

**Investigating the mechanism of action of cell division proteins
FtsZ and FtsA of cell wall-less bacteria**

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

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द्वारा / By
सौम्यजित् दत्त / Soumyajit Dutta

पंजीकरण सं. / Registration No.:
२०१९३६२५ / 20193625

शोध प्रबंध पर्यवेक्षक / Thesis Supervisor:
डॉ. गायत्री पनांगट / Dr. Gayathri Pananghat



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CERTIFICATE

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Dr. Gayathri Pananghat

Date:25/03/2025

Declaration by Student

Name of Student: Soumyajit Dutta

Reg. No.: 20193625

Thesis Supervisor(s): Dr. Gayathri Pananghat

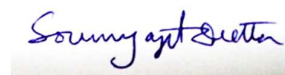
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Soumyajit Dutta

Date: 25.03.2025

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Abbreviations

% mol	Mole percent
μM	Micro Molar
μL	Micro Liter
Å	Angstrom
$^{\circ}\text{C}$	Degree celsius
cc	Apparent critical concentration
CCTP	Conserved C-terminal peptide
CTAH	C-terminal amphipathic Helix
CRAC	Cholesterol recognition amino acid consensus
CTD	C-terminal domain
CV	Column volume
Cryo EM	Cryogenic electron microscopy
CL	1,3-bis[1,2-dioleoyl-sn-glycero-3-phospho]- glycerol
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPG	1,2-dioleoyl-snglycero-3-phospho-rac-1-glycerol
Ec	<i>Escherichia coli</i> (<i>E. coli</i>)
EcFtsZ	FtsZ from <i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
GDP	Guanosine diphosphate
GMPPNP	Guanosine 5'-[(β , γ)-imido] triphosphate
GMPCPP	Guanosine-5'-[(α , β)-methyleno] triphosphate
GTP	Guanosine triphosphate
GTP γ S	Guanosine 5'-O-[gamma-thio] triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KCl	Potassium chloride
kDa	Kilo Dalton
k_{obs}	Apparent specific activity
KpFtsZ	FtsZ from <i>Klebsiella pneumoniae</i>
LB	Luria Bertani
mL	Milli Liter

mM	Milli Molar
MgCl ₂	Magnesium chloride
nN	Nano Newton
NTD	N-terminal domain
nm	Nano Meter
NaCl	Sodium chloride
OD ₆₀₀	Optical density at 600 nanometer
pN	Pico Newton
RMSD	Root mean square deviation
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
Sm	<i>Spiroplasma melliferum</i> (<i>S. melliferum</i>)
SmFtsA	FtsA from <i>Spiroplasma melliferum</i>
SmFtsZ	FtsZ from <i>Spiroplasma melliferum</i>
SM	Sphingomyelin
TIRF	Total Internal Reflection Fluorescence
TEM	Transmission electron microscopy
Tris	Tris(hydroxymethyl)aminomethane

Abstract

FtsZ and FtsA are the two primary components of the Z-ring involved in bacterial cell division. Despite extensive research, their roles in driving cell constriction are not well defined. In this context, FtsZ and FtsA from mollicutes or the cell wall-less bacteria can serve as a model system to investigate the role of Z-ring in the division process in the absence of a cell wall synthesis machinery.

In this work, we have employed an *in vitro* approach to study FtsZ and FtsA proteins from *Mycoplasma* and *Spiroplasma*, the two most studied genera under mollicutes. In *Mycoplasma*, we discovered a phylogenetically separate FtsZ group containing putative membrane-binding amphipathic helix at the N-terminal or C-terminal extensions. We showed the proposed C-terminal amphipathic helix (CTAH) in *Mycoplasma genitalium* FtsZ can bind membrane *in vitro*. Furthermore, a cholesterol recognition motif was identified within the CTAH region of *M. genitalium* FtsZ. Bacterial FtsZs are not so far known to bind membrane or cholesterol, which makes these observations quite interesting and novel as well.

We also characterized FtsA from *Spiroplasma melliferum* (SmFtsA) and its interaction with FtsZ. Here, we found SmFtsA to be inactive for ATP and GTP hydrolysis unlike many other reported homologs. We observed stimulation in GTPase activity of SmFtsZ along with higher order lateral assembly of SmFtsZ protofilaments in the presence of FtsA (a property generally shown by FtsZ stabilizers in cell walled bacteria). Based on the observations, we proposed a plausible mechanism for GTPase stimulation and higher order assembly which will be validated further in future studies.

Finally, we determined the structure of SmFtsZ filament in a GTP bound state using cryo-EM at 3.5 Å resolution. Our preliminary analysis suggests that the filament is composed of T state monomers with an inter-subunit distance of 45 Å. We observed an intrinsic curvature of the filament with the N-terminal domain facing the concave side. Additionally, the ordered T3 loop indicates a possible correlation with the presence of gamma phosphate in the nucleotide pocket and interface stabilization. Currently, more structural analyses are underway to gain structural insights into the native state conformational changes of the two domains that drive polymerization dynamics. Altogether, our results give an overview of the cell division genes in mollicutes and a new perspective of the diverse functions of FtsZ and FtsA in *Mycoplasma* and *Spiroplasma*.

Synopsis

Investigating the mechanism of action of cell division proteins FtsZ and FtsA of cell wall-less bacteria

Name: Soumyajit Dutta

Registration number: 20193625

Supervisor: Dr. Gayathri Pananghat

Department: Biology, IISER Pune

Date of Registration: 1 January 2019

Cell division in bacteria is initiated by constriction of the Z-ring comprising two essential proteins FtsZ and FtsA. Despite our knowledge about the crucial function of the Z-ring in bacterial division, the precise roles and mechanism of how FtsZ and FtsA drive cell constriction remain elusive. FtsZ/FtsA in wall-less bacteria like mycoplasmas is an ideal model system for obtaining mechanistic insights into Z-ring constriction in the absence of cell wall machinery. FtsZ and FtsA are the two major proteins involved in bacterial cell division (Margolin, 2005; Adams and Errington, 2009). The tubulin-like protein FtsZ forms short patches of overlapping filaments called the Z-ring which treadmills and gradually leads to constriction of the cell (Bisson-Filho et al., 2017; Yang et al., 2017). The presence of FtsZ is essential to initiate constriction but seems to be dispensable at the later stages of bacterial cell division (Whitley et al., 2021; Squyres et al., 2021). Treadmilling FtsZ filaments act as a guide for incorporating peptidoglycan strands in the newly forming septum during constriction (Xiao and Goley, 2016; Bisson-Filho et al., 2017; Coltharp and Xiao, 2017; Yang et al., 2017). In most bacteria, the actin-homolog FtsA, with the help of its C-terminal amphipathic helix, functions as the primary membrane anchor for FtsZ (Pichoff and Lutkenhaus, 2005; Juarez and Margolin, 2012). FtsZ and FtsA interact with other cell division-related proteins to further regulate the downstream events in the constriction process (Corbin et al., 2004; Busiek et al., 2012; Du et al., 2016; Vega and Margolin, 2019; Park et al., 2020). Other than FtsA, there are proteins such as ZipA (in Gram-negative gamma proteobacteria) and SepF (in Gram-positive bacteria and cyanobacteria)

that act as membrane anchors for FtsZ (Hale and de Boer, 1997; Hamoen et al., 2006, Duman et al. 2013).

Cell division is a complex process that relies on coordinated and collective action of a number of proteins involved in Z-ring assembly, cell wall synthesis and their regulations (McQuillen and Xiao, 2020). Overall, in bacteria, there are at least twenty different proteins that play an important role in the formation of the divisome, a multiprotein complex that coordinates different steps of the division in a highly regulated and organized manner (den Blaauwen et al., 2017). Notably, very often in bacteria, many of the division-related proteins are transcribed from a single operon called division and cell wall (*dcw*) cluster (Margolin, 2000). The *dcw* operon in cell walled model organisms *Escherichia coli* and *Bacillus subtilis* comprises sixteen and seventeen genes, respectively, which include FtsZ, FtsA and their downstream interactor proteins involved in cell wall synthesis. While much of the information regarding the function of cell division proteins has been deciphered based on studies carried out in walled bacterial species, our understanding of cell wall-less bacteria is limited. In cell wall-less bacteria, genes related to peptidoglycan synthesis are lost and a concomitant shorter *dcw* operon is the result of an extensive genome reduction (Bové, 1993). Mycoplasmas, for example, only five genes namely *mraZ*, *mraW* (*rsmH*), *ftsA*, *ftsZ* and *sepF* can be found in the *dcw* operon (Alarcón et al., 2007; Pelletier et al., 2021). The highly conserved nature of these genes across the bacterial kingdom implies their important function in cell division in a cell wall less milieu. In cell-walled bacteria, cell wall synthesis by the peptidoglycan synthesis machinery is supposedly the major player for constriction force generation as the inward force generated by the Z-ring is not sufficient to overcome the turgor pressure (Xiao and Goley, 2016). However, how constriction occurs in cell wall-less bacteria where FtsZ, FtsA and SepF possibly are the only structural components of a ‘minimal’ divisome, is an enigma. Most of the work so far in the field has been focused on cell walled model organisms where ring constriction and cell wall synthesis processes are coupled for dividing the cell. In this context, studying FtsZ/A in a wall-less system can provide valuable insights to understand the precise role of these proteins in cell constriction in the absence of cell wall machinery. Despite its significant potential, proteins involved in cell division in wall-less bacteria have largely been overlooked until now. My Ph.D. thesis work primarily focuses on this research gap related to this ‘minimal’ division machinery. This thesis work is divided in to seven chapters with specific aims and objectives as discussed below:

Chapter wise summary:

Chapter 1: Introduction

This chapter gives an overview of the cell division in bacteria and briefly summarizes the existing literature on cell division proteins. We have primarily focused on current understanding and consensus about the roles of the two major proteins FtsZ and FtsA in bacterial division. Investigating cell division proteins from wall-less bacteria can shed light on the precise functions of these proteins in cell division in the absence of peptidoglycan synthesis machinery. Overall, this section will enable the readers to know the current concepts and understanding of cell division principles in a concise manner and help them to build the fundamentals for understanding the subsequent chapters.

Chapter 2: Bioinformatics analysis of cell division proteins in wall-less bacteria

Division and cell wall (*dcw*) cluster comprises about fifteen genes including FtsZ, FtsA and peptidoglycan synthesis related genes. However, in mollicutes, the cell wall-less bacterial class, peptidoglycan genes are lost resulting in a short *dcw* cluster comprising only five genes namely *mraZ*, *mraW*, *ftsA*, *ftsZ* and *sepF*. Currently, limited data is available in the literature regarding cell division in wall-less bacteria. Here, we aimed to analyse the presence of different genes in the *dcw* cluster across different genera from genome data deposited in the NCBI database. Interestingly, we found that not all five division related genes are always present across genera. In Entomoplasma and Mesoplasma, all five genes are found to be present. In Spiroplasma, *mraZ*, *mraW*, *ftsA*, *ftsZ* are always present while *sepF* is absent in some species of the apis group. In Mycoplasma, *mraZ* and *mraW* are present but *ftsZ*, *ftsA* and *sepF* are not always present. Interestingly, Phytoplasma has only *mraW* whereas Ureaplasma has only *mraZ* and *mraW*. Acholeplasmas have *mraW*, *ftsZ* and *sepF* while *mraZ* and *ftsA* are absent. Thus, it is possible that the cell division operon genes are lost during evolution depending on its essentially in a particular organism.

Chapter 3: Characteristic features of cell division proteins FtsZ and FtsA in Mycoplasma

In this chapter, sequence features of cell division proteins FtsZ, FtsA and SepF have been explored through bioinformatics approaches and various modes of membrane attachment of

the Z-ring have been proposed in different Mycoplasma groups. We have analysed FtsZ, FtsA and SepF sequences in 113 Mycoplasma species and compared the conserved features with cell-walled model bacteria. We report a phylogenetically distinct group of 12 species that possess FtsZs without the canonical FtsA interacting conserved C-terminal peptide (CCTP) motif. Interestingly, these FtsZs contain putative membrane-binding amphipathic helix as an N-terminal or C-terminal extension to the globular FtsZ domain. As a proof-of-concept, we experimentally show that the proposed C-terminal amphipathic helix of *M. genitalium* FtsZ binds liposomes. Additionally, we identify a putative cholesterol recognition motif within the C-terminal amphipathic helix region of *M. genitalium* FtsZ. This study catalogues the variability in the modes of membrane attachment by the FtsZ and FtsA system in cell wall-less mycoplasmas and provides a new perspective to study novel functions of the FtsZ/A system in cell division.

Chapter 4: Characterization of FtsA from *Spiroplasma melliferum*

FtsA is the primary membrane adapter for FtsZ in bacteria. FtsZ makes short protofilaments which attach to the membrane through an amphipathic helix present at the C-terminal end of FtsA. Recent studies on cell-walled bacteria indicate that FtsA plays a significant role not only in anchoring FtsZ but also in regulating the dynamics of the Z-ring. In this scenario, we aimed to characterize FtsA protein *in vitro* from *Spiroplasma melliferum* (Sm) and explore its role and effect on FtsZ in cell wall-less bacteria. We cloned the *ftsA* gene from Sm genome, expressed heterologously in *E. coli* and purified the protein for *in vitro* characterization. We checked for GTP and ATP hydrolysis activity of SmFtsA and found it to be inactive. The protein also did not show polymerization in the pelleting assay as well as negative staining transmission electron microscopy in the conditions tested. Membrane binding was observed and possibly the entire C-terminal region rather than a conserved C-terminal amphipathic helix is responsible for membrane binding in SmFtsA. Interestingly, we observed in our sequence analysis that the ATP binding residues are not conserved indicating a possibility that this protein may not bind and hydrolyse nucleotide. We are currently standardizing purification of various mutants to check the nucleotide binding effect and explore more about the novel nucleotide binding phenomenon of SmFtsA.

Chapter 5: Interaction studies of FtsZ and FtsA from *Spiroplasma melliferum*

We have investigated the interaction between FtsZ and FtsA from *S. melliferum* using in vitro biochemical approaches to understand the effect of FtsA on the function of FtsZ. The GTPase activity of SmFtsZ was found to be stimulated by FtsA with lowering of the apparent critical concentration for polymerization and hydrolysis activity. Additionally, we observed that FtsA induces higher order assembly formation (i.e. straight bundled structure) of FtsZ protofilaments possibly by promoting lateral inter-protofilament interaction. Interestingly, the bundled assembly was only observed at a sub-stoichiometric to equimolar concentration range of FtsA to FtsZ, while excess amount of FtsA prevents higher order bundle formation. However, stimulation of GTPase activity was observed above a critical concentration of FtsA, particularly when FtsA concentration becomes excess (i.e. approximately 1.5 times higher than FtsZ). These results indicate a possible correlation among FtsZ/FtsA concentration, GTPase activity and bundle formation. Based on our observations we propose a model of the molecular mechanism of GTPase stimulation and bundle formation which will be investigated further in future experiments.

Chapter 6: Filament structure of *Spiroplasma melliferum* FtsZ: a cryo-EM study

In this chapter, I report the filament structure of SmFtsZ obtained by cryo-EM. FtsZ filaments are flexible, unstable, and furthermore, and its non-helical configuration poses challenges for getting high resolution cryo-EM structure. Here, we have captured SmFtsZ in a GTP bound T state conformation within filament in contrast to the earlier cryo-EM structure of FtsZ filament (3 Å resolution) from *Klebsiella pneumoniae* (KpFtsZ) which was in GMPCPP bound state. We could achieve an overall 3.5 Å resolution with higher local resolution in regions of the C-terminal domain and GTP pocket, reaching ~3 Å. We also observed an intrinsic curvature of the SmFtsZ filament with N-terminal domain facing the concave side. We performed a comparative structural analysis of SmFtsZ and KpFtsZ focusing on the conformational changes in and around the nucleotide pocket. The GTP orientation in SmFtsZ matches more with the GMPCPP conformation (i.e. gamma phosphate is near to the T3 loop) in KpFtsZ-ZapA complex rather than GMPCPP of KpFtsZ (gamma phosphate is away from the T3 loop). We also confirm the T3 loop in SmFtsZ structure to acquire ordered conformation in the presence of gamma phosphate which is otherwise sometimes found to be disordered/unstructured in GDP or apo state crystal structures. We believe our current effort to obtain filament structure

of SmFtsZ has provided a good starting ground with standardization of sample preparation and data collection parameters. Our next objective is to collect additional datasets for both wild-type and various mutant constructs to understand GTP hydrolysis and polymerization of FtsZ in greater detail.

Chapter 7: Conclusions and future prospects

This chapter summarizes major findings and conclusions from my PhD work. Future experimental plans for each project have also been mentioned here.

Here is a list of publications from the projects I worked on during my PhD tenure:

1. Chakraborty, J., Poddar, S., **Dutta, S.**, Bahulekar, V., Harne, S., Srinivasan, R., & Gayathri, P. (2024). Dynamics of interdomain rotation facilitates FtsZ filament assembly. *Journal of Biological Chemistry*, 300(6). doi: <https://doi.org/10.1016/j.jbc.2024.107336>.
2. **Dutta, S.**, Poddar, S., Chakraborty, J., Srinivasan, R., & Gayathri, P. (2023). An amphipathic helix facilitates direct membrane binding of Mycoplasma FtsZ. *bioRxiv*, 2023-08. doi: <https://doi.org/10.1101/2023.08.29.555414>. (Accepted for publication in *Biochemistry*).
3. Kumari, J., Uthaman, A., Kundu, A., Dhar, A., Sharma, V., Bose, S., **Dutta, S.**, Roy, S., Srinivasan, R., Pande, S. and Kutti, V.R., Gayathri, P., & Palani, S. (2025). Distinct filament morphology and membrane tethering features of the dual FtsZs in *Odinarchaeota*. *bioRxiv*, 2025-02. doi: <https://doi.org/10.1101/2025.02.03.636245>.

Manuscript under preparation:

1. **Dutta, S.**, Chakraborty, J., & Gayathri, P. Characterization of cell division protein FtsA from *Spiroplasma melliferum* and its effect on FtsZ polymerization.
2. **Dutta, S.**, & Gayathri, P. Cryo-EM filament structure of GTP bound FtsZ from *Spiroplasma melliferum* at 3.5 Å resolution.

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Chapter 1

Introduction

1.1 Overview of cell division in bacteria

Bacteria divide by a process called binary fission, in which cells first grow in size and then split into equal halves, giving rise to two equal sized daughter cells. Division in bacteria follows a specific sequence of events, tightly regulated in spatial and temporal manner. While a cell grows in size, the nucleoid gets duplicated followed by its segregation. Once the nucleoid is segregated, division proteins start to accumulate at the mid-cell. One of the first proteins to localize at the mid-cell at the onset of division is FtsZ. FtsZ is a tubulin homolog which polymerizes in the presence of GTP and forms short overlapping filaments to form a ring-like structure (called the Z-ring) that treadmills along the inner cytoplasmic membrane. Z-ring anchors to the membrane with the help of membrane tethering proteins like FtsA or ZipA resulting in formation of a division initiation complex called proto-ring. The proto-ring gradually recruits other proteins to finally form the divisome, a multiprotein complex involved in coordinating cell division, cell wall synthesis, signal transduction and its regulation between proto-ring and cell wall synthesis machinery. The mature divisome then constricts the cytoplasmic membrane accompanied by new peptidoglycan synthesis at the septum to achieve cytokinesis, the separation of cytoplasm into two daughter cells (Figure 1.1) (Haeusser and Margolin 2016).

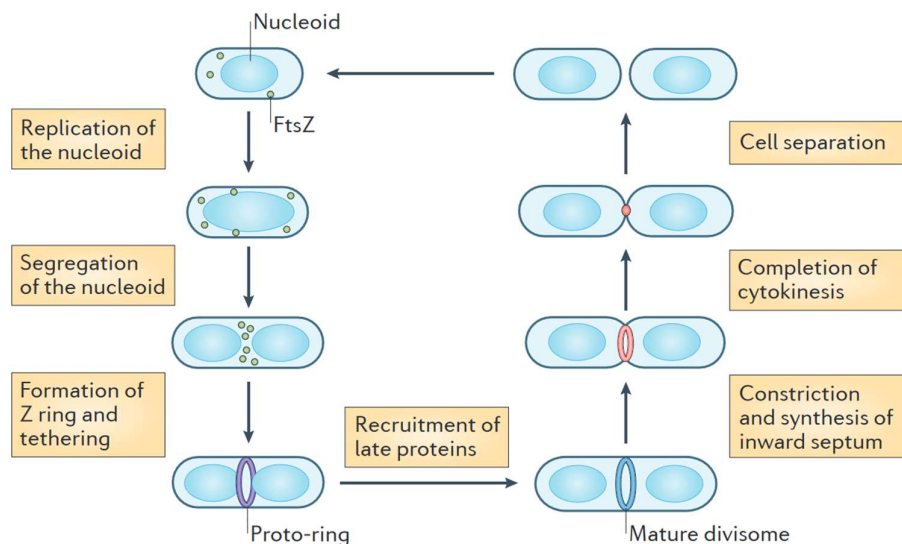


Figure 1.1 Schematic of steps involved in bacterial cytokinesis. Duplication and segregation of nucleoid and separation of cytoplasm are the two primary events of cell division. FtsZ acts as the master regulator to initiate the division process by forming a proto-ring complex which matures to form divisome. Divisome proteins coordinates the septum cell wall synthesis and ring constriction for a successful cytokinesis. Image adapted from Haeusser and Margolin 2016.

1.2 The divisome

Divisome is a multiprotein complex composed of more than thirty different proteins which assembles at the mid cell for dividing the cell (Radler and Loose 2024, Lutkenhaus et al. 2012 (Rowlett and Margolin, 2015, Levin and Janakiraman, 2021). Different components of the divisome complex in *Escherichia coli* and their organization inside the cell have been shown as a schematic diagram in Figure 1.2a. One of the first events for centring the divisome involves localizing the FtsZ filaments which occurs by MinCDE system and nucleoid occlusion mechanisms (de Boer 1989, Wu and Errington 2004). FtsZ then binds to membrane anchor proteins like FtsA or ZipA to attach to the membrane and form the proto-ring complex which is an initiation checkpoint for the assembly of downstream proteins (Haeusser and Margolin 2024). Peptidoglycan hydrolysis related proteins FtsE and FtsX and several other peptidoglycan remodelling proteins are recruited to the proto-ring in the following order: FtsK–(FtsQ–FtsL–FtsB)–FtsW–FtsI–FtsN (Attaibi and den Blaauwen 2022). Other major category of divisome proteins involves FtsZ associated proteins (Zaps) like ZapA, ZapB, ZapC, ZapD which control the FtsZ filament dynamics and stability (Hale and de Boer, 1997; Ma *et al.*, 1997; Durand-Heredia *et al.*, 2012; Buss *et al.*, 2017; Caldas *et al.*, 2019). Various categories of divisome proteins according to their functions are tabulated Figure 1.2b.

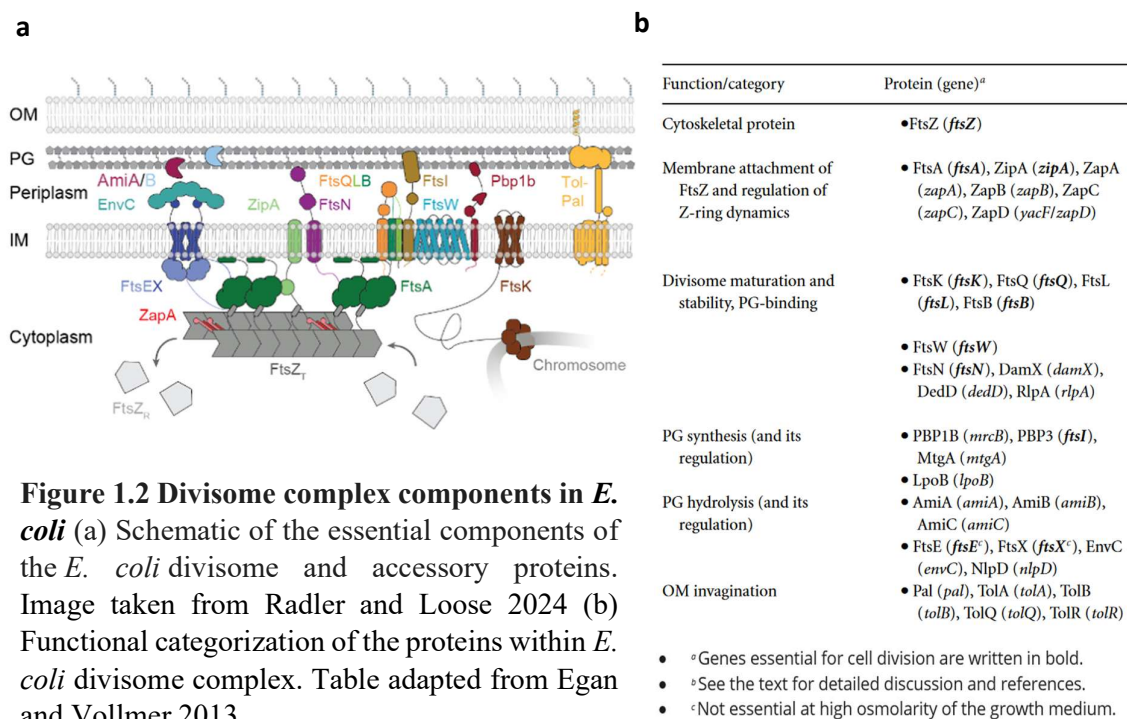


Figure 1.2 Divisome complex components in *E. coli* (a) Schematic of the essential components of the *E. coli* divisome and accessory proteins. Image taken from Radler and Loose 2024 (b) Functional categorization of the proteins within *E. coli* divisome complex. Table adapted from Egan and Vollmer 2013.

1.3 Division and cell wall (*dcw*) cluster in bacterial genome

Cell division genes including *ftsZ* and *ftsA* and many cell wall synthesis related genes are located together as a cluster in the genome calling it as *dcw* cluster and are often transcribed as a single operon. In *E. coli* and *Bacillus subtilis*, the *dcw* operon consist of sixteen and seventeen genes respectively (Dewar and Dorazi 2000) (Figure 1.3). As the *dcw* operon starts with *mraZ* gene, a transcriptional regulator for the entire operon, it is alternatively named as *mraZ* operon. *mraZ* gene is followed by *mraW* and other peptidoglycan synthesis related genes which ends with *ftsA* and *ftsZ*, the two major structural/cytoskeletal components of the divisome. This is called a cluster and not operon because in some species this group of genes, although located together, are transcribed into more than one transcripts (Francis et al. 2000, Real et al. 2006). It is interesting that the *dcw* cluster is highly conserved across bacteria in terms of gene content and the gene neighbourhood order in which they are present (Megrian et al. 2022). One striking pattern related to the position of *mraZ*, *mraW* is that they are always found next to each other; the same is true for *ftsA* and *ftsZ* as well. It is also interesting that in a few phyla the *dcw* genes are rearranged in the cluster which can be linked to the cell envelope specifications and not to the cell shape (Megrian et al. 2022).

Escherichia coli



Bacillus subtilis



Figure 1.3 Genomic locus of division and cell wall cluster (*dcw*) operon genes of *E. coli*, and *B. subtilis*. The arrowhead indicates gene orientation 5' → 3'. Cell wall synthesis related protein coding genes are colored in grey. The rest are structural components (*ftsA*, *ftsZ* and *sepF*) and transcriptional regulators (*mraZ* and *mraW*). *sepF* is shown as a break as it is not a part of the *dcw* cluster, instead it is transcribed from the next operon in the *B. subtilis* genome.

1.4 FtsZ and FtsA: the major players in bacterial division

fts genes were first discovered in 1960s by genetic screening of mutations in *E. coli* cells which grow normally in a permissive temperature of 30 °C but gives rise to filamentous cells when

growth temperature shifted to 42 °C. Since mutations in these genes are sensitive to higher temperature and resulted in filamentous phenotype, they were named as Filamentous Temperature Sensitive (fts) genes (van de Putte et al. 1964, Hirota et al. 1968, Allen et al. 1974). These mutations were traced within multiple genes which were named *fts-A, B, H, K, L, Q, W, Z* etc. Among these, the mutant formally called PAT84, was unable to constrict at the division site however could segregate their DNA normally. Later Lutkenhaus and colleagues mapped this gene to the filamentous temperature sensitive gene Z (*ftsZ*) (Lutkenhaus et al. 1980, Yi and Lutkenhaus 1985, Bi and Lutkenhaus 1990). *ftsZ* and *ftsA* were also identified as essential genes for division and conserved across bacterial species except some mollicutes, chlamydiae and planctomycetes which can divide without FtsZ (Bi and Lutkenhaus 1991, Miyagishima et al. 2014). Most other *fts* genes are part of the divisome and involved in the various steps of peptidoglycan synthesis and hydrolysis, and regulation during division.

1.5 Conserved features of FtsZ and FtsA

The domain organization of FtsZ protein, a tubulin homolog, contains a GTP binding globular core, an N-terminal extension, a C-terminal disordered linker, and a conserved C-terminal peptide (CCTP) (Löwe and Amos 1998) (Figure 1.4). The globular core domain contains two highly conserved tubulin signature motifs, (1) GTP binding motif- [S/A/G]-G-G-T-G-[S/A/T]-G, and (2) GTP hydrolysis motif- N-X-D-X-X-D, (where X can be any residue) (Erickson 1995, Nogales et al. 1998). The CCTP motif also shows a high degree of conservation across different bacterial species with a consensus sequence of D/E-I/V-P-X-F/Y-L-R/K, (where X can be any residue) (Ma and Margolin, 1999, Yan et al. 2000). The CCTP is required for interaction between FtsZ and many of its interacting membrane tethering proteins like FtsA, and the less conserved ZipA or SepF (Ma et al. 1997, Mosyak et al. 2000; Duman et al. 2013). Structurally, the globular core of FtsZ is formed by an N-terminal domain (NTD, blue colored) and C-terminal domain (CTD, green colored) separated by a long helical region (H7 helix, yellow colored) (Figure 1.4 b). The C terminal end of the H7 helix contains a loop region, called the T7 loop. The GTP binding pocket is in the NTD and the hydrolysis occurs once another monomer sits at the top providing the catalytic residues located at the T7 loop. Thus, GTP hydrolysis occurs when two monomers interact and stack on top of the other, forming an open-ended dimer. As per convention, the side on which nucleotide binds is

considered as the top surface and the opposite end where the T7 loop is located is the bottom surface of the FtsZ molecule.

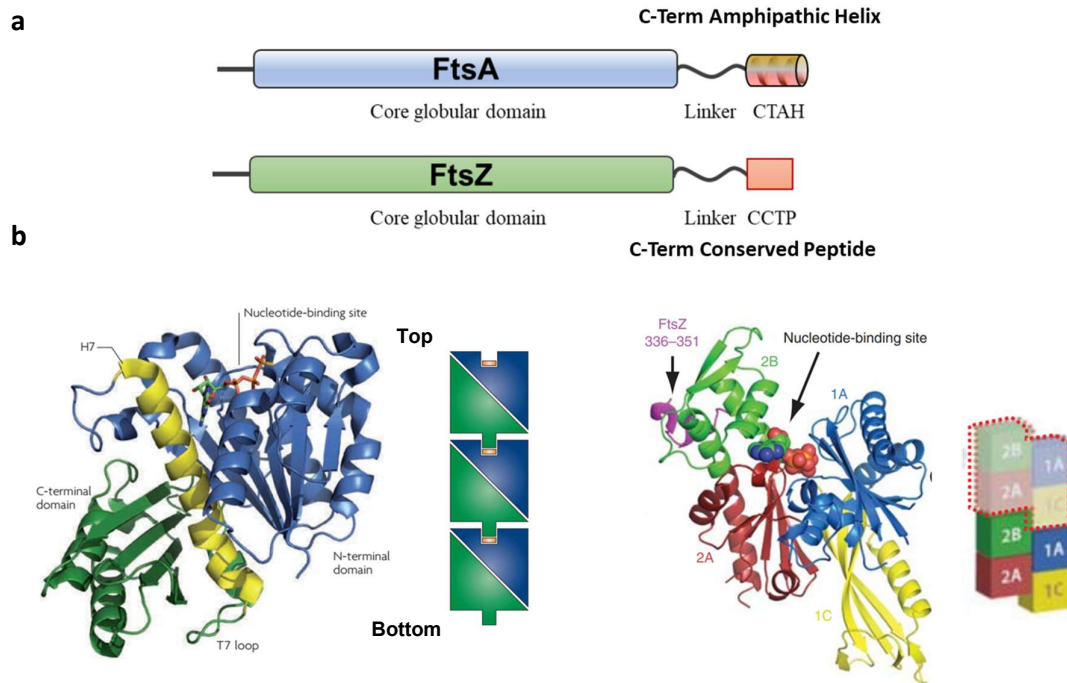


Figure 1.4 Domain architecture of FtsZ and FtsA. (a) Schematics of the conserved domain architecture of FtsA and FtsZ proteins. (b) Crystal structure of globular domain of FtsZ (left, Adams and Errington 2009) and FtsA bound to FtsZ CCTP (right, Szwedziak et al. 2012) with main subdomains highlighted. Polymerization interfaces are shown schematically by the side of the crystal structures of FtsZ and FtsA.

The second essential component of Z-ring is FtsA which is an Hsc70/sugar kinase/actin family protein. Structurally, FtsA contains a globular core domain followed by a disordered linker and C-terminal amphipathic helix (CTAH) (Pichoff and Lutkenhaus 2005). It acts as the primary membrane anchor for FtsZ and it is widely conserved in most bacterial species (Pichoff and Lutkenhaus 2005; Busiek and Margolin 2015). Unlike other actin homologs, FtsA has a unique domain architecture, with the absence of canonical 1B domain and presence of 1C domain on the opposite side of the 1A subdomain (Bork et al. 1992, Szwedziak et al. 2012) (Figure 1.4). The ATP binding pocket is formed by subdomains 1A, 2A, and 2B. Three conserved motifs that interact with ATP and possibly responsible for ATP hydrolysis are (a) Phosphate loop1 (D/E-X-G), (b) Phosphate loop 2 (D-X-G-X-G) and (c) Adenosine pocket

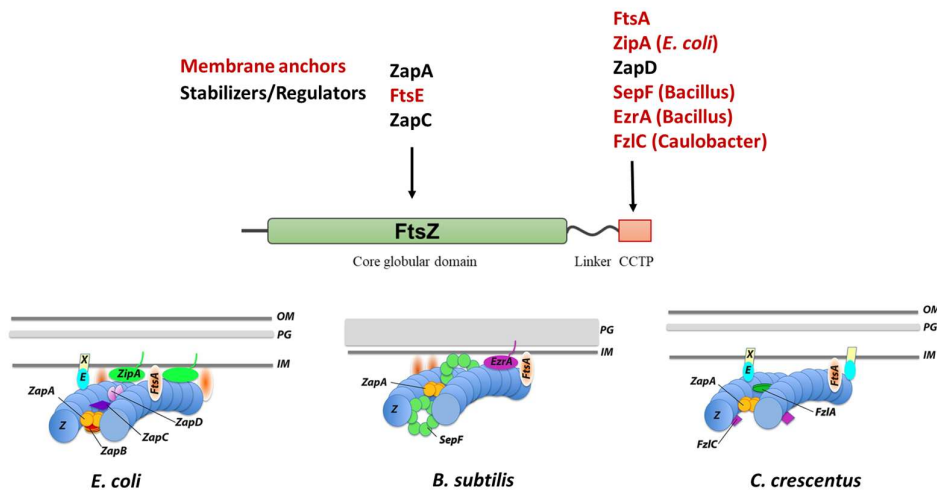
(G–G), where X represents any residue. Crystal structures of FtsA from various organisms suggests that although putative ATP binding pocket may be conserved, the activity may vary in *in vitro* condition (Morrison et al. 2023). ATPase activity of *Thermotoga maritima* or *Streptococcus pneumoniae* FtsAs have not been observed despite the presence of ATPase domain (Szwedziak et al. 2012, Fujita et al. 2014). FtsA from *E. coli* (Conti et al. 2018), *Bacillus subtilis* (Feucht et al. 2001) and *Pseudomonas aeruginosa* (Paradis-Bleau et al. 2007) also showed ATPase activity *in vitro*. Interestingly, *E. coli* FtsA's ATPase activity was found to be stimulated in the presence of phospholipids (Conti et al. 2018). One of the reasons many FtsAs were not found to be active could be because they require other accessory proteins to induce conformational changes in the structure to stimulate activity which can not be detected *in vitro* by checking activity of FtsA alone (Fujita et al. 2014). Moreover, the role of ATP hydrolysis for the functionality of FtsA is not clear yet as the Z-ring dynamics has been found unaffected *in vitro* by the ATPase activity of FtsA (Loose and Mitchison 2014). Instead, polymerization has been found to be a conserved property of FtsA across different species. FtsA has been shown to form protofilament structures *in vitro* by negative staining transmission electron microscopy from different organisms like *T. maritima* (Szwedziak et al. 2012), *S. pneumoniae* (Lara et al. 2005, Krupka et al. 2014), *V. cholerae* (Nag et al. 2020), and *E. coli* (Krupka et al. 2017, Morrison et al. 2022) as well as by *in vivo* electron tomographic study in *C. crescentus* and *E. coli* cells (Szwedziak et al. 2014).

1.6 Membrane anchors for the Z ring

FtsZ filaments require attachment to the cytoplasmic membrane for constriction. Since FtsZ does not have intrinsic membrane binding ability, it requires other membrane anchor proteins to serve the purpose (Naha et al. 2023). The main purpose of the membrane anchors is to tether the FtsZ ring to the inner cytoplasmic membrane thus helping to condense the FtsZ filaments and form the proto-ring complex to initiate divisome maturation. Though FtsA is mostly conserved, it is not the only membrane anchor found in bacteria. Membrane anchors show a wide diversity both within species and across species. Interestingly, although the anchor proteins are structurally diverse, they all have evolved to bind mostly to the constant CCTP motif (or in specific cases with the globular domain) of FtsZ protein (Figure 1.5). The non-essential nature of these accessory membrane anchors suggests their overlapping functions. Interestingly, *E. coli* possesses two essential membrane tethers of FtsZ, FtsA and ZipA.

However, the requirement of multiple anchors is not clearly understood. A hyperactive mutation R286W in FtsA (also called FtsA*) can bypass the requirement of ZipA. Thus, it is possible that two essential anchors have distinct roles. Firstly, ZipA facilitates the interaction between FtsN and FtsA (Weiss, 2015; Söderström et al., 2018; Pichoff et al., 2018). It can also help to stabilize FtsZ from Clp protease mediated degradation which is not achieved by FtsA or FtsA* alone (Pazos et al., 2013). ZipA potentially regulates Z-ring dynamics in a different manner where it organizes FtsZ filaments *in vitro* into static bundles instead of a rapidly reorganizing filament network as observed in presence of FtsA (Loose and Mitchison 2014). Other than FtsA, few additional membrane anchors present in some model organisms are SepF (Gram positive bacteria and some archaea e.g. *Haloferax volcanii*), EzrA (*B. subtilis*), and FzlC (*C. crescentus*) (Meier et al., 2016, Pichoff and Lutkenhaus, 2005, Singh et al., 2007). Importantly, in some organisms which lack FtsA (e.g. genus *Mycobacterium*), SepF acts as the primary essential membrane anchor for FtsZ (Gola et al. 2015).

a



b

Anchor	General Distribution	Essentiality (Typical)
FtsA	Gram-positive and Gram-negative bacteria	Essential
SepF	Gram-positive bacteria, Cyanobacteria, Euryarchaeota	Nonessential except in species lacking FtsA
ZipA	γ -Proteobacteria	Essential
EzrA	Bacillota (Firmicutes)	Nonessential
FzlC	α -Proteobacteria	Nonessential
FtsEX	Gram-negative and Gram-positive bacteria	Nonessential
GpsB (potential)	Bacillota (Firmicutes)	Nonessential
MapZ/LocZ	Streptococci, Lactococci, and Enterococci	Nonessential
SsgB	Streptomyces	Nonessential
CrgA (potential)	Actinomycetota	Nonessential

Figure 1.5 Membrane anchors for FtsZ (a) A few examples of membrane anchors with interaction site in FtsZ present in various species of bacteria. Some regulatory proteins involved in filament stability are also highlighted. Images adapted from Huang et al. 2013. (b) Distribution and diversity of FtsZ anchors and their essentiality. Table adapted from Naha et al. 2023

1.7 Structure of the Z-ring and its dynamics

One of the first observations of FtsZ localization at the mid-cell was from an immune-gold labelling electron microscopy experiment by Bi and Lutkenhaus 1991 of *E. coli* cells. Later, fluorescence microscopy study with fluorescently tagged FtsZ protein also confirmed the localization of FtsZ at the constricting septum (Erickson 2010). These early experiments proposed that the FtsZ possibly forms a ring-like structure (thus named as Z-ring) by forming a long protofilament that gradually constricts to divide the cell. However, with the advancement of high-resolution microscopy techniques, the Z-ring was found to be made up of short, discontinuous, overlapping, heterogeneously distributed clusters of FtsZ protofilaments (Fu et al. 2010) (figure 1.6a). *In vivo* cryo-electron tomographic studies in *Caulobacter* cells also provided evidence for the presence of individual arc-like filaments (approximately 40–160 nm long) of FtsZ are localized at the constriction site (Li et al. 2007). Recent *in vivo* TIRF imaging suggests FtsZ protein to form dynamic clusters of short dynamic filaments which treadmill along the inner surface of the cytoplasmic membrane (Niu and Yu 2008, Buss et al. 2013, Bisson-Filho et al. 2017, Yang et al. 2017) (Figure 1.6b). Current model of *in vivo* Z ring structure based on the existing high-resolution data suggests a non-continuous heterogenous arrangement of single layered FtsZ filaments mostly localized in a confined zone approximately 13–16 nm beneath the inner cytoplasmic membrane (Figure 1.6c). These filaments are dynamic and can be loosely interacting with each other through filament stabilizing proteins and membrane anchors (McQuillen and Xiao 2020).

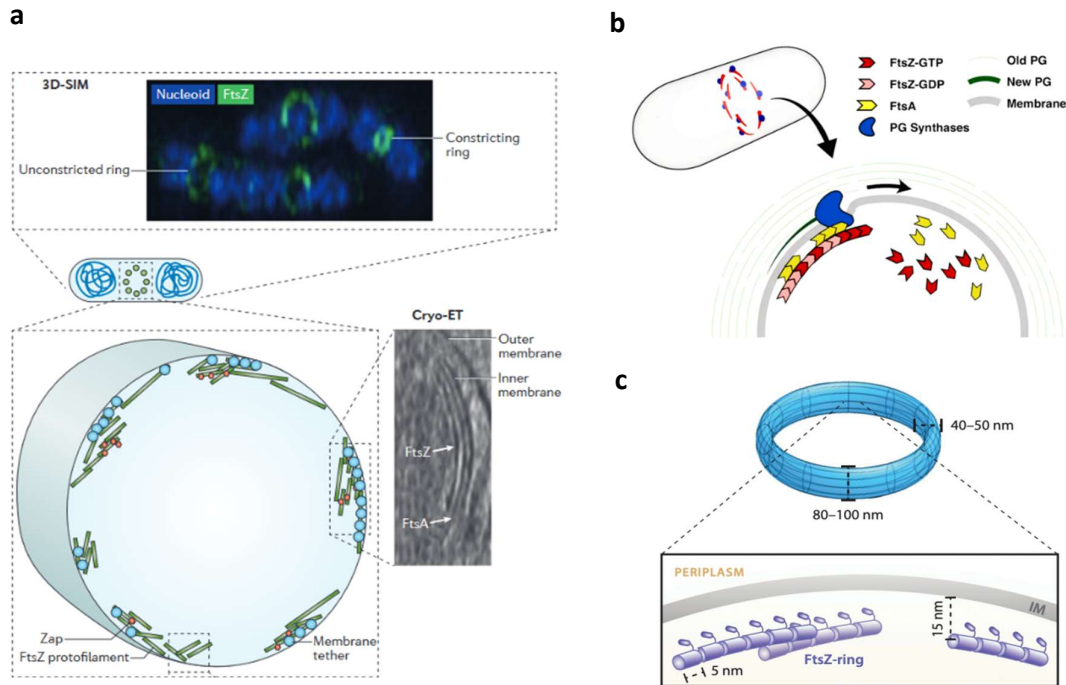


Figure 1.6 Structure of the Z-ring (a) Localization of FtsZ proteins and its patchy distribution observed through 3D-structured illumination microscopy (3D-SIM) of dividing *E. coli* cells. Blue and green represents DAPI stained nucleoid and anti-FtsZ antibody respectively. Model for the localization of membrane attached short discontinuous FtsZ filaments laterally interacting by Zap protein. Inset shows electron tomography image of FtsZ and FtsA filament structure in *E. coli* cell. Image adapted from Haeusser and Margolin 2016. (b) Treadmilling model of FtsZ filaments that guides PG synthesis along the inner membrane of the division septum. Image adapted from Bisson-Filho et al. 2017. (c) Current model of the Z-ring ultrastructure which is located in a confined toroidal zone beneath the membrane. Image adapted from McQuillen and Xiao 2020.

1.8 Role of Z-ring in bacterial cell division

Since the discovery of FtsZ protein, its role in cell division remains one of the major questions in the field. Till date, the exact role of the Z-ring in the constriction process remains unclear. There are recent theories proposed that the inward force generated by the Z-ring is not enough to overcome the turgor pressure to deform the thick peptidoglycan layer of a cell walled bacteria (Whatmore and Reed 1990, Lan et al. 2009, Deng et al. 2011, Erickson et al. 2010, Hsin et al. 2012, Xiao and Goley 2016). However, recent models support the idea that Z-ring

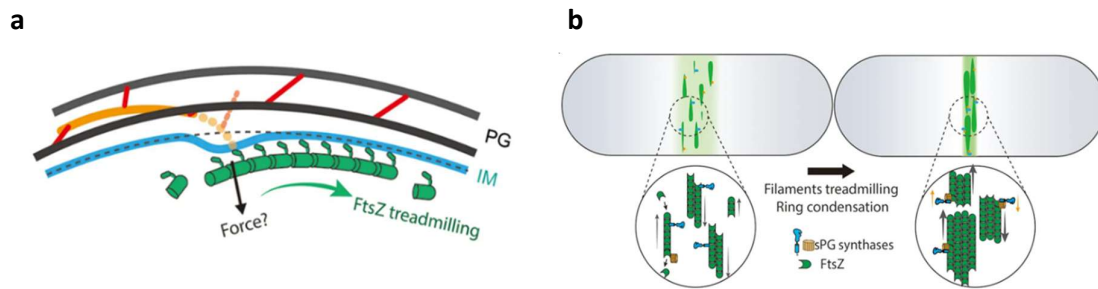


Figure 1.7 Models of potential role of Z-ring treadmilling in cell constriction. (a) Contractile force generated by FtsZ filament can deform the inner membrane towards the center. This pulls the cell wall synthesis complex inside the older one for building new septum. Images adapted from Yang and Liu 2022. (b) Treadmilling helps in condensation of the Z-ring that can concentrate and activate peptidoglycan synthases in the septum.

generated inward force plays an essential role for correct positioning of the peptidoglycan synthesis enzymes at the septum. The idea of a mechanical force generated by FtsZ filament came from *in vitro* reconstitution experiments where liposomes were found to be constricted by *E. coli* FtsZ protein (modified with a membrane binding motif) from inside in the presence of GTP (Osawa et al. 2008). Importantly, liposome membrane deformation was dependent on the position of the membrane binding surface of the FtsZ filament suggesting that the filament has an inherent curvature upon bending (Osawa et al. 2009). These observations were additionally supported by negative staining transmission electron microscopy data of FtsZ filament which showed curved GDP bound filament compared to a straighter GTP bound filament (Erickson 2009). Though the force generation model from bending of FtsZ filament upon GTP hydrolysis was intriguing, questions remain whether the force is sufficient to deform both inner membrane and peptidoglycan layer against turgor. Later, Coltharp et al. 2016 questioned this idea by showing that the constriction rate of a dividing *E. coli* cell depends on cell wall synthesis activity rather than the GTPase activity of FtsZ. Additionally, the maximum force generated by the Z-ring was estimated to be 8-80 pN range which is not enough to overcome turgor pressure of 50 -300 nN (Whatmore and Reed 1990, Lan et al. 2009, Deng et al. 2011, Erickson et al. 2010, Hsin et al. 2012, Xiao and Goley 2016). Thus, the exact role of the Z-ring in force generation and cell constriction remained elusive.

Recent studies have found that treadmilling of FtsZ filament can generate torsional stress upon GTP hydrolysis which can deform membrane and this is essential for cell constriction (Ramirez-Diaz et al. 2021, Whitley et al. 2021). It is possible that an inward force generated by the treadmilling FtsZ is necessary to pull the PG synthases inward along with the inner membrane. This inward membrane deformation is possibly essential to change the direction of the new PG synthases inside the older one as proposed from simulation studies by Nguyen et al. 2019 (Figure 1.7a). According to the current understanding, treadmilling of FtsZ filaments helps to encounter each other in a dynamic pattern which laterally interact giving rise to a condensed narrow ring. Then the condensed Z-ring acts as a scaffold to recruit PG synthesis enzymes and guide them to evenly construct the new septum (Walker et. al. 2020, Squyres et al. 2021, Whitley et al. 2021) (Figure 1.7b). Though recent advancement of high-resolution microscopy experiments and simulation studies have greatly improved our understanding of the Z-ring dynamics *in vivo*, we still do not have a consensus model of force generation mechanism by FtsZ filament and more importantly how it is important for cell constriction. More experimental evidence particularly from a diverse class of bacteria will be needed in future to get a clearer picture of the constriction mechanism.

1.9 Structural basis of treadmilling

Treadmilling is an apparent movement of the filament during polymerization from one end and depolymerization from the other end while each monomer remains static. As discussed in the previous section, FtsZ filament has been shown to treadmill *in vivo* and *in vitro* and it plays an essential role for divisome functioning. One important feature of treadmilling is directionality, meaning new monomers can only be added from one direction (for FtsZ, bottom addition is always favourable compared to top, Du et al. 2018, Knossow et al. 2020). However, the mechanism is not clearly understood why there is preference for bottom addition. Analysis from available crystal structures suggests that FtsZ molecule undergoes a structural transition from R state to T state during polymerization and filament assembly (Figure 1.8). R state is the relaxed conformation which is favoured by free monomers while T state is the tensed conformation which is energetically less favourable in solution and found in a filament state (or in monomer in a crystal lattice) (Huecas *et al.*, 2008; Dajkovic *et al.*, 2008; Wagstaff et al., 2017, Corbin and Erickson 2020, Chakraborty et al. 2024). Two major structural changes that

take place during R to T transition are: 1. Downward movement of the H7 helix with respect to NTD, and 2. Rotation of CTD resulting in opening of the cleft between H7 and CTD (Fujita et al. 2017, Wagstaff et al. 2017). These conformational changes are thought to be triggered by polymerization and depolymerization of the monomer (cytomotive switch model, Wagstaff et al. 2023). Another factor which could possibly facilitate T state conformation is the T3 loop conformation which upon GTP binding gets ordered and the peptide bond of a glycine residue flips to interact with the next subunit favouring inter-monomer interaction (Wagstaff 2017, Chakraborty et al. 2024). However, since flipped out ordered T3 loop can be seen in both GTP and GDP bound crystal structures and it can even be disordered in a GTP bound state, it is difficult to propose a consistent general mechanism of T3 loop involvement in monomer to filament transition (Wagstaff et al. 2017).

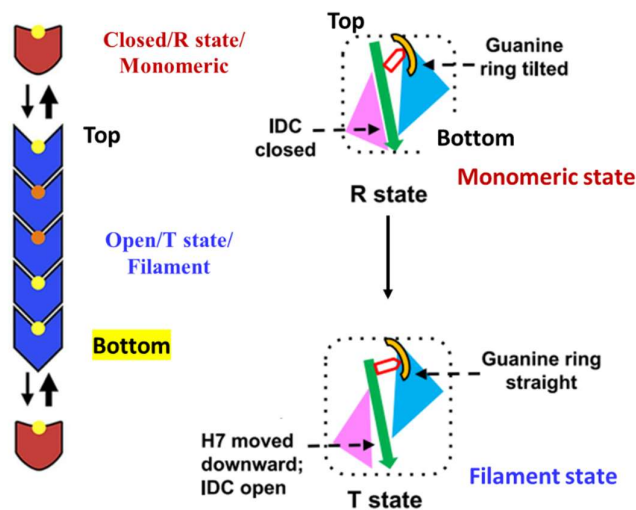


Figure 1.8 Structural basis of FtsZ treadmilling (left) R state monomers are added from the bottom (T7 loop face) and dissociate from the top (nucleotide pocket face). T state exists within the filament. Conformational switch between R and T state occurs during polymerization and depolymerization within the filament. Image adapted from Wagstaff et al. 2017. (right) Major conformational changes during R to T state transitions- (1) tilt in the guanine ring orientation, (2) downward movement of H7 helix, and (3) opening of interdomain cleft (IDC). Image adapted from Chakraborty et al. 2024.

However, it is clear from the structural data that required asymmetry of the FtsZ filament end necessary for treadmilling arises from the differences in the conformation of R and T state. GTP binding may play an important role by forming longitudinal interactions while hydrolysis induced structural change may weaken these interactions to fall off from the filament. But a lot more structural evidence will be needed to fully understand the role of nucleotide binding and hydrolysis and the sequential events during R to T state switch and vice-versa during polymerization and depolymerization respectively. Especially, capturing FtsZ filament in native state bound to different nucleotides can provide substantial information about the nucleotide specificity of FtsZ polymerization and treadmilling. This will also shed light on the second question of why FtsZ filament assembly always occurs at the bottom and disassembly from the top of the filament.

1.10 Role of FtsA in cell division

Since the discovery of FtsZ and FtsA proteins essential for cell division in bacteria, most of the focus has gone into understanding the role of FtsZ, the primary component of the Z-ring. However, our understanding about FtsA functions is not very clear because of the lack of consistency in the results from FtsA related studies. FtsA possesses an amphipathic helix at the C-terminus which facilitates membrane anchoring for FtsZ filaments (Pichoff and Lutkenhaus, 2005). Membrane tethering role of FtsA is essential as deleting the amphipathic helix halts cell division (Shiomi and Margolin, 2008). Other than just acting as Z-ring anchor, FtsA also regulates the dynamics of FtsZ. One of the early experiments by Osawa and Erickson 2013 showed complete septation of *in vitro* reconstituted liposomes in the presence of FtsZ and FtsA* (R286W mutant, also known as hyperactive FtsA) were present. In contrast, only FtsZ (with membrane attachment sequence) can deform the liposome but is unable to constrict fully (Osawa et al. 2008). These indeed suggest that the function of FtsA is possibly beyond just membrane anchoring. Later, FtsA was proposed to disassemble FtsZ filament from the membrane and thereby reorganise the network treadmilling Z-ring network (Loose and Mitchison 2014). Thus, interaction of FtsZ and FtsA plays a significant role for Z-ring functioning. However, self-organization dynamics of FtsZ can be seen without FtsA supporting the role of FtsA could be in filament disassembly and not in treadmilling (Ramirez Diaz et al. 2018). Interestingly, GTPase activity of FtsZ was found to be stimulated for *S. aureus* in the

presence of FtsA (Fujita et al. 2014) whereas negative or no effect of FtsA was observed for *E. coli* or *Deinococcus radiodurans* FtsZ activity (Loose and Mitchison 2014, Modi and Misra 2014, Conti et al. 2018). Additionally, it was also found in *in vitro* sedimentation assays that excess of FtsA over FtsZ can destabilize and disrupt FtsZ filaments (Modi and Misra 2014, Conti et al. 2018).

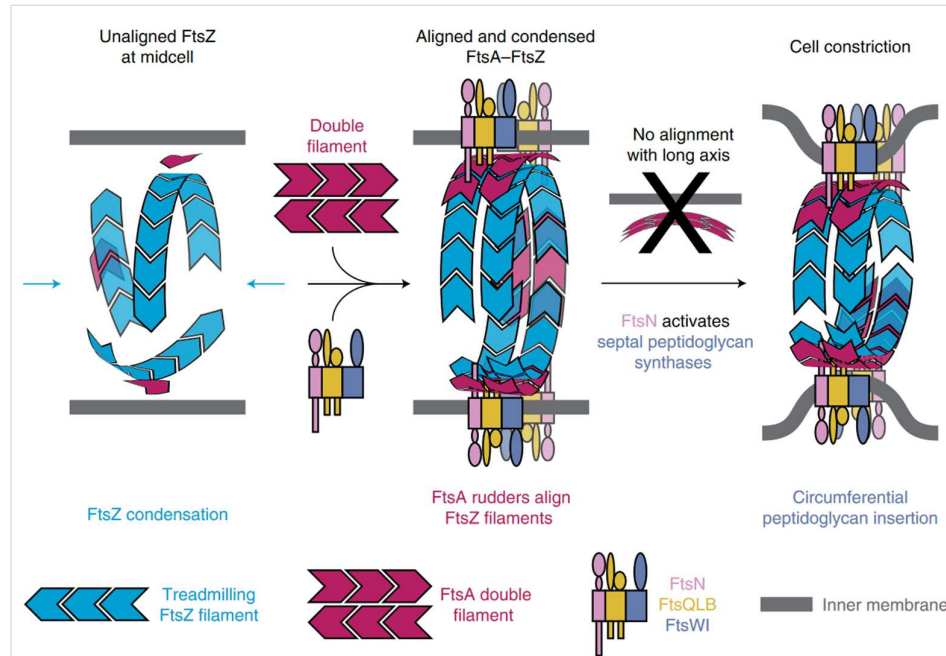


Figure 1.9 Model of FtsA guided divisome activation and cell constriction. FtsZ filaments recruited at the mid-cell are unaligned at the initial stage. Treadmilling filaments then condenses the ring along with activated FtsA double filaments (left). Downstream divisome proteins also gets recruited to the mid-cell by treadmilling Z-ring. FtsA aligns the divisome machinery along the short axis of the cell by curvature sensing mechanism of activated double filament (middle). FtsN helps in the activation of septal peptidoglycan synthesis as well to initiate cell constriction (right). Model and image adapted from Nierhaus et al 2022.

The other interesting aspect of FtsZ-FtsA interaction is FtsA-oligomerization dependent bundling of FtsZ filaments. Wild type *E. coli* FtsA was found to form mini-rings which prevents FtsZ filaments to laterally interact while certain FtsA mutants (which forms arcs or double-stranded filaments) promote laterally interacting filament to bundle (Krupka et al. 2017, Schoenemann et al. 2018, Cameron and Margolin 2024). The oligomeric state of FtsA has been proposed to serve as a potential checkpoint for divisome activation based on some recent data

from *in vitro* reconstitution experiments (Radler et al. 2022, Nierhaus et al. 2022). These experiments suggest that upon interacting with accessory proteins like ZipA, FtsX and FtsN, FtsA oligomeric conformation changes from min-ring (inactive) to short antiparallel double stranded filaments (active) which acts a possible divisome checkpoint for the activation of septal peptidoglycan synthases. FtsN-activated FtsA double filaments tend to orient (by means of its inherent curvature sensing mechanism) along the short axis of the cell and aligns the divisome components and works like a rudder for circumferential peptidoglycan synthesis at the septum. Thus, treadmilling FtsZ filaments along with the activated FtsA double protofilament coordinate with each other for the condensation of the division machinery by promoting the formation of a dense array of dynamic FtsZ filaments (Figure 1.9). However, the molecular details of the activation process, the components involved in this activation mechanism or its *in vivo* relevance are yet to be unveiled.

1.11 Cell wall-less bacteria as a model for minimal divisome

One of the outstanding questions related to cell division is what role does Z-ring play in the constriction process. As discussed in the previous section, the Z-ring acts as a scaffold to recruit different components of the divisome and as the filaments treadmill, it guides the PG synthases along the invaginating septum. Till date, most of the cell division work has been solely focused on cell walled model bacteria where Z-constriction and cell wall synthesis are coupled. Hence, it is difficult to speculate the individual contribution of these two machineries and delineate their individual roles. Mollicutes or the cell wall-less class bacteria can be considered as a good model system to characterize the functions of FtsZ and FtsA in the absence of a cell wall synthesis machinery. However, until now there are very limited studies on cell division proteins in wall-less bacteria.

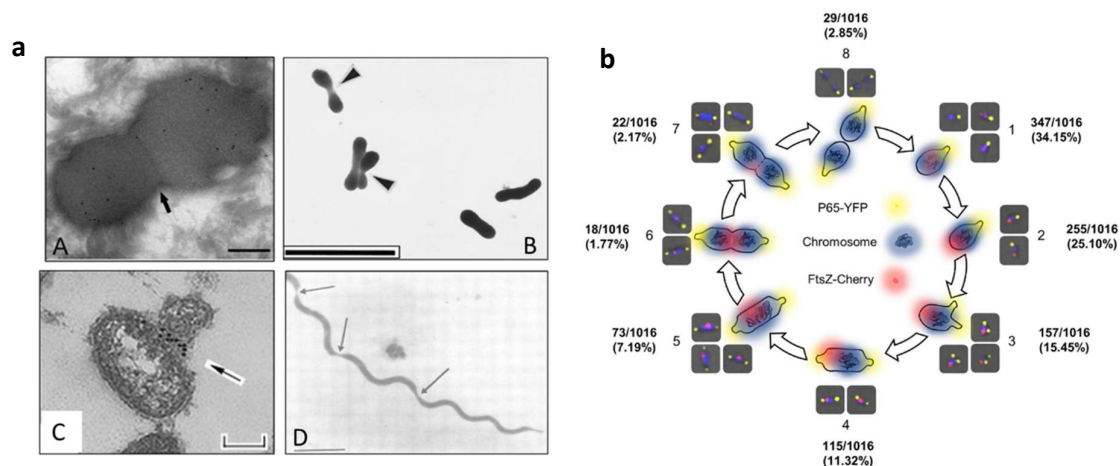


Figure 1.10 Examples of constriction as a division mechanism in mollicutes. (a) Various species of mollicutes showing division by constriction- A. *Mycoplasma salivarum* (scale bar- 0.5 μm) Mizuki et al. 2017, B. *Mycoplasma capricolum* (scale bar- 1 μm) (Seto and Miyata. 1998), C. Localization of FtsZ protein at the constriction site of dividing *Mycoplasma hominis* (scale bar- 200 nm) (Vishnyakov et al. 2009), D. *Spiroplasma citri* (scale bar-2 μm) (Garnier et al. 1981). (b) Stages of *M. genitalium* cell cycle and FtsZ localization dynamics (Martínez-Torró et al. 2021).

Mollicutes are evolved from cell walled Firmicutes (a Gram-positive bacterial phylum with low GC content) by extensive genome reduction (Wolf et al., 2004). Interestingly, the *dcw* cluster in mollicutes is only comprises of five genes namely, *mraZ*, *mraW*, *ftsA*, *ftsZ* and *sepF* and all other peptidoglycan synthesis related genes are lost from the *dcw* cluster (Alarcón et al. 2007). As mollicutes mostly have a parasitic lifestyle inside the host under a constant osmotic environment, it is possible that the cell wall became redundant for their survival (Miyagishima et al. 2014). Constriction has been observed in various species of mycoplasmas and spiroplasmas suggesting that these organisms too undergo binary fission (Garnier et al. 1981, Vishnyakov et al. 2009, Seto and Miyata 1998, Mizuki et al. 2017) (Figure 1.10a). While FtsZ gene is essential in most of the bacteria, it is found to be not essential in *Mycoplasma genitalium* and other wall-deficient L-form bacteria (Mercier et al., 2014). Recently, FtsZ localization dynamics have been observed *in vivo* in *M. genitalium* showing mid-cell localization of FtsZ proteins during cytokinesis (Martínez-Torró et al., 2021) (Figure 1.10b). *ftsZ* deleted strain of *M. genitalium* has been found to be capable of division with a same doubling rate as wild type (Lluch-Senar et al., 2010). Cell wall-less strains of bacteria including the L-form have been found to utilize processes other than FtsZ dependent binary fission such as membrane blebbing, budding, or extrusion-resolution for division (Briers et al., 2012; Mercier et al., 2014). In *Mycoplasma pneumoniae*, a close relative of *M. genitalium*, motility-driven mechanical force appears to facilitate constriction, a mechanism alternatively termed as cytofission (Hasselbring et al., 2006). Thus, if FtsZ is not essential, why it remains conserved in the genome of cell wall-less bacteria, remains unexplained. More interestingly, a recent discovery on mycoplasma JCVI-syn3a, the minimal cell with synthetic genome, shows the requirement *ftsZ* and *sepF* for normal cell morphology and division (Pelletier et al., 2021). These findings motivated us to look into the distribution of *dcw* genes across cell wall-less species to understand their role in cell wall-less bacteria.

1.12 Motivation and Objectives

Till date, most of the studies on cell division have been performed in cell walled bacteria where the function Z-ring constriction and the peptidoglycan synthesis machinery are interdependent. In this scenario, investigating cell division proteins from wall-less bacteria can give us new insights to the function of a minimal divisome machinery which possibly comprises three structural components - FtsZ, FtsA and SepF, in the absence of any influence from cell wall specific components. Currently there is a significant gap in our understanding of cell division in cell wall-less bacteria due to the challenges associated with culturing these organisms and a limited availability of genetic tools for genome manipulation. Nevertheless, there is a considerable potential to uncover many interesting and fundamental properties which might be driving cell division in these non-model bacterial systems. This greatly motivated us to pursue this project to study cell division protein *in vitro* from cell wall-less *Mycoplasma* and *Spiroplasma*, which are the two relatively more characterized genera within the mollicute class with available genomic information. The broad objectives of my PhD research work involve characterizing the *dcw* cluster genes in mollicutes with a particular focus on the roles of FtsZ and FtsA, the two essential components of bacterial divisome.

To achieve this goal, I have divided the entire thesis into five sections with specific objectives as follows:

1. Analysis of *dcw* cluster genes across in mollicute class: Here, we have undertaken a bioinformatics approach to identify the distribution of the *dcw* genes in the genomes from the NCBI database.
2. Characterization of the function of FtsZ and FtsA in *Mycoplasmas*, the most studied genera so far: Combining bioinformatics analysis and *in vitro* biochemical studies, we sought to identify characteristic sequence features of FtsZ and FtsA and their putative roles in cell division.
3. Biochemical characterization of FtsA, the primary membrane anchor for FtsZ, from *Spiroplasma melliferum*.
4. Characterization of interaction between FtsZ and FtsA from *Spiroplasma melliferum* to understand the role of these two major structural components in a minimal divisome machinery.

5. Determination of filament structure of *Spiroplasma melliferum* FtsZ by cryo-electron microscopy to understand the structural basis of nucleotide hydrolysis and polymerization.

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Chapter 2

Division and cell wall (*dcw*) cluster in mollicutes: A bioinformatics study

Some sections reprinted from Dutta et al. 2025; *Biochemistry*

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2.1 Introduction

Cell division in bacteria is a coordinated and collective action of multiple proteins involved in Z-ring assembly, peptidoglycan synthesis, remodelling and its regulations (McQuillen and Xiao, 2020). More than twenty different proteins play an important role in the formation of the divisome, a multiprotein complex that coordinates different steps of the division in a highly regulated and organized manner (den Blaauwen *et al.*, 2017, McQuillen and Xiao, 2020). Notably, very often in bacteria, many of the division-related proteins are transcribed from a single operon called division and cell wall (*dcw*) cluster (Margolin, 2000). The *dcw* cluster in cell walled model organisms *E. coli* and *Bacillus subtilis* comprises sixteen and seventeen genes, respectively, which include FtsZ, FtsA and their downstream interactors involved in the division process. While most information regarding the function of cell division proteins is known based on studies carried out in cell walled bacteria, our understanding of cell wall-less systems is limited so far. In mollicutes or the cell wall-less bacterial class, genes related to peptidoglycan synthesis are lost and a concomitant shorter *dcw* operon is the result of an extensive genome reduction (Bové, 1993). In mycoplasmas, for example, only five genes namely *mraZ*, *mraW* (*rsmH*), *ftsA*, *ftsZ* and *sepF* can be found in the *dcw* operon (Alarcón *et al.*, 2007; Pelletier *et al.*, 2021). The highly conserved nature of these genes across the bacterial kingdom implies their important function in cell division in a cell wall-less milieu. Recently it has also been shown that FtsZ in *M. genitalium* localizes at the mid cell during constriction (Martínez-Torró *et al.* 2021). Moreover, *ftsZ* and *sepF* genes have been found to be essential for normal cell morphology and division of mycoplasma JCVI-syn3a, a minimal cell with synthetic genome derived from *M. mycoides* subspecies capri (Pelletier *et al.*, 2021). Despite FtsZ being essential in most bacteria, there are instances of FtsZ independent division. *M. genitalium* can divide in the absence of FtsZ without affecting its doubling time (Lluch-Senar *et al.* 2010). In the FtsZdeleted *M. genitalium* strain, gliding motility plays an essential role for the division process (Lluch-Senar *et al.* 2010). In *M. pneumoniae*, a close relative of *M. genitalium*, motility-driven mechanical force probably facilitates constriction, a mechanism alternatively termed as cytofission (Hasselbring *et al.* 2006, Erickson and Osawa 2010). Some other cell wall-less strains of bacteria, like the L-forms, utilize processes such as membrane blebbing, budding, or extrusion-resolution for division (Briers *et al.* 2012, Mercier *et al.* 2014). The above examples suggest a non-essential nature of FtsZ for cell division. These discoveries motivated us to look into the conservation of proteins in the *dcw* cluster across mollicute genomes to understand how a minimal divisome functions.

Here I have taken a bioinformatics approach to check the occurrence and conservation of these five *dcw* cluster genes across seven different genera of the mollicutes class. I have also checked conservation of three main functional motifs found in FtsZ namely, GTP binding, GTP hydrolysis and FtsA binding conserved C-terminal peptide (CCTP) motif.

2.2 Materials and methods

The division and cell wall (*dcw*) cluster locus was identified by checking the presence of the cluster of genes in genomes of each organism for which complete genome assembly was available in the NCBI database. We performed a keyword-based search with ‘FtsZ’, ‘FtsA’, ‘rsmH’ (gene annotation for MraW protein) or ‘MraZ’. The neighbourhood genes (-1 and +1) of the possible *dcw* cluster were also identified. Additionally, to confirm the absence of a particular protein in the genome we carried out tBLASTn search against genome sequences with *Mycoplasma mycoides* FtsZ (WP_017697740.1) and FtsA (WP_020862814.1) and SepF (WP_020862813.1) as query. For mycoplasma, we analysed a total 113 species from the NCBI genome database (number of genomes present as in April 2023). We had prior information about these four mycoplasma genes present in the *dcw* operon from the existing literature. We found that the upstream coding sequence to the *ftsZ* gene was annotated as a hypothetical protein-coding gene except for *Mycoplasma mycoides*, where it is annotated as *ftsA*. Indeed, in earlier published literature, the hypothetical gene has been reported as ‘*ftsA*’. Hence, we downloaded the protein sequences of the *ftsZ* gene along with the hypothetical protein-coding gene upstream of *ftsZ* for our analysis. We verified all the FtsA sequences by multiple sequence alignment with known and already characterized FtsA sequences from cell-walled model bacteria, followed by assignment of secondary structures and domains present in FtsA using PROMALS 3D (<http://prodata.swmed.edu/promals3d/promals3d.php>) and PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>). For spiroplasmas, we analysed 27 species with complete sequence information in the NCBI genome database and followed the similar steps for identifying presence and absence of the *dcw* cluster. The sequence logo from the multiple sequence alignment was created using the WebLogo server (<https://weblogo.berkeley.edu/logo.cgi>). Phylogenetic trees were constructed using the maximum likelihood method in MEGA 11 software with default parameters and visualized in MEGA 11 and iTOL (<https://itol.embl.de/>). There are few other genera except mycoplasma and spiroplasma were also included in this analysis: Acholeplasma (6 species), Phytoplasma

(5 species), Mesoplasma (7 species), Entomoplasma (1 species), and Ureaplasma (1 species). Details are mentioned in the results section.

2.3 Results

2.3.1 Sequence analysis of FtsZ and FtsA in Mycoplasma genomes

We analysed 113 mycoplasma genomes in the NCBI database for division and cell wall (*dcw*) operon to check the presence of division related genes. While searching the NCBI genome database, we found *ftsA* as an unannotated gene present in between *mraW* and *ftsZ* in most mycoplasmas. After checking the domain organization of its protein coding sequence, we confirmed this as FtsA (Figure 2.1). Importantly, in mycoplasma, the four division related genes namely *mraZ*, *mraW*, *ftsA*, and *ftsZ* were present in the same sequential manner as found in cell walled bacteria. As expected, all additional genes related to the cell wall synthesis present in the *dcw* operon in cell-walled bacteria were absent. We observed that even FtsZ and FtsA were not conserved across all mycoplasma species. Accordingly, based on the presence of FtsA and FtsZ, we divided them into four groups (Figure 2.2a, b). Majority of them i.e., 82 out of 113 species, were classified into group 1 where both FtsZ and FtsA are present. Group 2, having only FtsZ but no FtsA, includes 10 species while group 3 has 7 species where FtsA is present but FtsZ is absent. Finally, the 14 species of group 4 lack both FtsZ and FtsA. Further, we analysed the sequences systematically to find out the characteristic features of these two proteins in the various groups and to further correlate with their role in cell division. Based on the comparison with the 16S rRNA based phylogenetic tree, we observed that the presence of FtsZ/FtsA was correlated to the species organization, except for a few species that were outliers (highlighted by ‘*’; Figure 2.2b).

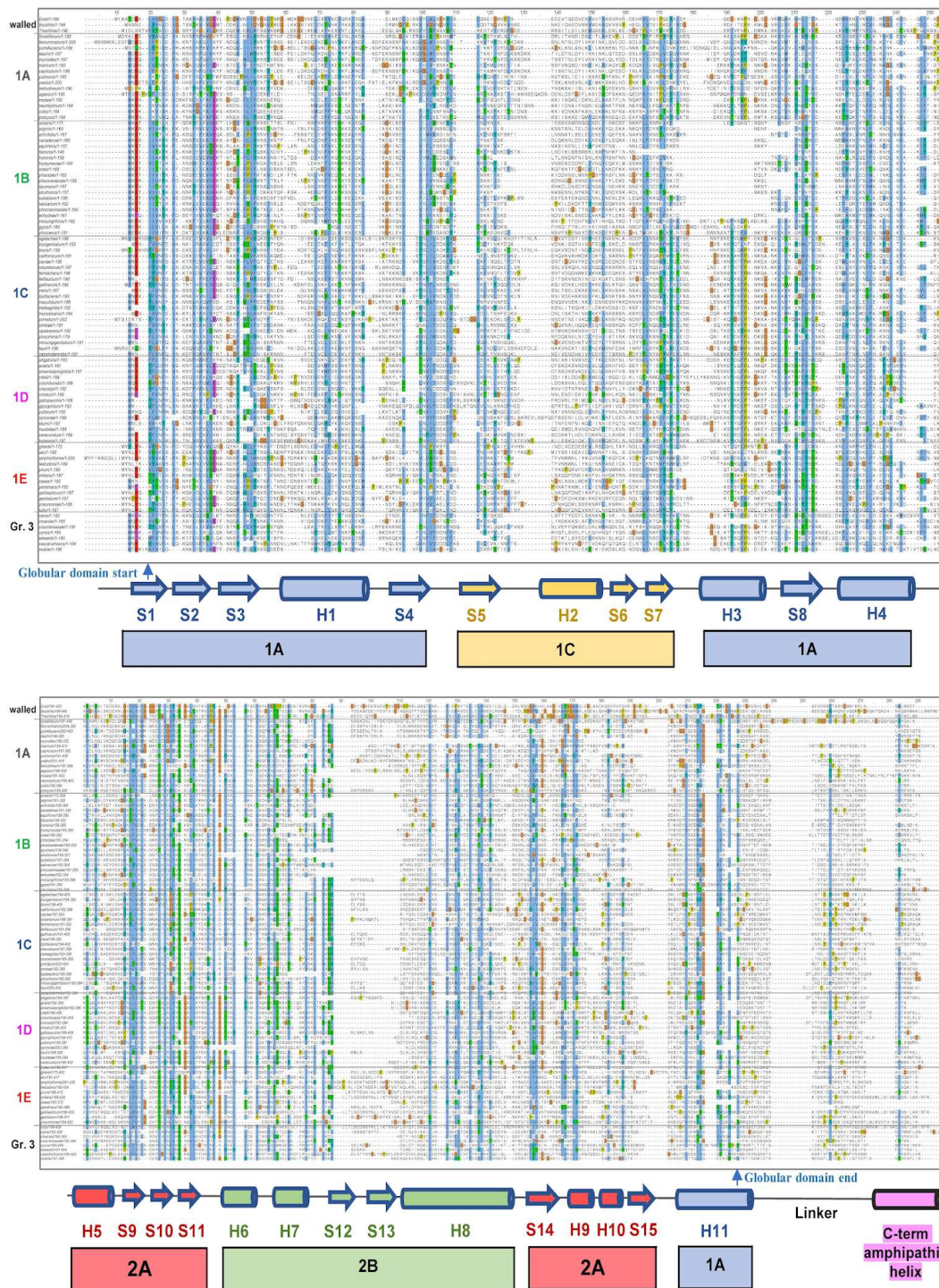


Figure 2.1 Domain assignment of hypothetical FtsA proteins in mycoplasma

Multiple sequence alignment of hypothetical protein sequences upstream of FtsZ. The secondary structures and domain assignment of FtsA are shown below the alignment showing the presence of canonical FtsA domain architecture.

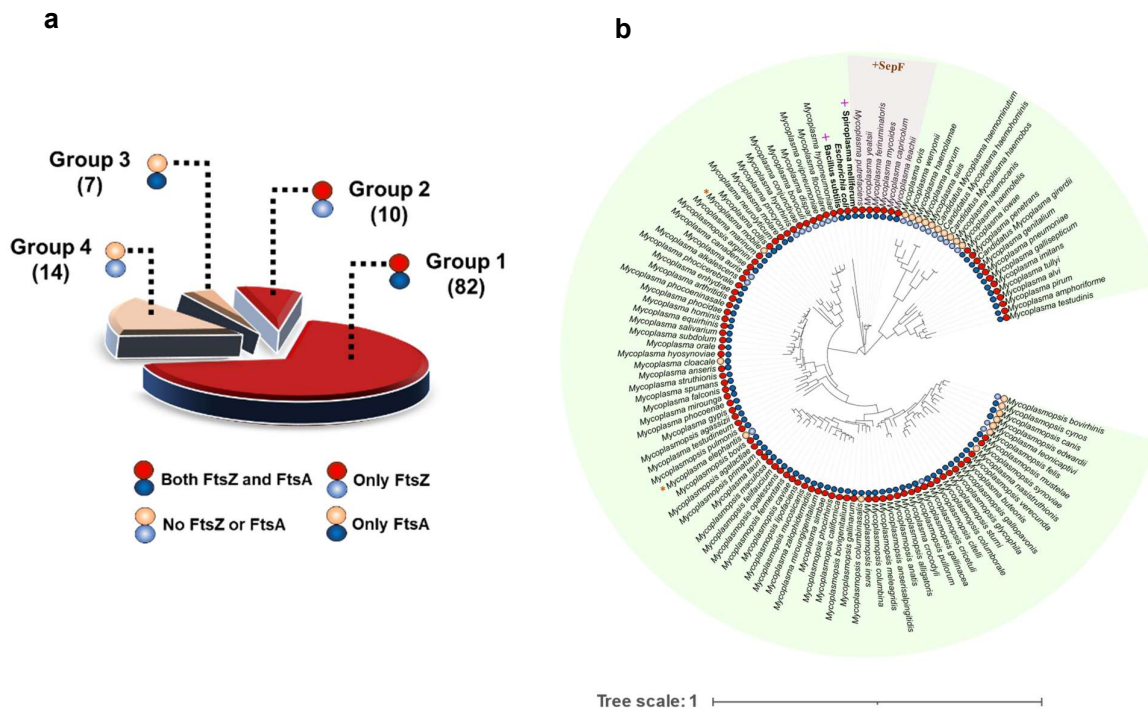


Figure 2.2 Classification of mycoplasma species based on the presence of *ftsA* and *ftsZ* in their genomes

(a) 16S rRNA-based tree of 113 mycoplasma species was constructed using maximum likelihood analysis and the distribution of FtsZ and FtsA proteins are highlighted. Solid red (for FtsZ) and solid blue (for FtsA) circles represent the presence of the proteins and transparent colored circles indicate their absence. Presence of SepF has been marked with a '+' sign.

(b) Grouping of mycoplasmas based on the presence of FtsZ and FtsA. The number of organisms consisting each group is mentioned within brackets.

We next checked for the presence of the alternative membrane attachment adaptor SepF. We found that *sepF* was present in only six species of group 1A (no hit was obtained in the tBLASTn search against the genomes for the rest) and located next to the *ftsZ* gene of the *dcw* cluster (Figure 2.3).

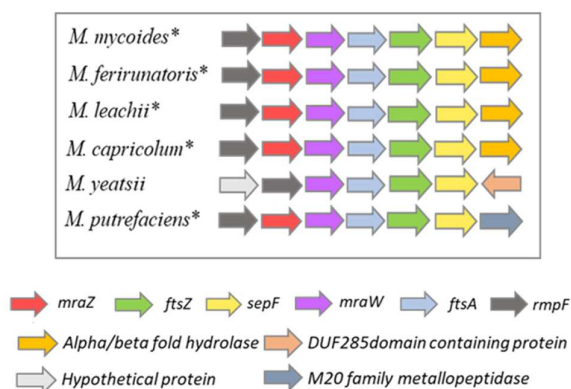


Figure 2.3 Schematic representation of *dcw* cluster genes in group 1A mycoplasmas containing SepF

Neighbouring genes (+1 and -1 positions) of *dcw* operon genes are also marked. The colour code and annotations are mentioned. The direction of the arrows indicates gene orientation 5' → 3'. Organisms marked as '*' do not possess an amphipathic helix in their FtsAs.

2.3.2 FtsZ sequence analysis reveals variable CCTPs among mycoplasma species

To compare the FtsZ protein sequences among group 1 mycoplasma species, we checked for the conservation of three characteristic motifs of FtsZ, viz. GTP binding motif, GTP hydrolysis motif and CCTP (Löwe and Amos 1998, Nogales et al. 1998). We found the GTP binding and hydrolysis motifs were well conserved across species whereas the CCTPs were variable (Figure 2.4a). We divided them into five subgroups based on the signature amino acid sequences of CCTP. The cladogram constructed from the FtsZ sequences interestingly matched with the CCTP based classification and these groups also formed clusters in the 16S rRNA based cladogram (Figure 2.5b). 14 organisms out of 82 possessed a canonical FtsZ CCTP and were classified into group 1A (6 of them matches with the "Mycoides" cluster). Sequences that showed conservation of charges similar to canonical CCTP residues, but missed the conserved proline, were classified into group 1B. Group 1C sequences were more divergent where proline and phenylalanine were conserved while the negatively charged stretch of residues were not. The rest of the sequences did not show any conservation in their CCTP (group 1D and 1E). Multiple sequence alignment of the C-terminal FtsZs of 1D and 1E groups showed conservation of negative charges and hydrophobic residues (Figure 2.5a), although different

from the canonical motif signature. Importantly, the invariant proline of canonical CCTP was missing in groups 1B, 1D and 1E.

a

Conserved FtsZ motifs			Group
GTP binding [S/A/G]-G-G-T-G-[S/A/T]-G	GTP hydrolysis N-X-D-X-X-D	CCTP D/E-I/V-P-X-F/Y-L-R/K	
			Walled (6)
			1A (14)
			1B (20)
			1C (21)
			1D (15)
			1E (12)

b

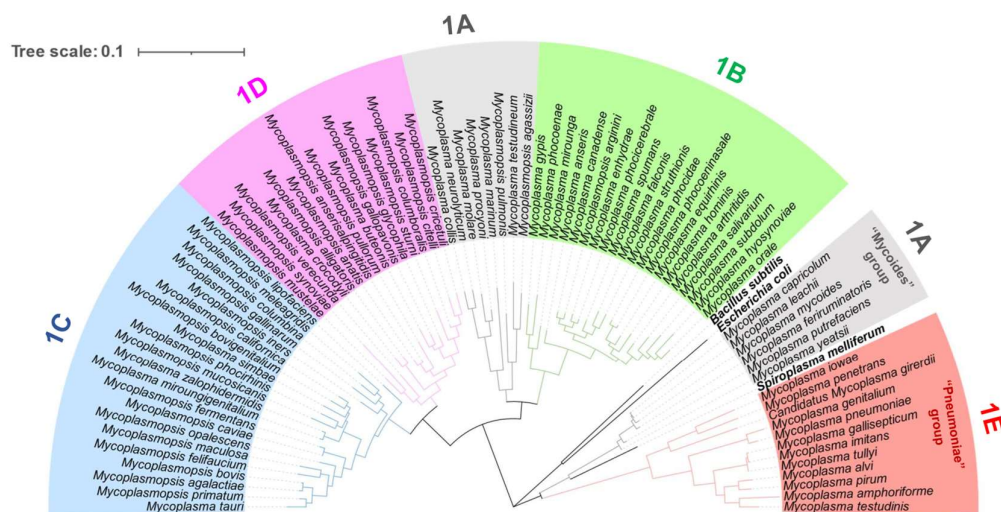


Figure 2.4 Classification based on conserved C-terminal peptide (CTTP) sequences matches with the phylogenetic relationship of mycoplasma FtsZs

(a) Cladogram of FtsZ from group 1 mycoplasma species is constructed using the maximum likelihood method. Different clusters are highlighted and labelled as 1A – E.

(b) Group 1 mycoplasmas having FtsZ and FtsA are grouped into five categories based on the cladogram and the type of CCTP present. Groupwise WebLogos are shown for three conserved FtsZ motifs: GTP binding, hydrolysis, and CCTP. The number within the bracket for a group represents number of sequences used for generating the WebLogo.

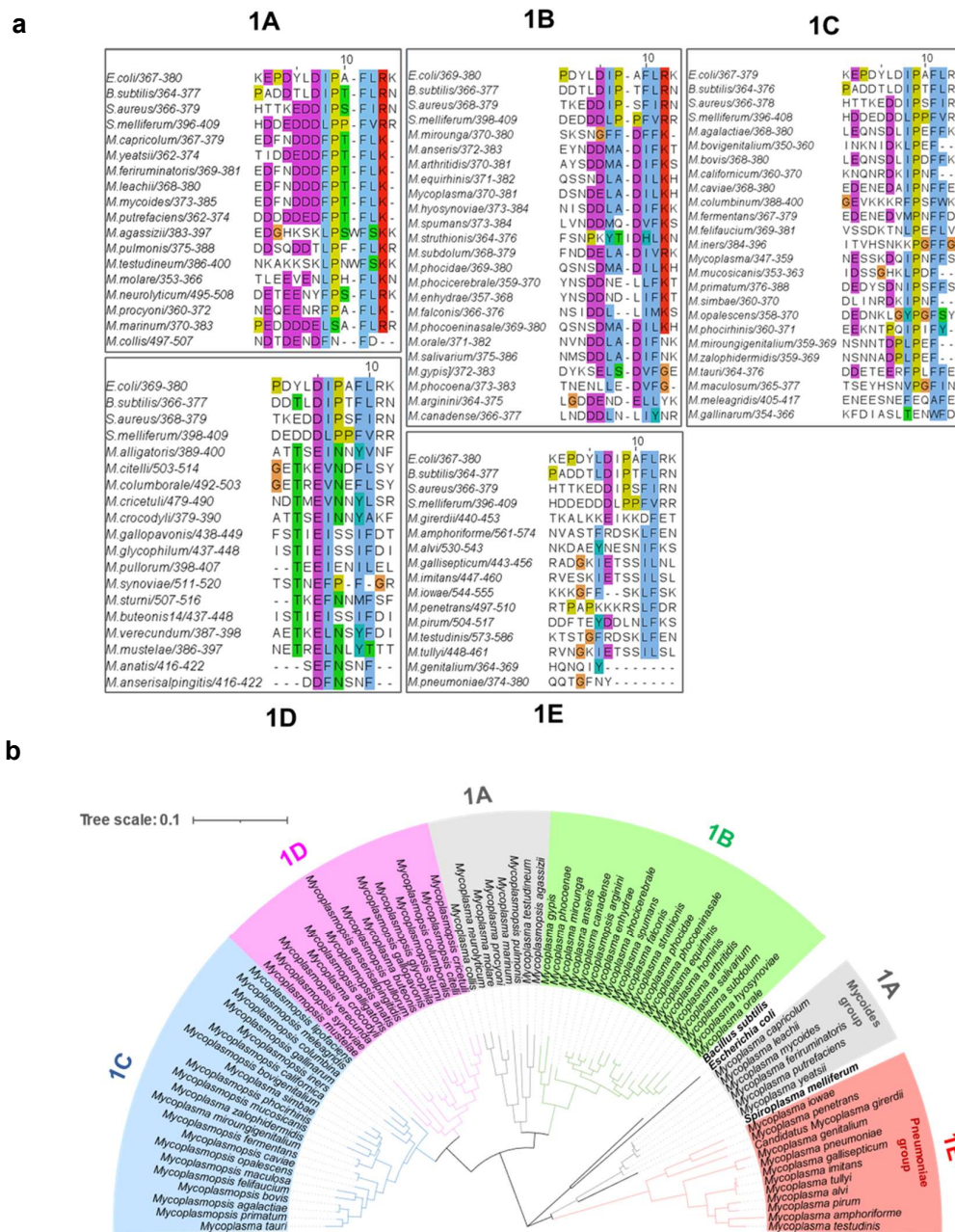


Figure 2.5 Subgrouping of group 1 mycoplasma species

(a) Sequence alignment of CCTP region of FtsZ proteins from group 1 species of mycoplasma. Various types of CCTPs were divided into five sub-groups of mycoplasma FtsZs. While group 1A-1C CCTPs show more similarity to the conserved canonical CCTP of cell-walled bacteria, groups 1D and 1E CCTPs do not. The alignment of CCTP motifs shown here was used to generate the WebLogos in Figure 2.4a (b) 16S rRNA based cladogram of group 1 mycoplasma species.

We also analysed group 2 FtsZ sequences (here FtsZ was present but FtsA was not) and found that the GTP binding motif was conserved in all of them. However, the GTP hydrolysis and FtsA binding (CCTP) motifs were conserved in only a few species namely *M. gallinacea*, *M. auris*, and *M. alkalescens* (Figure 2.6). Rest of the species do not possess consensus signature sequences of the respective motifs.

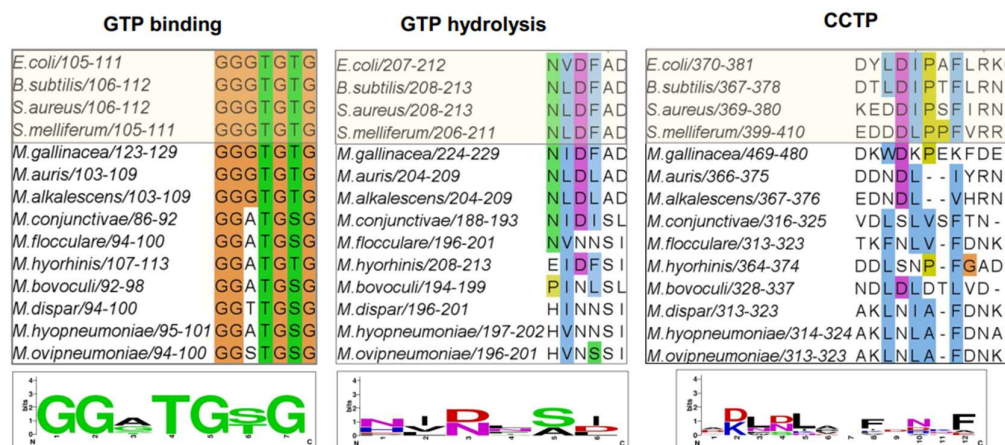


Figure 2.6 Sequence alignment of GTP binding, GTP hydrolysis, and CCTP region of FtsZ proteins of group 2 mycoplasma species

Some species show conserved motifs however in most of them the conservation is lost. WebLogos are shown below each alignment.

2.3.3 Occurrence of *dcw* cluster genes in Spiroplasma genomes

We analysed 27 Spiroplasma genomes in the NCBI database and checked for the presence of five cell division cluster related genes found in mollicutes. 19 out of 27 species possess all five genes namely *mraZ*, *mraW*, *ftsA*, *ftsZ* and *sepF* whereas in the rest eight species (marked with a star in the phylogenetic tree) *sepF* is absent (Figure 2.7). Notably, spiroplasmas can be divided into two groups - apis clade (comprising 17 species) and citri clade (comprising 10 species) based on their phylogenetic relatedness (Ku et al. 2014, Paredes et al 2015, Tsai et al. 2018). Interestingly, all eight species lacking *sepF* were found to belong to the apis clade.

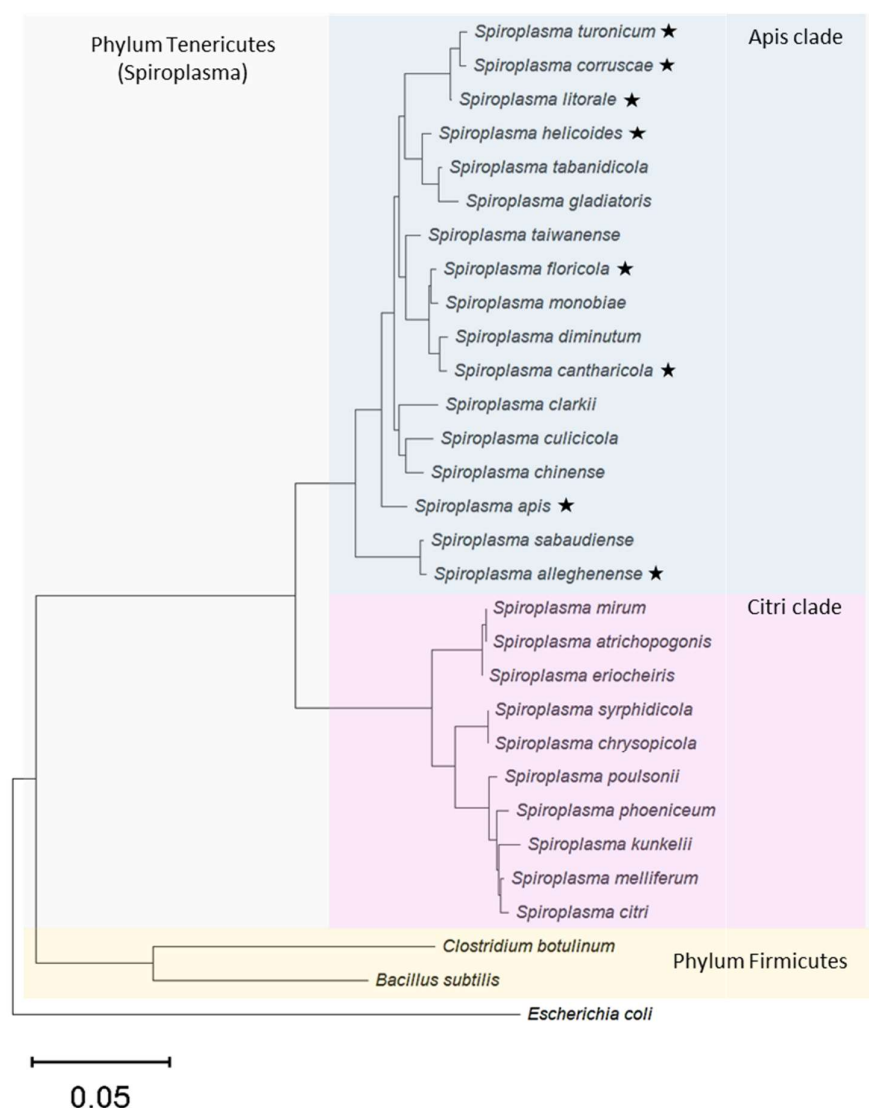


Figure 2.7 16S rRNA based phylogeny of *Spiroplasma* species. Organisms without *sepF* in the *dcw* cluster are marked with black colored star.

2.3.4 FtsZ motif conservation in *Spiroplasma*

To check the conservation of canonical motifs in the FtsZs of *Spiroplasma*, multiple sequence alignment of all 27 FtsZ protein sequences along with the cell walled model bacteria (*E. coli*, *B. subtilis*, *S. aureus*, *C. botulinum*) were done. All the three motifs of FtsZ were found to be conserved across all the sequences (Figure 2.8). This possibly indicates the presence of functional FtsZ protein in *Spiroplasmas*.

Conserved FtsZ motifs			Group
GTP binding [S/A/G]-G-G-T-G-[S/A/T]-G	GTP hydrolysis N-X-D-X-X-D	CCTP D/E-I/V-P-X-F/Y-L-R/K	
			Walled bacteria
			Spiroplasma

Figure 2.8 Motif conservation in spiroplasmas species. WebLogos for the respective motifs vis. GTP binding, GTP hydrolysis, and CCTP region of FtsZ proteins are compared with cell walled bacteria.

2.3.5 *dcw* cluster in others mollicutes

We have checked the presence of five genes namely *mraZ*, *mraW*, *ftsA*, *ftsZ* and *sepF* in the *dcw* cluster in other five genera of mollicutes namely Acholeplasma, Phytoplasma, Entomoplasma, Mesoplasma, and Ureaplasma (Figure 2.9a). We found that across these genera not all five *dcw* genes are always present. Phytoplasma has only *mraW* whereas Ureaplasma has only *mraZ* and *mraW*. Acholeplasmas have *mraW*, *ftsZ* and *sepF* while *mraZ* and *ftsA* are absent. Entomoplasma, Mesoplasma show presence of all five genes. In spiroplasma, *mraZ*, *mraW*, *ftsA*, *ftsZ* are always present while *sepF* is absent in some species of the apis group. In Mycoplasma, *mraZ* and *mraW* are present but presence of *ftsZ*, *ftsA* and *sepF* are not always present. Additionally, we have performed gene neighbourhood analysis to compare it with the cell walled *dcw* operon. We found that in Entomoplasma, Mesoplasma, Spiroplasma, and Mycoplasma five genes are present in the same sequential manner as there in the walled bacteria. In Acholeplasma, *mraW* is present at a distant location in the genome whereas *ftsZ* and *sepF* are neighbours with unrelated genes in between (Figure 2.9b).

Next, we checked FtsZ motifs across three groups where FtsZ is present i.e. Acholeplasma, Entomoplasma and Mesoplasma. We saw GTP hydrolysis, GTP binding and the CCTP motifs are highly conserved which are similar to the canonical motifs of walled bacteria (Figure 2.10), indicating the presence of a functional FtsZ.

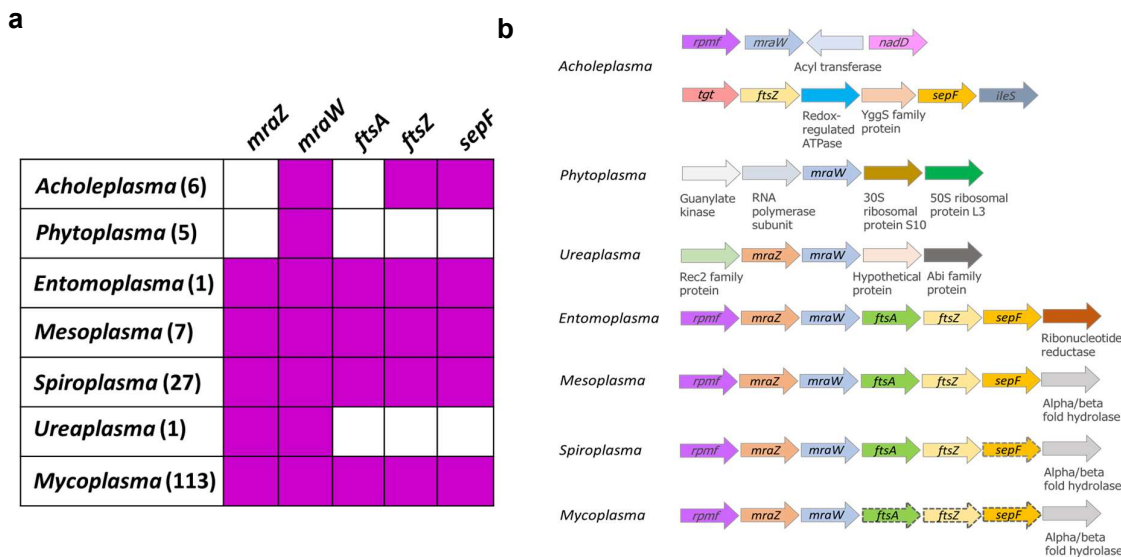


Figure 2.9 Occurrence of *dcw* genes and gene neighbourhood analysis.

(a) Table showing the distribution of five *dcw* cluster genes in different genera of mollicutes. Filled and empty boxes represent presence and absence respectively of the corresponding genes. Numbers in the brackets indicates number of genomes analysed within a particular genus. (b) Schematic representation of the gene neighbourhood of the *dcw* cluster.

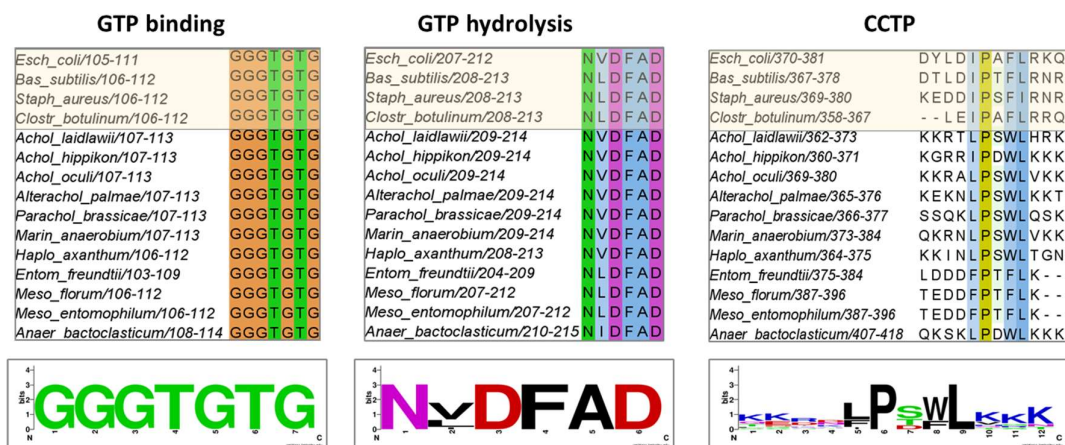


Figure 2.10 Multiple sequence alignment of GTP binding, GTP hydrolysis, and CCTP region of FtsZ proteins of *Acholeplasma*, *Mesoplasma* and *Entomoplasma*. Conservation of the three motifs of FtsZ are shown in the sequence alignment. Cell walled bacterial sequences are highlighted in tallow shade. Weblogos are shown below each alignment.

2.4 Discussion

In this chapter, we have summarised our bioinformatics analysis of *dcw* cluster genes in mollicute genomes to check its occurrence across cell wall-less class of bacteria. We have catalogued a species level information of the presence of the cell division genes. It was observed that the *dcw* cluster is not conserved across mollicute class and the presence of the division genes is species specific. Importantly, most of the FtsZs when present in the genome was found to have conserved GTP binding, hydrolysis and CCTP motifs indicating its possible active role. In order to understand the evolutionary correlation of the emergence and loss of the *dcw* cluster genes, we correlated the presence and absence in a 16S rRNA based phylogenetic tree with representative species from different genera. Figure 2.11 summarises the combinations of cell division genes present across all the relevant clades discussed in our analysis.

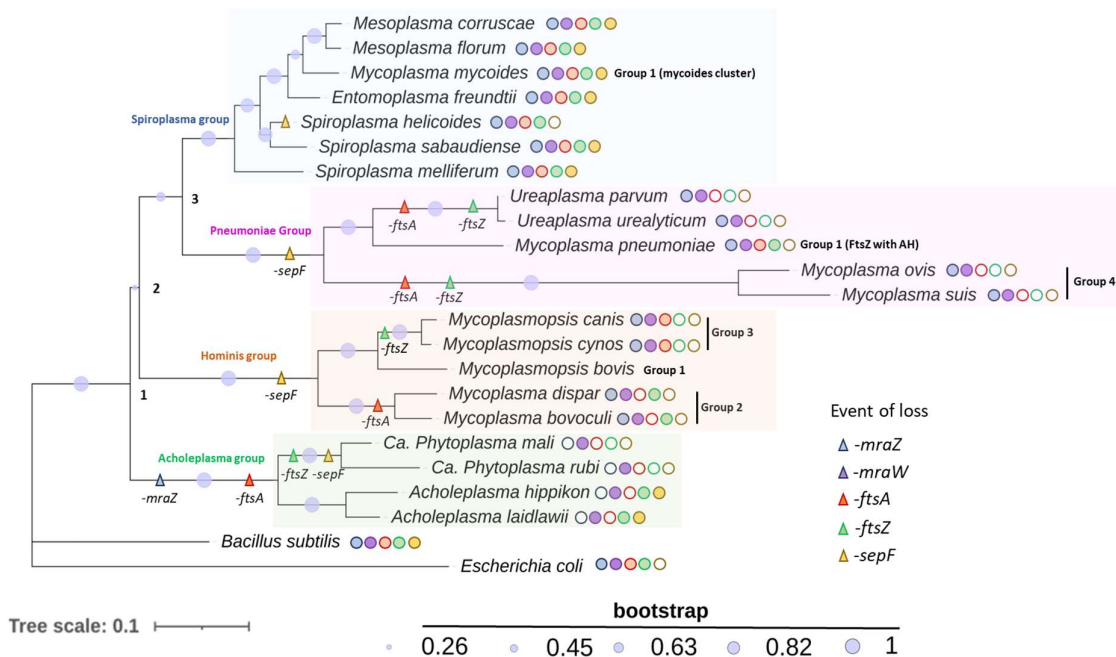


Figure 2.11 Tracing possible gene loss events in mollicutes in the 16S rRNA phylogeny.

16S phylogenetic tree depicts the diversification of various genera which forms separate cluster. Bootstrap values are drawn as circular symbols for each clade. Possible gene loss events are marked for five genes. Presence and absence of genes is shown as filled and open colored circles against each representative species. Major diversification events are marked with numbers.

In the phylogenetic tree we observed three major diversification events across the mollicutes class (Figure 2.11). The first branch gives rise to the Achleoplasma group where *mraZ* and *ftsA* are lost. Within the Achleoplasma group, further loss of *ftsZ* and *sepF* is correlated with the formation of Phytoplasma clade. In the next diversification to the Hominis group, loss of *sepF* is observed. Importantly, the group 2 and group 3 of Mycoplasma are part of this group which originate with the loss of *ftsA* and *ftsZ* respectively. The third and final diversification event results in the formation of Pneumoniae group and Spiroplasma group. Pneumoniae group lacks *sepF*, whereas in the Spiroplasma group *sepF* is retained with a few exceptions in the spiropasma genera. Within the Pneumoniae group we observed the loss of both *ftsZ* and *ftsA* giving rise to Group 4 Mycoplasma and Ureaplasma genera. Notably, *Mycoplasma pneumoniae* (with potential membrane binding amphipathic helix in its FtsZ, discussed in detail in the next chapter) falls in this cluster. The spiropasma group is formed by three major genera of mollicutes namely Spiroplasma, Mesoplasma and Entomoplamsa. All five genes of the *dcw* cluster are retained except eight species in the apis clade of Spiroplasma as mentioned earlier. The mycoides cluster of Mycoplasma (with six species containing *sepF*) also falls within the Spiroplasma group.

Overall, our analysis of the *dcw* cluster across the mollicute genomes indicates multiple independent and clade specific gene-loss events. However, its functional and evolutionary significance remain unclear. As mollicute species show a variety of cell division strategies, it is plausible that certain cell division genes could become redundant. Additionally, host specific factors might also be correlated with the presence and absence of certain genes as the optimal cell division strategy may vary depending on the host environment. Further experimental evidence on cell division mechanisms across different mollicutes species could possibly shed more light on the evolution of the *dcw* cluster in future.

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Chapter 3

A novel membrane binding and cholesterol sensing motif in *Mycoplasma genitalium*

FtsZ

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3.1 Introduction

FtsZ and FtsA are two primary components of the Z-ring involved in bacterial cell division. Despite numerous studies in the past, the exact mechanism of how they execute cell division is still not clearly understood. Majority of the work in the field is focused on cell walled bacteria where ring constriction and cell wall synthesis are coupled for cell division. In this context, FtsZA from cell-wall less bacteria can serve as a model system to comprehend the precise role of the Z-ring in the division process in the absence of cell wall synthesis machinery. Here, we explore the characteristic features of FtsZ and FtsA in Mycoplasmas, which is also the most studied genera among the mollicutes. In cell wall-less bacteria, genes related to peptidoglycan synthesis appear to be lost during extensive genome reduction, resulting in a shorter *dcw* cluster (Bove 1993). For example, in mycoplasmas, only five genes are present in the *dcw* cluster, namely *mraZ*, *mraW* (*rsmH*), *ftsA*, *ftsZ* and *sepF* (Alarcon et al. 2007, Pelletier et al. 2021). The highly conserved nature of these genes across the bacterial kingdom implies their important function in cell division in cell wall-less bacteria. Despite FtsZ being essential in most bacteria, there are instances of FtsZ independent division. *Mycoplasma genitalium* can divide in the absence of FtsZ without affecting its doubling time (Lluch-Senar et al. 2010). In the *M. genitalium* strain with FtsZ deleted, gliding motility plays an essential role for the division process (Lluch-Senar et al. 2010). In *M. pneumoniae*, a close relative of *M. genitalium*, motility-driven mechanical force probably facilitates constriction, a mechanism alternatively termed as cytofission (Hasselbring et al. 2006, Erickson and Osawa 2010). Some other cell wall-less strains of bacteria, like the L-forms, utilize processes such as membrane blebbing, budding, or extrusion-resolution for division (Briers et al. 2012, Mercier et al. 2014). The above examples suggest a non-essential nature of FtsZ for cell division. FtsZ in *Mycoplasma genitalium* has been found to localize at the mid cell during constriction (Martínez-Torró et al. 2021). Moreover, recent studies in JCVI-syn3a, a minimal cell with synthetic genome derived from *M. mycoides* subspecies capri (Pelletier et al. 2021) has proposed that *ftsZ* and *sepF* genes to be essential for normal cell morphology and division of mycoplasma. In this scenario, the conservation of FtsZ in the genomes of cell wall-less bacteria remains enigmatic.

As discussed in the previous chapter, mycoplasmas can be divided into four groups based on the presence and absence of FtsZ and FtsA. Most of the species have both the proteins, and are classified into group 1. However, the canonical FtsA binding CCTP motif is not conserved in the 12 species of group 1E (the Pneumoniae clade), possibly indicating independent roles for FtsZ and FtsA. These observations were very intriguing and motivated

us to take a deeper look at the sequences of FtsZ and FtsA of group 1E organisms. Moreover, the group 2 FtsZs and group 3 FtsAs were also checked for their characteristic features.

Here, we report the presence of an amphipathic helix with potential membrane binding characteristics in group 1E. We experimentally checked our hypothesis for *M. genitalium*, the bacterium with a minimal genome, to possess a C-terminal amphipathic helix (CTAH). CTAH will possibly allow a direct membrane attachment for FtsZ bypassing the requirement of FtsA as membrane anchor. We also identified a cholesterol recognition amino acid consensus (CRAC) sequence in the C-terminal of *M. genitalium* and *M. pneumoniae* FtsZ. Overall, our identification of unique membrane binding and cholesterol interacting properties of bacterial FtsZ provide us with examples of naturally existing minimal divisome components, a powerful handle to obtain new insights to the fundamental mechanism of cell division.

3.2 Materials and methods

3.2.1 Sequence analysis of FtsZ and FtsA

We analysed a total of 113 Mycoplasma species with complete sequence information of the FtsZ and FtsA from the NCBI genome database (genomes present as in April 2023). For secondary structure prediction of protein sequences, we used PSIPRED server (McGuffin et al. 2000, <http://bioinf.cs.ucl.ac.uk/psipred/>). HeliQuest (Gautier et al. 2008, <https://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py>) was used for helical wheel representation and calculation of hydrophobic moment and hydrophobicity parameters. In order to identify a potential amphipathic helix (AH), we first checked the secondary structure from PSIPRED and took the helical region present at the N- and C-terminal extension of FtsZ and FtsA sequences. We input the sequence in the HeliQuest server to get the helical wheel plot and respective amphipathic parameters. The AHs were predicted by comparing the hydrophobic moment plot of known AHs whether they fall in the same cluster or not. Multiple sequence alignments were performed in Jalview (Waterhouse et al. 2009) using MUSCLE (Edgar 2004). Phylogenetic analysis was done in MEGA11 software (Tamura et al. 2021) and iTOL (Letunic and Bork 2021) was used for tree visualization.

3.2.2 Cloning and protein purification

SmftsZ gene was PCR amplified from the chromosomal DNA of *Spiroplasma melliferum* KC3 followed by restriction digestion and ligation at the NdeI and BamHI sites into a (His)₆ tag containing pHis17 vector as reported earlier (Chakraborty et al. 2024). Restriction free cloning (van den Ent and Lowe 2006) was used for cloning of untagged SmFtsZ construct using specific primers. A detailed list of plasmid constructs and oligonucleotide primers used in our study is given in Table 2. SmFtsZ^{MgCTAH} was created by replacing 45 base pairs from the 3' end of the untagged SmFtsZ sequence (i.e. 397-411 residues) with respective 69 base pairs of *M. genitalium* *ftsZ* using PCR (corresponding to residue numbers 347 to 366 of *M. genitalium* FtsZ). The amplified product was then inserted into (His)₆-SUMO-pET28a vector at the NdeI and BamHI sites by restriction digestion and ligation. The construction of SmFtsZ^{Mgnd-NTAH} was done using restriction free cloning in the (His)₆-SUMO-pET28a vector at the NdeI and BamHI site.

Untagged SmFtsZ was expressed in *E. coli* BL21-AI cells by 0.2% arabinose induction at OD₆₀₀ = 0.6-0.8 and was grown at 30 °C for 5 hours. Cells were harvested by centrifugation at 4,000 x g at 4 °C for 20 mins and cell pellet was flash-frozen in liquid nitrogen and stored at -80 °C until processed. On the day of purification, cell pellet was taken out from -80 °C and thawed in ice for 10 mins and then resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10% glycerol) followed by sonication to lyse the cells. The total lysate was spun at 30,000 xg for 1 hour to remove the cell debris and the clear supernatant was loaded into HiTrap Q HP 5 ml column (Cytiva) equilibrated with Buffer A50 (50 mM Tris-HCl pH 8.0, 50 mM NaCl). Protein was eluted in 5 mL fractions using a linear KCl gradient from 50 mM to 1000 mM over 20 column volumes. Peak fractions containing proteins were confirmed from SDS PAGE, collected and pooled together for dialysis against A50 buffer and loaded into MonoQ column (cytiva) to increase the purity. Finally, the pure fractions were confirmed from SDS PAGE gel and pooled for dialysis in HK50 buffer (50 mM HEPES-KOH adjusted pH 7.4, 50 mM KCl). After dialyzing for 2 hours, the protein was concentrated using centricon (with 10 kDa membrane cut off), flash-frozen in aliquots and stored at -80 °C.

SmFtsZ^{MgCTAH} (in pET28a SUMO vector) was expressed in *E. coli* Rosetta (DE3) pLysS at OD₆₀₀ = 0.5-0.6. by inducing with 0.6 mM IPTG and grown at 18 °C for 16 hours. Cells were harvested by centrifugation at 4,000 xg for 20 mins at 4 °C and cell pellet was flash-frozen in

liquid nitrogen and stored at -80 °C until purification. Cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM KCl, 10% glycerol) and sonicated to lyse the cells. The lysate was centrifuged at 30,000 xg at 4 °C for 1 hour and the supernatant was loaded to a 5 mL His-Trap column (GE), equilibrated with Buffer A (50 mM Tris-HCl pH 8.0, 200 mM KCl). Protein was eluted using a step gradient of 2%, 5%, 10%, 20%, 50% and 100% Buffer B (50 mM Tris-HCl pH 8.0, 200 mM KCl, 500 mM Imidazole). To cleave the N-terminal (His)₆-SUMO tag, peak protein fractions were pooled and Ulp1 protease was added in a 1:100 (protease:protein) molar ratio and dialysis was set up against the cleavage buffer (50 mM Tris-HCl pH 8.0, 200 mM KCl) for 10 hours at 4 °C. (His)₆-SUMO tag and uncleaved protein was removed by passing the dialysed sample mixture through a His-Trap column and flowthrough containing the SmFtsZ^{MgCTAH} protein was collected. Finally, the protein was dialysed in HK50 for 2 hours, concentrated using centricon (with 10 kDa membrane cut off), flash-frozen in aliquots and stored at -80 °C. SmFtsZ^{MgCTAH(M-CRAC)}, SmFtsZ^{Mgrd-NTAH} and Untagged SmFtsZ^{ACT} protein purifications were done using the same protocol as SmFtsZ^{MgCTAH}.

Table 3.1: List of primers and construct details

Oligo name	5'-3' sequence	Construct made	Vector backbone
SmFtsZ_vF	GTTTAACTTTAAGAAGGAGATATACAT ATGGACAATTTGATAATTATG	Untagged SmFtsZ	pHis17- Amp ^R
SmFtsZ_stop_vR	GCTTTTAATGATGATGATGATGATGGG ATCCTTACCAACTACGACGAACAAAT G	Untagged SmFtsZ	pHis17- Amp ^R
SmFtsZ-CTAH-RP1	CTTAACATATTTTCATGCCAAGTTCCTT CAGCTCATCTAAAAGTTTCAACTTATG TTCAACTACTTGATTATTA	SmFtsZ ^{MgCTAH}	pET28a- Kan ^R
SmFtsZ-CTAH-RP2	GCTTTTAATGATGATGATGATGATGGG ATCCGTAGATTTGGTTTTGGTGCTTAA CATATTCATGCCAAGTTC	SmFtsZ ^{MgCTAH}	pET28a- Kan ^R
MCRAC Rev1	GGTTCTGATGTTCAACAACCTTTCATGC CAAGTTTCTTCAGCTCATC	SmFtsZ ^{MgCTAH(M-CRAC)}	pET28a- Kan ^R
MCRAC Rev2	CTCGAATTCGGATCCTCAATAAATCTG GTTCTGATGTTCAACAAC	SmFtsZ ^{MgCTAH(M-CRAC)}	pET28a- Kan ^R
NTAH-SmFtsZ FP1	CAAAATCTTGATGAAATTATTAACGG TGGTGGTATGGACAATTTTGATAATTA TG	SmFtsZ ^{Mgrd-NTAH}	pET28a- Kan ^R
NTAH-SmFtsZ FP2	CACCGCGAACAGATTGGTGGCCATAT GAGCATAAACAAAATTCTTGATGAAA TTAT	SmFtsZ ^{Mgrd-NTAH}	pET28a- Kan ^R
SmFtsZ-314 pET RP	GTCGACGGAGCTCGAATTCGGATCCT CAATCAAACCCAGTCGCAATTACAG	SmFtsZ ^{Mgrd-NTAH} , Untagged SmFtsZ ^{ACT}	pET28a- Kan ^R

3.2.3 Liposome preparation and sedimentation assay

Liposomes were prepared by using synthetic lipids DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPG (1,2-dioleoyl-snglycero-3-phospho-rac-1-glycerol), DPG/cardiolipin (1,3-bis[1,2-dioleoyl-sn-glycero-3-phospho]- glycerol) and SM (sphingomyelin), all purchased as chloroform solutions from Avanti Polar Lipids, Inc. Cholesterol powder was purchased from Sigma. To prepare 4 mM stock liposomes, the required volume of individual components (as per molar ratio) was first taken in a glass tube. The chloroform was evaporated under a stream of nitrogen and further kept for drying at room temperature for 15 mins. A thin layer of lipids was visible at the bottom wall of the tube once the chloroform was dried. The dried lipid mixture was hydrated with the required volume of reaction buffer containing HK50. After vortexing well, the mixture became turbid and was extruded 15-20 times through a 100 nm pore-size polycarbonate membrane until the solution became translucent.

For liposome sedimentation assay, purified flash-frozen protein was taken out from -80 °C, thawed in ice for 10 mins and spun at 21,000 xg for 1 hour at 4 °C to remove any precipitation and supernatant was used in the assay. We set up a 100 µL reaction with 2 µM of protein and a varying concentration of liposome from 0 µM (i.e., no liposome control) to 1000 µM. The reaction mixture was incubated at 30 °C for 20 mins and spun at 21,000xg for 40 mins to pellet down the liposomes (for pelleting cholesterol containing liposomes, ultracentrifugation at 100,000 xg was used for 30 mins). Immediately after the spin, we took out the supernatant, washed the pellet with 100 µL of buffer and resuspended it in the same volume. Next, the supernatant and the pellet samples were mixed with 2X Laemmli buffer and equal amounts of both were loaded and run into a 12% SDS-PAGE gel followed by staining with Coomassie Brilliant Blue to check the presence of protein on the gel. Experiments were performed in triplicates and representative gel images are shown. The percentage intensities of protein bands in pellet and supernatant fractions were calculated as intensity of pellet (or supernatant) divided by sum of the two intensities. The intensity of the bands was quantified using the ImageJ (Schindelin et al. 2012) software by measuring the area of the respective bands and percentage of protein in pellet fractions were calculated by dividing the pellet band intensity by the sum of the pellet and supernatant band intensity. All the plots were generated in GraphPad Prism 8 software.

3.3 Results

3.3.1 Pneumoniae group (group 1E) of mycoplasmas possess amphipathic helix in FtsZ

One of the interesting findings of our analysis is the presence of a potential amphipathic helix in the FtsZ sequences of Pneumoniae group (group 1E in our analysis), where CCTP motifs for FtsA interaction were probably absent. Ten FtsZ sequences were identified to possess an amphipathic helix at the long N-terminal extension and the other two (*M. genitalium* and *M. pneumoniae*) at the C-terminal end (Figure 3.1, 3.2). The helical wheel diagram highlights the amphipathic nature of the proposed N-terminal and C-terminal helical regions (Figure 3.3a). We also calculated two parameters related to amphipathic helix, namely, hydrophobicity and hydrophobic moments for all the proposed amphipathic helices, and compared them with known membrane binding amphipathic helices. Importantly, a plot of the parameters for all the twelve proposed mycoplasma amphipathic helices occupied the same region as that of known amphipathic helices from FtsA, SepF and MinD, indicating a capability of these helices to bind membrane (Figure 3.3b).

Group 2 FtsZ sequences did not have N-terminal extensions, nor an amphipathic helix at their C-terminal end. We confirmed this from PSIPRED secondary structure prediction along with helical wheel plot (Figure 3.4). Among the seven species in the group 3 (where FtsA is present but FtsZ is absent) all except *M. mobile* and *M. cloacale* possessed an amphipathic helix at the C-terminal end of the FtsA sequences (Figure 3.5).

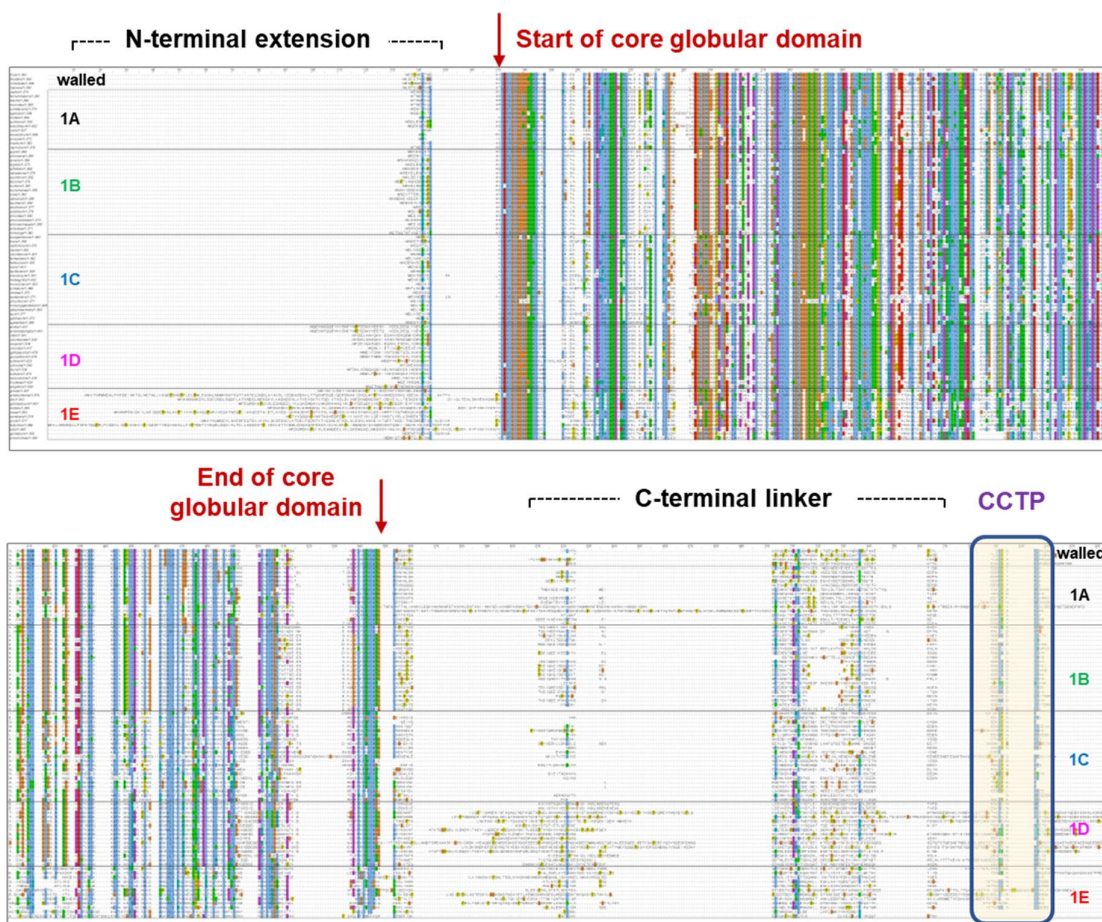


Figure 3.1 Sequence alignment of FtsZ proteins from group 1 species of mycoplasma

Multiple sequence alignment of FtsZ sequences highlights the differences in conservation at the C-terminal end compared to cell-walled bacterial FtsZs. Arrow indicates start and end of the globular core domain followed by a C-terminal disordered linker and CCTP. The CCTP region is highlighted within the yellow-shaded box. Multiple sequence alignment of mycoplasma FtsZ sequences and its homologs from cell-walled bacteria shows the presence of a longer N-terminal extension region in group 1D and 1E

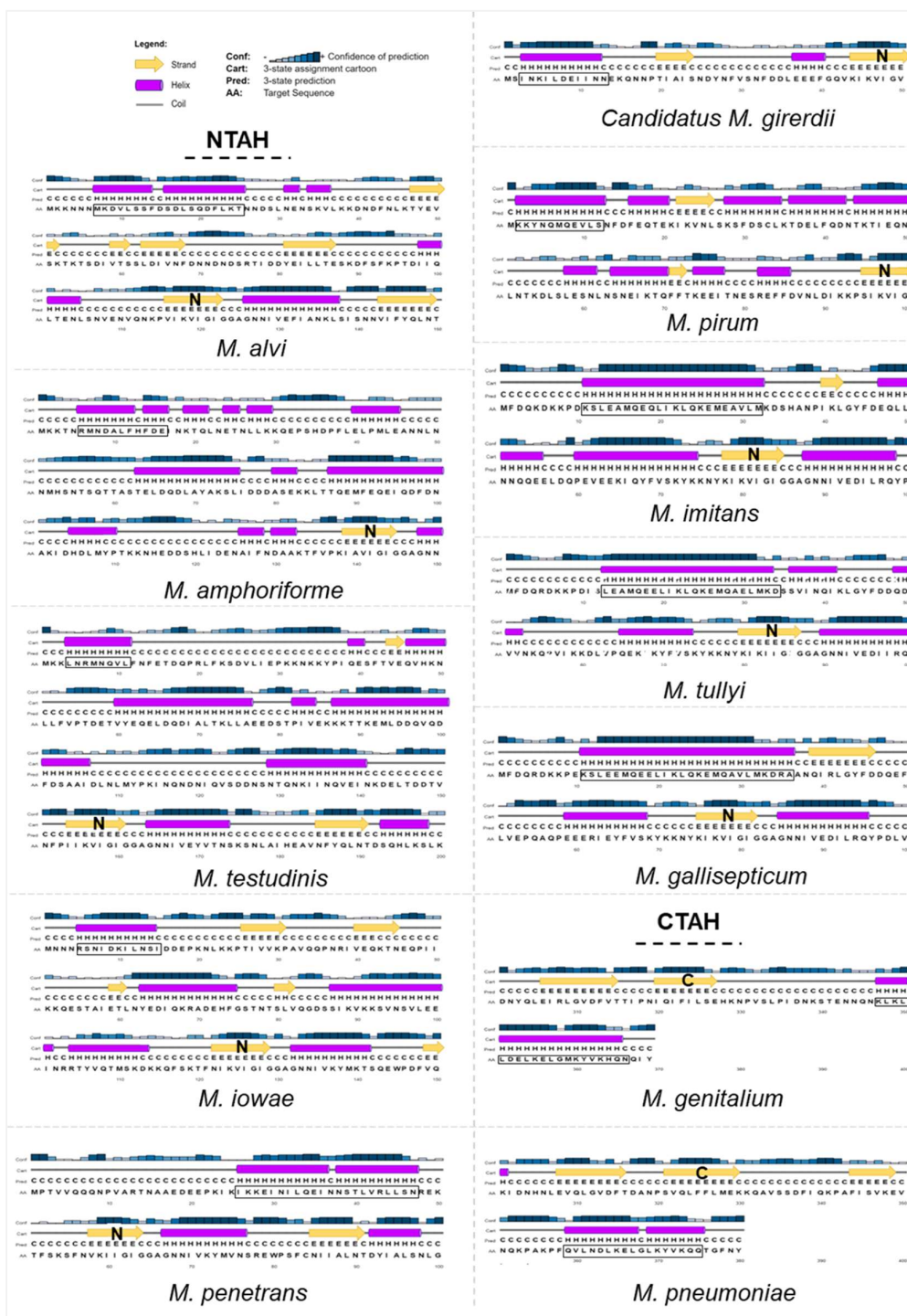
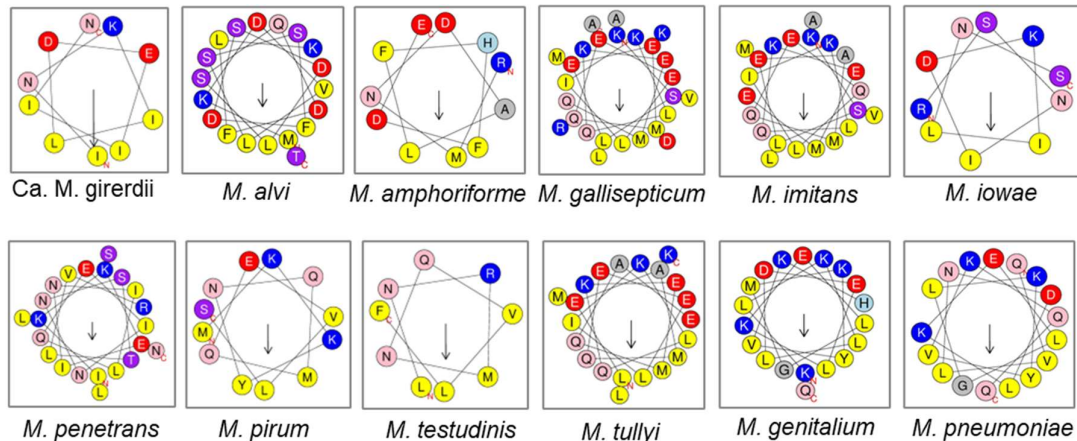


Figure 3.2 Prediction of secondary structures of N- and C-terminal extensions of FtsZ in group 1E mycoplasma species.

N-terminal amphipathic helix (NTAH) and C-terminal amphipathic helix (CTAH) containing FtsZ sequences are shown with secondary structures as predicted through PSIPRED. Helical regions with potential amphipathic nature are highlighted within the black rectangular box. The start and end of the globular domain of FtsZ are marked as 'N' and 'C' respectively.

a



b

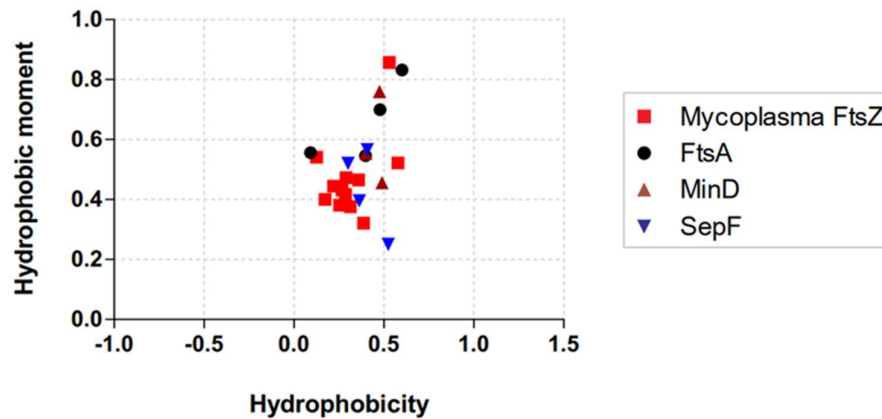
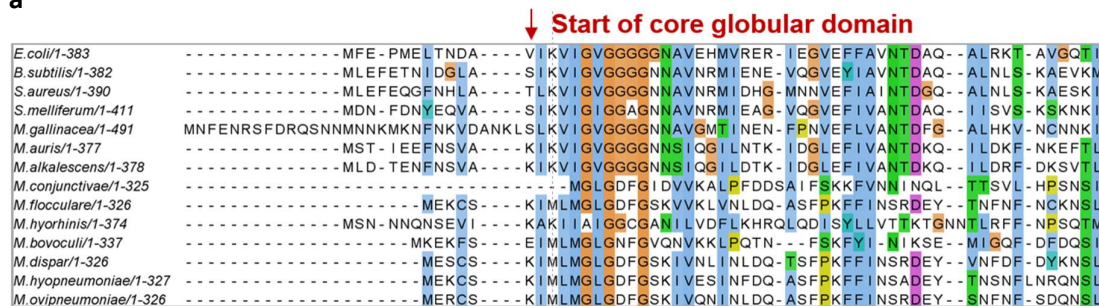


Figure 3.3 Mycoplasma FtsZ representative of Group 1E binds to membrane directly through an inherent amphipathic helix

(a) Helical wheel projections show the amphipathic nature of the helix-forming amino acids at the FtsZ terminals. Hydrophobic residues are depicted in yellow showing the amphipathic face. Arrows indicate the hydrophobic moments of the respective helices.

(b) Hydrophobic moment plot of the putative N-terminal and C-terminal α -helices of group 1E FtsZs. The hydrophobic moment is plotted against hydrophobicity to predict its potential to bind to the membrane. Known amphipathic helices from FtsA, MinD, and SepF also plotted simultaneously for comparison.

a



b

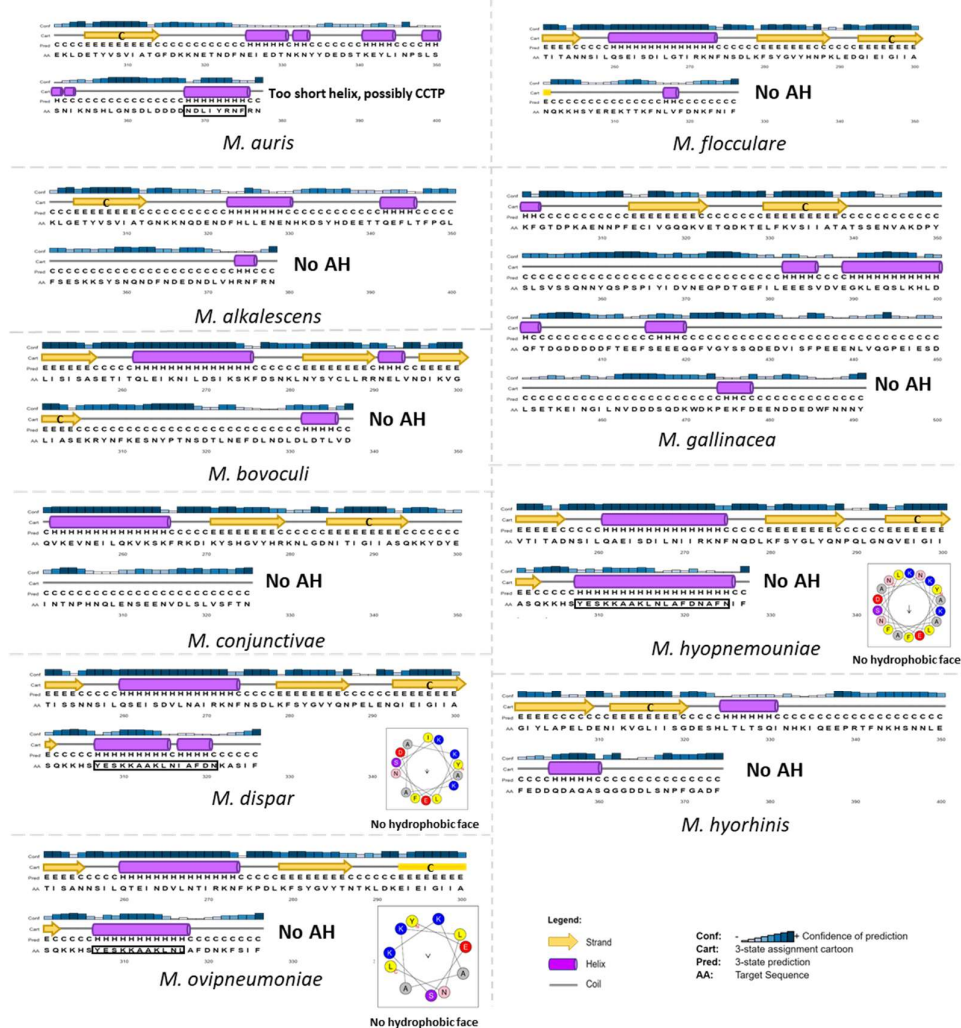


Figure 3.4 N- and C-terminal regions of group 2 FtsZ sequences showing absence of AH

(a) Multiple sequence alignment of group 2 FtsZ sequences shows the absence of N terminal extensions. Arrow indicates start of the globular core domain. (b) Secondary structures of the C-terminal regions of FtsZ sequence were predicted through PSIPRED. The black rectangular box highlights the helical region and were used for generating helical wheel plot (inset).

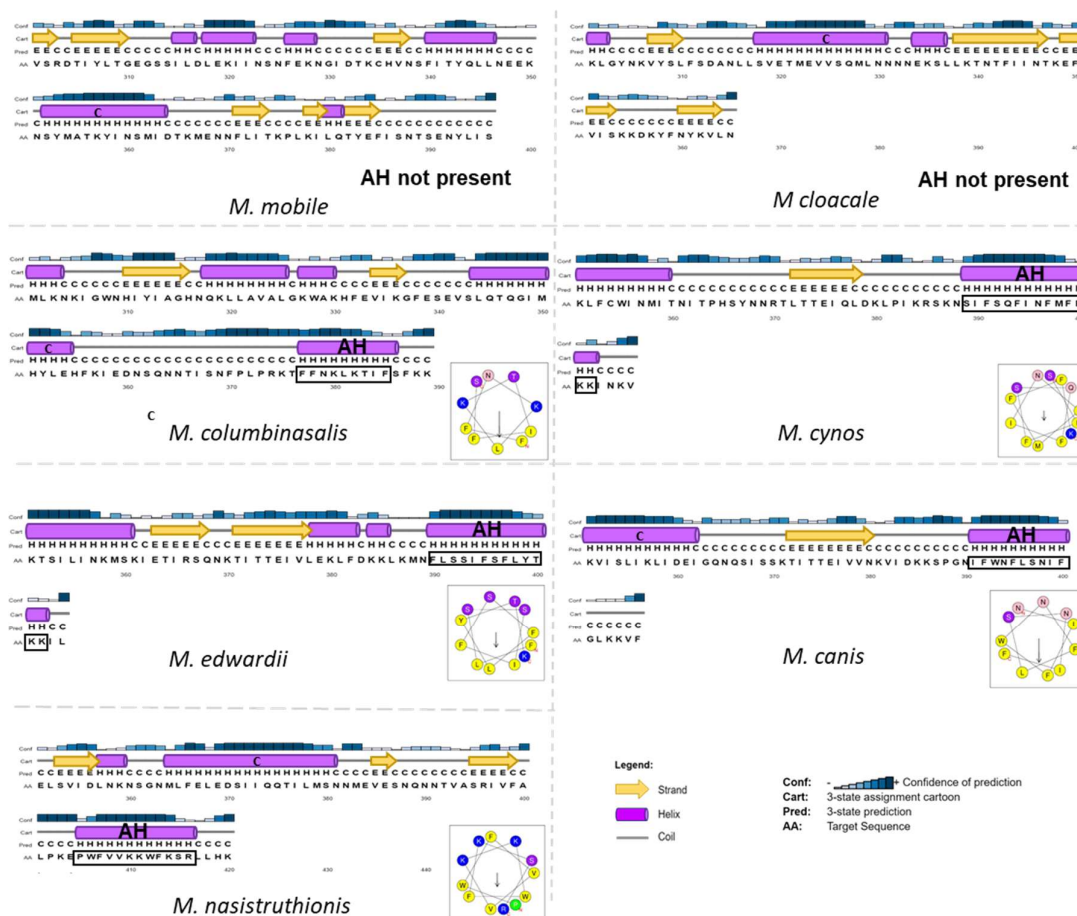


Figure 3.5 Prediction of secondary structure and amphipathic helix (AH) at the C-terminal end of FtsAs in group 3 mycoplasma species.

C-terminal amphipathic helix (CTAH) containing FtsA sequences are shown with secondary structures as predicted through PSIPRED. The end of the globular domain of FtsA are marked as 'C'. The black rectangular box highlights the helical region with potential amphipathic nature and were used for generating helical wheel plot (inset).

3.3.2 Prediction of membrane attachment strategies through FtsA and SepF in *Mycoplasma*

Because group 1E FtsZs lack the canonical CCTP motif and contain an inherent amphipathic helix, we checked whether an amphipathic helix is present in their respective FtsAs. All FtsA sequences (except *M. iowae* in which the FtsA sequence consists of the core globular domain only) from group 1E possess the canonical CTAH (Figure 3.6a). There were a few sequences from groups 1A and 1B which do not have amphipathic helix in their FtsA sequences,

compared to groups 1C and 1D in which most sequences of FtsA possess CTAH. We found that FtsAs from group 1A and group 1B without CTAH formed a separate branch in the phylogenetic tree. We next checked for the presence of the alternative membrane attachment adaptor SepF. Interestingly, five out of six SepF containing species lacked the membrane binding CTAH in their FtsAs (Figure 3.6b). We also checked for potential membrane binding region at the N-terminal end of the SepF sequences using helical wheel plot. Though no specific hydrophobic face or amphipathic helix was identified similar to that of *B. subtilis* SepF (Figure 3.7), there were multiple conserved hydrophobic amino acids (e.g. phenylalanine, tryptophan) that might be responsible for membrane binding (Figure 3.7b). So, FtsZs in mycoplasma groups plausibly anchor to the membrane in different ways: using its inherent amphipathic helix, via FtsA's amphipathic helix or through SepF when FtsA does not have a CTAH.

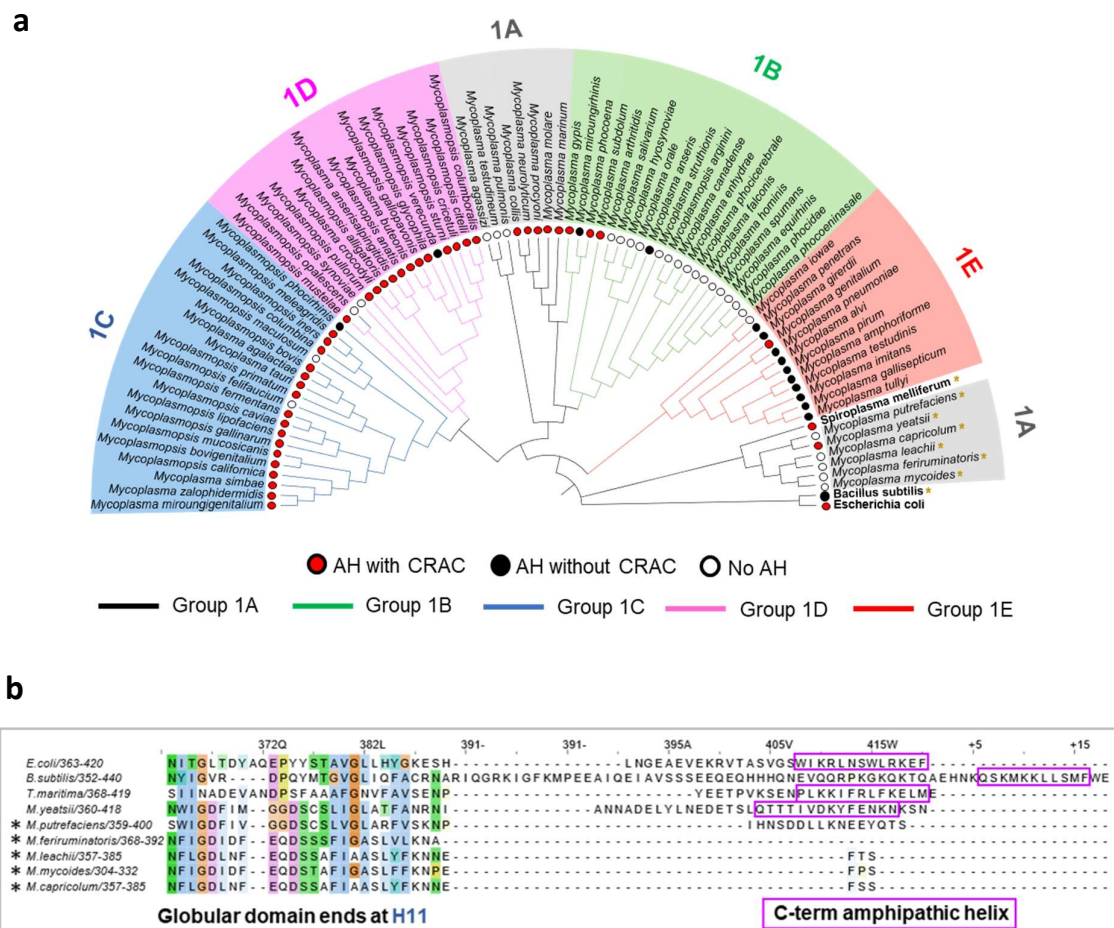


Figure 3.6 Prediction of amphipathic helix in FtsA

(a) A cladogram based on the FtsA sequences was constructed using the maximum likelihood method. The presence and absence of CTAH in FtsAs across mycoplasma species are depicted as filled and open circles respectively. Red color filled circle is used to show presence of CRAC motif in the respective CTAH. The branches of the organisms are colored according to their respective groups. Mycoplasma species that contain SepF protein are marked with yellow star (*).

(b) Sequence alignment of FtsA highlighting the absence of a C-terminal amphipathic helix for organisms marked as black colored star (*). Residues within the pink colored box represent canonical C-terminal amphipathic helix in cell-walled bacteria.

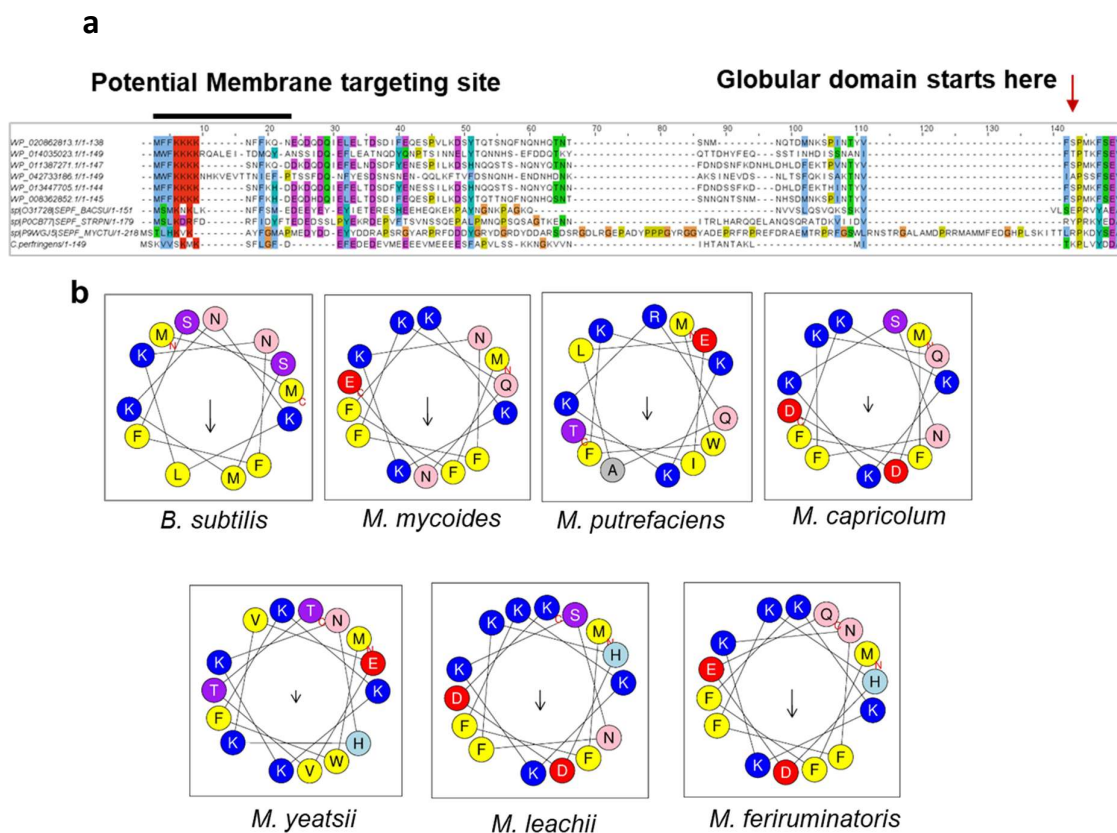


Figure 3.7 Prediction of amphipathic helix in mycoplasma SepF

(a) Multiple sequence alignment of mycoplasma SepF proteins showing potential membrane binding region at the N-terminal end similar to what is present in *B. subtilis*, *S. pneumoniae*, *M. tuberculosis* and *C. perfringens* SepF.

(b) Helical wheel diagram of predicted membrane binding sites showing the presence of hydrophobic residues. Known membrane binding regions of SepF in *B. subtilis* has been included for comparison.

3.3.3 *M. genitalium* FtsZ CTAH binds membrane *in vitro*

To experimentally verify the membrane binding ability of the proposed amphipathic helix in group 1E FtsZs, we chose *M. genitalium* FtsZ CTAH as a representative for the amphipathic helix at the C-terminal end. The 347-366 residues of *M. genitalium* FtsZ were attached at the C-terminal linker end (i.e. after 396 residue) replacing the CCTP of *Spiroplasma melliferum* (Sm) FtsZ to obtain a chimeric construct (named as SmFtsZ^{MgCTAH}) (Figure 3.8).

Liposomes were made with polar lipids (in molar ratio of 25% DOPG, 20% DOPC, 30% cardiolipin, and 25% spingomyelin) according to their relative abundance in mycoplasma membrane (Gaspari et al. 2020). Liposome sedimentation assay confirmed that SmFtsZ^{MgCTAH} bound liposomes. The protein was present in the pellet fraction which increased with increasing liposome concentrations (Figure 3.9). In contrast, for the wild type untagged SmFtsZ protein (where instead of *M. genitalium* FtsZ CTAH motif, *S. melliferum* FtsZ CCTP motif is present), no detectable protein band was observed upon pelleting in the presence of liposomes (Figure 3.9a). Furthermore, we checked the membrane binding ability of the N-terminal 12 amino acid stretch (1-12; N-terminal amphipathic helix (NTAH)) from *Ca. M. girerdii* FtsZ sequence. The NTAH sequence and a tri-glycine linker were included at the N-terminal of SmFtsZ globular domain (1-314 residues) to get the SmFtsZ^{Mgrd-NTAH} construct (Figure 3.8). Here again, we found NTAH sequence of *Ca. M. girerdii* bound liposomes, consistent with our prediction from sequence analysis (Figure 3.9 b, d)

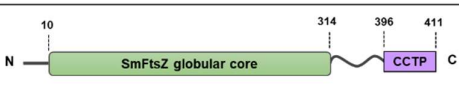
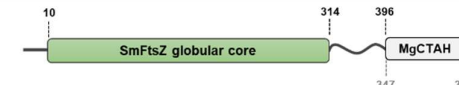
Construct name	Schematic of domain organization	Purpose
Untagged SmFtsZ		In vitro liposome pelleting assay as a negative control for membrane binding
SmFtsZ ^{MgCTAH}		In vitro liposome pelleting assay to check binding of MgCTAH motif
SmFtsZ ^{MgCTAH(M-CRAC)}		In vitro liposome pelleting assay to check binding of MCRAC motif
SmFtsZ ^{Mgrd-NTAH}		In vitro liposome pelleting assay to check binding of Mgrd NTAH motif
Untag SmFtsZ ^{ΔCT}		In vitro liposome pelleting assay as a negative control for Mgrd NTAH motif

Figure 3.8 Schematic of the constructs used in the study

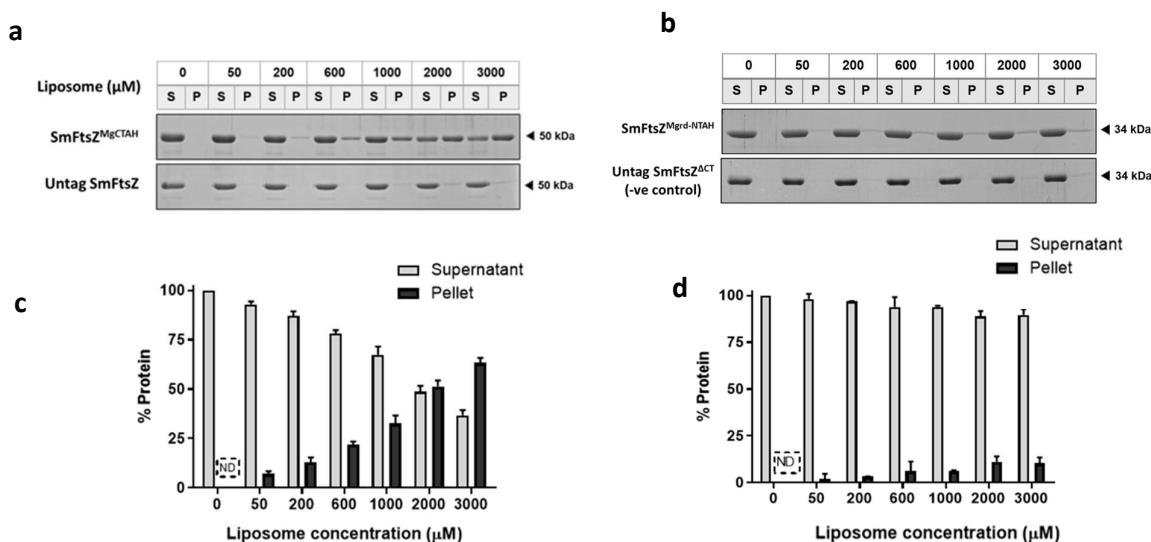


Figure 3.9 Liposome pelleting assay of SmFtsZ^{MgCTAH} and SmFtsZ^{Mgrd-NTAH}. Representative 12% SDS-PAGE gel showing the presence of (a) SmFtsZ^{MgCTAH}, Untagged SmFtsZ (negative control) and (b) SmFtsZ^{Mgrd-NTAH}, Untag SmFtsZ^{ΔCT} (negative control) in the supernatant (S) and pellet fraction (P) with increasing concentrations of liposomes from 0 μM (i.e. no liposome control) to 3000 μM. (c, d) Quantification plot for the gels. The fraction of protein was calculated from the SDS PAGE gel band intensity in the pellet fraction divided by the sum of band intensities in pellet and supernatant. Error bar denotes standard deviation calculated from two independent experiments. ND: Not detectable.

3.3.4 Sequence analysis identifies a cholesterol recognition motif in *M. genitalium* and *M. pneumoniae* FtsZ

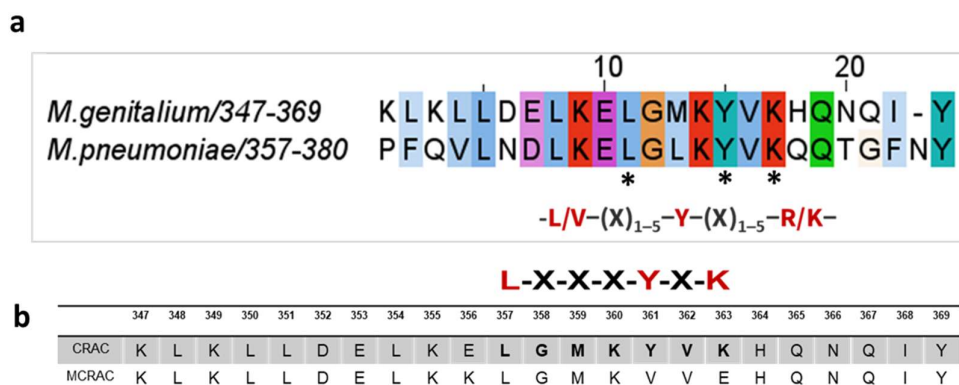
A careful analysis of the *M. genitalium* and *M. pneumoniae* FtsZ sequences identified a putative Cholesterol Recognition Amino acid Consensus (CRAC) motif located within the C-terminal helix (Fig. 3.10). The CRAC motif is defined as (L/V)-X₁₋₅-Y-X₁₋₅-(R/K) where “X” represents any residue. The central tyrosine has been shown to be essential for cholesterol binding (Jamin et al. 2005). We found that *M. genitalium* and *M. pneumoniae* FtsZs possessed the CRAC motifs L³⁵⁷-X-X-X-Y³⁶¹-X-K³⁶³ and L³⁶⁷-X-X-X-Y³⁷¹-X-K³⁷³ in their respective C-terminal ends (Figure 3.10a). These CRAC motifs may give specificity for binding to cholesterol and

thereby to the membrane in mycoplasmas, where 35-50% of total membrane lipid is cholesterol (Dahl 1993). Other than *M. genitalium* and *M. pneumoniae*, *M. alvi* and *M. pirum* FtsZs from group 1E contain CRAC/CRAC like motif in the NTAH region (Table 3.2).

We also checked the presence of the CRAC motif across all amphipathic helices in FtsAs. Among 56 FtsA AH motifs in Groups 1 and 3, 41 FtsAs were found to have a CRAC/CRAC like motif. The group wise distribution is as follows - Group 1A: 6 out of 6, Group 1B: 3 out of 5, Group 1C: 17 out of 18, Group 1D: 14 out of 15 and lastly only 1 out of 12 from group 1E (Figure 3.6a). To experimentally confirm the proposed CRAC motif confers a preference for cholesterol, we performed pelleting assay of SmFtsZ^{MgCTAH} construct with liposomes containing cholesterol (in molar ratio of 30% DOPG, 30% DOPC, 40% cholesterol). We then disrupted the proposed CRAC motif by mutating three residues E356K, Y361V and K363E (named SmFtsZ^{MgCTAH(M-CRAC)}) to see if it abolishes cholesterol specific binding (Figure 3.10b). The choice of the mutations was in such a way that the CRAC motif was disrupted while the hydrophobicity (CRAC: 0.280, MCRAC: 0.294), hydrophobic moment (CRAC:0.415, MCRAC: 0.427) and net charge (CRAC: +2, MCRAC: +2) remain similar to CTAH sequence.

We observed significantly increased membrane binding in liposomes containing cholesterol compared to liposomes made without cholesterol (i.e. 30% DOPG and 70% DOPC) (Figure 3.10c, d). Binding of both SmFtsZ^{MgCTAH} and SmFtsZ^{MgCTAH(M-CRAC)} was higher in presence of cholesterol. However, in the presence of cholesterol, SmFtsZ^{MgCTAH(M-CRAC)} showed significantly reduced membrane binding compared to SmFtsZ^{MgCTAH} (Figure 3.10c, d) while they both have similar binding to liposomes with no cholesterol content.

All purified proteins used in this study were run on a 12% SDS PAGE for checking the purity level and quality (Figure 3.11).



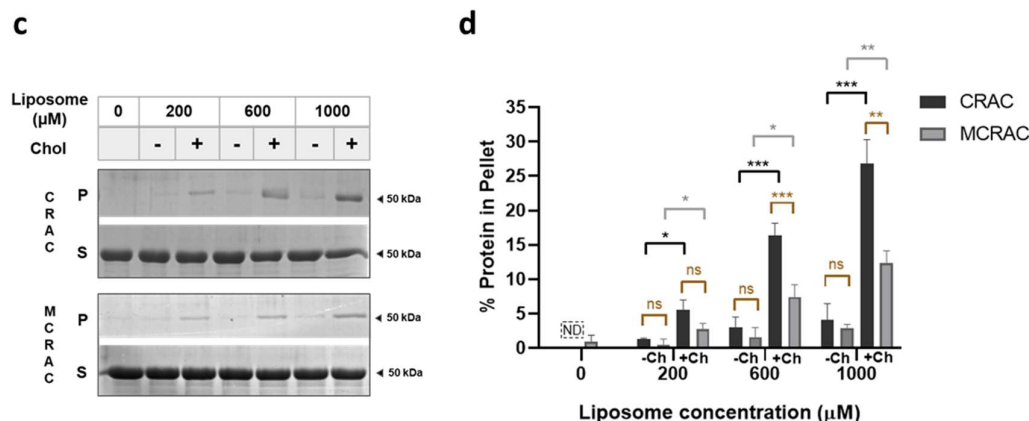


Fig. 3.10 Cholesterol Recognition Amino acid Consensus (CRAC) motif in *M. genitalium* FtsZ provides it a preference for cholesterol rich liposome binding

(a) Sequence alignment of C-terminal end region of *M. genitalium* and *M. pneumoniae* FtsZ shows the presence of CRAC motif specific conserved residues (marked with '*'). The consensus motif is mentioned below the alignment.

(b) Sequence of SmFtsZ^{MgCTAH} (denoted as CRAC) and SmFtsZ^{MgCTAH(M-CRAC)} (denoted as MCRAC) C-terminal motifs. The consensus amino acid sequence specific to CRAC motif are highlighted in bold. The mutated residues in MCRAC are underlined.

(c) Representative 12% SDS-PAGE gels showing cholesterol mediated induction of membrane binding in CRAC (i.e SmFtsZ^{MgCTAH}) and MCRAC (i.e. SmFtsZ^{MgCTAH(M-CRAC)}) in liposome pelleting assay. Liposome containing cholesterol (+Chol) has higher amount of protein in pellet fraction compared to the liposome without cholesterol (-Chol).

(d) Quantification of protein amount in pellet fraction in liposome pelleting assay. CTAH and MCRAC both show more binding with liposomes containing cholesterol. However, a significant reduction in binding to cholesterol was observed in case of MCRAC. +Ch and -Ch denote liposomes made with and without cholesterol respectively. Comparisons within CRAC and MCRAC are marked in black and grey color line respectively. Yellow line shows comparisons between CRAC and MCRAC. Error bars indicate standard deviation from three independent experiments. p values are obtained from an unpaired two tailed t-test using the function 'T.TEST' in Microsoft Excel. Significance level is denoted by *, p < 0.05; **, p < 0.01; ***, p < 0.005; ns, not significant; ND, not detected.

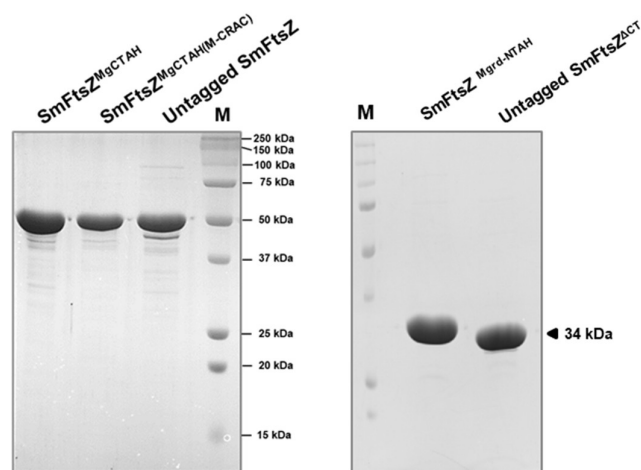


Figure 3.11 Representative 12% SDS PAGE gel showing the purity of final purified proteins used in this study

Table 3.2: Amphipathic parameters of Group 1E mycoplasma FtsZ CTAH/NTAH motifs

Organism	NTAH/CTAH sequence	Hydrophobicity	Hydrophobic moment	Is CRAC/CARC motif present?
<i>Candidatus Mycoplasma girerdii</i>	INKILDEIIN (3-12)	0.530	0.857	No
<i>Mycoplasma alvi</i>	MKDVLSFFSDLSQDFLKT (7-25)	0.313	0.376	Yes
<i>Mycoplasma amphoriforme</i>	RMNDALFHFDE (6-17)	0.287	0.417	No
<i>Mycoplasma gallisepticum</i>	KSLEEMQEELIKLQKEMQAV LMKDRA (11-37)	0.173	0.4	No
<i>Mycoplasma imitans</i>	KSLEAMQEQLIKLQKEMEAV LM (11-32)	0.359	0.465	No
<i>Mycoplasma iowae</i>	RSNIDKILNS (5-14)	0.125	0.541	No
<i>Mycoplasma penetrans</i>	IKKEINILQEINNSTLVRLLSN (26-47)	0.387	0.321	No
<i>Mycoplasma pirum</i>	MKKYNQMGEVLS (2-12)	0.220	0.445	Yes
<i>Mycoplasma testudinis</i>	LNRMNQVLF (4-12)	0.579	0.522	No
<i>Mycoplasma tullyi</i>	LEAMQEELIKLQKEMQAE LMK (13-33)	0.290	0.473	No
<i>Mycoplasma genitalium</i>	KLKLLDELKELGMKYVKHQ (347-366)	0.254	0.381	Yes
<i>Mycoplasma pneumoniae</i>	QVLNDLKELGLKYVKQQ (354-375)	0.268	0.432	yes

3.4 Discussion

Here, we provide evidence that a phylogenetically separate FtsZ clade (the “Pneumoniae” group) among mycoplasmas possesses an inherent amphipathic helix that might facilitate FtsA-independent membrane attachment. We experimentally show that putative CTAH of *M. genitalium* and NTAH from *Ca. M. girerdii* FtsZ bind liposomes. Also, when we expressed the chimeric protein containing *M. genitalium* CTAH linked to *E. coli* FtsZ in *E. coli* cells (FtsZ depleted condition), membrane localization was observed (Dutta et al. 2025, in collaboration with Dr. Ramanujam Srinivasan). This confirms the membrane binding ability of MgFtsZ^{CTAH} motif.

The possibility of direct membrane binding in cell division protein FtsZ, bypassing the requirement of additional adaptor proteins, is very relevant in the context of a minimal genome containing bacteria like mycoplasma. FtsZ with a membrane binding helix provides a minimal system for mechanistic insights into the process of cell constriction and division. It is interesting that eukaryotic organelle FtsZs have probably evolved a similar mechanism of an inherent amphipathic helix directly binding to the membrane, making the anchoring role of FtsA redundant. Recently eukaryotic chloroplast FtsZ1 from *Arabidopsis thaliana* has been shown to interact with membranes via its C-terminal amphipathic β -strand motif (Liu et al. 2022). Presence of amphipathic helix at the N-terminal extension of archaeal FtsZ based on sequence analysis has been reported earlier (Liao et al. 2021). Another report on a novel species of *Atribacteria* predicted a similar amphipathic helical region at the N-terminal extension of FtsZ (Katayama et al. 2020). However, the membrane tethering role of the proposed amphipathic helices from FtsZ has not been experimentally demonstrated till date.

We found the putative direct membrane attachment sequences in mycoplasma FtsZs either at the N- or C-terminal ends. The functional significance of such oppositely oriented membrane anchors from the perspective of FtsZ filament structure and requirements for mycoplasma cell division is currently not clear. Earlier, a chimeric construct of FtsZ fused to a membrane tethering sequence (from MinD protein) was shown to constrict tubular liposomes (Osawa et al. 2008). It has been also shown that changing the position of membrane targeting sequence in FtsZ from the conventional C-terminal end to N-terminal end results in convex bulge rather than concave depression (Osawa et al. 2009). In both cases, bending force could be generated leading to the tubulation of the liposomes *in vitro*. However, experiments with membrane targeting sequence attached to FtsZ did not show full constriction. Septation was

only sometimes observed upon addition of FtsA indicating a requirement of FtsA and its role beyond Z-ring anchoring (Osawa and Erickson 2013).

It is still an enigma how the other groups (group 2, 3 and 4) with only FtsZ (without an evident AH) or only FtsA (without FtsZ) undergo cell division. It is interesting that in the group 2, FtsA as well as the FtsA binding CCTP motif from FtsZ are lost. Moreover, in our sequence analysis we did not find the presence of an amphipathic helix which could anchor these FtsZs to the membrane. Thus, the role of FtsZs in group 2 organisms and the mechanism of action remains to be studied in future experiments. The most probable hypothesis is that these organisms could have different modes of cell division such as blebbing, cytofission etc. and not the conventional FtsZA mediated ring constriction.

M. genitalium and *M. pneumoniae* among mycoplasmas were additionally found to contain a CRAC motif within the C-terminal amphipathic helix region. It is interesting to observe CRAC motif in membrane anchoring motifs of bacterial species which possess a membrane composition with a high level of cholesterol. CRAC motif has been known to exist in numerous proteins spanning across eukaryotic and prokaryotic organisms (Jafurulla et al. 2011, Fantini et al. 2011, Boesze-Battaglia et al 2009). In bacteria, CRAC motif has been so far identified solely in pathogenic bacterial toxins that specifically bind to the cholesterol present in the membrane of eukaryotic host cells (Brown et al. 2013, Koufos et al. 2016). CRAC sequence containing peptides within bacterial leukotoxin has been shown previously to have higher affinity towards cholesterol (Koufos et al. 2016). Notably, the presence of a CRAC motif within FtsZ might be of significance as this could imply its membrane localization and subsequent role in cell division can be cholesterol dependent especially when 35-50% total mycoplasma membrane lipids is composed of cholesterol. We also checked if similar cholesterol recognition sequences are present in the other Mycoplasma FtsZs (containing N-terminal AH) and FtsAs (containing C-terminal AH) and did not find any. Thus, the membrane attachment of other AH containing FtsZ and FtsAs appear to follow distinct mechanisms. Future *in vivo* studies will possibly give us more information about how membrane cholesterol composition affects cellular functioning of mycoplasma divisome machinery.

Overall, our analysis highlights different possible modes of FtsZ-anchoring to the mycoplasma membrane, which are shown schematically in Figure 3.12a-e. Group 1A FtsZ binds to FtsA in a similar mechanism as found in cell walled bacteria with its canonical CCTP (Figure 3.12). Importantly, we find the presence of SepF within group 1A organisms where CTAH in their respective FtsAs are lost. This is possibly an instance where the role of SepF in membrane attachment and thus anchoring FtsZ to the membrane is maintained in the course of

evolution when FtsA has lost the membrane anchoring property. Similarly in mycobacteria, SepF acts as the membrane anchor for FtsZ and becomes an essential protein in this organism which lacks homologs of membrane tethers like FtsA or ZipA (Gola et al. 2015).

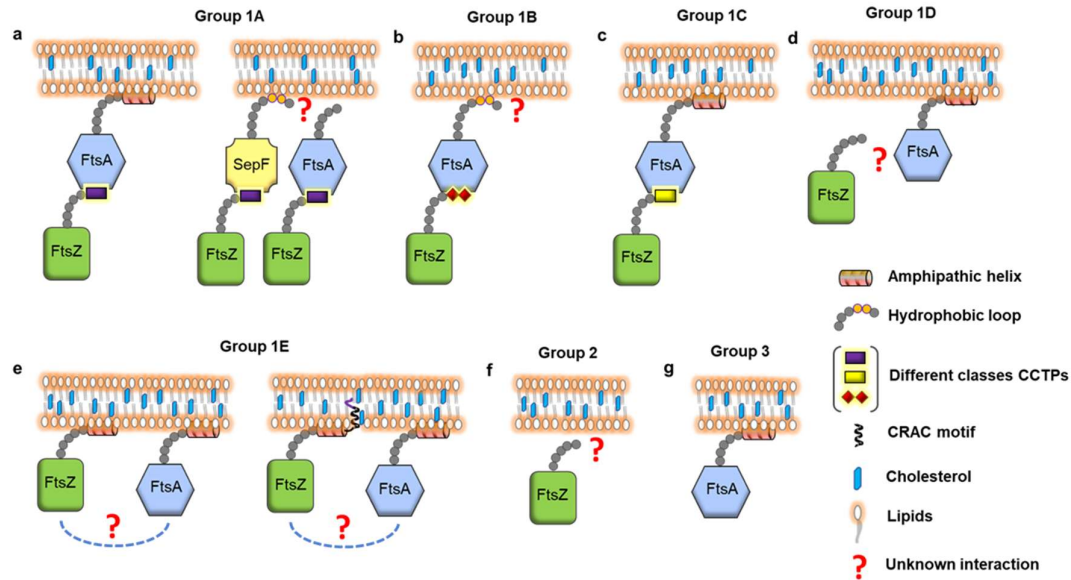


Figure 3.12 Modes of FtsZ-FtsA interactions across different mycoplasma groups

The possible modes of FtsZ-FtsA interaction and their membrane-binding potentials are shown schematically for different mycoplasma groups.

The co-occurrence of SepF with FtsZ has also been found in most archaea and its role in membrane attachment has also been established (Pende et al. 2021). Importantly, in mycoplasmas, SepF mediated membrane binding possibly occurs through hydrophobic loops rather than amphipathic helix. Next, groups 1B and 1C have deviant type of CCTPs for binding FtsA. However, because group 1B FtsAs do not possess a canonical amphipathic helix, their mechanism of membrane targeting is unclear (Figure 3.12b, c). It is possible that smaller motifs of one or two amino acids can contribute to membrane binding, as has been observed for DivIVA (Olivia et al. 2010) and bactofilins (Deng et al. 2019). Finally, group 1D and 1E do not have a consensus CCTP and experiments are needed to show whether the non-canonical CCTP can still bind FtsA (Fig 3.12d, e). Group 1E FtsZs with N-or C terminal amphipathic helix raises the possibility of direct membrane attachment making FtsA's membrane tethering

function dispensable. Based on our results here, we hypothesise that the C-terminal amphipathic helix in *M. genitalium* and *M. pneumoniae* FtsZ will help it to directly attach to the membrane. The CRAC motif contributes to the membrane binding dynamics in an organism with a large percentage of cholesterol in the membrane (Fig. 3.12e right). We believe our observations on membrane-targeting strategies of mycoplasma FtsZs set the stage for studying individual and cooperative roles of FtsZ and FtsA in ring constriction in the absence of cell wall synthesis machinery.

3.5 References

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Chapter 4

Characterization of cell division protein FtsA from *Spiroplasma melliferum*

4.1 Introduction

FtsA is one of the essential early divisome components involved in bacterial cell division. It serves as the primary membrane anchor for FtsZ in *E. coli* and many other model cell walled bacteria (Pichoff and Lutkenhaus 2002). FtsA interacts with FtsZ and helps the Z-ring filaments to attach to the inner membrane of the cytoplasm to initiate the formation of divisome assembly during constriction. FtsA belongs to the actin/HSP70 family of protein (Bork et al. 1992). Importantly, FtsA fold is different from canonical actin fold by a domain-swap-like arrangement of sub-domains (van den Ent and Lowe 2000). Structurally, it consists of a globular core domain of actin fold at the N terminal, followed by a disordered linker and a membrane binding C-terminal amphipathic helix (CTAH) which localizes FtsA and thus FtsZ filaments to the membrane (Pichoff and Lutkenhaus, 2005). In addition to the membrane anchoring role of the Z ring, FtsA is important for recruiting other divisome proteins which in turn activates the downstream constriction process (Corbin et al. 2004, Rico et al. 2004, Jensen 2005).

The role of FtsA in the division process can vary across different bacterial species. FtsA protofilament assemblies, similar to the actin family protofilaments, have been observed *in vitro* for *Thermotoga maritima* (Szwedziak et al. 2012), *Streptococcus pneumoniae* (Lara et al. 2005, Krupka et al. 2014), *Escherichia coli* (Krupka et al. 2017, Morrison et al. 2022), and *Vibrio cholerae* (Nag et al. 2020). However, no ATPase activity was observed for *P. aeruginosa* (Paradis-Bleau et al 2005), *T. maritima* (Szwedziak et al. 2012) and *S. pneumoniae* (Lara et al. 2005) FtsAs. *E. coli* and *B. subtilis* both show detectable ATP hydrolysis (Feucht et al. 2001). Interestingly, ATPase activity increased in the presence of phospholipids for *E. coli* (Conti et al. 2018) whereas no enhancement in activity was observed in a separate study when *E. coli* FtsA was assembled on lipid monolayers (Schoenemann et al. 2018). Furthermore, *in vitro* experiments suggested that the ATPase activity of FtsA is not required for Z-ring dynamics (Loose and Mitchison 2014). While a passive membrane anchoring role is important, it is not yet clear if FtsA plays an additional active role in divisome machinery involving its ATP hydrolysis or oligomerization. FtsA was found to form different oligomeric structures like mini-rings, arcs, short double filament and long linear polymers *in vitro* (Szwedziak et al. 2012, Schoenemann et al. 2018, Nierhaus et al. 2022). In *E. coli*, the active form of FtsA is proposed to be the short open-ended oligomers which can interact with FtsN to promote divisome activation (Nierhaus et al. 2022). In this scenario, more studies on the structural and functional

aspects of FtsA protein from diverse species are needed to understand its multifaceted role in cell division.

In this chapter, we aim to characterise *in vitro* the FtsA protein from *Spiroplasma melliferum*, a cell wall-less bacterium with minimal genome content. Importantly, only five genes namely, *mraZ*, *mraW*, *ftsA*, *ftsZ* and *sepF* comprise the division and cell wall (*dcw*) cluster in this species. Studying FtsA in a minimal system like spiroplasma will help us to better understand its role in the absence of other division proteins commonly present in many characterized cell walled bacteria. Here, we have cloned the *ftsA* gene for heterologous expression in *E. coli* and successfully purified it for *in vitro* characterization. We characterize this protein's ATPase activity and polymerization. We attempted to obtain insights into the role of ATP binding, if any, through structure predictions using AlphaFold and sequence and structure analysis. Our attempts contribute to the basis for future experiments to characterise the role of FtsA in FtsZ assembly, in the absence of other cell wall synthesis machinery.

4.2 Materials and methods

4.2.1 Cloning

ftsA gene was PCR amplified from the chromosomal DNA of *S. melliferum* KC3 followed by restriction free cloning to insert into a (His)₆ tag containing pHis17 vector between NdeI and BamHI sites. In the *S. melliferum* genome, TGA codes for tryptophan whereas it is a stop codon for *E. coli* genome. Therefore, to use *E. coli* as a heterologous expression system for spiroplasmas genes, TGA stop codons have to be changed to the codon corresponding to tryptophan in *E. coli*. Using specific primers and restriction free cloning we changed three TGAs to TGGs. Finally, the codon optimized gene was subcloned into (His)₆-SUMO-pET28a vector using gene specific primers and vector specific overhangs by restriction free cloning.

A detailed list of plasmid constructs and oligonucleotide primers used in our study is given in Table 4.1.

Table 4.1: List of primers used in this study

Oligo name	5'-3' sequence	Construct made	Vector backbone
SmFtsA_FP	ATGGATTATAAGTTAAAAGAA GTATATGC	SmFtsA-H ₆	pHis17-Amp ^R
SmFtsA_RP	TTTAATATTTTGGTAGTATTTT TG TTG	SmFtsA-H ₆	pHis17-Amp ^R
W194/211_F P	GTTTAGTCTTGCGTGGAATAT TGCATCACCAGTTGATTTGCA AAATGGGATTACAATTATTAA TTGGGATTATG	SmFtsA-H ₆	pHis17-Amp ^R
W362_R	GCAAAAGAAACATGGTGTACT GGC	SmFtsA-H ₆	pHis17-Amp ^R
SmFtsA_VF	GTTTAACTTTAAGAAGGAGAT ATACATATGGATTATAAGTTA AAAGAAGTATATGC	SmFtsA-H ₆	pHis17-Amp ^R
SmFtsA_VR	GCTTTTAATGATGATGATGAT GATGGGATCCTTTAATATTTT GGTAGT	SmFtsA-H ₆	pHis17-Amp ^R
SmFtsA SUMO FP	CACCGCGAACAGATTGGTGGC CATATGGATTATAAGTTAAAA GAAGTATATGC	SmFtsA	pET28a-Kan ^R
SmFtsA SUMO RP	GCTTGTGCGACGGAGCTCGAAT TCGGATCCTTATTTAATATTTT GGTAGTATTTTG	SmFtsA	pET28a-Kan ^R
SmFtsZ (CTAH)FtsA RP	AATATTTTGGTAGTATTTTGT TGATATTGTTGTGAACCATGT TCAACTACTTGATTATT	SmFtsZ396 ^(439 CTAH FtsA)	pET28a-Kan ^R
SmFtsZ-FtsA FLCTAH FP	GTAAGTGTAAATTGCGACTGGG TTTGATAATATTCAATCGGTT GATCATCG	SmFtsZ314 ^(383 CT FtsA)	pET28a-Kan ^R
FtsA FLCTAH pETSUMO RP	GTCGACGGAGCTCGAATTCGG ATCCTCATTTAATATTTTGGTA GTATTTTGTG	SmFtsZ314 ^(383 CT FtsA)	pET28a-Kan ^R

SmFtsA 382 STOP pET Rev	GTCGACGGAGCTCGAATTCGG ATCCTCATGTTGCACTAACTTT ATTGTC	SmFtsA ^{ΔCT}	pET28a-Kan ^R
D212G FP	CAATTATTAATTGGGGTTATG ATAATATTACCG	SmFtsA ^{ΔCT D212G,E328G}	pET28a-Kan ^R
E328G RP	CCACTAATTCGTAAAATACCA CCAACAACAACCGC	SmFtsA ^{ΔCT D212G,E328G}	pET28a-Kan ^R

4.2.2 Expression and protein purification

SmftsA-(His)₆ was expressed in *E. coli* BL21-AI cells by inducing with 0.2% arabinose OD₆₀₀=1-1.2 and was grown at 18 °C for 16 hours. Cells were harvested by centrifugation at 4,000xg at 4°C for 20 mins and the cell pellet was flash-frozen in liquid nitrogen and stored at -80°C until processed. On the day of purification, 2 L cell pellet was taken out from -80 °C and thawed in ice for 10 mins and then resuspended in 80 mL lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 10% glycerol) followed by sonication to lyse the cells. Two cycles of sonication (3 min each with 1 sec ON and 3 sec OFF) was performed with a 5 min gap after each cycle. The total lysate was spun at 30,000 xg for 1 hour to remove the cell debris and the clear supernatant was loaded into a 5 mL Ni-NTA column pre-equilibrated with Buffer A (50 mM Tris-HCl pH 8.0, 200 mM NaCl). Protein was eluted using a step gradient 5%, 10%, 20% and 100% Buffer B (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 500 mM Imidazole). For each concentration of buffer B, a total of 40 mL (corresponding to 8 column volumes) was collected as 5 mL fractions. The eluted fractions (without heat denaturing during SDS PAGE sample preparation; as this possibly makes the proteins to form aggregates which gets stuck in the gel well) were run on a 12% SDS PAGE and stained with Coomassie blue to visualize the proteins. Next, the protein was concentrated using a 10 kDa centricon till 1 mL and injected into Superdex 200 column pre-equilibrated with HK50 (50 mM HEPES pH 7.4, 50 mM KCl) buffer. The eluted fractions were concentrated and stored in 10 µL aliquots at -80 °C until further use.

SmftsA (with N-terminal (His)₆-SUMO) was expressed in *E. coli* BL21-DE3 cells by inducing with 1 mM IPTG at OD₆₀₀ = 1-1.2 and was grown at 18 °C for 16 hours. The first of Ni-NTA affinity chromatography step was the same as described for SmFtsA. Next, for cleaving the N-terminal (His)₆-SUMO tag, peak Ni-NTA protein fractions were pooled and Ulp1 protease was added approximately in a 1:100 (protease:protein) molar ratio. The cleavage reaction was set up with the eluted pooled proteins in 15 mL centrifuge tubes each containing 10 mL protein

and incubated for 1.5 hours in ice with intermittent gentle mixing. We observed that after SUMO cleavage the untagged SmFtsA protein still binds to the Ni-NTA column, so we did not pass it through Ni-NTA to separate SUMO. Instead, the cleaved protein was precipitated with 35% ammonium sulfate, resuspended in 2 mL HK50 buffer and loaded onto Superdex75 column to separate SUMO tag and increase purity. Fractions corresponding to a monomeric peak were pooled and flash frozen and stored at -80 °C until further use.

The purification protocol for SmFtsA382 and SmFtsA382^{D212G,E328G} is the same as SmFtsA.

4.2.3 Thermal Shift assay

Thermal shift assay was used for checking protein stability in the storage buffer conditions. In a reaction volume of 25 μ L containing 2 μ M of protein with different conditions of buffers was mixed. Then SYPRO Orange dye was added to a final concentration of 5X in the reaction mixture (5000X SYPRO Orange dye stock solution was available from Sigma-Aldrich, USA). Once the dye was added, the reaction was put in a 96-well PCR plate, sealed with sealing tape and short spun for proper mixing. Bio-Rad CFX96 real-time PCR machine was used to monitor the fluorescence of SYPRO Orange. The reaction was then heated from 4 °C to 90 °C with an increment of 1.2 °C per min with an initial incubation at 4 °C for 10 mins. Readings were recorded in every 0.4 °C. T_m was estimated from the minima by plotting the change in fluorescence with temperature ($-dF/dT$) versus temperature.

4.2.4 Pelleting Assay with nucleotides

Protein was first spun at 100,000xg at 4 °C to remove any precipitate. 50 μ L of reaction was set up in HK50 buffer with 10 μ M of the protein, 5 mM MgCl₂ and 1 mM of the respective nucleotides. Then reaction mixtures were incubated at 30 °C for 10 minutes, and centrifuged at 100,000xg at 30 °C for 15 minutes. The supernatant was taken out and the pellet was washed with HK50 buffer. Pellet was then resuspended in 50 μ L of 5X TGS buffer. Samples for the supernatant and pellet fractions were mixed with 2x Laemmli buffer and run on 12 % SDS-PAGE gel.

4.2.5 Liposome preparation and cosedimentation assay

Liposomes were prepared by using synthetic lipids DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPG (1,2-dioleoyl-snglycero-3-phospho-rac-1-glycerol), Cardiolipin (1,3-bis[1,2-dioleoyl-sn-glycero-3-phospho]- glycerol) and SM (sphingomyelin) in as per *S. melliferum* lipid composition in 7:43:12:38 mol% ratio respectively (Davis et al. 1985). All the lipid stocks were purchased as chloroform solution from Avanti Polar Lipids, Inc. To prepare a 2 mM stock liposome, the required volume of individual components (as per molar ratio) was first taken in a glass tube. The chloroform was evaporated under a stream of nitrogen and further kept for drying at room temperature for 15 mins. A thin layer of lipids was visible at the bottom wall of the tube once the chloroform was dried. The dried lipid mixture was hydrated with the required volume of reaction buffer containing HK50. After vortexing well, the mixture became turbid and it was extruded 15-20 times through a 100 nm pore-sized polycarbonate membrane until the solution became translucent.

For liposome sedimentation assay, purified flash-frozen protein was taken out from -80 °C, thawed in ice for 10 mins and spun at 100,000xg for 30 mins at 4 °C to remove any precipitation and supernatant was used in the assay. We set up a 100 µL reaction with 6 µM of protein and a varying concentration of liposome from 0 µM (i.e., no liposome control) to 1000 µM. The reaction mixture was incubated at 30 °C for 20 mins and spun at 100,000xg for 30 mins to pellet down the liposomes. Immediately after the spin, we took out the supernatant, washed the pellet with 100 µL of buffer and resuspended it in the same volume. Next, the supernatant and the pellet samples were mixed with 2X Laemmli buffer and equal amounts of both were loaded and run into a 12% SDS-PAGE gel followed by staining with Coomassie Brilliant Blue to check the presence of protein on the gel.

4.2.6 Negative staining TEM

For visualizing protein sample under TEM, 5 µL of reaction samples was loaded on a glow discharged (15 mA for 25 sec) carbon coated copper grid and incubated for 2 minutes before blotting out the protein from the side. Then 5 µL 1.5% (w/v) uranyl acetate solution was added on the grid and blotted after 30 seconds. Grid was air-dried and imaging was performed in TEM (JEM- 2200FS Jeol Ltd.) operated at 200 kV.

4.2.7 GTPase/ATPase activity measurement

The GTPase/ATPase activity was measured using malachite green assay (Feng *et al.*, 2011). The protein was spun at 21,000 xg at 4 °C and 20 µL reaction was set with the required concentration of protein in HK50 reaction buffer, 2 mM of GTP or ATP, and 5 mM MgCl₂. The reaction mixture was incubated at 30 °C for 15 minutes and stopped by addition of 100 mM of EDTA (5 µL of 0.5 M of EDTA). The reaction mixture was added to a 96 well plate and 100 µL of the malachite green mixture was added into the reaction mixture. After 10 minutes of incubation, absorbance was measured at 630 nm wavelength. A set of phosphate solutions of known concentrations was prepared which served as standard for phosphate concentrations.

4.2.8 Sequence analysis and structure prediction

FtsA sequences from different bacterial species were retrieved from UNIPROT KB database and multiple sequence alignment was performed and aligned in Jalview (Waterhouse et al. 2009) using MUSCLE tool (Edgar 2004). Sequence logos were generated from multiple sequence alignment using WebLogo (Crooks et al. 2004). AlphaFold 3 (Abramson 2024) was used for sequence-based structure prediction of *T. maritima* and *S. melliferum* FtsA sequences with ATP as ligand. Structures were visualized in ChimeraX software. For helical wheel plot and amphipathic parameters, we used HeliQuest server (Gautier et al. 2008).

4.3 Results

4.3.1 Purification of SmFtsA constructs

SmFtsA-His₆ (hexa histidine tag at the C-terminal end) was purified from 2L culture pellet using two steps - Ni NTA affinity chromatography and size exclusion chromatography. The details of buffer compositions are mentioned in the material and methods section. In the Ni NTA affinity chromatography, the protein was eluted in 100% of buffer B fractions. As the protein did not get eluted before 100%, we washed with 20% buffer B to remove impurities. This was followed by eluting in 100% buffer B to minimize the elution volume as the protein tends to precipitate during the concentrating step (Figure 4.1a). In gel filtration using Superdex

200 column, the protein eluted majorly at 17.5 mL with a minor peak 19.5 mL (Figure 4.1b, c). Notably, the elution volumes do not correspond to the molecular weight of SmFtsA i.e. 51 kDa. The unusual gel filtration profile of SmFtsA could be because of the presence of a long disorder linker (about 70 aa) in the protein. The final concentrated protein was run on a 12% SDS PAGE for a purity check. Final protein concentration was 0.8 mg/mL, total 400 μ L (yield = 0.32 mg) (Figure 4.1d). Thermal shift assay of SmFtsA showed more stability in lower pH buffer (50 mM HEPES pH 6.5, 50 mM KCl), melting temperature 62 °C compared to higher pH Tris NaCl buffer (50 mM Tris pH 8.0, 200 mM NaCl), melting temperature 58 °C (Figure 4.1e). The effect of salt and nucleotides in the folding of the protein was minimal as no significant changes in the melting temperature were observed in the thermal shift assay (Figure 4.1f, g).

The SmFtsA-H₆ construct contains a (His)₆ tag at the C-terminal end for the purpose of affinity-based purification. We decided to make an untagged construct as one of our objectives was to check membrane interactions and any C-terminal tag could affect the interaction. Thus, we cloned *SmftsA* gene in a His-SUMO containing pET vector as described in the materials and methods. SUMO-SmFtsA was purified from 2L culture pellet using three steps:- Ni NTA affinity chromatography, ammonium sulphate precipitation followed by size exclusion chromatography. The details of buffer compositions are mentioned in the material and methods section. In the affinity chromatography, the protein was eluted majorly in 50% of buffer B fractions. The sizes of SmFtsA protein and SUMO tag are 51 kDa and 12 kDa respectively adding to 63 kDa which runs between 75 kDa and 50 kDa marker as visualized in the 12% SDS gel (Figure 4.2a). Peak fractions (15th-22nd fraction) were pooled and SUMO cleavage reaction was set up. During 1.5 hours of incubation with Ulp1 protease, the SUMO tag was completely cleaved and ammonium sulphate precipitation was performed with 35% saturation concentration. The SUMO tag mostly got separated in the supernatant leaving the untagged SmFtsA protein in the pellet fraction. Ammonium sulfate precipitation step greatly saved concentrating step thus reducing precipitation and increasing yield. The pellet was resuspended in 1 mL HK-50 buffer and injected into the Superdex 75 column to increase purity. The protein was eluted in a single peak at 10.1 mL corresponding to monomeric size (Figure 4.2b, c). The peak fractions (18th-25th fractions, each of 0.5 mL volume) was pooled and concentrated till 6 mg/mL to a total volume of 500 μ L. The final concentrated protein was run on 12% gel for checking purity (Figure 4.2d). We got a relatively good yield (3 mg from 2 liter of bacterial culture) and the protein purity was good. This protein (purified by SUMO cleavage is called

SmFtsA henceforth) was used in further characterization. Thermal shift assay was performed with this protein in HK50 buffer which showed a melting temperature of 59 °C indicating its stability in the HK50 buffer (Figure 4.2e).

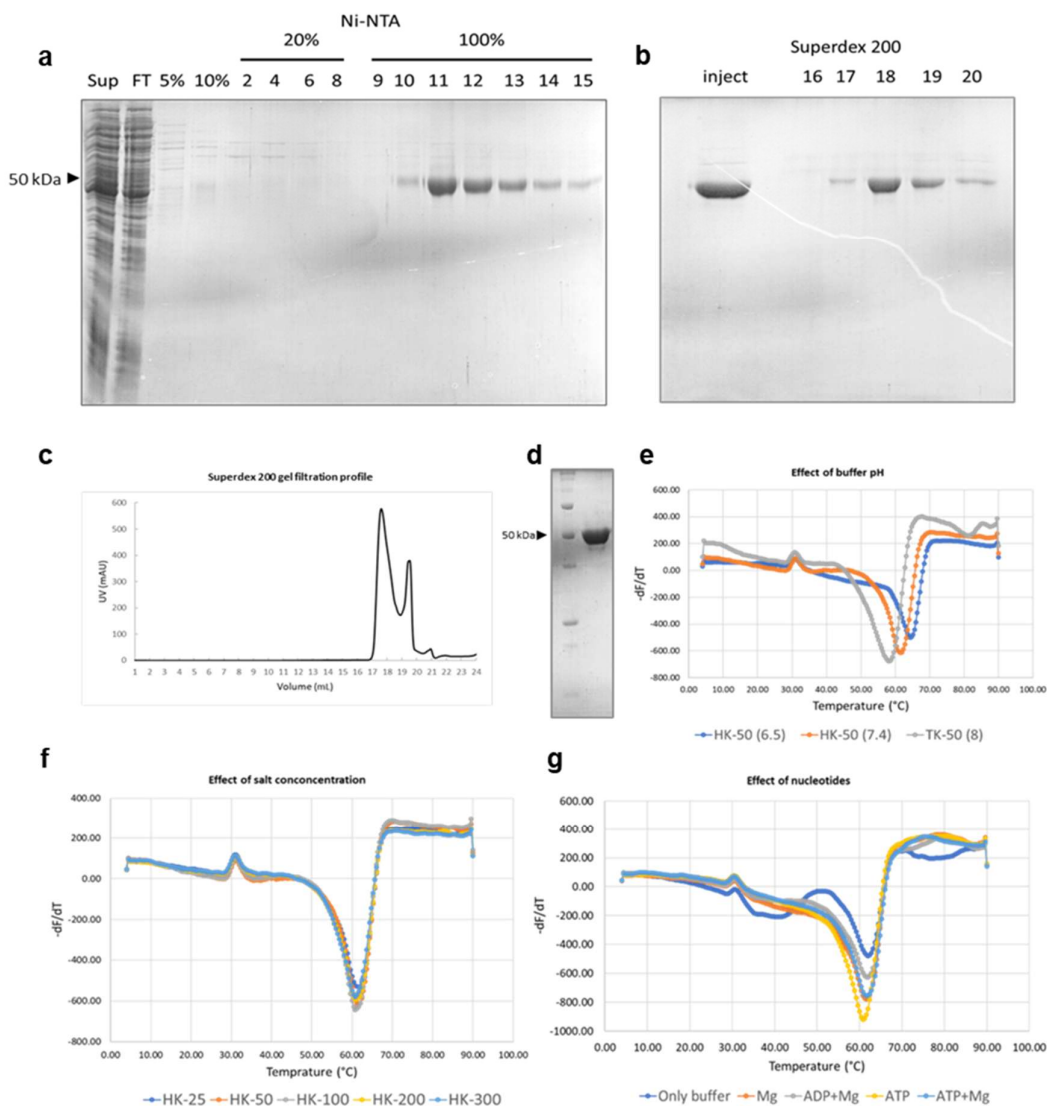


Figure 4.1 Purification and thermal shift assay of SmFtsA-H₆. (a) Ni-NTA affinity chromatography and (b) Superdex 200 gel filtration gels showing the elution fractions for SmFtsA-H₆. (c) Superdex 200 gel filtration profile of SmFtsA protein showing elution peak at 17.5 mL and 19.5 mL (d) 12% SDS PAGE gel for protein purity check. (e, f, g) Thermal shift assay profile of SmFtsA-H₆ with different buffer pH, salt concentrations and nucleotides.

4.3.2 Polymerization of SmFtsA with different nucleotides

Ultracentrifugation based pelleting assay was performed to check polymerization potential of SmFtsA in the presence of different nucleotides. We did not observe protein in the pellet fraction in different nucleotide conditions like ATP (and its non-hydrolysable analog AMPPNP) or GTP (Figure 4.2f). To confirm that the protein indeed does not form filaments in the above conditions, negative staining transmission electron microscopy (TEM) grids were prepared for different conditions and checked under TEM. Here again, we did not observe any regular filament type organization on the grid. Protein seemed to form aggregate-like structures in the presence of ATP and GTP (Figure 4.3).

4.3.3 ATPase/ GTPase activity assay

Next, we checked the ATP and GTP hydrolysis ability of SmFtsA protein using malachite green based colorimetric assay. We did not observe any GTPase or ATPase activity for SmFtsA in our buffer condition. ScMreB5 (Pande et al. 2022) and SmFtsZ (Chakraborty et al. 2024) were kept for ATPase and GTPase control respectively (Figure 4.2 g). As previously reported by Conti et al. 2018, *E. coli* FtsA show rapid ATP hydrolysis in the presence of lipid compared to without lipid condition. We tested ATPase activity of SmFtsA in the presence of liposomes (made of polar lipid mix equivalent of *S. melliferum* i.e. 7:43:12:38 mol% ratio (Davis et al. 1985)) but we did not see any activity.

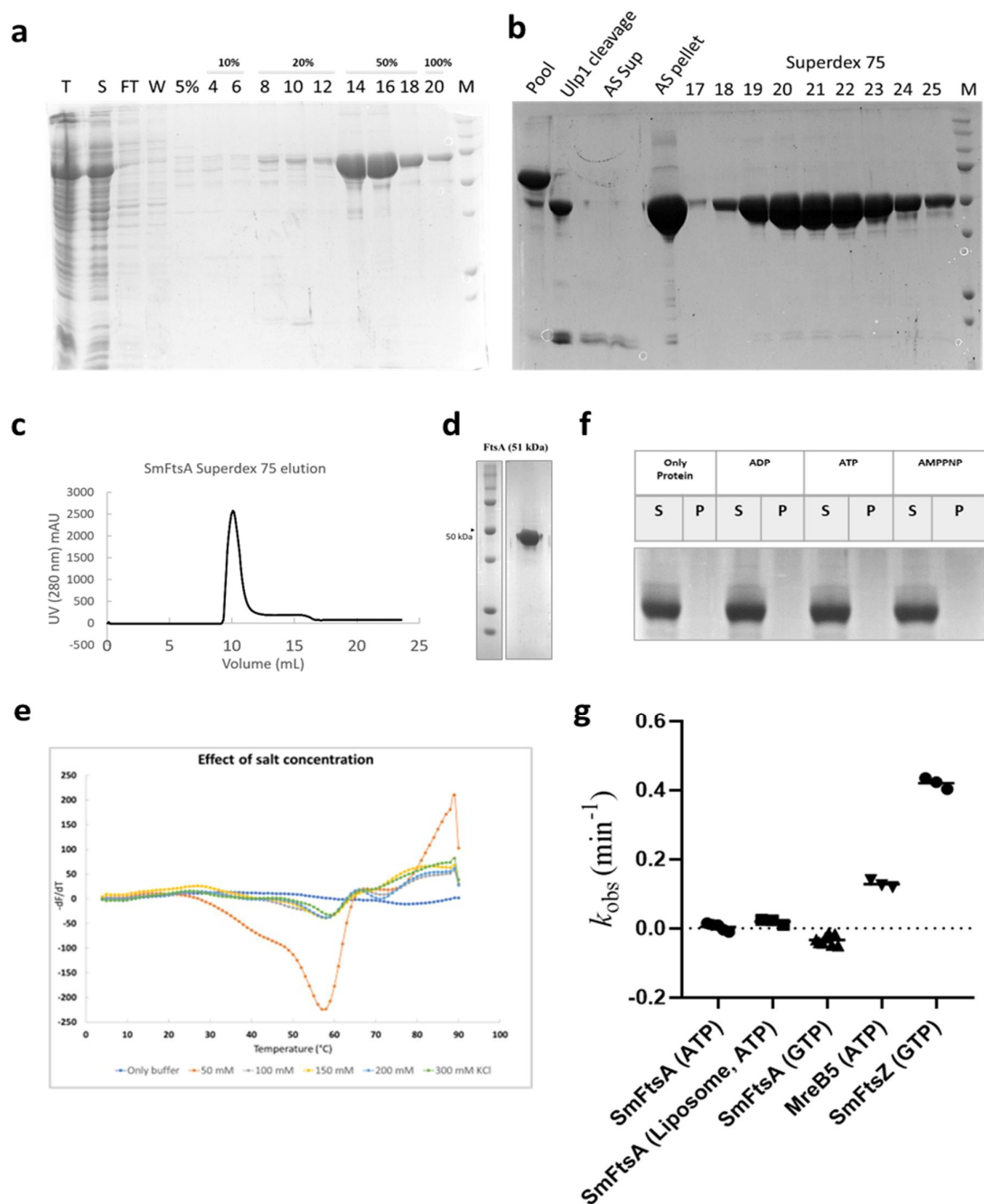


Figure 4.2 Purification of SmFtsA and assay for characterization (a) Ni-NTA affinity chromatography and (b) Superdex 75 gel filtration gels showing the elution fractions of SmFtsA. (c) Superdex 75 gel filtration profile of SmFtsA protein showing monomeric elution peak at 10.1 mL (d) 12% SDS PAGE gel for protein purity check. (e) Thermal shift assay profile of SmFtsA with different salt concentrations in 50 mM HEPES pH 7.4 buffer. (f) Pelleting assay of SmFtsA for checking polymerization in the presence of different nucleotides. (g) GTPase and ATPase activity assay of SmFtsA.

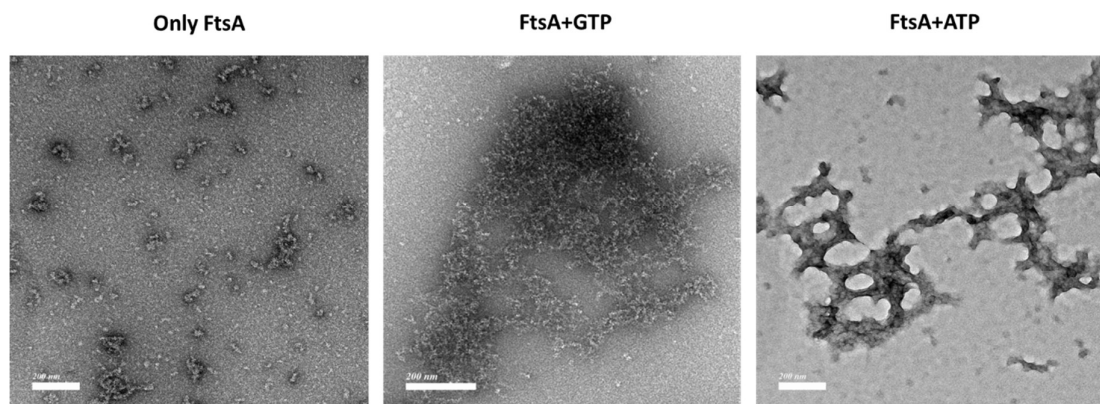


Figure 4.3 Negative staining TEM of SmFtsA in the presence of GTP and ATP. Scale bar 200 nm.

4.3.4 Membrane binding of SmFtsA *in vitro*

The membrane binding potential of SmFtsA was analysed by multiple sequence alignment with FtsA sequences from different bacterial species. Importantly, we observed conservation of several hydrophobic and charged residues at the C-terminal end region (within about fifteen residue stretches) for FtsA. This region contains an amphipathic helix which is known to bind membrane. However, we could not identify a possible amphipathic helix for SmFtsA as the C-terminal residues are not conserved (Figure 4.4a). We have compared the amphipathic parameters of the known membrane binding motif of *E. coli* FtsA (EcFtsA) with SmFtsA. The hydrophobicity and hydrophobic moments were less than the known membrane binding motif of EcFtsA (Figure 4.4a). Thus, the potential membrane binding region in SmFtsA was not conclusive from the sequence analysis.

To experimentally verify membrane binding ability of SmFtsA, liposome cosedimentation assay was performed using spiroplasma membrane specific lipid composition as mentioned in the materials and methods. In the presence of liposomes SmFtsA was found in the pellet fraction suggesting membrane binding potential of the protein (Figure 4.4c). Additionally, liposomes were found to aggregate in the presence of FtsA indicating a possible interaction (Figure 4.4d). To identify the membrane binding region within SmFtsA, we made two chimeric constructs: 1. SmFtsZ396^{439-CTAH} FtsA, where SmFtsZ CCTP region was replaced with the 12

residues from C-term end of FtsA and 2. SmFtsZ314^{383-CT-FtsA}, where the entire C-terminal region after the globular domain of SmFtsA was attached to the globular domain of SmFtsZ (Figure 4.4e, f). The results of the sedimentation assays suggest that SmFtsZ396^{439-CTAH FtsA} has negligible binding with 1 mM liposomes compared to SmFtsZ314^{383-CT FtsA}. The binding to liposomes is expected to be the effect of insertion of the FtsA regions as the SmFtsZ construct does not bind to liposomes (Figure 4.4g).

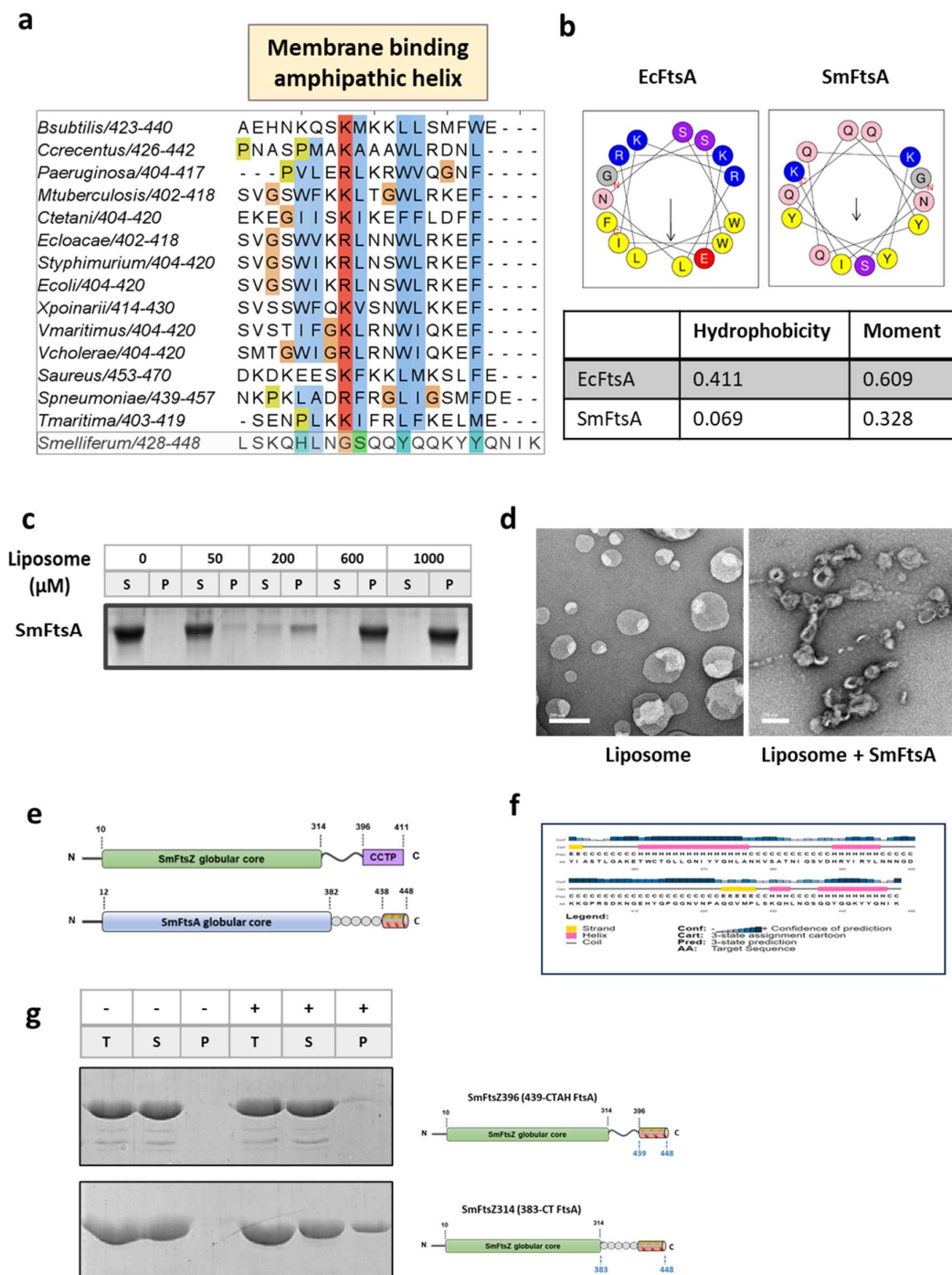


Figure 4.4 Membrane binding of SmFtsA (a) Multiple sequence alignment of FtsA C-terminal end region from different bacterial species showing the conservation of hydrophobic residues in the amphipathic helix (b) Helical wheel plot of the C-terminal amphipathic helix of EcFtsA compared to SmFtsA sequence (c) Representative 12% SDS PAGE gel for liposome pelleting assay with SmFtsA protein. Increasing protein concentration in the pellet fraction from 50 μ M of liposomes confirms its membrane binding ability. (d) Negative staining TEM images of liposomes showing the aggregation in the presence of SmFtsA. Scale bar 200 nm. (e) Schematics of domain organization of FtsZ and FtsA protein. (f) PSIPRED predicted secondary structure of SmFtsA C-terminal region (g) Representative gel of liposome pelleting assay of SmFtsZ 314^{383-CT FtsA} and SmFtsZ 396^{439-CTAH FtsA}. ‘+’ and ‘-’ denote presence and absence of 1 mM liposomes respectively. Total (T), Supernatant (S) and Pellet (P) fractions are loaded on the gel.

4.3.5 Sequence analysis and structure prediction of SmFtsA

Multiple sequence alignment of FtsA from different bacterial species shows the conservation of two glycine residues located in the ATP binding pocket (G214 and G337 in *Thermotoga maritima* FtsA) (Figure 4.5a). From the analysis of *Thermotoga maritima* (PDB 4A2B) FtsA structure, G214 and G337 residues were found to interact with ATP. Importantly, small size of the glycine side chain helps ATP to fit inside the pocket. In contrast, in *S. melliferum* FtsA sequence, bulky residues like D212 and E328 are located in place of G214 and G337 respectively which can possibly inhibit ATP binding.

To study ATP binding in SmFtsA, we first predicted SmFtsA structure using AlphaFold 3 and compared it with the crystal structure of *T. maritima* FtsA (PDB 4A2B) (Figure 4.5b). The SmFtsA model showed high pLDTT score and the domain organization was also similar to other FtsA crystal structures. As predicted from the multiple sequence alignment, the superimposed SmFtsA model with TmFtsA (PDB 4A2B, ATP bound) crystal structure showed the two identified residues D212 and E328 clash with ATP in the canonical binding conformation (Figure 4.5c). Next, FtsA structures with ATP as ligand were predicted for *T. maritima* (as positive control) and *S. melliferum* using AlphaFold 3 (Figure 4.5d). The predicted structure of TmFtsA showed ATP to bind to the canonical site as found in crystal structure. However, for SmFtsA the predicted ATP binding pocket is not the same. It was predicted in a different location (between subdomain 1C and 2A) opposite to the known ATP binding pocket i.e. between subdomain 1A and 2B of FtsA (Figure 4.5d). The pTM and ipTM

scores were 0.9 and 0.82 respectively which is considered to be a high confidence prediction. Interestingly, the ATP binding pocket for SmFtsA^{D212/E328G} was predicted in the canonical ATP binding site of FtsA similar to what is observed in the crystal structure of TmFtsA. These AlphaFold 3 predicted results of SmFtsA and its double mutant SmFtsA^{D212G,E328G} motivated us to experimentally check the effect of mutating D212, E328 to G212, G328 in ATP binding.

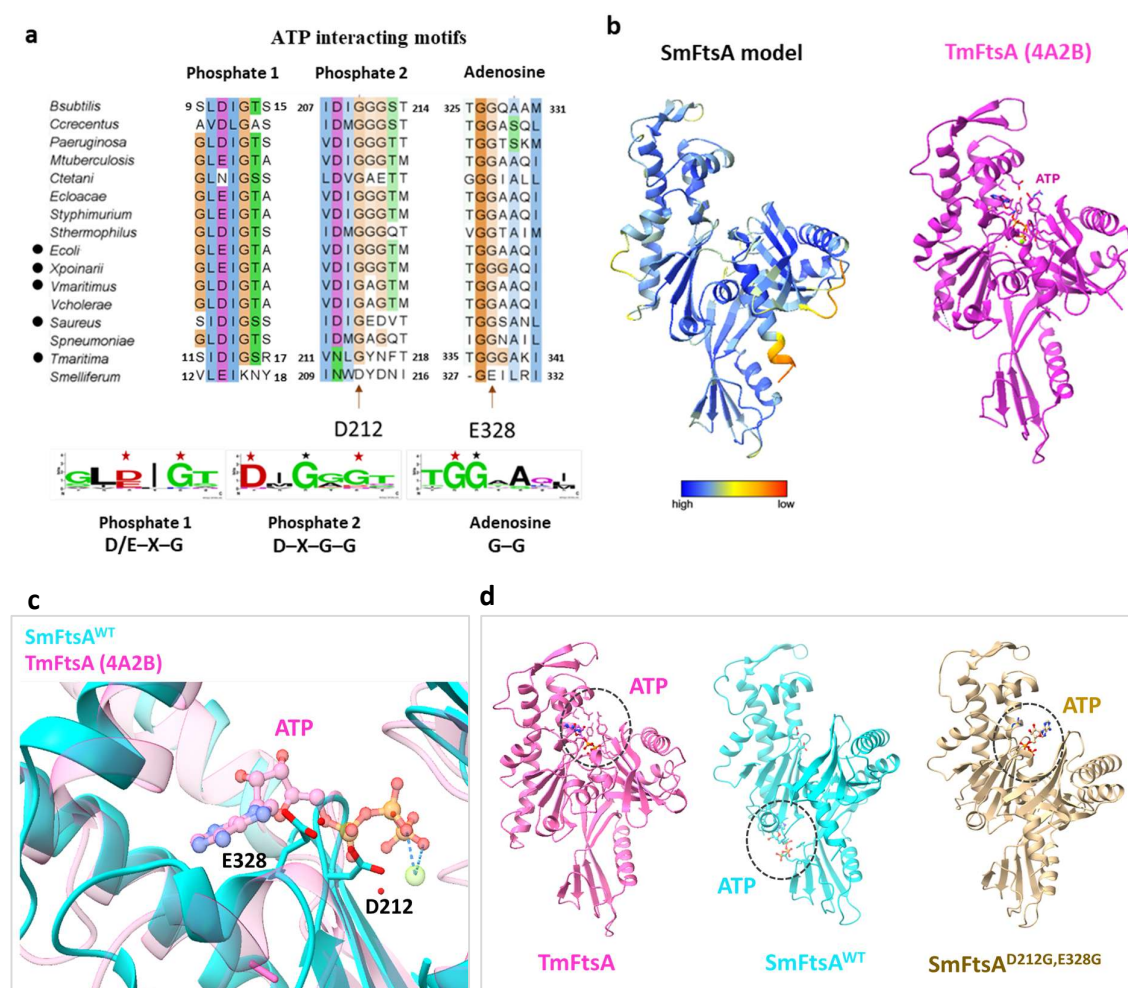


Figure 4.5 Sequence analysis and structure prediction for ATP binding in SmFtsA. (a) Multiple sequence alignment of FtsA sequences showing the conservation of ATP binding residues across organisms. Organisms having crystal structures for FtsA proteins have been indicated with filled black circles. Weblogos have been shown below the sequence alignment. (b) AlphaFold 3 predicted globular domain FtsA (pLDDT color coded, left) structure showing similar domain organization with *T. maritima* (Tm) FtsA (pink, right) crystal structure (4A2B). (c) Superimposition of AlphaFold 3 predicted SmFtsA (cyan) structure with TmFtsA (pink, 4A2B) showing possible clashes between SmFtsA D212 and E328 with ATP molecule. (d) AlphaFold 3 predicted structures for FtsA with ATP as ligand for TmFtsA, SmFtsA and SmFtsA^{D211G,E328G} showing the difference in the ATP binding pocket prediction.

4.3.6 Purification of SmFtsA^{ΔCT} and SmFtsA^{ΔCT D212G,E328G}

To experimentally verify the structure and nucleotide binding in SmFtsA, we aim to crystallize SmFtsA globular domain (1-382 residues) SmFtsA^{ΔCT} as well as SmFtsA^{ΔCT D212G/E328G}, a double mutant construct which we hypothesize to bind to ATP. We cloned these two constructs in the His-SUMO containing pET28 vector and performed purification following the protocol mentioned in the materials and methods section. However, during the first step of Ni-NTA purification, we noticed that both SmFtsA^{ΔCT} and SmFtsA^{ΔCT D212G,E328G} tend to precipitate with very less yield. We are currently in the process of standardizing the purification condition for these two constructs.

4.4 Discussion

In this chapter, we have discussed the standardization of the purification protocol of FtsA protein of *S. melliferum* (SmFtsA). We have characterized it *in vitro* for nucleotide hydrolysis, polymerization and membrane binding ability. In our assays, SmFtsA did not show hydrolysis activity for GTP and ATP. The presence of liposomes did not have any effect in the activity. Notably this observation is different from *E. coli* FtsA where ATPase activity was shown to increase in presence of lipids (Conti et al. 2018). Similar to our observation, FtsAs without any ATPase activity were found in other organisms like *S. aureus* (Fujita et al 2014), *T. maritima* (Szwedziak et al. 2012) and *S. pneumoniae* (Lara et al. 2005).

The variation in activity of FtsA in *in vitro* conditions could be attributed to the buffer conditions. It is also possible that FtsA activity may be dependent on other cell division proteins which upon interaction induce a change in FtsA structure favorable for ATP hydrolysis (Fujita et al 2014). To understand the ATP binding and hydrolysis phenomenon better, we took a bioinformatics approach and looked into the conservation of the ATP binding motif in SmFtsA. We found that several residues are not conserved in SmFtsA in the phosphate loop 1, phosphate loop 2 and adenosine motifs. We think these residues (e.g. K16, D212, E328) could hinder the ATP binding in a canonical manner. In *E. coli* FtsA, mutation in the adenosine motif i.e. Gly336 to Asp has been reported earlier to reduce ATP binding (Sanchez et al. 1994). Introduction of a bigger side chain of aspartic acid in the place of a glycine possibly inhibits the nucleotide binding (Sanchez et al. 1994, van den Ent and Lowe 2000). For SmFtsA, the ATP binding pocket residues are different from the known ATP binding FtsAs which possibly precludes nucleotide binding. Interestingly, our Alphafold3 prediction supports this idea that SmFtsA is

possibly a non-ATP binding actin homolog protein. However, we can not rule out a non-canonical ATP binding motif where slight conformational change in the canonical pocket can still hold ATP. Our next objective is to get the crystal structure of SmFtsA^{ΔCT} and SmFtsA^{ΔCT D212G,E328G} which will confirm this prediction and give us detailed structural insights on the nucleotide binding motif in SmFtsA.

The polymerization of SmFtsA was checked by ultracentrifugation based pelleting assay and TEM imaging. Filament formation by FtsA could not be observed in either of these experiments. FtsA in earlier studies have been shown to polymerize and form filaments on lipid monolayers (Lara et al. 2005, Szwedziak et al. 2012, Krupka et al. 2014, Krupka et al. 2017, Nag et al. 2020, Morrison et al. 2022). It is possible that the FtsA might require additional adaptor proteins or membrane support for efficient polymerization which we will be investigating in the future by checking FtsA filament formation on lipid coated grids under TEM. Earlier studies had shown that presence of ATP is necessary for membrane localization and recruitment of FtsZ *in vitro* (Loose and Mitchison 2014, Krupka et al. 2014, Morrison et al. 2022, Morrison et al. 2023). Moreover, FtsA deforms lipid vesicles in ATP bound states (Conti et al 2018). Thus, nucleotide states possibly regulate polymerization, membrane binding and FtsZ interaction, but mechanisms are still not clear.

According to our results from *in vitro* liposome sedimentation assay, SmFtsA binds membrane. However, interestingly, the C-terminal end of SmFtsA does not contain canonical amphipathic helix for membrane binding (Pichoff and Lutkenhaus 2005) as predicted from the multiple sequence alignment. We observed membrane binding only when the entire C-terminal region of FtsA was present (i.e. SmFtsZ396^{439-CTAH} FtsA showed negligible binding compared to SmFtsZ314Z^{383-CT} FtsA). From the current study we can not confirm the specific region or motif responsible for membrane binding in SmFtsA. Still, we can confirm that the conventional idea of C-terminal amphipathic helix mediated membrane binding possibly is not true in the case of SmFtsA.

FtsA has been known to be a difficult protein to work *in vitro* because of challenges with heterologous expression, instability and low yield upon purification (Martos et al. 2012). This could be a possible reason behind less number of studies for FtsA compared to FtsZ. This is evident from the earlier studies of FtsA from *E. coli* where a better behaving hyperactive mutant was chosen to work *in vitro* compared to wild type protein (Osawa and Erickson 2013, Chen et al. 2017, Schoenemann et al. 2018). The limited data available in the literature suggest a

wide variability across different species for activity, nucleotide binding, nucleotide hydrolysis, oligomerization property, filament architecture etc. Thus, characterization of new FtsAs is particularly important to shed light on its diverse functions across bacteria. We got some significant insights with respect to standardization of purification conditions, thermal stability of SmFtsA in different buffer pH, salt and nucleotide conditions as well as the differences in the conservation in nucleotide pocket from model FtsAs studied till date. The immediate next step will be to standardize purification for other constructs mentioned before and determine the crystal structure. Another important aspect of SmFtsA which we will be discussing in the next chapter is the interaction between SmFtsA and SmFtsZ. We believe these studies will deepen our understanding of how the FtsA might function in a “minimal” divisome where peptidoglycan machinery is not present.

4.5 References

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Chapter 5

Characterization of interaction between cell division proteins FtsZ and FtsA from *Spiroplasma melliferum*

5.1 Introduction

FtsZ and FtsA are two essential components of the divisome complex in bacterial division. FtsZ forms a ring-like dynamic structure at the mid-cell which tethers to the membrane with the help of anchor proteins like FtsA, ZipA (specific to Gram negative bacteria), SepF (specific to Gram positive bacteria) etc. Membrane interaction of the Z-ring is essential to successfully constrict the cell. However, the role of anchor proteins is not just membrane tethering, it also regulates the dynamics of the Z-ring assembly and disassembly process (Loose and Mitchison 2014, Naha et al. 2023). Until now, all the studies related to interaction between FtsZ and FtsA have been centered on cell walled bacteria. Importantly, in cell wall-less bacteria the *dcw* operon consists of only five genes namely *mraZ*, *mraW*, *ftsA*, *ftsZ*, and *sepF* while all other peptidoglycan synthesis related genes are lost in the course of evolution (Alarcon 2007). How a minimal divisome machinery consisting of only three components performs cell division remains unknown. Thus, studying FtsZ and FtsA in cell wall-less bacteria will shed light on their novel roles in division in the absence of other accessory proteins.

Earlier, in vitro experiments with *Escherichia coli* FtsZ and FtsA showed that the two proteins together can constrict liposomes, while without FtsA no constriction was observed (Osawa et al. 2008, Osawa and Erickson 2013). These results suggest a possible role of FtsA in driving constriction. Loose and Mitchison's experiments with wild type *E. coli* FtsA and FtsZ suggested that FtsA preferentially interacts with polymerized FtsZ to recruit it to the membrane. It was also found that FtsA might be able to destabilize the FtsZ filament on membrane and thus reorganize the FtsZ filament network (Loose and Mitchison 2014, Conti et al. 2018). Additionally, no change in the GTPase activity was found in the presence of FtsA. More recently, a contradictory observation has been reported for *E. coli* FtsZ where decrease in GTP hydrolysis was observed when FtsA was added in the reaction (Conti et al. 2018). Interestingly, for *Staphylococcus aureus* FtsZ, presence of FtsA resulted in an enhanced GTPase activity (Fujita et al. 2014). However, the mechanism behind stimulation or inhibition of GTPase mediated by FtsA is currently unknown. FtsZ has also been found to form higher order filament assemblies like bundles, tubules, crosslinks in the presence of anchor proteins like ZapA, ZapD, ZipA, FtsA* (a hyperactive mutant of FtsA that can bypass the requirement of ZipA protein in *E. coli*) (Huang et al. 2013). These higher order structures have been

observed *in vitro* and could play an important role in the Z-ring constriction. However, its physiological significance or mechanism of action is still not clear.

Here, we have investigated *Spiroplasma* FtsZ-FtsA interactions through *in vitro* biochemical and biophysical assays and electron microscopy studies. We have found that the GTPase activity of FtsZ gets stimulated in the presence of FtsA. Furthermore, FtsA promotes higher order assembly of FtsZ filaments. We hypothesize a possible mechanism behind the stimulation and bundle assembly. Our results give new insights about how FtsZ-FtsA together might play an important role, and highlight the unique features of the cell wall-less bacterial proteins and common features across all FtsZ and FtsA.

5.2 Materials and methods

5.2.1 Cloning and protein purification

A detailed description of the cloning methodology of SmFtsZ and SmFtsA constructs has been mentioned in the previous chapters. Briefly, *SmftsA* or *SmftsZ* gene was PCR amplified from the chromosomal DNA of *Spiroplasma melliferum* KC3 and inserted into pHis17 vector by restriction free cloning. After changing the TGA codons with TGG (as TGA codes for Trp in *Spiroplasma*) the full-length gene was subcloned into (His)₆-SUMO-pET28a vector using restriction free cloning and specific primers as mentioned in the earlier chapters.

Purification of (His)₆-SUMO tagged SmFtsA (called as SmFtsA) constructs were performed using 5 mL His-Trap column (Cytiva), equilibrated with Buffer A (50 mM Tris-HCl pH 8.0, 200 mM NaCl). Protein was eluted using a step gradient of 2%, 5%, 10%, 20%, 50% and 100% Buffer B (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 500 mM imidazole). To cleave the N-terminal (His)₆-SUMO tag, peak protein fractions were pooled and Ulp1 protease was added in a 1:100 (protease:protein) molar ratio. The cleaved protein was precipitated with 35% ammonium sulphate, resuspended in 1 mL HK50 buffer (50 mM HEPES pH 7.4; 50 mM KCl) and loaded into Superdex75 column to separate SUMO tag and improve purity. Fractions corresponding to a monomeric peak were pooled and flash frozen and stored at -80°C until further use.

Untagged SmFtsZ construct was purified using two steps of anion exchange chromatography. The cell lysate was loaded into HiTrap Q HP 5 ml column (Cytiva) equilibrated in Buffer A50 (50 mM Tris-HCl pH 8.0, 50 mM NaCl). Protein was eluted in 5 mL fractions using a linear

KCl gradient from 50 mM to 1000 mM over 20 column volumes. Peak fractions containing proteins were confirmed from SDS PAGE, collected and pooled together for dialysis against A50 buffer and loaded into the MonoQ (Cytiva) column to increase the purity level. Finally, the pure fractions were confirmed from SDS PAGE gel and pooled for dialysis in HK50 buffer (50 mM HEPES pH 7.4; 50 mM KCl). After dialyzing for 2 hours, the protein was concentrated using centricon (with 10 kDa membrane cut off), flash-frozen and stored at -80 °C.

Purification of SmFtsZ^{D211G} was done following the protocol described in Chakraborty et al. 2024.

5.2.2 GTPase activity measurement

The GTPase activity was measured using malachite green assay (Feng et al. 2011). The protein was spun at 21,000xg at 4°C for 10 mins and 20 µL reaction was set with the required concentration of protein in the reaction HK50 buffer containing 2 mM of GTP, and 5 mM MgCl₂. Then this reaction mixture was incubated at 30°C for 15 minutes. To stop GTPase reaction, 5 µL of 0.5 M EDTA was added in 20 µL reaction. 100 µL of the malachite green mixture was added into the 25 µL final mixture and waited for 10 mins and absorbance was measured at 630 nm wavelength. Phosphate solutions of known concentrations were used as standard to estimate the released phosphate amount in the reaction.

5.2.3 FtsZ-FtsA interaction study

A 60 µL reaction mixture containing FtsZ and FtsA in specific concentration ratio was prepared in HK50 buffer. 2 mM GTP and 5 mM MgCl₂ were added when required. Then the reaction mixture was incubated at 30 °C for 15 mins and centrifugation was done at 14,000 xg in room temperature for 10 mins. The supernatant was carefully taken out and the pellet was gently washed with buffer. Finally, the pellet was resuspended in 5X TGS buffer and prepared samples for running 10% SDS PAGE gel (1.5 mm thick). The intensities of the bands were quantified using the ImageJ software by measuring the area of the respective bands and percentage of protein in pellet fractions were calculated by dividing the pellet band intensity by the sum of the pellet and supernatant band intensity.

5.2.4 Negative staining Transmission Electron Microscopy

For visualizing protein samples under TEM, 5 μ L of reaction samples was loaded on a glow discharged (15 mA for 25 Sec) carbon coated copper grid and incubated for 2 minutes before blotting out excess liquid from the side. Then 5 μ L 1.5% (w/v) uranyl acetate stain solution was added on the grid and blotted from side after 30 seconds. Grid was air-dried and imaging was performed in TEM (JEM- 2200FS Jeol Ltd.) operated at 200 kV. Fourier transform and fiber diffraction image analysis of the TEM images were done in ImageJ (Schindelin et al. 2012).

5.3 Results

5.3.1 FtsA enhances GTPase activity of FtsZ and lowers critical concentration

Previously, *S. melliferum* (Sm) FtsZ i.e. SmFtsZ (His)₆ construct was shown to hydrolyse GTP with a specific activity (k_{obs}) of 0.67 ± 0.03 (mean $\pm$ SEM) which is approximately threefold lower than that of *E. coli* (Chakraborty et al. 2024). Here, for checking interaction of FtsZ with FtsA, we used an untagged SmFtsZ construct as the C-terminal (His)₆ tag might interfere in the interaction process. We found the untagged construct to have less GTPase activity 0.25 ± 0.04 min⁻¹ (mean $\pm$ SD) compared to the SmFtsZ (His)₆ construct as reported earlier. In the presence of SmFtsA, the activity increases to 3.7 ± 0.24 min⁻¹ (mean $\pm$ SD) (Figure 5.1a). Protein concentration of SmFtsZ and SmFtsA was taken as 10 and 20 μ M respectively i.e. 1:2 ratio of FtsZ:FtsA.

To understand if the stimulation is dependent on FtsA concentration, we measured GTPase activity with a varying concentration of FtsA from 0 to 20 μ M with a constant FtsZ concentration (i.e. 5 μ M and 10 μ M of FtsZ) (Figure 5.1 b, c). Interestingly, the activity reaches maximum when the FtsZ:FtsA ratio becomes about 1:1.5 and thereafter becomes saturated. To find out the effect of FtsA on apparent change in critical concentration (cc, the monomeric concentration of the protein above which it forms filaments and hence show consistent GTPase activity), if any, on FtsZ GTPase, rate of phosphate release was measured at different concentrations of protein from 2 μ M to 15 μ M with and without FtsA (Figure 5.1d, e). FtsZ and FtsA protein concentrations were used at a constant ratio of 1:2. The apparent critical concentration (cc) for GTPase activity was calculated by plotting rate vs concentration of

SmFtsZ, and extrapolating to the x-axis. In the presence of FtsA, the cc becomes much lower for FtsZ. Additionally, the GTPase mutant SmFtsZ^{D211G} (which potentially interacts with FtsA but cannot hydrolyse GTP) in the presence of SmFtsA or only SmFtsA were not GTPase active which confirms that hydrolysis is SmFtsZ specific.

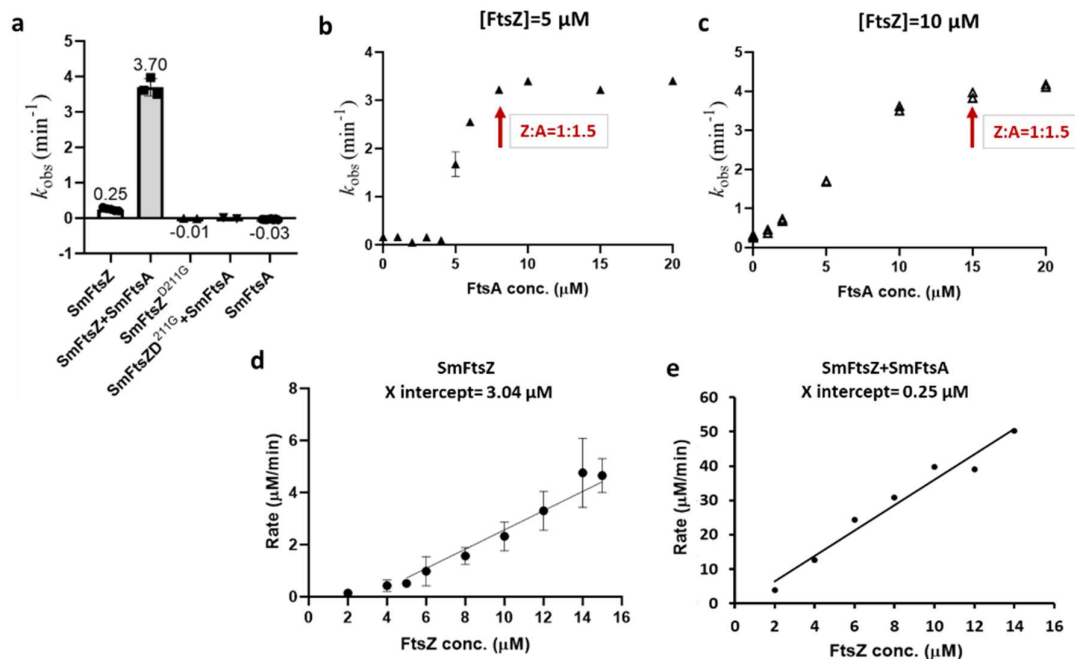


Figure 5.1 GTPase activity enhancement of SmFtsZ in presence of FtsA. (a) Comparison of GTPase activity of SmFtsZ and SmFtsZ+SmFtsA. GTPase activity of (b) 5 μM and (c) 10 μM of SmFtsZ with varying SmFtsA concentrations. (d) Critical concentration estimation of only SmFtsZ and (e) SmFtsZ in the presence of SmFtsA.

5.3.2 FtsA promotes formation of higher order assemblies of FtsZ filaments

One of the possible reasons behind GTPase stimulation and lowering of cc could be that FtsA bound FtsZ is more likely to polymerize leading to lower cc and more hydrolysis. To check this hypothesis and the effect of FtsA on FtsZ polymerization, we analysed the FtsZ+FtsA+GTP reaction mixture under TEM with negative staining. SmFtsZ (His)₆ was previously shown to form single protofilaments in the presence of GTP (Chakraborty et al. 2024). Here we confirmed untagged SmFtsZ also to form single protofilament in the presence of GTP. In the presence of FtsA, we found higher order assemblies which appeared as straight

bundles (Figure 5.2, 5.4). Notably, single short protofilaments were also observed in the background. Importantly, this higher order assembly was not observed in the sample containing the same concentrations of only SmFtsZ or only SmFtsA suggesting the bundle formation is FtsA mediated. Fourier transform of the image of the bundle was done to find out the repeating distance of and filament subunits inside the bundles. The first layer line was clearly visible at a 4.5 nm distance in the diffraction pattern which corresponds to a longitudinal intermonomer distance of a FtsZ filament (KpFtsZ filament structure, PDB 8IBN whereas FtsA monomer has a longitudinal distance of 4.8 nm, PDB 7Q6F). However, no prominent equatorial spots were identified, possibly due to the non-heterogeneity and irregular lateral arrangements of the filaments inside the bundle.

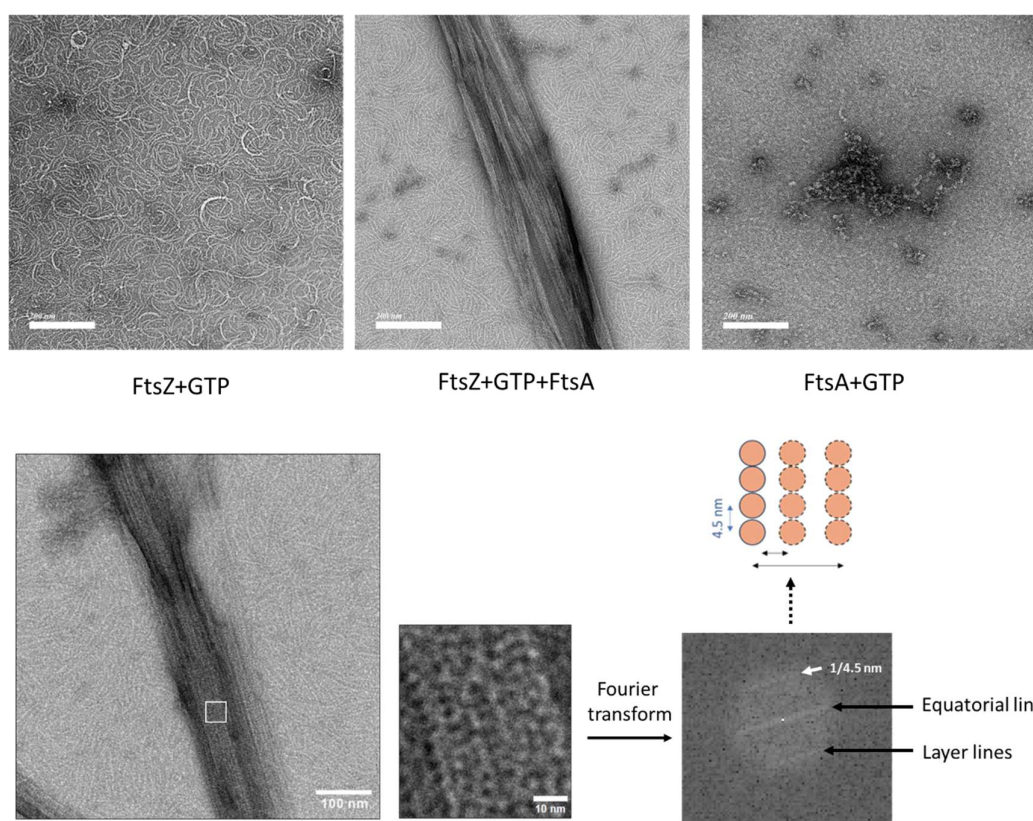
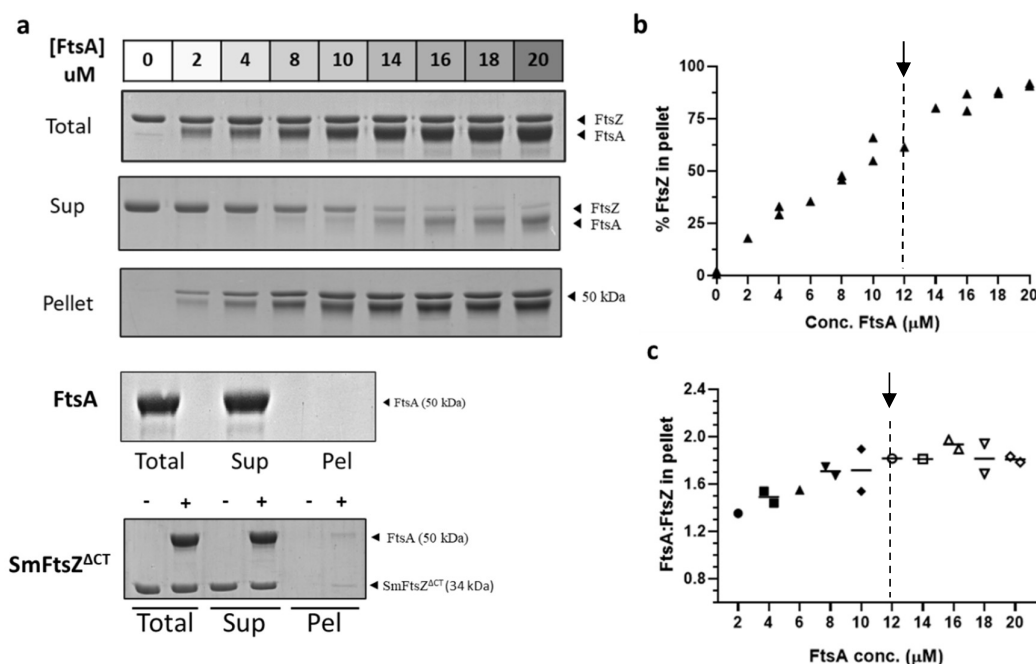


Figure 5.2 TEM images of bundle formation by FtsZ filaments in the presence of FtsA. Fourier transform of the bundle showing repeating distance between two FtsZ monomers in the filament as first layer line. (Scale bar: 200 nm (top panel images), 100 nm (bottom panel), 10 nm (inset))

To further characterize the interaction between FtsZ and FtsA, we performed a low-speed centrifugation based pelleting assay. Low-speed sedimentation assay allows study of the

interaction of FtsZ and other proteins that form higher order assembly (Król, E., Scheffers 2013) as only FtsA or only FtsZ do not pellet during centrifugation. The presence of each protein in the pellet and supernatant fractions were confirmed by SDS PAGE gel. For this analysis, 8 μM of FtsZ with varying FtsA was used to get a wide distribution of ratio of the two proteins. From the gel, we quantified the protein band intensities using ImageJ and observed an increasing amount of FtsZ in the pellet as the concentration of FtsA increases in the reaction. Furthermore, the reaction mixture containing SmFtsZ^{ACT} (construct of only globular domain with C-terminal linker and CCTP deleted) and SmFtsA does not pellet. Both these controls suggest that the pelleting down of FtsZ is because of its interaction with FtsA. We also calculated the ratio of FtsA:FtsZ in the pellet fraction to estimate an approximate stoichiometry of interaction. The ratio initially increases and becomes constant at 12 μM of FtsA. Interestingly this trend matches with the GTPase activity when the maximum activity of FtsZ was observed when the FtsA concentration reached ~ 1.5 times of FtsZ). The estimated interaction stoichiometry of FtsA:FtsZ was 1.8, implying that approximately twice the amount of FtsA pelleted along with FtsZ.



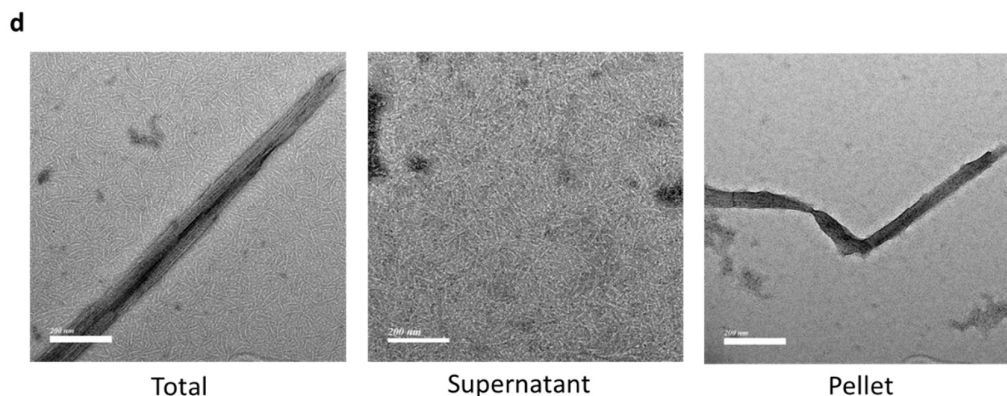


Figure 5.3 SmFtsZ-FtsA interaction study by centrifugation based pelleting assay. (a) Representative 10% SDS PAGE gel for total, supernatant, and pellet fraction of pelleting assay of 8 μ M FtsZ with varying FtsA concentrations. Only FtsA (20 μ M) and SmtsZ^{ACT} as negative control for FtsZ-A interaction. (b) Quantification of percentage of FtsZ protein coming in pellet with varying FtsA concentration. (c) Ratio of FtsA to FtsZ in the pellet with varying FtsA concentration. (d) Negative staining TEM images total, supernatant and pellet fraction of the Z-A pelleting assay. Scale bar 200 nm.

To confirm higher order bundles of FtsZ-FtsA complex indeed come in the pellet fraction in low-spin centrifugation, we prepared a negative staining TEM sample from the pellet and supernatant fractions of the reaction mixture. The supernatant and pellet fraction of the reaction with GTP contain single protofilament and bundle respectively (Figure 5.3d) whereas no distinguishable structural features were observed in reaction without GTP.

5.3.3 Higher order assembly depends on the relative concentrations of FtsZ and FtsA

We observed different types of bundle morphology in transmission electron microscopy depending on relative concentrations of FtsZ and FtsA in the reactions. At low concentration of FtsZ (e.g. at 5 μ M of FtsZ and 1 μ M of FtsA), long filament bundles were seen which became shorter and less populated over the grids as the concentration of FtsA increases (at 5 μ M each of FtsZ and FtsA). Bundle formation was not observed with excess of FtsA (e.g. at 6 μ M of FtsZ and 1 μ M of FtsA). Instead, numerous ‘aster’ like structures were noticed. Additionally, we found that the bundle formation happens above a certain minimum concentration of FtsA. Because we could not detect bundles at 1 μ M each of FtsZ:FtsA and only single protofilaments were observed all over the grid. However, as we increased FtsA concentration to 2 μ M (i.e. FtsZ:FtsA 1:2) short bundles were found. As we increased FtsA concentration further to 5 μ M

(i.e. FtsZ:FtsA 1:5), the grid seemed overcrowded with samples with no bundles or any distinguishable structures. Overall, our data suggests higher order filament assembly occurs only within a particular concentration ratio of FtsZ:FtsA, specially a substoichiometric amount of FtsA is favorable for bundle formation. An excess amount of FtsA prevents FtsZ filaments from forming higher order structures probably due to increased nucleation, as observed from the EM images (Figure 5.4). Similar results were observed earlier in sedimentation assay where FtsA was found to disassemble and destabilize polymeric FtsZ (Loose and Mitchison 2014, Modi and Misra 2014, Conti et al. 2018, Morrison et al. 2022, Morisson et al. 2023). In our experiments, an excess amount of SmFtsA is possibly affecting lateral interactions of FtsZ filaments creating a distance restraint for FtsZ higher order assembly. However, more data will be needed to find out the molecular mechanism or physiological relevance in future.

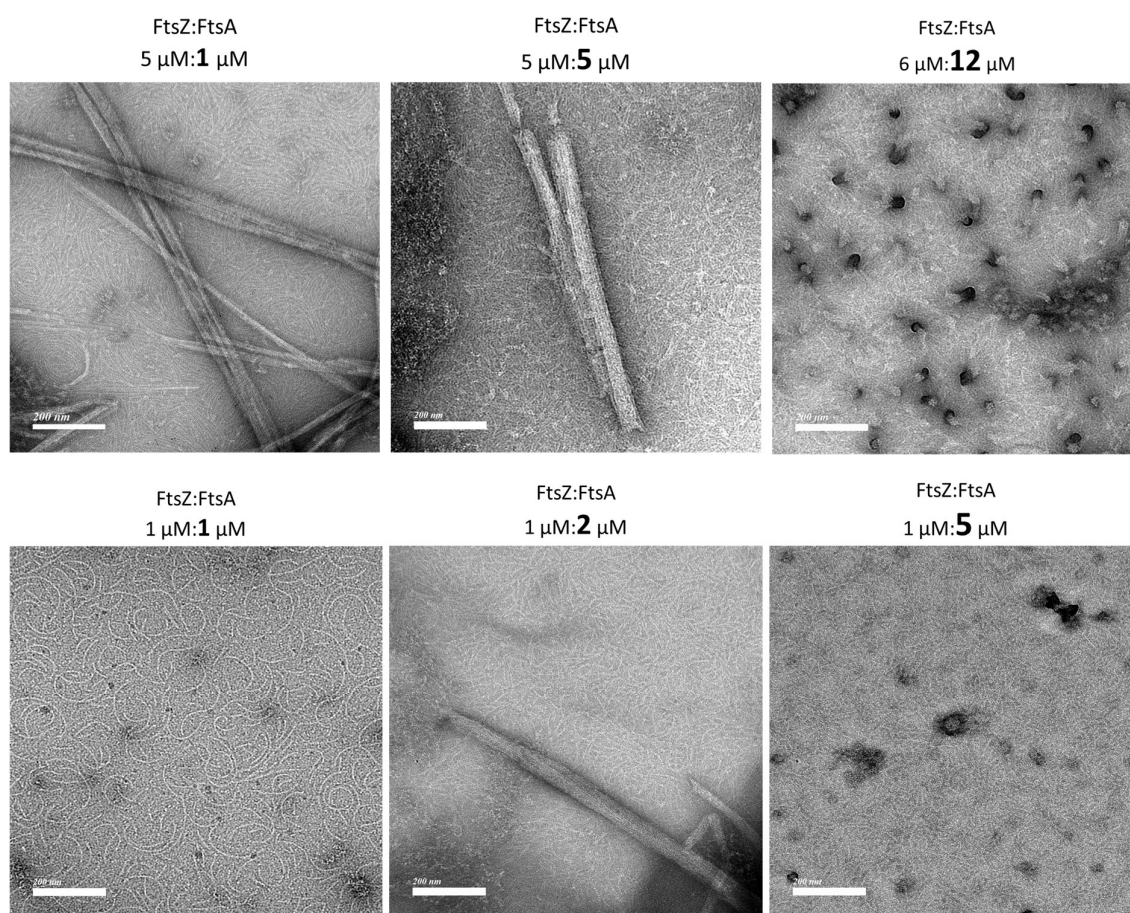


Figure 5.4 Bundle morphology of FtsZ-FtsA assembly at different relative concentrations of FtsZ and FtsA. Scale bar 200 nm.

5.4 Discussion

FtsZ and FtsA are two essential proteins for bacterial cell division. The interaction between these two proteins is crucial for the Z-ring to attach to the membrane and regulate downstream processes for divisome assembly functioning. Earlier studies on *E. coli* FtsA suggest its possible role in FtsZ filament destabilization and disassembly (Loose and Mitchison 2014, Conti et al. 2018, Morrison et al. 2022, Morisson et al. 2023) other than just membrane anchoring. In cell wall-less bacteria with a minimal divisome content, the role of FtsA and its interaction with FtsZ remain an unexplored area. In our study we have used FtsZ and FtsA from *S. melliferum* to characterize their interaction and consequent higher order assembly. The two main interesting observations of our study are:

1. The enhancement of GTPase activity of FtsZ in presence of FtsA, and
2. Higher order assembly formation of FtsZ filaments induced by FtsA.

Earlier, stimulation in GTPase activity of FtsZ was reported for *S. aureus* in presence of FtsA when concentration of FtsZ was above 5 μ M and FtsZ:FtsA is ratio less than 2:1, i.e. FtsA concentration should be at least half of FtsZ (Fujita et al. 2014). In our experiments, we observed stimulation when a FtsA concentration dependent stimulation where maximum activity reaches when FtsA concentration is about 1.5 times that of FtsZ. The reason behind the concentration dependency of FtsA could be related to the varying binding affinity of FtsA to FtsZ across species. Our pelleting assay suggests that *Spiroplasma* FtsZ can interact with FtsA in monomeric state without any requirement of nucleotides unlike *E. coli* FtsA that preferentially interacts with polymerized FtsZ (Loose and Mitchison 2014). Thus, structural investigation on the FtsZ-FtsA interaction is needed to have a clear picture on how interaction and GTP hydrolysis are correlated.

Another interesting property of FtsZ-FtsA complex is the formation of higher order structures of FtsZ filament that have been shown *in vitro* in the presence of various crosslinking proteins like ZapA, ZapD, SepF, FzlA from different bacterial species (reviewed by Huang et al. 2013). These proteins are thought to be the positive regulators of the Z-ring which assembles at the mid-cell for divisome functioning. Additionally, FtsA* (a hyperactive mutant of FtsA in *E. coli*) and few other FtsA* like mutants have also been found to induce FtsZ filament bundling (Schoenemann et al. 2018). Recently cryo-tomography studies showed higher order assemblies of FtsZ filaments in complex with ZapD (Merino-Salomón 2024). FtsZ was found to form bundles, torroid or straight bundle depending on the density of ZapD proteins which forms the

connections within FtsZ protofilament (Merino-Salomón 2024). The common feature of these FtsZ interactors is to regulate the Z-ring stability and filament dynamics. However, the mechanistic details of the formations of dynamic yet stable bundles remain unclear. Moreover, the relevance of the bundle structure of the FtsZ filaments *in vivo* is not understood. It is generally thought that lateral interactions between FtsZ filaments could be important to stabilize and strengthen higher order structures of the contractile Z-ring during division (Haeusser and Margolin 2016). Several bundling proteins like ZipA, ZapA, ZapC, ZapD or SepF possibly help to maintain the Z-ring integrity at various stages during division.

Our *in vitro* experiments with FtsZ and FtsA from the cell wall-less bacterium *S. melliferum* revealed FtsA induced bundled architecture of the FtsZ filaments. It is possible that in a cell wall-less system with minimal divisome machinery, FtsA itself can take up the role of the conventional FtsZ regulators and stabilizers found in cell walled bacteria. Furthermore, our negative staining TEM data suggest that the relative concentrations of FtsZ and FtsA is crucial for higher order assembly or the bundle formation. A sub-stoichiometric concentration of FtsA (e.g. FtsZ:FtsA ratio 5:1 to 5:5) is required for inducing higher order assembly while excess FtsA concentration such as (5:10 or 6:12 FtsZ to FtsA ratio) prevent bundling possibly by inhibiting lateral interaction. Similar results of FtsZ filament disassembly were observed earlier in *in vitro* experiments with *E. coli* FtsA in excess. However, excess FtsA concentration causes maximum stimulation of GTPase indicating a faster hydrolysis rate that is possibly induced by more nucleation events. The concentration dependency is particularly intriguing because the relative concentrations of FtsZ and FtsA is tightly regulated inside the cell for proper division and septum formation (Dewar et al 1992, Dai and Lutkenhaus 1992). In cells like *E. coli*, *B. subtilis*, cellular FtsZ:FtsA ratio is roughly 5:1 (Rueda et al. 2003, Wang and Gayda 1992). Though the concentration of FtsZ and FtsA proteins is so far not determined for spiroplasma, the bundling promoting concentration in our *in vitro* assay interestingly matches with known FtsZ:FtsA ratio. In future, high resolution structural studies using Cryo EM and tomography will be helpful to understand details about the structure function relationship of the FtsZ architecture. Studying how the assembly could be different in the presence of a lipid bilayer is also necessary.

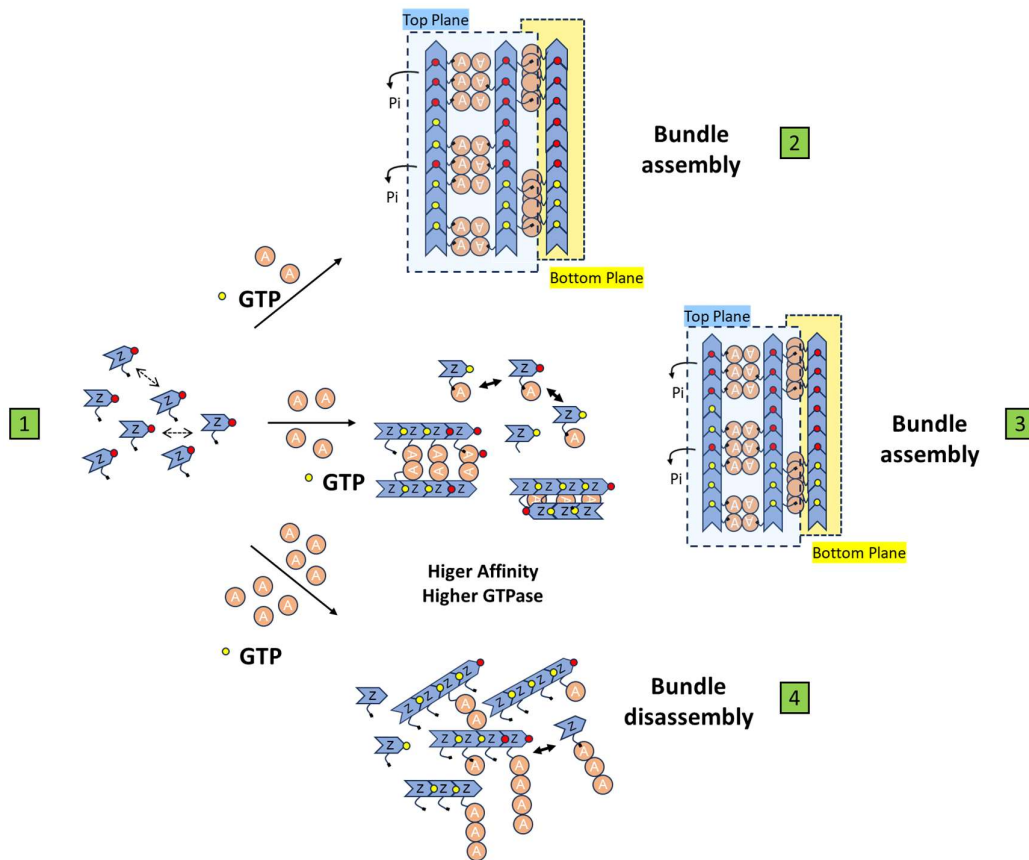


Figure 5.5 Model of a possible mechanism of GTPase stimulation and bundle formation of SmFtsZ protofilaments in the presence of FtsA.

Finally, we propose a plausible model for GTPase stimulation and bundle formation by FtsA (Figure 5.5). FtsZ protein is monomeric when GTP is not present (case 1). In the presence of GTP, it polymerises to form filaments and simultaneously hydrolyse GTP. When the concentration of FtsA reaches a critical level, it induces FtsZ filaments to laterally interact producing bundles (case 2). The filaments are possibly held in a bundled structure by both FtsA-FtsA and FtsZ-FtsZ intermonomer lateral interaction. To note, only above a concentration threshold, FtsA induced the bundle structure of the FtsZ filaments. The diffused lateral spacing of the FtsZ filaments as found in our TEM image analysis could be because of the multilayer arrangements of the filaments inside bundles. FtsZ binds to FtsA with the help of CCTP located at the C-terminal end. Because FtsA could also polymerise or oligomerise (though not demonstrated for the SmFtsA), as FtsA concentration goes higher, FtsA bound FtsZ has a

higher probability to interact with each other compared to a scenario without FtsA. This leads to an increase in local concentration and thus lowering of critical concentration of polymerization and enhancement of GTPase activity (case 3). The bundle formation could happen simultaneously at this stage. It is possible that FtsA binding can induce conformational change in FtsZ structure which facilitates transition from monomeric to polymeric state. Another possibility is that FtsA promotes realigning of residues in the catalytic pocket formed at the interface of two FtsZ molecules such that the GTP hydrolysis and Pi release is faster. However, this hypothesis can be envisaged to be true only if the FtsZ-FtsA interaction involves the globular domain of FtsZ, which has not been observed till date. To distinguish these mechanistic possibilities, structural analysis of FtsZ-FtsA assembly is required in future. Next, when FtsA concentration becomes excess (i.e. more than 1:1 ratio), bundle formation is inhibited (case 4). One possible reason is at very high concentration of FtsA, FtsA self-polymerizes, preventing lateral interaction of FtsZ filaments leading to disassembly. However, a detailed characterization of SmFtsA oligomerization or polymerization as well as the FtsZ-FtsA complex will be required to shed light on the precise mechanism.

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Chapter 6

Filament structure of *Spiroplasma melliferum* FtsZ: a cryo-EM study

6.1 Introduction

FtsZ is a tubulin homolog which polymerizes in the presence of GTP. The dynamic phenomenon of polymerization at the one end and depolymerization from the other end of a filament called treadmilling leads to an apparent movement of the filament in the forward direction while individual monomers remain static. Treadmilling of FtsZ is essential for the divisome assembly and function in bacterial division (Whitley et al. 2021). Though the treadmilling behaviour of FtsZ has been observed both *in vivo* and *in vitro*, structural basis of this process remains elusive. In the past, crystal structures analysis of FtsZ protein from different bacterial species revealed that FtsZ molecule can exist in two different conformational states: R (relaxed) state or the monomeric state and T (tensed) state or the filament state (Huecas et al., 2008; Dajkovic et al., 2008; Wagstaff et al., 2017). These two conformational states make the ends of the FtsZ filament asymmetric, which is required for maintaining directionality for treadmilling. A series of studies thus propose a cytomotive switch model of treadmilling where R to T transition in the FtsZ molecule is induced by polymerization and depolymerization and is independent of the nucleotide states (Wagstaff et al. 2017, Corbin and Erickson 2020, Wagstaff et al. 2023). Interestingly, T state structures in a protofilament-like architecture were only obtained from FtsZs of *Staphylococcus aureus* (Matsui et al 2014, Fujita et al. 2017, Wagstaff et al. 2017, Ruiz et al. 2022) whereas FtsZ structures from other organisms are only captured in R state. Though the presence of R and T state can account for the directionality of treadmilling, two major questions regarding the FtsZ dynamics remain unanswered: 1. why the monomer addition is preferred from the bottom end of the filament, instead from the top, and 2. what is the mechanistic trigger for R to T transition and vice versa during polymerization and depolymerization respectively. Additionally, the role and the effect of nucleotide state on the filament assembly remains another open question in the field. To understand the mechanism underlying dynamics of FtsZ filaments, in-depth structural analysis of native filament in different nucleotide states is required rather than relying solely on crystallographic data as crystal structures might have artifacts of crystal packing. One of the promising methods for obtaining high resolution native structure of protein filaments is cryo electron microscopy. However, because of the flexible nature of the FtsZ filament, it is difficult to obtain a high-resolution structure. One of the first attempts of capturing *Escherichia coli* FtsZ (with GMPCPP, a slowly hydrolysable GTP analog) in filament state was limited to a resolution of 7-8 Å. This low-resolution EM map was found to fit better with open conformation or the T-state of the FtsZ crystal structure compared to the closed conformation

or the R-state. Similarly, the cryo-EM structure of *Mycobacterium tuberculosis* FtsZ (with GMPCPP) filament has been determined at about 7 Å resolution (Wagstaff 2023). The primary reasons for such low resolution are filament flexibility and low twist of the helical filament accompanied by the preferred orientation problem of the filament in the vitrified ice (Wagstaff et al. 2017, Wagstaff et al. 2023). Recently, the first high resolution filament structure of FtsZ from *Klebsiella pneumoniae* (KpFtsZ) has been captured at an overall 3 Å resolution. In this structure where FtsZ was bound to GMPCPP, the residue level interactions of the nucleotide pocket were not well-resolved. Moreover, GMPCPP freezes the filament in a stable state. Another cryo-EM structure of the FtsZ double protofilament (GMPCPP bound) in complex with ZapA has been reported at 2.73 Å resolution (Fujita et al. 2024). This double protofilament arrangement of FtsZ-ZapA structure primarily revealed details of lateral interactions of FtsZ protofilaments and the effect of ZapA binding in protofilament assembly.

Here, we attempted to determine the filament structure of *Spiroplasma melliferum* FtsZ by cryo-electron microscopy. Earlier, we had obtained the crystal structure of *S. melliferum* FtsZ in a domain swapped dimeric state. This domain swapped dimer is not polymerization competent and thought to be a possible intermediate state of FtsZ domain movement during polymerization that got stabilized by crystal packing (Chakraborty et al. 2024). This in turn motivated us to capture the native state of FtsZ filament in presence of GTP and look into the structural changes during GTP hydrolysis. Here, we could determine the filament structure of SmFtsZ in the GTP-bound state at 3.5 Å using cryo-EM single particle analysis. Our preliminary data shows that the SmFtsZ monomers within the filament are in GTP bound T state conformation. We have also performed a comparative structural analysis of GTP binding pocket in SmFtsZ filament, KpFtsZ filament and *S. aureus* FtsZ (SaFtsZ) crystal structure. We believe our present filament model will be useful for understanding GTP binding, hydrolysis and conformational transition mechanism of FtsZ polymerization.

6.2 Material and Methods

6.2.1 Cloning and protein purification

Untagged SmFtsZ (referred to as SmFtsZ in this chapter) was used for cryo-EM study. Details of the cloning and purification procedures of untagged SmFtsZ construct has been described in

the materials and methods section of chapter 3. Briefly, the construct was expressed in *E. coli* BL21-AI cells by 0.2% arabinose induction at $OD_{600} = 0.6-0.8$ and was grown at 30 °C for 5 hours. Cells were harvested by centrifugation at 4,000 xg at 4 °C for 20 mins and the cell pellet was flash-frozen in liquid nitrogen and stored at -80 °C until processed. The cell pellet was thawed in ice for 10 mins and then resuspended in a lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10% glycerol) followed by sonication to lyse the cells. The total lysate was spun at 30,000 xg for 1 hour to remove the cell debris and the clear supernatant was loaded into HiTrap Q HP 5 ml column (Cytiva) equilibrated in Buffer A50 (50 mM Tris-HCl pH 8.0, 50 mM NaCl). Protein was eluted in 5 mL fractions using a linear KCl gradient from 50 mM to 400 mM over 40 column volumes. Peak fractions containing proteins were confirmed from SDS PAGE, collected and pooled together for dialysis against A50 buffer and loaded into 10/100 GL MonoQ column (Cytiva) to improve purity. Finally, the pure fractions were confirmed from SDS PAGE gel and pooled for dialysis in the HK50 buffer (50 mM HEPES pH 7.4, 50 mM KCl). After dialyzing for 1.5 hours, the protein was concentrated using centricon (with 10 kDa membrane cut off), flash-frozen in aliquots and stored at -80 °C.

6.2.2 Negative staining sample preparation for transmission electron microscopy

Polymerization reaction was set up in HK 50 buffer with 2 μ M protein, 5 mM $MgCl_2$ and 2 mM nucleotide (GTP, GMPPNP or $GTP\gamma S$ as mentioned), incubated for 10 min at 30 °C. For visualizing protein sample under TEM, 5 μ l of reaction samples was loaded on a glow discharged (15 mA for 25 Sec) carbon coated copper grid and incubated for 2 minutes before blotting out excess sample from the side. Then 5 μ l 1.5% (w/v) uranyl acetate stain solution was added on the grid and blotted from side after 30 seconds. Grid was air-dried and imaging was performed in TEM (JEM- 2200FS Jeol Ltd.) operated at 200 kV.

6.2.3 Cryo-EM sample preparation and data collection

For SmFtsZ cryo-EM sample preparation, 0.1 mg/mL (~2 μ M) protein was used in HK50 buffer with 5 mM $MgCl_2$ and 2 mM GTP. The reaction mixture was incubated for 10 min at 30 °C before applying on the grids. Quantifoil grids (R1.2/1.3 Au 200 mesh) were glow-discharged at 25 mA for 60 sec. Upon 3 μ L of sample application, grid was blotted using Vitrobot Mark IV with a force of 0 and a time of 3.5 s and then immediately flash frozen by plunging into liquid ethane. Cryo-EM image datasets were acquired using Titan Krios (Thermo

Fisher Scientific, USA) operated at 300 kV. The nominal magnification was 75,000 $\times$, corresponding to 1.07 Å pixel size. Each movie was fractionated into 25 frames with a total dose of 25.67 e/Å².

6.2.4 Cryo-EM image processing and model building

Cryo-EM data processing was done in cryoSPARC software ver. 4.1.1. A total of 1,080 movies were imported and motion corrected, and the contrast transfer functions (CTFs) were estimated. 965 micrographs with CTF resolutions above 5 Å were selected to process further for particle picking. Initially particle picking was done using template free filament tracer with a filament diameter of 40 Å. After running a 2D classification step, best classes were chosen for template-based filament picking. In the filament tracer job, separation distance was kept as 0.5 and hysteresis threshold (min, max) was optimized as (85, 90) to increase the number of particles. All the other parameters were set as default. Next, the particles were extracted with a box size of 256 pixels. After two rounds of 2D classification, best 2D class averages containing 906,054 particles with single filaments with features of secondary structure were selected for an ab-initio 3D reconstruction. After heterogenous refinement and 3D class averaging, the best class (75,654 particles) with straight filament orientation and low B factor value was processed for a non-uniform refinement to reach the map resolution of 3.5 Å (FSC = 0.143), with a B factor of 130.3.

The initial models of SmFtsZ T and R state were generated in SWISS-MODEL (Waterhouse et al. 2020) using crystal structure of *Staphylococcus aureus* FtsZ as template (PDB 3VOA for T state and 5MN6 for R state). The T state SmFtsZ model was used for further refinement using COOT ver. 0.9.8.7 (Emsley et al. 2010). GTP molecule was fitted in the map in the canonical nucleotide binding pocket during model building. Final validation and real space refinement was performed in PHENIX ver. 1.21.2 (Adams, P. D. et al. 2010). After the refinement process, the final atomic model of a monomer was fitted into the map using UCSF ChimeraX ver. 1.8 (Meng et al. 2023) to generate a filament model composed of three GTP bound SmFtsZ monomers. All structure figures were made in ChimeraX ver. 1.8.

6.3 Results

6.3.1 SmFtsZ filament morphology depends on nucleotide states

To check polymerization of SmFtsZ, we observed filament formation by negative staining TEM in three different nucleotide conditions i.e. GTP, GTP γ S, and GMPPNP. In the presence of GTP single separated protofilaments were observed whereas GTP γ S produced very long bundle like thick filaments (Figure 6.1). Filament formation was not efficient in the presence of GMPPNP. This indeed suggests that nucleotide state can impact polymerization and depolymerization efficiency and thereby filament formation in SmFtsZ. As well separated single protofilament was observed in GTP condition, we decided to use this condition for cryo-EM sample preparation.

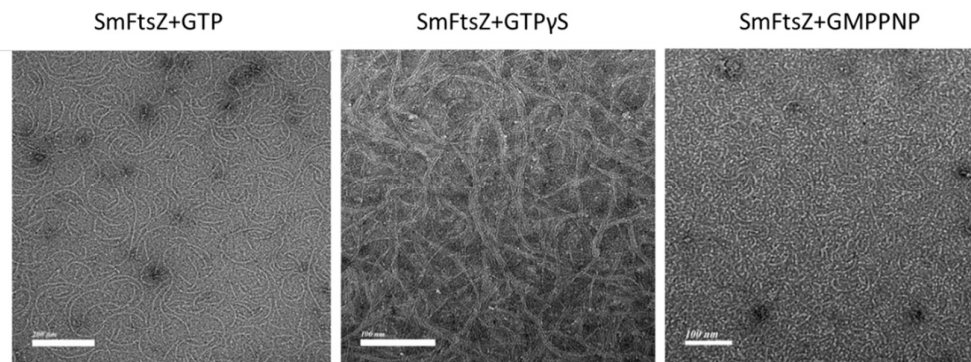


Figure 6.1 Negative staining TEM images of SmFtsZ in the presence of different nucleotides. Filament formation for SmFtsZ was tested in three different nucleotide conditions: GTP, GTP γ S, and GMPPNP. Scale bar 200 nm (left), 200 nm (middle), 100 nm (right).

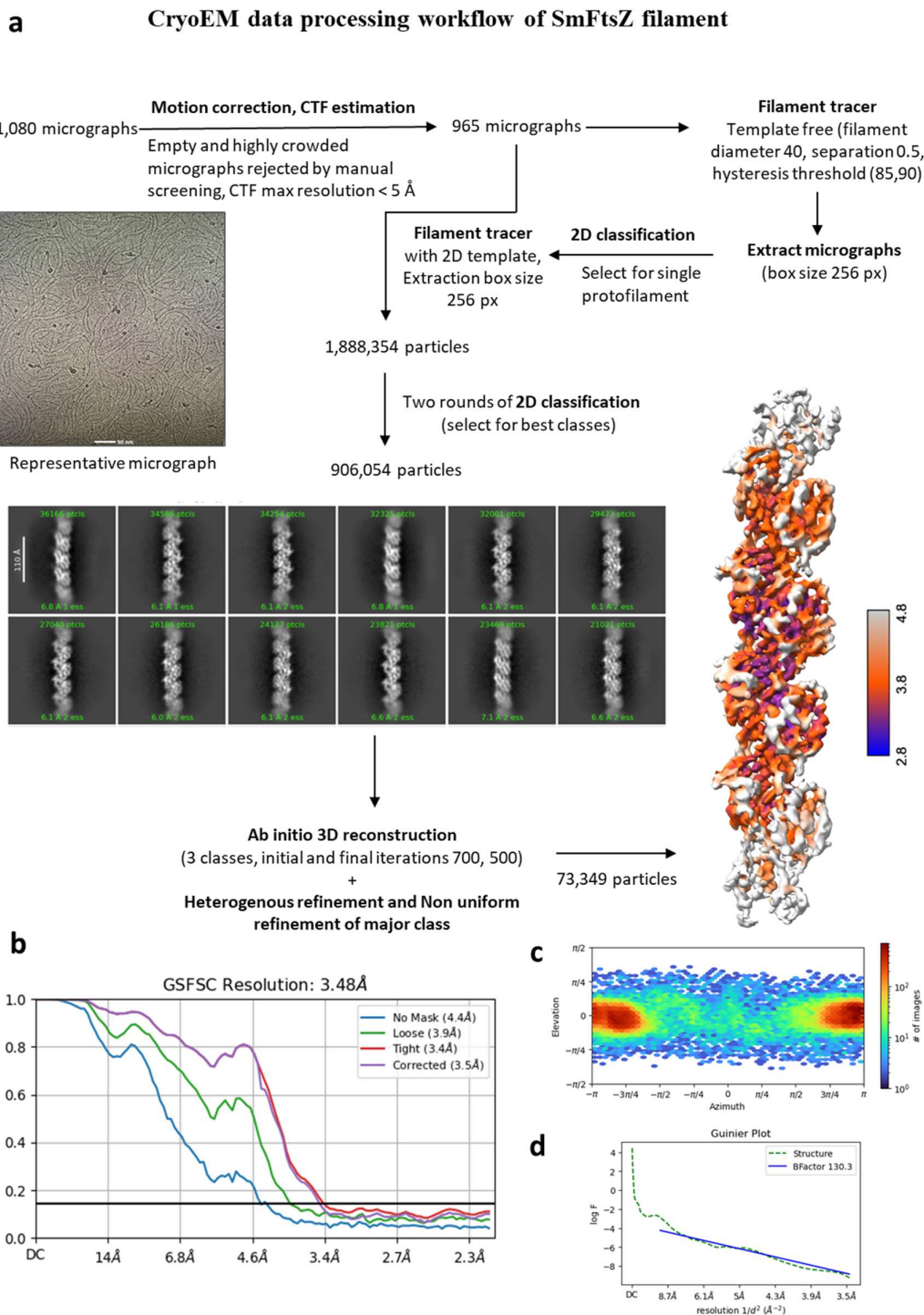


Figure 6.2 Workflow of cryo-EM data processing and final reconstructed map. (a) Sequential steps followed for data processing has been shown as a flowchart. The final map is shown here with 0.5 contour level with local resolution estimates. The color coding is according to the local resolution indicated on the right side of the map. Scale bar of representative cryo-EM micrograph is 50 nm (b) FSC curve of the final refined map for resolution estimation as generated from cryoSPARC. (c) Angular distribution of particles shown as heatmap across all micrographs. (d) Guinier plot for B factor estimation.

Table 6.1 Refinement statistics of SmFtsZ model

Initial model used (PDB code)	3VOA
Resolution estimates (Å)	
• d model	4.0
• d FSC model (0/0.143/0.5)	2.8/3.6/4.8
Model vs. Data	
CC (mask)	0.64
(volume)	0.64
Model composition	
• Non-hydrogen atoms	2243
• Protein residues	305
• Ligands	1 (GTP)
Ramachandran plot	
• Favored (%)	95.05
• Allowed (%)	4.95
• Disallowed (%)	0.00
R.m.s. deviations	
• Bond lengths (Å)	0.003
• Bond angles (°)	0.756
Validation	
• MolProbity score	1.91
• Clashscore	8.87
Rotamer outliers (%)	1.30

6.3.2 Cryo-EM structure of SmFtsZ filament was obtained at 3.5 Å resolution

Based on our observations of the negative staining data, we proceeded for Cryo-EM sample preparation with SmFtsZ in GTP condition. After standardization, the final data was collected in 0.1 mg/mL concentration of the protein as higher concentrations showed heterogeneous filament population with bundling of laterally interacting filaments. After one first round of 2D classification, the best classes with straight filament morphology were chosen for 3D reconstruction. The final 3D map after non-uniform refinement represents a single protofilament of SmFtsZ constituting five molecules (three molecules at the centre of filament have better density compared to filament ends) at a 3.5 Å resolution (FSC threshold 0.143). The entire workflow is shown in Figure 6.2 and the refinement statistics for the final SmFtsZ-GTP model has been mentioned in Table 6.1.

Angular distribution of the particles used for final reconstruction shows orientation bias as seen in the heatmap with a non-uniform distribution around the azimuth i.e. more particles with viewing angles from $\frac{\pi}{2}$ to π region compared to a lesser population between $-\frac{\pi}{2}$ to $+\frac{\pi}{2}$ (Figure 6.2c). It could be possible that the particle orientation bias along with the flexibility of the filament limited a higher map resolution. However, we were able to clearly identify secondary structural features, especially the central β -sheet in the C-terminal domain (CTD) and H7 helix were very prominent (Figure 6.3a). We also noticed that the local resolution of the CTD β sheet and the GTP pocket was near 3 Å. The lower resolution of the N-terminal is possibly an indication of the dynamic nature of this region with respect to the CTD. We could also confirm that the filament is in the T state conformation, by fitting T and R state atomic models of SmFtsZ in the EM map (Figure 6.3b). The intermonomer distance in the filament model was measured to be 45 Å (Figure 6.3c) which is similar to earlier reports in EcFtsZ and KpFtsZ (Wagstaff et al. 2017, Fujita et al. 2023).

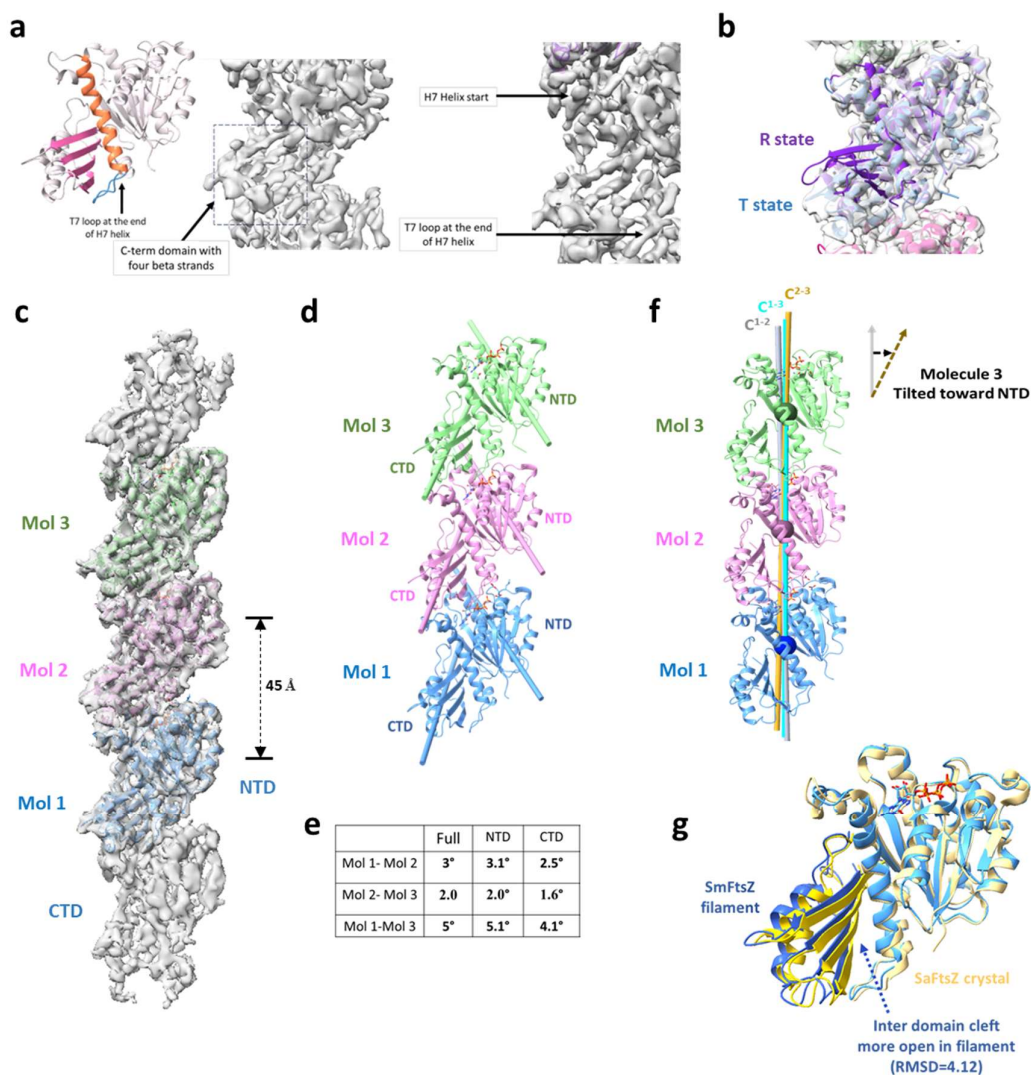


Figure 6.3 Final refined cryo-EM map and atomic model of SmFtsZ filament structure. (a) Final 3D refined EM map showing clear density H7 helix, T7 loop and C-terminal domain with characteristic beta strands. (b) Superimposition of R and T state structure in the map. (c) Final refined map (0.5 contour level) fitted with three monomers shown in different colours. (d) Calculation of relative domain movement across the molecules in the filament. Solid lines in respective molecule's color represent principal axes of respective domains. (e) Angles between domains and full molecules are measured for each pair of molecules along the filament. (f) Measurement of curvature of SmFtsZ filament showing a tilt of molecule 3 toward NTD. (g) Comparison of domain movement between SmFtsZ filament and SaFtsZ crystal structure.

Next, we checked the bending of the filament by calculating relative rotation angles of N-terminal domain (NTD), CTD across three subunits within the filament (Figure 6.3d, e). The angles were calculated in ChimeraX by defining individual axes along NTD, CTD and GTP for all three molecules in SmFtsZ filament model. We observed about 5° bending of molecule 3 with respect to molecule 1, indicating the inherent curvature of the filament. We measured the direction of the curvature by drawing two axes along the centroid of molecule 1-2 (C^{1-2}) and molecule 2-3 (C^{2-3}) (Figure 6.3f). We observed C^{2-3} axis to be oriented towards the NTD suggesting the filament (i.e. molecule 3) is tilted towards the NTD. To find out if there is a difference in the orientation of the NTD and CTD between filament and crystal structures, we superimposed the NTD of one SmFtsZ monomer of the filament with NTD of SaFtsZ (PDB 3WGN). Then we calculated the RMSD for the CTDs. We observed that the CTD of SmFtsZ filament is moved away from the NTD resulting in a wider inter-domain cleft between NTD and CTD compared to crystal structure (RMSD = 4.12) (Figure 6.3g).

6.3.3 SmFtsZ is bound to GTP in the filament

SmFtsZ molecules in the filament were found in a GTP bound state which was confirmed by fitting GTP in the extra density between two subunits (Figure 6.4). A clear density for the gamma phosphate was observed in the canonical binding pocket of the SmFtsZ molecule (Figure 6.4a). This is interesting because the previously published two cryo-EM structures of KpFtsZ and KpFtsZ-ZapA complex were bound to GMPCPP, a slowly hydrolysable GTP analog which might stabilize the filament conformation thus provide a non-native conformation

of the molecule. A high resolution GTP bound SmFtsZ filament, in contrast, can potentially give us more structural insights of conformational changes in the molecule associated with GTP binding and hydrolysis. Presently, we achieve a medium level resolution of 3.5 Å where we could confidently place the GTP molecule and the T3 loop (Figure 6.4). The ordered structure of the T3 loop supports one of our previous predictions from GMPPNP bound SmFtsZ crystal structure (PDB 7YOP) where we hypothesized that T3 loop gets ordered in the presence of GTP to accommodate the gamma phosphate in place (Chakraborty et al. 2024).

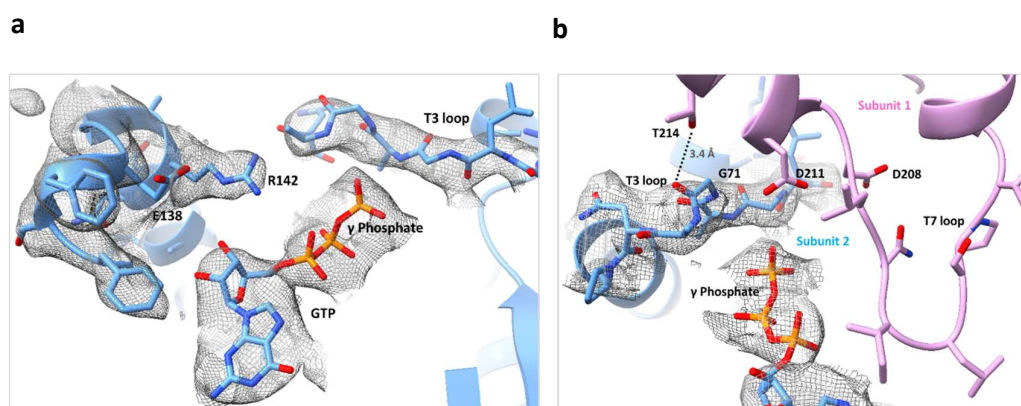


Figure 6.4 SmFtsZ is bound to GTP in the filament. Close-up view of the GTP density (a) and the T3 loop (b) in the cryo-EM map in between two monomer subunits of SmFtsZ filament. The map contour levels are set at 0.6 for both (a) and (b). Subunit 1 and subunit 2 in SmFtsZ filament are coloured in pink and blue respectively.

Next, to check if there is any difference in nucleotide orientation in a GTP vs GMPCPP bound state, we superimposed the NTD of SmFtsZ with KpFtsZ filament structure (GMPCPP bound, PDB 8IBN). We observed the gamma phosphate position to be different in GMPCPP from GTP. The gamma phosphate is placed closer to the T3 loop in GTP bound SmFtsZ whereas in KpFtsZ, the gamma phosphate position is away from the loop as evident from the map density (Figure 6.5a). Additionally, when we compared the position of T7 loop and we observed gamma phosphate position of SmFtsZ is closer Asp211 while the KpFtsZ gamma phosphate is closer to the Asp209 (Asp208 in SmFtsZ) (Figure 6.5b). These two Asp residues are conserved across FtsZs and important for GTP hydrolysis. Importantly, ZapA-KpFtsZ complex filament structure shows both conformations of gamma phosphate, one being closer to Asp212 (Asp

211 in SmFtsZ) and the other closer to Asp209 (Asp208 in SmFtsZ) (Fujita et al. 2024). The T7 loop also was found to move along with gamma phosphate of GMPCPP in KpFtsZ (Figure 6.5b).

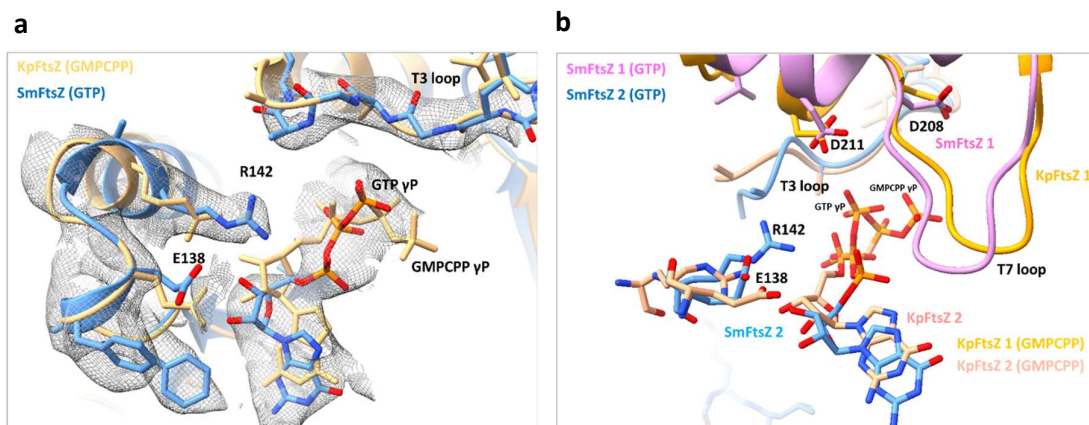


Figure 6.5 Comparison of nucleotide orientation between SmFtsZ and KpFtsZ (a) Close-up view of the nucleotide pocket in both the structures. Electron density of the cryo EM map has been shown for GTP (in SmFtsZ) and the two conserved ribose interacting residues-Asp138 and Arg142. Residue numbering is according to the SmFtsZ sequence. The contour level of the map is 0.6. (b) Orientation of nucleotide in bottom subunit (SmFtsZ/KpFtsZ 2) with respect to the top subunit (SmFtsZ/KpFtsZ 1) in filament structure of SmFtsZ and KpFtsZ.

We also compared GTP orientation in SmFtsZ filament with the existing crystal structures of SmFtsZ (GMPPNP bound, PDB 7YOP) and SaFtsZ (GTP γ S bound, PDB 3WGN) (Figure 6.6). The gamma phosphate position in the crystal structures were near to the T3 loop which was found in SmFtsZ filament structure as well in contrast to the KpFtsZ gamma phosphate orientation. Because of the resolution limitation, we could not specifically determine the direction of the peptide bond in Gly71 residue which was earlier proposed to flip outside to accommodate gamma phosphate and comes in contact with the top subunit. However, the clear density of the T3 loop indeed suggests the ordered conformation of the loop region which potentially can interact with the other subunit of the filament stabilizing interface interaction.

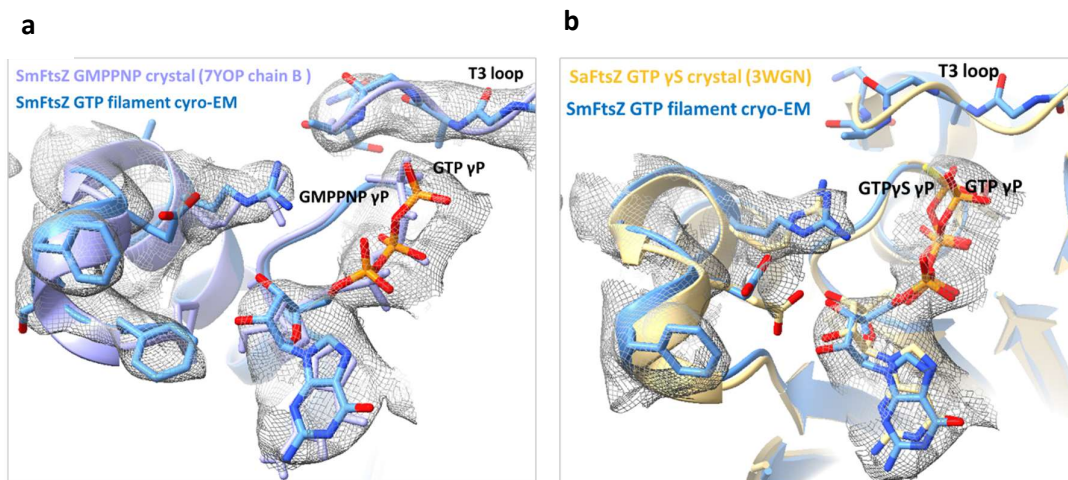


Figure 6.6 Comparison of nucleotide orientation between SmFtsZ filament and crystal structures (a) Close-up view of the nucleotide pocket SmFtsZ filament and SmFtsZ crystal structure. (b) Close-up view of the nucleotide pocket SmFtsZ filament and SaFtsZ crystal structure. Electron density of the cryo EM map has been at 0.6 contour level for SmFtsZ filament

6.4 Discussion

Until now, filament structure of high resolution FtsZ through cryo-EM has only been obtained from *K. pneumoniae* in a GMPCPP bound form (Fujita et al. 2023, Fujita et al. 2024). Earlier attempts with *E. coli* and *M. tuberculosis* FtsZs by Wagstaff and colleagues did not successfully achieve resolution beyond 7 Å possibly because of the unstable and high flexibility of the filaments (Wagstaff et al. 2017, Wagstaff et al. 2023). In this scenario, our present structure of FtsZ filament from *S. melliferum* would be quite useful for a comparative structural analysis of filament conformations. The overall resolution of our SmFtsZ filament is 3.5 Å (FSC = 0.143 cutoff) with local resolution of the CTD and GTP pocket reaching ~3 Å. One of the important features of the present SmFtsZ structure is that the monomers are GTP bound in contrast to the GMPCPP bound filament structures reported earlier. This is quite interesting because our GTP bound filament structure could possibly capture various dynamic states of hydrolysis and the conformational changes associated with it. In the current EM map, we can clearly identify the gamma phosphate density in all three subunits having highest resolution at the centre of the filament. We think the slow hydrolysis rate of SmFtsZ is possibly the reason we are able to

capture the monomer in its prehydrolysis state. Note that during processing of the current dataset we eliminated the curved filaments at the 2D class averaging step to improve overall resolution of the map. However, it is highly possible that bent filament structure could represent post hydrolysis state before the filament disassembles or even the intermediate states of hydrolysis. A detailed analysis of the entire dataset could enable us to capture the dynamics of the filament in a native state. It will be also interesting to obtain SmFtsZ filament structure in the presence of GDP and aluminium fluoride, $\text{AlF}_3/\text{AlF}_4^-$ (AlFx), to mimic the transition state of hydrolysis and capture conformational changes in the molecule upon hydrolysis.

In our analysis, we observe an inter-subunit tilt of about $2-3^\circ$ towards the NTD which is possibly an effect of intrinsic curvature of the FtsZ filament. The direction of the curvature towards the NTD in a native GTP bound state of a FtsZ filament is quite intriguing as this supports one of the earlier experimental observations by Osawa et al. 2009. In their liposome reconstitution experiment, they observed a change in the direction of the curvature of liposomes depending on which face of FtsZ protein (modified with a membrane targeting site (MTS) at the N- or C-terminal side) binds to the membrane i.e. concave depression was observed when MTS is at the C-terminal while a convex bulge when MTS is at the N-terminal. Based on these observations they proposed that the FtsZ protofilament has a preferential bend with C-terminal on the convex side and N-terminal on the concave side. Filament architecture of FtsZ (or in complex with monobody and other FtsZ associated proteins) has been observed in varied conformations and curvatures from straight protofilament to bent spirals and torroids within crystal packing as well as in solution when visualized under negative staining TEM and cryo-EM (Erickson and Osawa 2017, Fujita et al. 2023, Merino-Salomón et al. 2023). Our findings about the filament's intrinsic curvature towards N-terminal face could fit into a physiological scenario where short patches of treadmilling FtsZ-ring intrinsically orient its convex surface (i.e. the C-terminal side) towards the membrane to point the long flexible region outward of the ring for binding to FtsA through CCTP which is connected to the CTD. A more detailed structural analysis of the cryo-EM dataset, with an emphasis on the curved protofilament, its nucleotide states, and the associated domain movements, will provide valuable insights into this question.

Finally, while looking into the GTP orientation in SmFtsZ, we observed the gamma phosphate is positioned near the T3 loop unlike KpFtsZ structures which shows flexibility in the GMPCPP conformations in the filament. The gamma phosphate can be oriented either towards the T3 loop or away from it (Fujita et al. 2023, Fujita et al. 2024). Previous studies on the structural

analysis of T3 loop conformation in SmFtsZ crystal structures (PDB 7YOP, 7YSZ) proposed a possibility of ordering and flipping of the T3 loop in the presence of GTP (or its analog) which acts as an initial trigger for R to T transition during polymerization (Chakraborty et al. 2024). Our structure also shows strong electron density of the T3 loop which possibly is a dual effect of GTP and interaction from nearby subunit. In future, it will be interesting to carry out cryo-EM experiments to capture a native non-filamentous monomeric state, if possible, in GDP and GTP bound conformations to validate this hypothesis of GTP triggered polymerization.

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Chapter 7

Conclusions and future prospects

7.1 Summary of major findings

Cell division genes and their functions in wall-less bacteria have not been investigated earlier in detail which motivated us to look into the division related genes and their functions across a different genera of cell wall-less bacteria, the mollicute class. Here, I have summarized the major findings from my PhD research work.

Through genome analysis we first identified the occurrence of *dcw* cluster across different genera of mollicutes. Our results suggest that the presence and absence of the five *dcw* m cluster genes namely *mraZ*, *mraW*, *ftsA*, *ftsZ*, and *sepF* vary across species. In *Mycoplasmas*, based on the presence and absence of *ftsZ* and *ftsA* genes, we identified all four types of combinations. Importantly, the motif features of FtsZs, especially CCTP, which are known to be conserved among cell walled bacteria, show variations across species. We found the pneumoniae group FtsZs (containing twelve species) within the group 1 (where both *ftsZ* and *ftsA* are present) do not possess CCTP motif which is required for binding to FtsA. Surprisingly, all these twelve FtsZs contain putative membrane binding amphipathic helix. As a proof of concept, we demonstrate the proposed amphipathic helix of *Mycoplasma genitalium* FtsZ binds to liposome *in vitro* (Figure 7.1) and localizes to *E. coli* cell membrane *in vivo* (in collaboration with Dr. Ramanujam Srinivasan's lab).

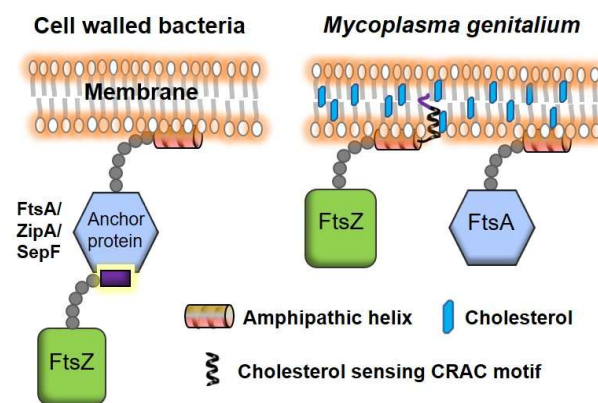


Figure 7.1 A novel mode of membrane attachment in *Mycoplasma genitalium* FtsZ in comparison with cell walled bacteria. Cell walled bacteria uses anchors like FtsA/ZipA/SepF which binds to the membrane with the help of their amphipathic helices. In contrast, *M. genitalium* FtsZ has been evolved with an inherent membrane binding amphipathic helix to bypass the requirement of FtsA for membrane attachment. Additionally, the amphipathic motif contains a CRAC motif that can potentially interact with membrane cholesterol.

We have also characterized FtsA from *Spiroplasma melliferum* through biochemical analysis and performed interaction studies with FtsZ. This is the first report of characterization of FtsA from cell wall-less bacteria, making our findings particularly important to understand its role in absence of a cell wall synthesis machinery. We have observed GTPase activity of SmFtsZ to be stimulated in the presence of FtsA. Moreover, FtsA induces higher order assembly formation of FtsZ filaments. We propose a model for GTPase stimulation and bundle assembly and disassembly process (Figure 7.2). SmFtsZ shows a basal level of GTPase and single protofilament formation. We hypothesise that in the presence of FtsA, FtsA-bound FtsZ appears to have higher intermolecular affinity which increases oligomerisation and thus hydrolysis. The bundle formation also occurs at this stage by lateral association of FtsZ protofilaments induced by FtsA. Excess concentration of FtsA prevents lateral interaction of FtsZ protofilaments possibly through extensive self-polymerization of FtsA. However, in the presence of excess FtsA, inter monomer interaction between FtsZ molecules becomes higher (because of increasing local concentration) causing more stimulation of GTPase. However, one of the caveats of this model is the assumption that SmFtsA polymerizes, which will be investigated in detail in our future studies. We have not yet demonstrated experimentally the multivalency of SmFtsA.

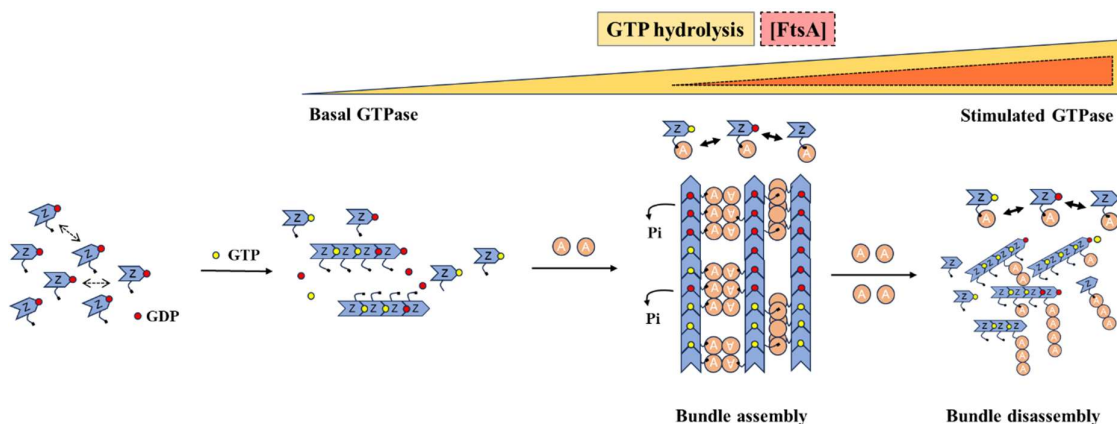


Figure 7.2 Model of FtsA dependent GTPase stimulation and bundle formation in FtsZ. Higher order assembly formation occurs at a sub-stoichiometric concentration of FtsA whereas excess FtsA enhances GTP hydrolysis and filament disassembly.

The final section of my thesis focuses on the cryo-EM structure of SmFtsZ filament captured in a GTP bound state. We could achieve a resolution of 3.5 Å where we can clearly identify electron density of secondary structures and many side chain residues as well. We could identify the gamma phosphate density in the canonical nucleotide binding pocket between two subunits and thus successfully place GTP molecule. We also confirm that SmFtsZ has a T state conformation within filament, similar to as observed for other filament structures earlier. In our filament structure, we can see good density for the T3 loop which is within interacting distance with the top subunit residues (carbonyl O of G71 (bottom) and OG1 of T214 (top)) that possibly stabilize the interface (Figure 7.3a). Earlier, we proposed from crystal structure analysis that GTP binding to an R state monomer helps to reorient the T3 loop so that it can interact with the top subunit which could be a possible trigger to R to T transition (Chakraborty et al. 2024). Our filament structure also supports our hypothesis of a possible role of GTP binding in polymerization. We also observed a filament curvature with concave side toward N-terminal domain (NTD) (Figure 7.3b). As a next step, we are in the process of collecting additional datasets to obtain filament structures with better resolution of wild type and several other mutants of SmFtsZ through cryo-EM to further validate our hypotheses.

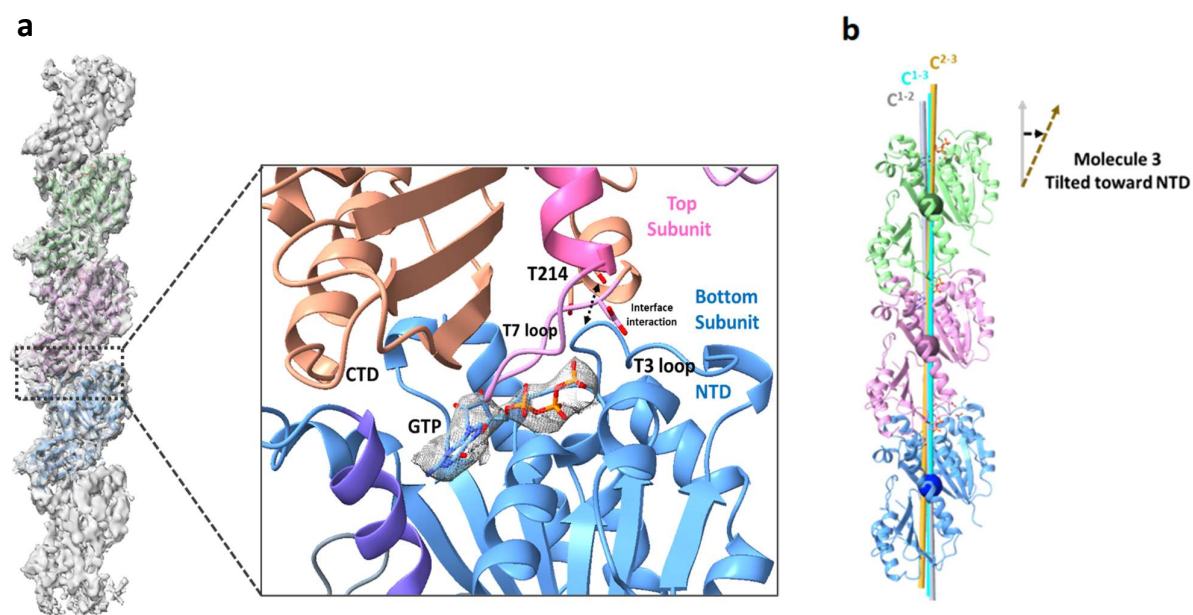


Figure 7.3 Cryo-EM map of SmFtsZ filament and final atomic model. Close-up view of the interface of two subunits is shown in right. Electron density for the GTP molecule is shown in 0.7 contour level. (b) SmFtsZ filament has a curvature towards the NTD.

7.2 Future prospects

7.2.1 *In vitro* and *in vivo* characterization of *Mycoplasma genitalium* FtsZ and FtsA

We have cloned *ftsA* and *ftsZ* of *M. genitalium* and currently we are standardizing the purification steps for checking membrane binding and cholesterol with wild type proteins *in vitro*. Our sequence analysis data shows the absence of a canonical CCTP motif in *M. genitalium* FtsZ thus possibly incapable of binding to FtsA. It will be really interesting to check this hypothesis and find out the possibility of other modes of FtsZ-FtsA interaction (e.g. possibly through globular domain). Next, we will also be looking into the activity of this novel FtsZ and the effect of FtsA on FtsZ function. Addressing these questions in future will shed light on the evolutionary significance of an inherent membrane anchor in FtsZ and the possible roles of FtsA proteins other than membrane anchoring.

7.2.2 To obtain crystal structure of SmFtsA and experimentally check nucleotide binding and polymerization

We have identified a few residues which are possibly hindering the nucleotide to bind at the canonical pocket of SmFtsA. We cloned these ATP binding mutant constructs and purification needs to be standardized for *in vitro* characterization. We are also aiming to obtain crystal structure of wild type and mutant proteins to validate our hypothesis. We could not observe polymerization for SmFtsA. However, there is a possibility that FtsA may require membrane support or other scaffold proteins for efficient polymerization. These possibilities will be checked in detail in future experiments.

7.2.3 To understand the molecular mechanisms behind GTPase stimulation and bundle formation in SmFtsZ

Based on the current results, we have proposed a model of GTPase stimulation and bundle formation in SmFtsZ induced by mainly FtsA's oligomerization property. However, the experimental validation is yet to be done. One of the ways to check is by mutating the polymerization interface of FtsA that is known from antiparallel double filament crystal structure of *E. coli* and *V. maritima* FtsA. We can check the effect of polymerization deficient FtsA in GTPase stimulation of FtsZ and bundling assembly. Additionally, we can confirm

whether the effect of FtsA is solely mediated by FtsZ-FtsA interaction by performing the same set of assays with CCTP deleted construct of SmFtsZ (i.e. SmFtsZ^{ΔCT}). Based on our pelleting assay, we observed that CCTP deleted SmFtsZ construct does not interact with SmFtsA, but since SmFtsZ^{ΔCT} itself forms bundled filaments we could not confirm it by negative staining TEM. In future, more data on TEM experiments and detailed image analysis will be required to compare the two types of bundles of SmFtsZ^{ΔCT} with and without SmFtsA.

7.2.4 To obtain high resolution filament structure of SmFtsZ

In our current study we have solved cryo-EM structure of SmFtsZ filament at 3.5 Å resolution but we need to achieve higher resolution for obtaining residue level structural information. The present data set contains about 1000 micrographs that were collected at 75,000x magnification and we used around 70,000 good particles for final reconstruction of the map. We believe by increasing the number of micrographs at higher magnification could help us to obtain higher resolution. We are currently in the process of collecting additional datasets for wild type as well as a few mutant constructs of SmFtsZ. T6 loop mutant constructs which show differential GTPase and polymerization can be one of the interesting targets for solving structure. We will also aim to capture transition states of nucleotide hydrolysis in the filament through cryo-EM with the help of GDP and aluminium fluoride, AlF₃/AlF₄⁻ (AlFx). This will be important to understand the conformational changes in FtsZ during GTP binding and hydrolysis. It will be also interesting to get the native structure of SmFtsZ-FtsA bundles through cryo-EM. FtsZ-FtsA filament complex structure will give us new insights about the detailed interactions and arrangement of the two proteins inside bundles which is unknown till date.

Overall, addressing these questions in future will enhance our understanding of the novel functions of FtsZ and FtsA in cell wall-less bacteria, while providing structural atomic level insights into the dynamics of FtsZ filaments and the FtsZ-FtsA complex in general, which remain unclear at present.