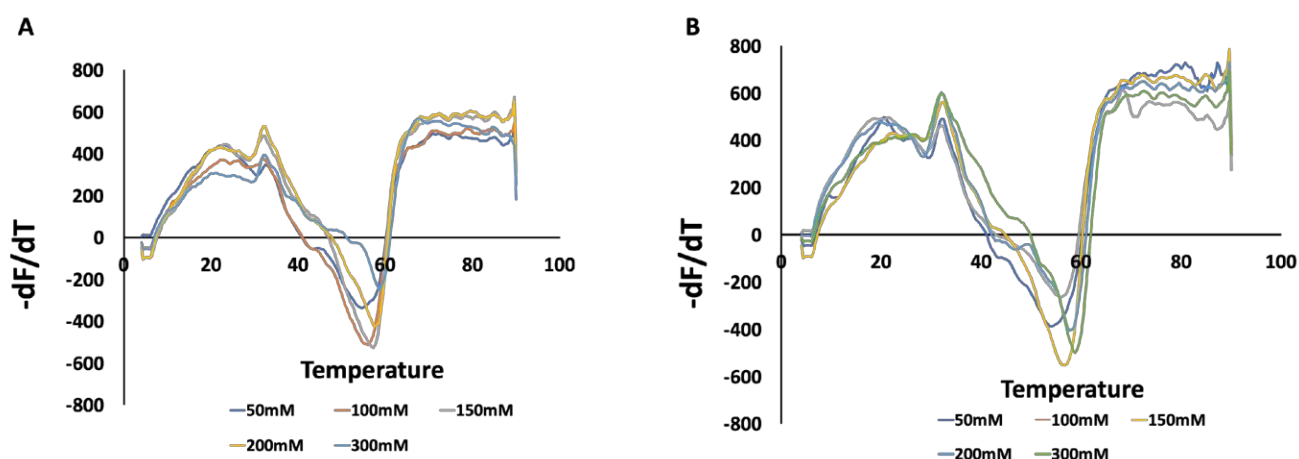
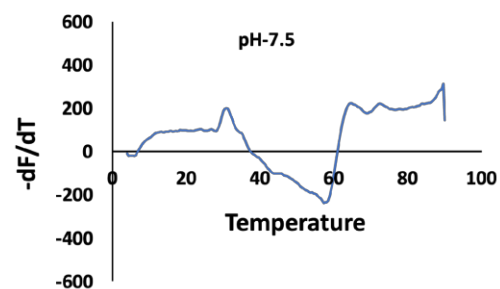
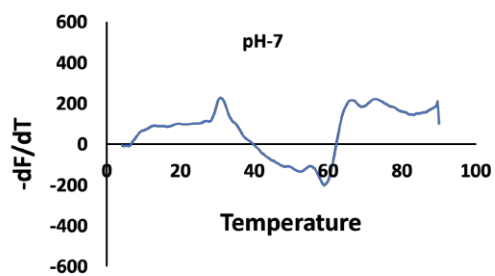
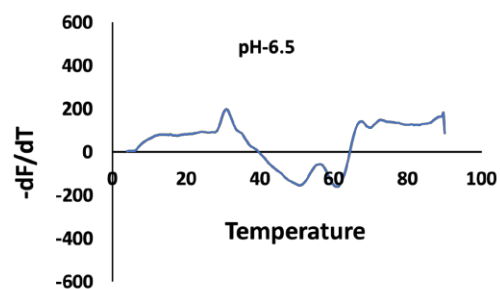
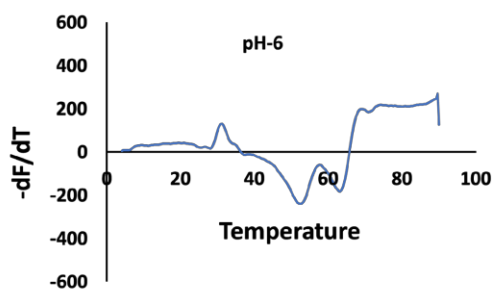
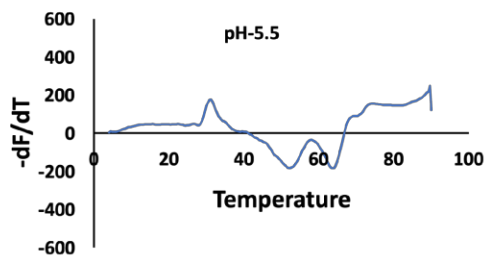
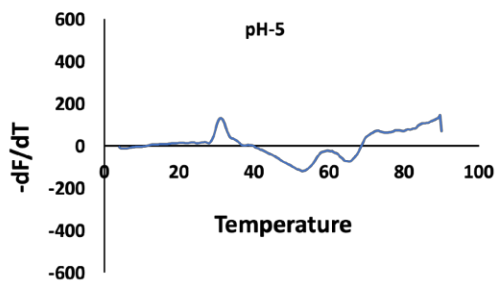
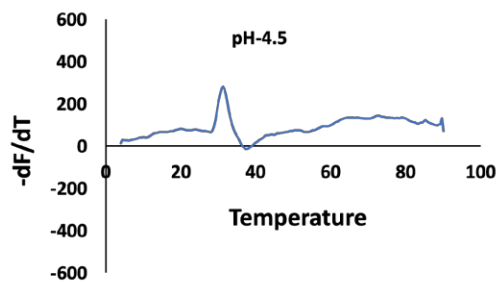
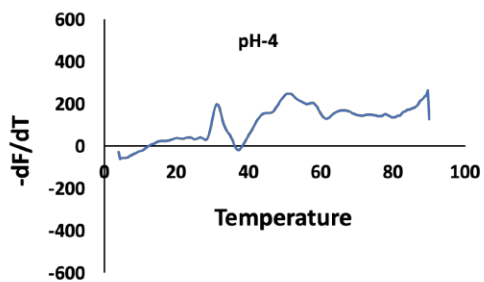


Figure 5.3. Thermal shift assay of MreB1^{T323I}-H₆ in the presence of different salts. A. Melting curves of MreB1^{T323I}-H₆ in the presence of different concentrations of KCl. **B.** Melting curves of MreB1^{T323I}-H₆ in the presence of different concentrations of NaCl. Highest T_m observed was 58.8 °C for 300 mM KCl and NaCl.

The T_m for both the salt conditions was around 58.8 °C. As ScMreB5^{WT} has been purified in KCl-containing buffer (Pande et al., 2022), 300 mM KCl was used for purification of MreB1-H₆ as well. Same batch of purified protein was used to screen different pH conditions for increased stability of the protein. A pH range from 4 - 10 was checked (Figure 5.4) for the assay.



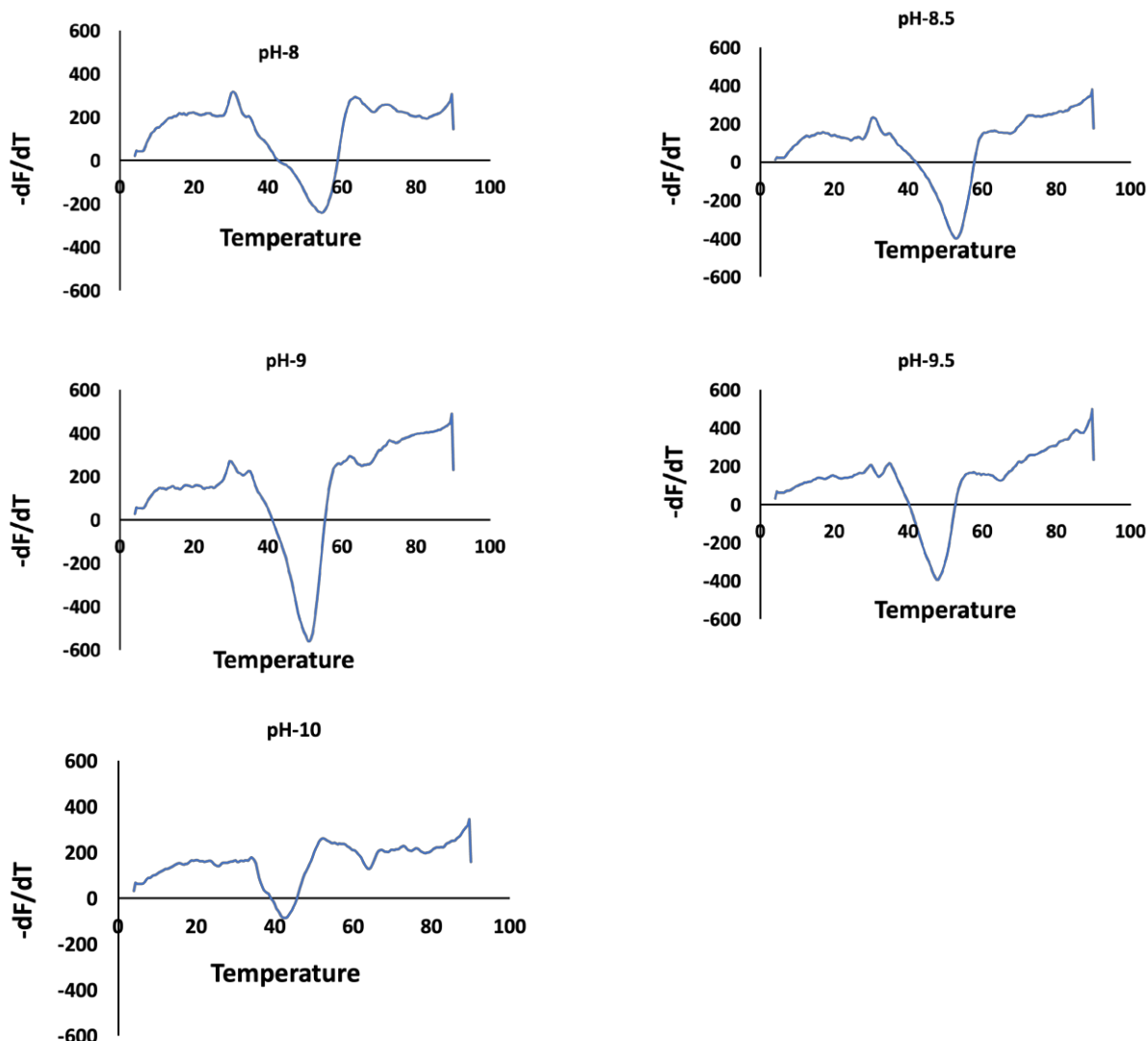


Figure 5.4. Melting curves of MreB1^{T323I}-H₆ at different pH. Stability of purified protein containing 2 mM ADP was seen highest in the pH range of 7.5 - 9. Two different species of protein were observed in the pH range of 4 - 7. pH 8 - 9.5 showed a single species. Highest T_m observed was 55.2 °C at pH - 8.0. T_m decreased as the pH was increased beyond 9. pH - 8.0 was used for further purifications.

Thermal shift assay indicates that in spite of possessing a point mutation to isoleucine at 323rd amino acid, the protein seems to be folded. The difference between the T_m values for 300 mM and 500 mM NaCl/ KCl were insignificant. Similarly, the protein seems stable in the pH range of 8 to 9.5. However, the highest T_m value (55.2 °C) was observed at pH - 8.0. Finally, 300 mM KCl, 50 mM Tris pH - 8.0 was used for the further purifications.

5.1.3.3 MreB1^{T323I}-H₆ and MreB1^{E137A,T323I}-H₆ might form higher oligomeric structures

Based on the sequence alignment, the threonine mutation of MreB1^{T323I}-H₆ at the 323rd amino acid does not take part in the hydrolysis of ATP. Purification trials were attempted for this construct (Figure 5.5). The purification strategy included affinity-based chromatography followed by size exclusion chromatography. The construct had a C terminal hexa-histidine tag which enabled the protein to bind to the Ni-NTA column. The purified protein was concentrated to approximately 500 to 800 μ L and the concentrated protein was injected in the Superdex 200 column for further purification and checking the oligomeric state of the protein.

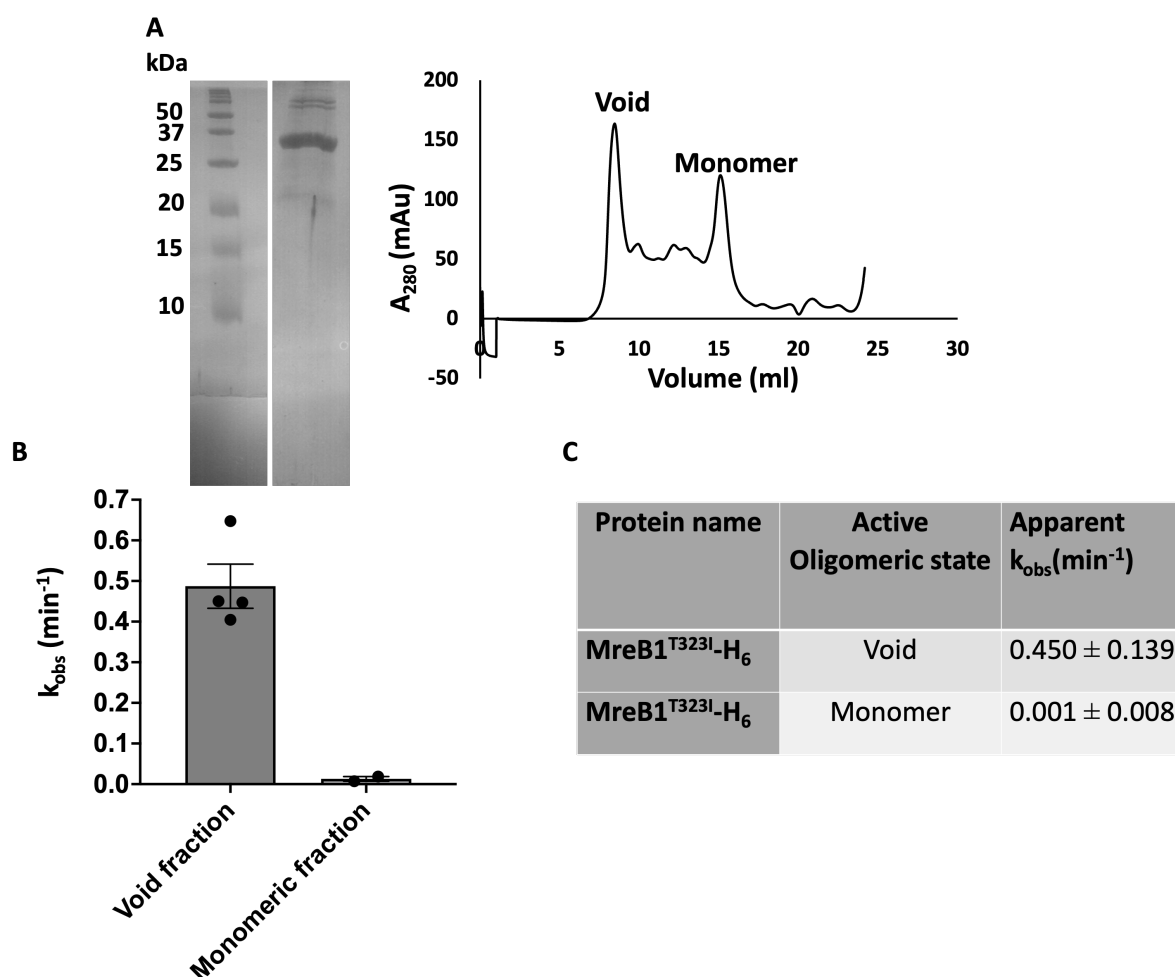


Figure 5.5. Purification of MreB1^{T323I}-H₆ shows two different oligomeric fractions of the purified protein. **A.** Size exclusion chromatography of MreB1^{T323I}-H₆ showed two peaks corresponding to void fraction and monomeric fraction. ATP hydrolysis activity was checked using malachite green assay for the fractions. **B and C.** Void fraction showed ATP hydrolysis whereas, monomeric fraction was ATPase dead. The purified protein showed non-specific higher bands.

When the protein was added onto the Superdex 200 Increase 10/300 GL column, most of the protein eluted as void fraction (Figure 5.5 A). To check whether the protein in void fraction were aggregates or filaments, we checked the ATPase activity of the void as well as monomeric fraction obtained from Superdex 200. Surprisingly, the void fraction of MreB1^{T323I}-H₆ showed ATP hydrolysis whereas there was no activity seen for the monomeric fraction (Figure 5.5 B and C).

During the purification, heavy precipitation was observed as soon as the protein eluted from the Ni-NTA column. The composition of salt and pH was kept constant for the purification trials; however, various additives were screened for the purification of MreB1^{T323I}-H₆ (Table 5.3). Addition of 10 % Glycerol and 2 mM DTT helped in reducing the precipitation. However, the final yield after adding glycerol or DTT was still very low.

Table 5.3. Optimization of buffer conditions for the purification of MreB1^{T323I}-H₆

Buffer	Additive	Precipitation	Yield	Activity
300 mM KCl, 30 mM Tris pH-8	None	Heavy precipitation	0.2 mg/ml	Not checked
300 mM KCl, 30 mM Tris pH-8	2 mM MgCl ₂	Less precipitation	0.2 mg/ml	Not checked
300 mM KCl, 30 mM Tris pH-8, 2 mM MgCl ₂	2 mM ADP	Heavy precipitation	0.7 mg/ml	Not checked
300 mM KCl, 30 mM Tris pH-8, 2 mM MgCl ₂	0.01% CHAPS	Very less precipitation	0.69 mg/ml	Less active
300 mM KCl, 30 mM Tris pH-8, 2 mM MgCl ₂	0.05% CHAPS	Very less precipitation	12.3 mg/ml	Inactive
300 mM KCl, 30 mM Tris pH-8, 2 mM MgCl ₂	10% Glycerol	Less precipitation	1.2 mg/ml	Active
300 mM KCl, 30 mM Tris pH-8, 2 mM MgCl ₂	2 mM DTT	Heavy precipitation	0.8 mg/ml	Active

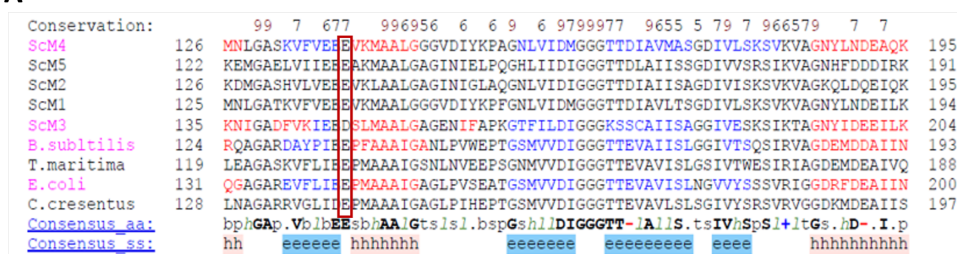
As seen from the purification gels, the protein purified from Ni-NTA was impure (Figure 5.5 A). To check if the ATPase activity shown was from our protein of interest and not a result of impurities, we created an ATPase hydrolysis mutant E137A. As seen from the sequence alignment, glutamate residue is fairly conserved amongst most of the MreB proteins of various organisms (Figure 5.6 A). If the mutant is able to abrogate the ATP hydrolysis activity of

MreB1^{T323I}-H₆, we can conclude that the activity was specific to MreB1^{T323I}-H₆ and not coming from any non-specific proteins purified along with it.

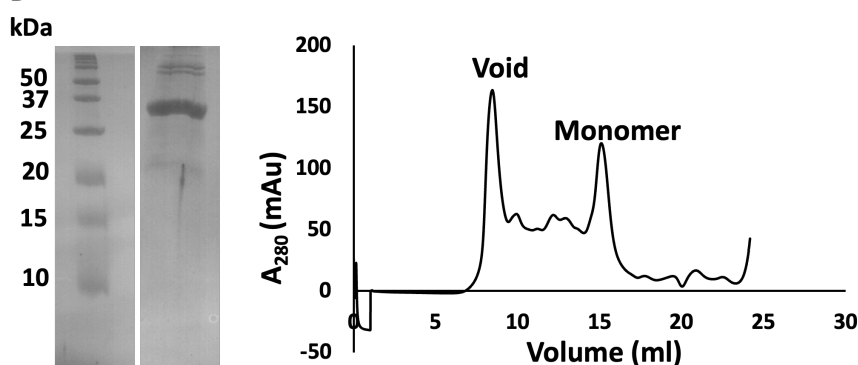
5.1.3.4 MreB1^{T323I}-H₆ is an active ATPase while MreB1^{E137A,T323I}-H₆ abrogates ATP hydrolysis

The ATPase mutant MreB1^{E137A,T323I}-H₆ was comparatively pure as compared to MreB1^{T323I}-H₆. The purified protein eluted as a monomer in Superdex 200 Increase 10/300 GL Figure 5.6 B). The activity of MreB1^{T323I}-H₆ and MreB1^{E137A,T323I}-H₆ was compared using malachite green assay. MreB1^{E137A,T323I}-H₆ showed no ATP hydrolysis (Figure C and D).

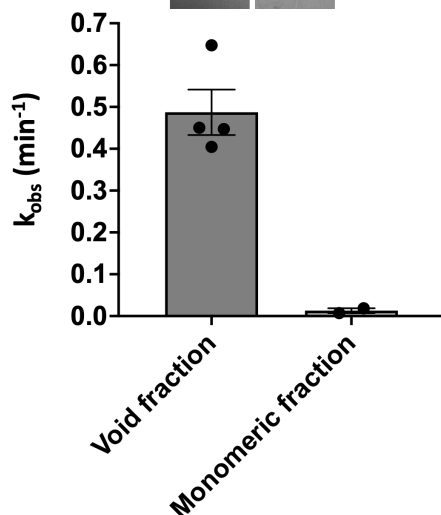
A



B



C



D

Protein name	Active Oligomeric state	Apparent k _{obs} (min ⁻¹)
MreB1 ^{T323I} -H ₆	Void	0.450 ± 0.139
MreB1 ^{T323I} -H ₆	Monomer	0.001 ± 0.008

Figure 5.6. ATP hydrolysis activity of MreB1^{T323I}-H₆ and MreB1^{E137A, T323I}-H₆. **A.** Purified MreB1^{E137A, T323I}-H₆ shows protein in monomeric fraction. **B.** Malachite Green assay of the purified proteins showed that MreB1^{T323I}-H₆ was ATPase active while the ATPase mutant MreB1^{E137A, T323I}-H₆ showed no activity as expected.

This might be an indication that the activity seen for MreB1^{T323I}-H₆ was from the protein of interest and not from the non-specific proteins purified with it. It is possible that the void fraction contains filaments and not aggregates. To further confirm this, we plan to visualize the proteins under transmission electron microscope.

5.1.3.5 Purification trials of MreB1-H₆ and MreB1^{E137A}-H₆

From the above results, we observed that buffer optimization failed to increase the stability of the protein. Hence, the threonine at 323rd position was corrected. It was suspected that the mutation might have an effect on the protein stability.

MreB1-H₆ and MreB1^{E137A}-H₆ were purified in the same buffer conditions as MreB1^{T323I}-H₆ and MreB1^{E137A, T323I}-H₆. The wild type protein as well as the ATPase were impure as compared to their corresponding threonine mutant. The size-exclusion runs showed the majority of MreB1-H₆ coming in void fraction. MreB1^{E137A}-H₆ eluted in void as well as monomeric fractions (Figure 5.7 A and B).

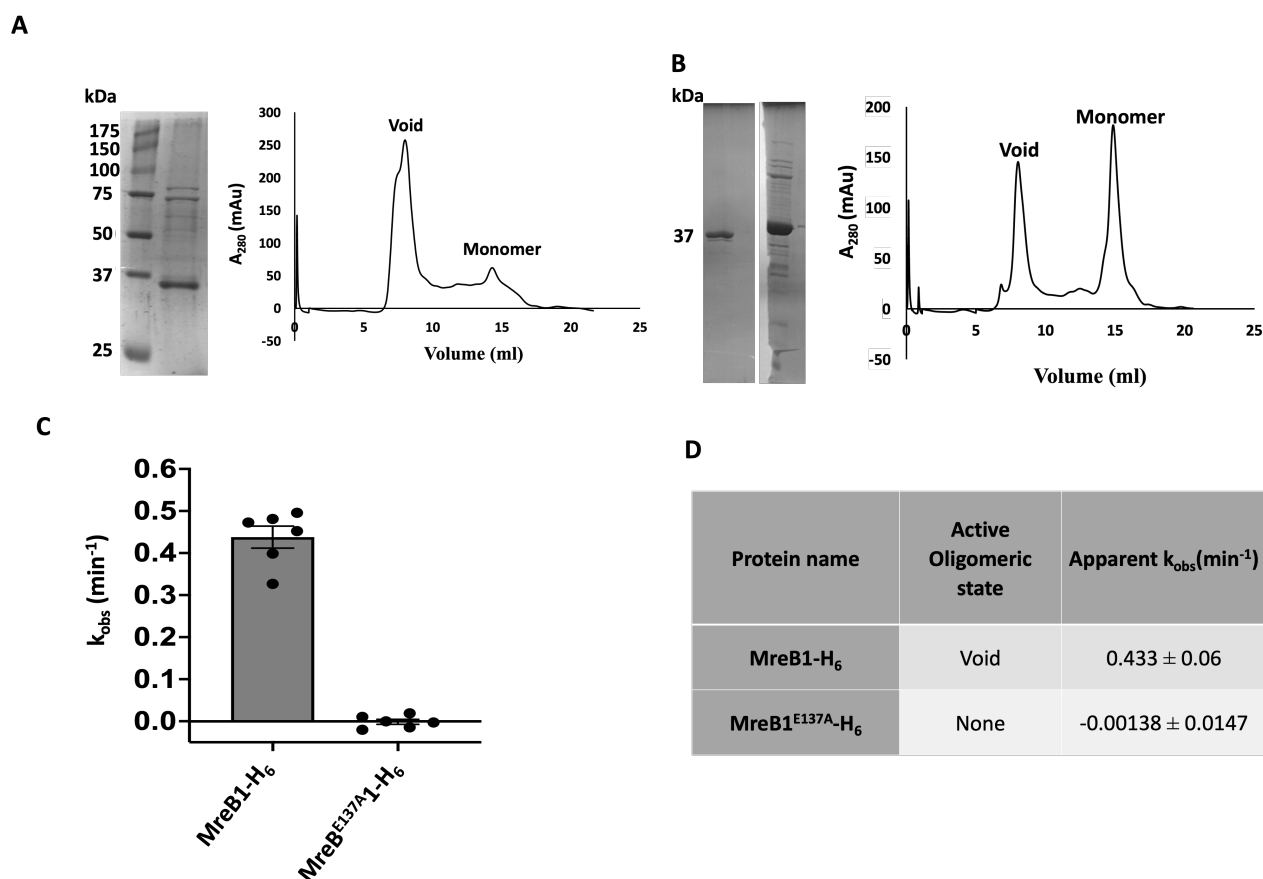


Figure 5.7. ATP hydrolysis activity of MreB1-H₆ and MreB1^{E137A}-H₆ was comparable to corresponding threonine mutants. **A.** Purification of MreB1-H₆ was done using Ni-NTA followed by size exclusion chromatography. Majority of the protein was seen in void fraction. **B.** MreB1^{E137A}-H₆ was purified similarly as MreB1-H₆. Almost 50% of the population eluted in void fraction and the other as monomeric fraction. **C.** ATP hydrolysis was compared between the wild type protein and the ATPase mutant. MreB1-H₆ was ATPase active whereas MreB1^{E137A}-H₆ did not show any activity.

However, the ATP hydrolysis activity was similar to that observed for the threonine mutants (Figure 5.7 C and D). This might indicate that the activity is specific to the proteins and does not result from the impurities co-purifying with MreB1.

5.1.3.6 Stability and folded state of MreB1-H₆

When thermostability of MreB1-H₆ was checked, the profile indicates that the protein might be misfolded (Figure 5.8). Surprisingly, the ATPase activity of MreB1-H₆ was similar to that observed for MreB1^{T323I}-H₆.

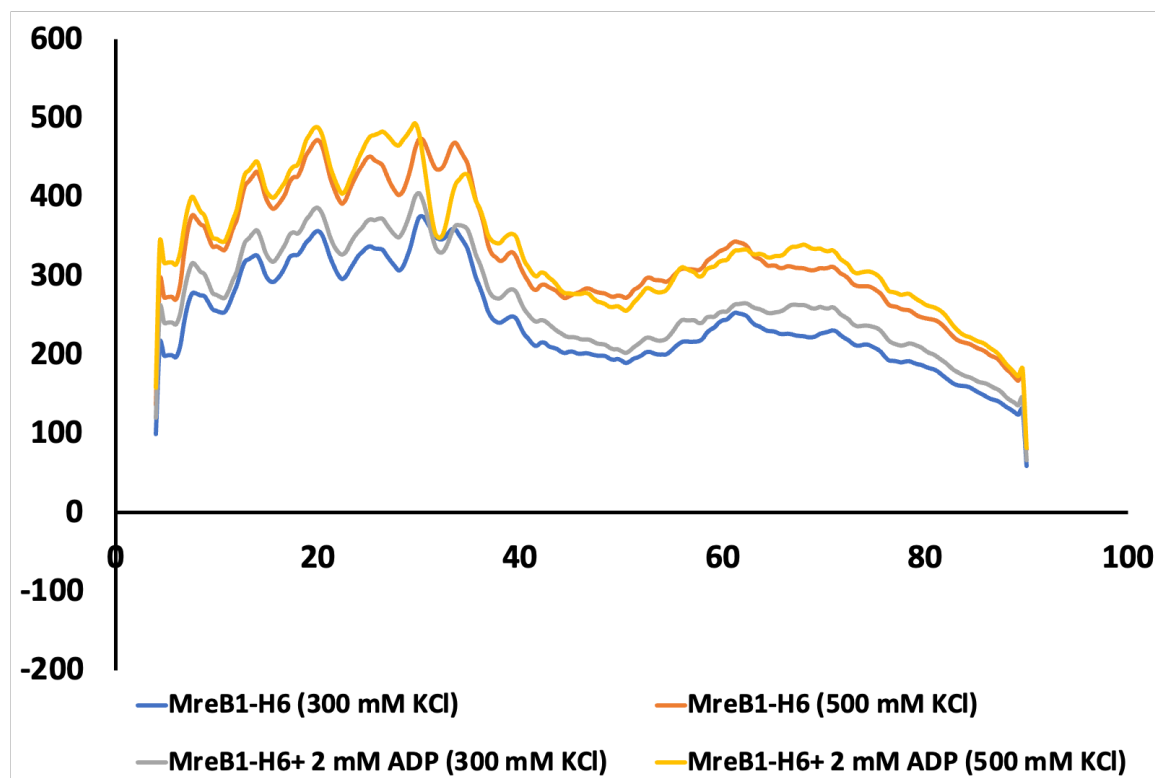


Figure 5.8. Thermal shift assay of MreB1-H₆. Salt concentrations of 300 mM and 500 mM KCl were tried to check stability of the protein. 2 mM ADP was added to check if there is any increase in the stability of the protein. The melting curve of MreB1-H₆ indicates that protein might be misfolded/unstable in the buffer conditions tried.

When the Threonine 323 mutation was reverted back to Isoleucine to get the wildtype gene, MreB1-H₆ precipitation decreased during purification steps. The ATP hydrolysis activity of MreB1-H₆ and MreB1^{E137A}-H₆ was similar with the corresponding threonine mutants. However, based on the thermal shift assay, MreB1-H₆ seems to be unstable and misfolded in the buffer conditions. As seen from the size exclusion profiles of MreB1-H₆, the protein eluted

in void fraction. This can be an indication of higher oligomeric structure or aggregated protein. There is a possibility that the purified protein might contain a portion of partially misfolded protein, hence giving high fluorescence in the thermal shift assay.

Purified proteins of all the constructs look highly impure. We suspect that during the thermal shift assay, Sypro Orange dye binds to other proteins as well and the fluorescence signal is an outcome of non-specific binding to impurities.

In order to get higher yield of the proteins, H₆-SUMO-MreB1 and H₆-SUMO-MreB1-Strep constructs were cloned and purification trials have been attempted for the same (<http://dr.iiserpune.ac.in:8080/xmlui/handle/123456789/7978>). Addition of SUMO tag increased the solubility of the protein. However, removal of the tag led to unstable protein. Further optimization in buffer conditions needs to be done. Cloning of polymerization mutants of MreB1 and further purification of the same will be beneficial to get a monomeric protein. Visualization of the purified proteins under transmission electron microscope will help in understanding the oligomeric state of each construct as well as the effect of different nucleotides on the polymerization of MreB1-H₆.

5.2 Purification optimization of Fibril

Heterologous expression and purification of Fibril and Fibril-EGFP in *E. coli* has been discussed in Chapter 3 (also in Harne and Gayathri, 2022). Overexpression of these constructs till now has resulted in Fibril bundles. In order to understand the filament assembly, well separated filaments were desired. Recent study has shown purification of Fibril from *S. eriocheiris* by using sucrose density gradient (Sasajima et al., 2022). Transmission electron microscopy images revealed single protofilaments using this strategy. Cryo-EM structure of Fibril has been obtained recently (Sasajima et al., 2023; <https://doi.org/10.1101/2023.06.28.546849>). We plan to obtain well separated protofilaments for interaction studies with MreB proteins. Attempts of visualization of MreB5 with Fibril were made. However, due to huge bundles of Fibril, identity of MreB5 filaments associated with them, if any, could not be established. In order to understand the filament assembly of Fibril and interacting MreBs, we used the purification strategy discussed in (Sasajima et al., 2022).

5.2.1 Purification optimization of Fibril helps in decreasing bundling of the filaments

Fibril purified by urografin density gradient gets separated into two distinct bands (Harne and Gayathri, 2022). As the lower band showed a higher amount of full-length protein, the upper

and lower bands were separated and only the lower band was subjected to sodium deoxycholate treatment. The insoluble protein when purified by a second round of urografin density gradient, protein was seen to elute at the same density as the lower band from the previous gradient. The purified protein shows a major band at ~ 59 kDa and degradation bands at ~ 37 kDa and ~ 25 kDa (Figure 5.9 A). This profile resembles the profile of purified Fibril discussed in (Harne and Gayathri, 2022).

To obtain single protofilaments, a sonication cycle of different time intervals ranging from 1 minute to 60 minutes were tried. Purified Fibril showed bundles even at 1 mg/mL. Huge overlapping bundles were seen post 1 minute of sonication (Figure 5.9 B). The bundles started separating post 4 minutes of sonication (Figure 5.9 C). Thinner bundles to separated protofilaments were observed beyond 4 minutes of sonication (Figure 5.9 D and E).

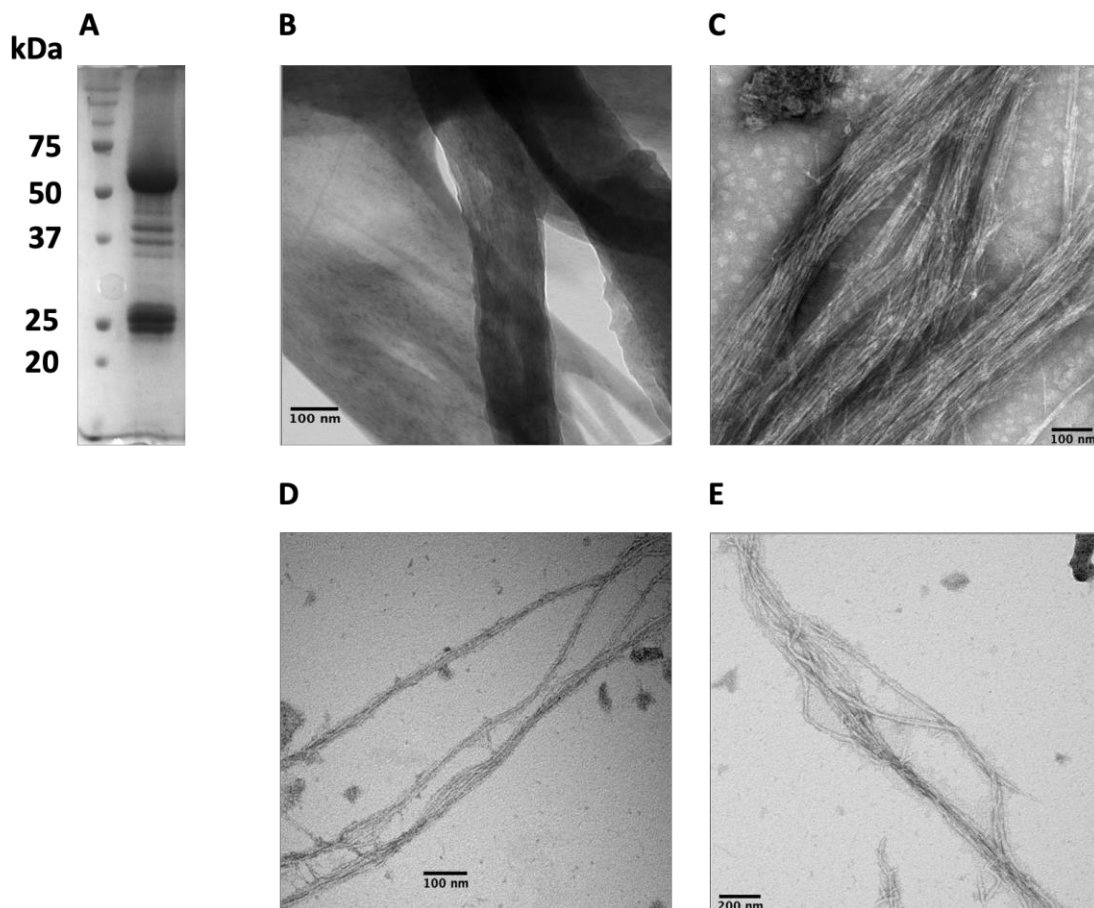


Figure 5.9. Transmission electron microscopy images show separation of Fibril filaments. A. Profile of Fibril wildtype purified by two rounds of urografin density gradient. Negatively stained images of Fibril filaments. The filaments were subjected to sonication of time intervals- 1 minute (B), 4 minutes (C), 20 minutes (D) and 30 minutes (E).

Treatment of the partially purified protein with sodium deoxycholate and preparation of samples for visualization under transmission electron microscope at lower concentrations have resulted in breaking and separating the bundles of Fibril.

In order to understand the filament assembly upon interaction of Fibril with MreB(s), the separated Fibril filaments will be used for the interaction and visualization of the same.

5.3. Future prospects

The six cytoskeletal proteins forming the ribbon lie close to each other and present just beneath the membrane (Kürner, 2005). It is likely that some of these proteins interact with each other in order to maintain the morphology of the cells. MreB5, one of the paralogs of *Spiroplasma citri*, has been shown to be essential for helicity and motility of the cells (Harne et al., 2020). However, the function of other MreB proteins and Fibril is not known. Out of the 6 filament types (Fibril and 5 MreBs), MreB5 and Fibril have been shown to interact (Harne et al., 2020). Co-sedimentation assays discussed in Chapter 4 reveal that Fibril and MreB5 interaction is nucleotide dependent. We wish to extend this study to check interaction of Fibril with other MreBs as well. In order to achieve this purification of MreB1 was tried. Further optimization is required to get pure and higher yield of the protein.

Visualization of the interacting proteins under transmission electron microscopy will help us in understanding filament assembly of the ribbon. For this purpose, separation of Fibril filaments has been tried by sonication of the purified protein. Sonication has helped us to achieve Fibril protein filaments that can be used for EM studies of Fibril interacting with MreBs.

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5.4 References

1. Ent, F. Van Den, Lo, J., 2006. Short note RF cloning : A restriction-free method for inserting target genes into plasmids 67, 67–74. <https://doi.org/10.1016/j.jbbm.2005.12.008>
2. Feng, J., Chen, Y., Pu, J., Yang, X., Zhang, C., Zhu, S., Zhao, Y., Yuan, Y., Yuan, H., Liao, F., 2011. An improved malachite green assay of phosphate: Mechanism and application. *Anal Biochem* 409, 144–149. <https://doi.org/10.1016/j.ab.2010.10.025>
3. Harne, S., Duret, S., Pande, V., Bapat, M., Béven, L., Gayathri, P., 2020. MreB5 Is a Determinant of Rod-to-Helical Transition in the Cell-Wall-less Bacterium *Spiroplasma*. *Current Biology* 30, 4753–4762.e7. <https://doi.org/10.1016/j.cub.2020.08.093>
4. Harne, S., Gayathri, P., 2022. Characterization of heterologously expressed Fibril, a shape and motility determining cytoskeletal protein of the helical bacterium *Spiroplasma*. *iScience* 25. <https://doi.org/10.1016/j.isci.2022.105055>
5. Huynh, K., Partch, C.L., 2015. Analysis of protein stability and ligand interactions by thermal shift assay. *Curr Protoc Protein Sci* 79, 28.9.1–28.9.14. <https://doi.org/10.1002/0471140864.ps2809s79>
6. Jones, L.J.F., Carballido-López, R., Errington, J., 2001. Control of Cell Shape in Bacteria. *Cell* 104, 913–922. [https://doi.org/10.1016/S0092-8674\(01\)00287-2](https://doi.org/10.1016/S0092-8674(01)00287-2)
7. Ku, C., Lo, W.S., Kuo, C.H., 2014. Molecular evolution of the actin-like MreB protein gene family in wall-less bacteria. *Biochem Biophys Res Commun* 446, 927–932. <https://doi.org/10.1016/j.bbrc.2014.03.039>
8. Kürner, J. et al, 2005. Cryo – Electron Tomography Reveals the Cytoskeletal Structure of *Spiroplasma melliferum* 436. <https://doi.org/10.1126/science.1104031>
9. Pande, V., Mitra, N., Bagde, S.R., Srinivasan, R., Gayathri, P., 2022. Filament organization of the bacterial actin MreB is dependent on the nucleotide state. *Journal of Cell Biology* 221. <https://doi.org/10.1083/jcb.202106092>
10. Sasajima, Y., Kato, T., Miyata, T., Kawamoto, A., Miyata, M., 2022. Isolation and structure of the fibril protein, a major component of the internal ribbon for *Spiroplasma* swimming Makoto Miyata Quick freeze replica electron microscopy, Electron tomography. 20. <https://doi.org/10.1101/2021.02.24.432793>
11. Sasajima, Y., Kato, T., Miyata, T., Kiyama, H., Kawamoto, A., Makino, F., Namba, K., Miyata, M., 2023. Cryo-electron microscopy of cytoskeletal “fibril” involved in *Spiroplasma* swimming. <https://doi.org/10.1101/2023.06.28.546849>

12. Soufo, D., Graumann, P.L., Chemie, F., 2003. Actin-like Proteins MreB and Mbl from *Bacillus subtilis* Are Required for Bipolar 13, 1916–1920.
<https://doi.org/10.1016/j.cub.2003.10.024>
13. van den Ent, F., Löwe, J., 2006. RF cloning: A restriction-free method for inserting target genes into plasmids. *J Biochem Biophys Methods* 67, 67–74.
<https://doi.org/10.1016/j.jbbm.2005.12.008>

Chapter 6 Conclusions and Future prospects

6.1 Conclusions

Cell wall synthesis during cell elongation and cell division has been widely studied in certain bacterial species like *E. coli* and *B. subtilis*. The cell wall synthesis machinery interacts with cytoskeletal proteins like MreB (for cell elongation) and FtsZ/FtsA (for cell division) for proper incorporation of cell wall precursors depending on the stage of the cell cycle (Kumar et al., 2022). Mutations or absence of these cell wall components interacting with MreB have shown to affect the cell morphology (Domínguez-Escobar et al., 2011; Garner et al., 2011). This indicates that a well-coordinated system between cell wall synthesis machinery and cytoskeletal protein is essential for maintenance of cell morphology. Most of the species in mollicutes possess pleomorphic cell shape. However, *Spiroplasma* shows a well-defined helical morphology. In spite of being a cell wall less bacteria, *Spiroplasma* shows the presence of five paralogs of MreB and a unique nucleotide independent polymerizing protein called Fibril (Ku et al., 2014; Kürner, 2005). Naturally occurring non-helical, non-motile strain of *Spiroplasma citri* shows absence of a 39 kDa protein (Townsend et al., 1977). This protein was identified as MreB5 (Harne et al., 2020). Recent studies have shown the significance of these cytoskeletal proteins in conferring helicity and motility in pleomorphic cells (Kiyama et al., 2022; Lartigue et al., 2022).

Biochemical and structural characterization of other paralogs of *Spiroplasma citri* will help us understand the filament dynamics and in checking the interaction between the cytoskeletal proteins.

Following section summarizes the results from each chapter.

6.1.1 Fibril does not show any enzymatic activity

The N-terminal half of Fibril shows ~24 % sequence similarity with MTAN (5'-methylthioadenosine nucleosidase). MreB possesses ~18 % sequence similarity with actin whereas FtsZ shows ~10% sequence similarity with tubulin. However, the bacterial homologs show high structural and functional similarity (Löwe and Amos, 1998; van den Ent et al., 2001). This inspired us to check the activity of Fibril and see if it resembles that of MTAN. Multiple sequence alignment of Fibril from all *Spiroplasma* species and plant and bacterial MTAN showed that the active residues of MTAN are not conserved in Fibril. Hydrolysis assay using SAH was performed to confirm this observation. No activity was seen for Fibril even at higher

concentration. This indicates that in spite of having sequence similarity with MTAN, Fibril has lost the enzymatic activity in the course of evolution.

6.1.2 Fibril does not hydrolyze ATP

Conformational changes in Fibril have been suspected to play an important role in change in the handedness of the cell (Cohen-krausz et al., 2011). To check the source of energy responsible for these conformational changes in the filament, ATP hydrolysis activity of Fibril was checked. No activity was seen for the protein. This suggests that change in Fibril filament might arise due to the energy transfer from interacting MreB proteins. MreB proteins are known to bind and hydrolyze ATP (Bork et al., 1992). Hence, the MreB paralogs and Fibril in the cytoskeletal ribbon show a well-coordinated arrangement which leads to helicity and motility of the cell.

6.1.3 Fibril-EGFP screen helped in the identification of probable interface residues

Fibril forms nucleotide independent filaments. Hence, there is no means of controlling its polymerization. In order to study the filament dynamics of Fibril and check interaction with other cytoskeletal components of the ribbon, we used an EGFP based screen to identify the polymerization interface. We aimed to obtain well folded fluorescently tagged filaments as well as non-polymerizing protein through this screen.

In vivo fluorescence and isolation of fluorescent protein from a heterologous expression system showed us variation of fluorescence signal for different Fibril-EGFP proteins. CD-spectroscopy and transmission electron microscopy showed that Fibril-EGFP proteins were well folded and formed filaments irrespective of the tag position and fluorescence intensities. However, we were able to identify residues which might be present at the interface of a protofilament or responsible for lateral interaction. Ideal positions for insertion of EGFP for further in vivo studies include the N-terminal end, and positions 27 and 304, which were among the positions of highest fluorescence intensities. Comparing our study with the recent Cryo-EM structure of Fibril (Sasajima et al., 2023) will help in the validation of our observations.

Fibril and MreB proteins form the cytoskeletal ribbon which lie underneath the cell membrane (Kürner, 2005). Interaction between the individual components will help understand the structure of ribbon in *Spiroplasma*. In this direction, Fibril and MreB5 have been shown to

interact in vitro (Harne et al., 2020). MreB5 being an ATPase, we checked the effect of different nucleotide bound states of MreB5 on the interaction with Fibril. Co-sedimentation assay of Fibril with MreB5 in different nucleotide states showed that the interaction is nucleotide dependent. Interestingly, our result was similar to the interaction pattern seen for MreB5-liposome interaction (Pande et al., 2022). This study was extended to check the interaction between ATPase mutants of MreB5 and Fibril. T161A mutant of MreB5 showed a high affinity to Fibril without addition of nucleotides, or with the addition of AMPPNP. Least interaction between the proteins was seen in the presence of ATP and ADP. In the case of the E134A mutant of MreB5, an overall increase in binding between the proteins was seen in all nucleotide conditions.

These results suggest that conformational changes in MreB5 filaments due to the nucleotide bound state makes it favorable/unfavorable for interacting with Fibril. The conformational states of binding with Fibril appear to be consistent with that of the binding states to liposomes as well. Hence, these might be the optimal condition at which MreB5 interacts with both liposomes and Fibril filaments. This observation might be of relevance to MreB5 function in vivo where it could co-exist with both Fibril and the membrane in the central cytoskeletal ribbon of *Spiroplasma*.

6.1.4 Optimization trials for purification of MreB1 and Fibril

With the purpose of biochemical characterization of different MreB proteins from *Spiroplasma citri* and characterizing filament-filament interactions among multiple MreBs and Fibril, I started with the purification of MreB1. Purification of MreB1 was tried using affinity-based columns. The purified protein was highly unstable and resulted in a very low yield. Several parameters like salt, pH and additives like ADP, DTT, glycerol, CHAPS were tried for stabilizing the protein. None of the factors helped in improving the yield of the protein. Further optimization for the purification of MreB1 is needed.

Heterologous expression and purification of Fibril has been tried using *E. coli* as the expression system (Harne and Gayathri, 2022). Visualization of the purified protein under TEM showed heavy bundles of Fibril. Purification optimization has been done by treating Fibril with sodium deoxycholate and purifying it with urografin density gradient. Lower concentrations of protein when subjected to sonication led to opening of the bundles and resulted in well separated filaments. We plan to use these filaments for checking interaction with MreB proteins from *S.*

citri. Visualization of these interacting partners under EM will shed light on the filament arrangement of the proteins while interacting.

6.2 Future Prospects

- i. Purification optimization and biochemical characterization of MreB proteins from *Spiroplasma citri*. Biochemical characterization includes ATP hydrolysis activity, membrane binding, polymerization kinetics etc. Crystal structure or Cryo-EM studies of the MreB filaments will help in understanding the structural properties of different paralogs of MreB.
- ii. Interaction between all the cytoskeletal proteins will help understand the structure and arrangement of the cytoskeletal ribbon. It will be interesting to understand the role of nucleotides in the interaction of these proteins.
- iii. Gene deletion studies of Fibril and MreBs from *Spiroplasma* will help in deciphering the role of each protein in the organism.

6.3 Open questions in the field

- i. Absence of functional MreB5 leads to loss of helicity and motility in *Spiroplasma citri* ASP1 (Harne et al., 2020). However, the cells do not completely lose morphology as seen in *Bacillus subtilis* (Jones et al., 2001) and maintain a rod shape. In absence of MreB5, do MreB1-4/Fibril have a role to play in maintaining cell morphology. Is it possible that some of the paralogs of MreB possess a role completely unrelated to cell shape maintenance as shown in some organisms (Gitai et al., 2005).
- ii. Most of the *Spiroplasma* species show the presence of 5-8 MreB paralogs (Joseph G. Tully, 1981; Williamson et al., 1997). Unlike the common characteristic of *Spiroplasma*s, few of the strains do not maintain a well-defined helical morphology. Despite having a high sequence similarity between the MreBs, the paralogs fail to complement the function of the other. Does the total protein content in the cell have an effect on the cell shape?
- iii. Fibril is a novel protein found only in *Spiroplasma* species. However, two of the strains of *Spiroplasma* have been reported to lack a functional *fibril* gene in their genome (Chang et al., 2014) whereas they show the presence of 5 MreB genes (Harne et al., 2020; Ku et al., 2014). These strains possess a helical morphology similar to other strains. This raises questions about the function of Fibril in these organisms.

6.4 References

1. Bork, P., Sander, C., Valencia, A., 1992. An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proceedings of the National Academy of Sciences* 89, 7290–7294. <https://doi.org/10.1073/pnas.89.16.7290>
2. Chang, T.-H., Lo, W.-S., Ku, C., Chen, L.-L., Kuo, C.-H., 2014. Molecular Evolution of the Substrate Utilization Strategies and Putative Virulence Factors in Mosquito-Associated *Spiroplasma* Species. *Genome Biol Evol* 6, 500–509. <https://doi.org/10.1093/gbe/evu033>
3. Cohen-krausz, S., Cabahug, P.C., Trachtenberg, S., 2011. The Monomeric , Tetrameric , and Fibrillar Organization of Fib : The Dynamic Building Block of the Bacterial Linear Motor of *Spiroplasma melliferum* BC3. *J Mol Biol* 410, 194–213. <https://doi.org/10.1016/j.jmb.2011.04.067>
4. Domínguez-Escobar, J., Chastanet, A., Crevenna, A.H., Fromion, V., Wedlich-Söldner, R., Carballido-López, R., 2011. Processive Movement of MreB-Associated Cell Wall Biosynthetic Complexes in Bacteria. *Science* (1979) 333, 225–228. <https://doi.org/10.1126/science.1203466>
5. Garner, E.C., Bernard, R., Wang, W., Zhuang, X., Rudner, D.Z., Mitchison, T., 2011. Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in *B. subtilis*. *Science* (1979) 333, 222–225. <https://doi.org/10.1126/science.1203285>
6. Gitai, Z., Dye, N.A., Reisenauer, A., Wachi, M., Shapiro, L., 2005. MreB Actin-Mediated Segregation of a Specific Region of a Bacterial Chromosome 120, 329–341. <https://doi.org/10.1016/j.cell.2005.01.007>
7. Harne, S., Duret, S., Pande, V., Bapat, M., Béven, L., Gayathri, P., 2020. MreB5 Is a Determinant of Rod-to-Helical Transition in the Cell-Wall-less Bacterium *Spiroplasma*. *Current Biology* 30, 4753–4762.e7. <https://doi.org/10.1016/j.cub.2020.08.093>
8. Harne, S., Gayathri, P., 2022. Characterization of heterologously expressed Fibril, a shape and motility determining cytoskeletal protein of the helical bacterium *Spiroplasma*. *iScience* 25. <https://doi.org/10.1016/j.isci.2022.105055>
9. Jones, L.J.F., Carballido-López, R., Errington, J., 2001. Control of Cell Shape in Bacteria. *Cell* 104, 913–922. [https://doi.org/10.1016/S0092-8674\(01\)00287-2](https://doi.org/10.1016/S0092-8674(01)00287-2)
10. Tully, J.G., Rose, D.L., Yunker, C.E., Cory, J., Whitcomb, R.F., Williamson, D.L., 1981. Helical Mycoplasmas (*Spiroplasmas*) from *Ixodes* Ticks. *Science* (1979) 212, 1043–1045.

<https://doi.org/10.1126/science.7233197>

11. Kiyama, H., Kakizawa, S., Sasajima, Y., Tahara, Y.O., Miyata, M., 2022. Reconstitution of a minimal motility system based on *Spiroplasma* swimming by two bacterial actins in a synthetic minimal bacterium. *Sci Adv* 8. <https://doi.org/10.1126/sciadv.abo7490>
12. Ku, C., Lo, W.S., Kuo, C.H., 2014. Molecular evolution of the actin-like MreB protein gene family in wall-less bacteria. *Biochem Biophys Res Commun* 446, 927–932. <https://doi.org/10.1016/j.bbrc.2014.03.039>
13. Kumar, S., Mollo, A., Kahne, D., Ruiz, N., 2022. The Bacterial Cell Wall: From LipidII Flipping to Polymerization. *Chem Rev* 122, 8884–8910. <https://doi.org/10.1021/acs.chemrev.1c00773>
14. Kürner, J. et al, 2005. Cryo – Electron Tomography Reveals the Cytoskeletal Structure of *Spiroplasma melliferum* 436. <https://doi.org/10.1126/science.1104031>
15. Lartigue, C., Lambert, B., Rideau, F., Dahan, Y., Decossas, M., Hillion, M., Douliez, J.P., Hardouin, J., Lambert, O., Blanchard, A., Béven, L., 2022. Cytoskeletal components can turn wall-less spherical bacteria into kinking helices. *Nat Commun* 13. <https://doi.org/10.1038/s41467-022-34478-0>
16. Löwe, J., Amos, L.A., 1998. Crystal structure of the bacterial cell-division protein FtsZ. *Nature* 391, 203–206. <https://doi.org/10.1038/34472>
17. Pande, V., Mitra, N., Bagde, S.R., Srinivasan, R., Gayathri, P., 2022. Filament organization of the bacterial actin MreB is dependent on the nucleotide state. *Journal of Cell Biology* 221. <https://doi.org/10.1083/jcb.202106092>
18. Sasajima, Y., Kato, T., Miyata, T., Kiyama, H., Kawamoto, A., Makino, F., Namba, K., Miyata, M., 2023. Cryo-electron microscopy of cytoskeletal “fibril” involved in *Spiroplasma* swimming. <https://doi.org/10.1101/2023.06.28.546849>
19. Townsend, R., Markham, P.G., PLASKITT, K.A., DANIELS, M.J., 1977. Isolation and Characterization of a Non-Helical Strain of *Spiroplasma citri*. *J Gen Microbiol* 100, 15–21. <https://doi.org/10.1099/00221287-100-1-15>
20. van den Ent, F., Amos, L.A., Löwe, J., 2001. Prokaryotic origin of the actin cytoskeleton. *Nature* 413, 39–44. <https://doi.org/10.1038/35092500>
21. Williamson, D.L., Adams, J.R., Whitcomb, R.F., Tully, J.G., Carle, P., Konai, M., Bove, J.M., Henegar, R.B., 1997. *Spiroplasma platyhelix* sp. nov., a New Mollicute with Unusual Morphology and Genome Size from the Dragonfly *Pachydiplax longipennis*. *Int J Syst Bacteriol* 47, 763–766. <https://doi.org/10.1099/00207713-47-3-763>