

Characterization of *Spiroplasma* FtsZ: Insights into
kinetic polarity and inhibitor binding

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By

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Dedicated to my parents and nanima

Certificate

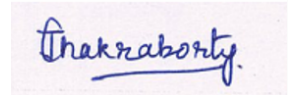
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Dr. Gayathri Pananghat

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Joyeeta Chakraborty

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List of abbreviations

Abbreviation	Full form
°C	Degree Celsius
nm	Nanometer
SMLM	Single-molecule localization microscopy
μM	Micrometer
NO	Nucleoid occlusion
DNA	Deoxyribonucleic acid
CCTP	C-terminal conserved peptide
sPG	Septal peptidoglycan
MTS	Membrane targeting sequence
GTP	Guanosine triphosphate
Mg ²⁺	Magnesium ion
GDP	Guanosine diphosphate
NTD	N-terminal domain
CTD	C-terminal domain
CTL	C-terminal linker
CCTP	Conserved C-terminal peptide
CTV	C-terminal variable region
PF	Protofilament
EcFtsZ	FtsZ from <i>E. coli</i>
SmFtsZ	FtsZ from <i>S. melliferum</i>
CC	Critical concentration
TEM	Transmission electron microscopy
NEB	New England Biolabs
LB	Luria Bertani
CV	Column volume
μg/ml	Micrograms per microliter

SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
KDa	Kilodalton
EDTA	Ethylenediaminetetraacetic acid
MgCl ₂	Magnesium chloride
k_{cat}	Specific activity
k_{obs}	Apparent specific activity
RMSD	Root mean square deviation
GSP	guanosine 5'-O-[gamma-thio] triphosphate
GMPPNP	Guanosine 5'- [(β , γ)-imido] triphosphate
Å	Angstrom
SDM	Site directed mutagenesis
HPLC	High Pressure Liquid Chromatography
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Tris	Tris(hydroxymethyl)aminomethane

Abstract

FtsZ, a tubulin homolog, marks the site of division and forms ring-like structure, the Z-ring, in bacteria. The Z-ring gets tethered to the membrane via FtsA, an actin homolog. Unlike the dynamic instability seen in tubulin, FtsZ undergoes treadmilling to guide the peptidoglycan synthesis machinery for the formation of the septum. However, the role of FtsZ filament dynamics in absence of peptidoglycan synthesis is unclear. Study of FtsZ filaments from cell wall-less bacteria could provide insights into these mechanistic aspects. A conformational switch in FtsZ from low affinity R state to high affinity T state favours cooperativity and filament elongation from one end and depolymerization from the other. The difference in rate of assembly and disassembly between two ends of the filaments decides kinetic polarity. However, the structural basis of the kinetic polarity of FtsZ polymerization is not fully understood. Since FtsZ is an essential protein in bacterial cell division, it can be the target for anti-microbial drugs. But the mechanism of the drug, PC190723, binding to FtsZ is not known.

In my PhD research, I have performed comparative biochemical characterization of FtsZ from a cell wall-less bacteria, *Spiroplasma melliferum* (SmFtsZ) and FtsZ from *Escherichia coli*, a cell walled bacterium (EcFtsZ). We observed that features like GTP binding, hydrolysis and filament formation are conserved for FtsZ from organism devoid of cell wall but SmFtsZ has lower GTPase activity and higher critical concentration of polymerization than that of EcFtsZ under similar *in vitro* conditions. We then determined the crystal structures of SmFtsZ, with GDP and GTP respectively, which captured the NTD in both R and T states and the CTD in an R state conformation, revealing an intermediate conformational state of FtsZ during R to T state transition. This led to the identification of a residue which could help in the cleft opening between N-terminal domain (NTD) and C-terminal domain (CTD) during R (closed) to T (opened) state transition. Our hypothesis was supported by structural, biochemical and *in vivo* experiments of the cleft mutant.

Further, structure and sequence analyses of the interdomain cleft of FtsZs helped us obtain insights into mechanism of binding of its inhibitor PC190723. We propose that the presence of occluding salt bridge interactions in the inter domain cleft of FtsZ is responsible for the resistance of PC190723 binding to certain FtsZs.

We finally propose a model for how the kinetic polarity of FtsZ filaments is opposite to that of microtubules, a polymer of its homolog tubulin and the model for mechanism of the drug, PC190723, binding to FtsZ.

Synopsis

Characterization of *Spiroplasma* FtsZ: Insights into kinetic polarity and inhibitor binding

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The cytoskeleton in bacteria plays an important role in various processes like cell division, cell shape regulation and cell polarity (Shih and Rothfield, 2006). One of the essential cytoskeletal proteins involved in bacterial cell division is the tubulin homolog FtsZ (Begg and Donachie, 1985; Nogales *et al.*, 1998). At the onset of division, FtsZ localizes at the mid-cell forming a ring-like structure known as the Z-ring (Bi and Lutkenhaus, 1991). The Z-ring binds to the membrane via proteins like FtsA, an actin homolog, or ZipA, and recruits other proteins required for cell division (Ma *et al.*, 1996; Hale and de Boer, 1997) forming a multiprotein complex called divisome that gradually constricts the cell, eventually leading to two daughter cells (Ma *et al.*, 1996; Hale and de Boer, 1997; Ma *et al.*, 1997). However, the precise mechanism of how ring constriction takes place is not fully understood. Earlier studies of FtsZ linked to a membrane targeting sequence (FtsZ-mts) showed that in presence of GTP, FtsZ forms rings which can constrict liposome membrane *in vitro* (Osawa *et al.*, 2008). Experiments with reconstituted FtsZ and FtsA* (a hyperactive mutant of FtsA) within liposomes showed efficient formation of constriction sites inside liposomes. Interestingly, unlike FtsZ-mts, FtsZ-FtsA* was found to perform complete scission of the reconstituted liposome membrane (Osawa and Erickson, 2013). Another mechanism of ring constriction could be attributed to the treadmilling of FtsZ filaments which have been recently found to be essential to initiate constriction during cell division (Whitley *et al.*, 2021). Treadmilling of FtsZ has also been shown to deform membranes by generating torsional stress upon hydrolysis of GTP (Ramirez-Diaz *et al.*, 2021) which can guide the cell wall synthesis machinery along the circumference of the inner membrane to insert new peptidoglycan at the septum (Bisson-Filho *et al.*, 2017).

Cell wall synthesis is also thought to be the primary force generator for constriction and the rate-limiting process in cytokinesis or septum closure (Coltharp *et al.*, 2016). This raises the intriguing question of how cell division could take place in bacteria that lack cell wall and cell wall production machinery. Thus, FtsZ from a cell-wall less bacterium which works independent of cell wall synthesis can serve as a model to study fundamental features of FtsZ in ring constriction and cell division.

Despite consisting of a single protofilament, FtsZ polymerization is a cooperative process that requires a critical concentration of the monomers for filament assembly (Mukherjee, 1998; Huecas *et al.*, 2008). In addition, FtsZ filaments exhibit treadmilling, as opposed to the typical dynamic instability observed for microtubules. The basis of treadmilling of FtsZ protofilament (PF) is the polymerization from one end and depolymerization from the other end (Artola *et al.*, 2017). For a homopolymer to assemble in a cooperative manner, it requires difference in the conformational states in the monomer form and the filament form. Earlier crystal structures have shown that FtsZ can exist in two states: the R state that is favored for monomers and T state that is favored for subunits within protofilament (Wagstaff *et al.*, 2017). In R state, the inter-domain cleft between the N-terminal domain (NTD) and the C- terminal domain (CTD) is closed whereas in T state, it is opened (Fujita *et al.*, 2017). The cryo-electron microscopy of the soluble filaments of FtsZ from *E. coli* is shown to fit better with the T state crystal structure of FtsZ from *S. aureus* than that of R state, confirming the T state conformation of FtsZ to be present in the filament form (Wagstaff *et al.*, 2017). Another important feature of FtsZ filament dynamics is kinetic polarity which is interestingly opposite to what is known for microtubules. Microtubules elongate from the top end, at which the N- terminal domain (NTD) of beta-tubulin is exposed, whereas FtsZ grows from the bottom end, the end with an exposed C-terminal domain (CTD) (Du *et al.*, 2018; Knossow *et al.*, 2020). However, the structural basis of this strikingly opposite directionality of growth for the two homologous proteins is unknown till date.

Since FtsZ is an essential protein for the process of cell division in bacteria, it becomes an attractive target for antimicrobial drug. Abrogating FtsZ would lead to cell division defects and eventually the death of the bacterial cells. The most efficient drug known to target FtsZ is PC190723. It is effective against all *Staphylococcus* species and strains, including the methicillin- and multidrug- resistant *Staphylococcus aureus*, *Bacillus subtilis*. But it is ineffective against from bacteria like *Streptococcus*, *E. coli*, and *Enterococcus*. The mechanism of action of this drug against FtsZ is not clear.

In order to decipher the aforementioned questions, the major goal of my thesis was to identify the unknown structural mechanism responsible for the kinetic polarity of FtsZ. In addition, my thesis seeks to understand the mechanism of FtsZ inhibitor binding.

To achieve this aim, my thesis is divided into the following objectives which are put into chapters:

Chapter 1: Introduction

This chapter summarizes the existing literature on the bacterial cell division protein FtsZ, which forms a ring like structure, Z-ring at the site of division. It summarizes the models which have been proposed for the constriction of the cell, the various factors for the positioning and regulation of the Z-ring, the molecular details of FtsZ and the open questions in the field. It also highlights the purpose of choosing FtsZ from a cell wall less bacterium for the study.

Chapter 2: Biochemical characterization of FtsZs from *Spiroplasma melliferum* and *Escherichia coli*

In order to identify the fundamental mechanism and conserved signatures for the FtsZ function that operates independently of the cell wall synthesizing machinery, it will be helpful to compare the FtsZs from organisms with and without cell walls. This chapter summarizes the cloning, purification, and biochemical characterization of FtsZs from cell wall less bacterium *Spiroplasma melliferum* (SmFtsZ) and from cell walled bacteria *Escherichia coli* (EcFtsZ). It was observed that SmFtsZ has lower GTPase and a higher critical concentration than that of EcFtsZ, under same *in vitro* conditions. SmFtsZ forms filaments only in presence of GTP as confirmed by pelleting assay and visualized using negative staining under TEM.

Chapter 3: Structure determination of conformational states of SmFtsZ and their structural analysis

We attempted to elucidate the molecular details of SmFtsZ in order to gain mechanistic insights into the lower GTPase activity of SmFtsZ. In this chapter, I summarize crystallization and structure determination of SmFtsZ with different nucleotides. It was observed that the structures we obtained are domain swapped dimers wherein one domain of one chain is packed against the other chain forming a globular FtsZ structure. Upon further analysing the structures it is seen that the N-terminal domain (NTD) is in T state and the C-terminal domain (CTD) is in R-state. We hypothesize this to be an intermediate state during the transition from R to T state. It is seen that the gamma phosphate of GTP indeed plays a role in the polymerization of

the protein by forming stable interactions with the T3 loop, which is shown to be at the polymerization interface, as seen in the filament structures. We propose that the top interface comprising the N-terminal nucleotide binding domain is compatible to bind to the bottom end, comprising the CTD, of the FtsZ filament upon GTP binding, independent of conformational change in the CTD. This aligns with the previous report that FtsZ polymerizes from the bottom end.

Chapter 4: Identification of an interdomain cleft residue in FtsZ important for R to T state transition

The transition of R to T state is accompanied by the opening of the interdomain cleft between NTD and CTD. We discovered a residue in the interdomain cleft located at the hinge which is responsible for the smooth opening of the cleft. SmFtsZ has a rigid residue, Phe224, at the hinge of the cleft which is hypothesized for the slower GTPase activity of the wild type. This chapter describes the biochemical characterization and structure determination of the cleft mutant of SmFtsZ. It is observed that upon replacing Phe224 with the conserved flexible residue, Met224, the GTPase activity increases as well as the critical concentration to form filaments decreases. We propose that the interdomain cleft opening is the rate limiting step for the transition from R to T state of FtsZ.

Chapter 5: Mechanistic insights on binding of the drug PC190723 to FtsZ

Since FtsZ is an essential protein in bacterial cell division, it can be the target for anti-microbial drugs. We chose the most potent anti-microbial drug that targets FtsZ, performed sequence and structural analyses and decipher the mechanism of this drug, PC190723, binding to FtsZ. Based on the sequence and structural analysis of FtsZs from different organisms, we demonstrate that the resistance or susceptibility of the drug to FtsZ is due to the presence or absence of salt-bridge interactions between the central H7 helix and beta-sheet S10 and S7 of FtsZ, respectively.

Chapter 6: Conclusion and future prospects

This chapter highlights the major conclusions from the work. We propose that that GTP binding triggers transition to the T-state of NTD, independent of the CTD conformation. This conformation is compatible for GTP-bound monomer addition to the CTD polymerization interface of FtsZ filament. However, for addition at the opposite end, the monomeric CTD has to undergo conformational change by cleft opening. Hence, addition from the NTD end of the

filament is responsive to GTP binding and hypothesized to be faster than from CTD end. Based on the structures of FtsZ conformational states, biochemistry and *in vivo* (done in collaboration) experiments, we thus provide a mechanistic basis for kinetic polarity of FtsZ.

We also provide the model for binding of the drug, PC190723, to FtsZ, which aligns well with the *in vitro* data (done in collaboration). Finally, this chapter provides the future analyses and experiments which can be done to gain mechanistic insights into opposite kinetic polarity of the two homologous proteins- FtsZ and tubulin and the cell division in cell wall less bacteria.

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Publications:

- 1) Sharma, A.K., Poddar, S.M., **Chakraborty, J.**, Nayak, B.S., Kalathil, S., Mitra, N., Pananghat, G., Srinivasan, R., (2023) A salt bridge-mediated resistance mechanism to FtsZ inhibitor PC190723 revealed by a cell-based screen. *Mol Biol Cell* mbcE22120538. <https://doi.org/10.1091/mbc.E22-12-0538>
- 2) **Joyeeta Chakraborty**, Sakshi Mahesh Poddar, Soumyajit Datta, Vaishnavi Bahulekar, Shrikant Harne, Ramanujam Srinivasan, Pananghat Gayathri. “Structures of FtsZ from a cell-wall less bacterium *Spiroplasma* provide a mechanism for kinetic polarity.” *bioRxiv* (2022), <https://doi.org/10.1101/2022.10.13.512043>; (Manuscript currently under revision)

Manuscripts under preparation:

- 1) Soumyajit Dutta, **Joyeeta Chakraborty**, Gayathri Pananghat. “An amphipathic helix facilitates direct membrane binding of Mycoplasma FtsZs.” (Manuscript ready to be submitted)
- 2) **Joyeeta Chakraborty**, Gayathri Pananghat. “A structure-based interpretation on opposite kinetic polarities of FtsZ and tubulin.” (Perspective under preparation for submission in the research topic “Shedding Light on Asymmetric Cellular Machinery”, *Frontiers in Cell and Developmental Biology*)
- 3) **Joyeeta Chakraborty**[#], Soumyajit Dutta[#], Vaishnavi Bahulekar, Gayathri Pananghat. “SAXS studies to elucidate the presence of an intermediate state of FtsZ.” ([#] indicates equal contribution; Manuscript under preparation)
- 4) Soumyajit Dutta[#], **Joyeeta Chakraborty**[#], Gayathri Pananghat. “Mechanism of FtsA along with FtsZ in cell-wall less bacterium.” ([#] indicates equal contribution; Manuscript under preparation)

Protein Data Bank (PDB) depositions:

7YOP, 7YSZ, 8GRW

Chapter 1

Introduction

1.1 Cytokinesis

Cell proliferation includes cell elongation, genome replication, chromosome segregation, and ultimately cytokinesis (Meunier *et al.*, 2020). The physical process of cell division known as cytokinesis entails the creation of a contractile ring that physically divides the cytoplasm of the parent cell into two daughter cells (Alberts *et al.*, 2002). The process of cytokinesis depends heavily on the cytoskeleton proteins (Erickson, 2007; Lowe, 2017). The cytoskeleton assembles into specific supramolecular assemblies in all cell types to promote cell division (Figure 1.1, Table 1.1) (Oliferenko *et al.*, 2009; Balasubramanian *et al.*, 2012). The cytoskeletal filaments that surround the area where the septum is formed beneath the membrane make up the contractile ring. A medial ring of actin and myosin, assisted by other proteins, undergo contraction to divide the cell in fungal and mammalian cells (Alberts *et al.*, 2002). Actin filaments and microtubules are used by plant cells as a cell plate. Eukaryotic cytoskeletal proteins have homologues in prokaryotes, which assist in cytokinesis of prokaryotic cells (Margolin, 2005).

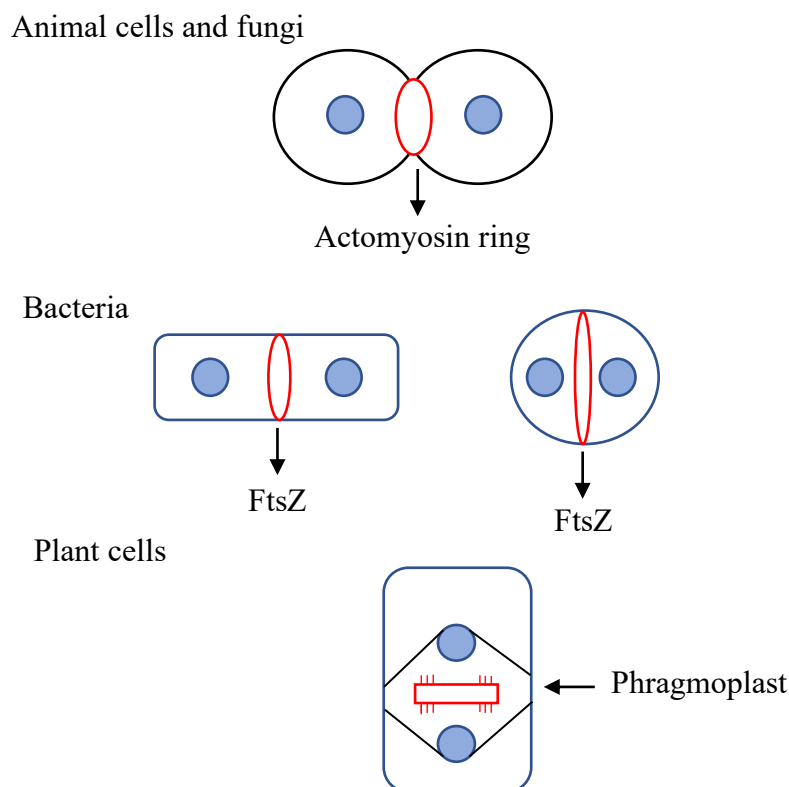


Figure 1.1 Cytoskeletal protein assembly in cell division of different cell types.

Animal cells and fungus both use a contractile ring that is mostly made of F-actin and myosin during cytokinesis. At the site of division, the tubulin-like protein FtsZ in bacteria forms a ring-structure. The phragmoplast, a microtubule-based apparatus in plant cells, is used for cell division. Adapted from Oliferenko *et al.*, 2012.

Table 1.1 Protein families involved in cytokinesis (Adapted from Oliferenko *et al.*, 2012)

	Actin		Myosin		Tubulin		Dynein		Kinesin	
	H	C	H	C	H	C	H	C	H	C
Bacteria	Yes	No	No	No	Yes	Yes	No	No	No	No
Yeast	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Plant	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes	Yes
Animal	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

*H denotes homolog, C denotes cytokinesis

1.2 FtsZ

The majority of prokaryotes divide using a protein called FtsZ, a tubulin homologue (Dai and Lutkenhaus, 1991; van den Ent *et al.*, 2001; Battaje and Panda, 2017). All chloroplasts and certain mitochondria need FtsZ to divide. Both of these are eukaryotic organelles that arose from bacteria (Glynn *et al.*, 2007; Johnson *et al.*, 2015; Chen *et al.*, 2018; Kraus *et al.*, 2021). The FtsZ is known to form a ring like structure known as the Z-ring at the site of division and helps in orchestrating the process of bacterial cytokinesis (Lutkenhaus and Addinall, 1997; Margolin, 2005; Haeusser and Margolin, 2016).

1.2.1 FtsZ and its discovery

In 1964, scientists began researching for the genes that bacteria needed to divide their cells. They isolated mutants with filamentous phenotypes as a result of temperature increase from 30° to 42 °C. This was brought on by temperature sensitivity causing harm to the proteins necessary for cell division. Since these genes are temperature sensitive and cause a filamentous phenotype in non-permissive temperatures, it was named as "Filamentous Temperature Sensitive Genes" (van de Putte *et al.*, 1964; Hirota *et al.*, 1968; Allen *et al.*, 1974). Lutkenhaus and colleagues later identified the filamentous temperature sensitive gene Z (*ftsZ*) gene as the site of the mutation in this strain (Lutkenhaus *et al.*, 1980). Cell division was observed to cease as the level of FtsZ started to drop after a change to the non-permissive temperature, demonstrating that septation is very sensitive to the amount of FtsZ (Dai and Lutkenhaus, 1991). The filaments were found to contain nucleoids along their whole length, indicating that nucleoid segregation is still normal even when the amount of FtsZ drops, but the cytokinesis stops as a result of the FtsZ decline (Dai and Lutkenhaus, 1991).

1.3 The Z ring

For the purpose of observing FtsZ localisation during cell division, immunoelectron microscopy was employed. *E. coli* cells that were exponentially expanding were fixed and immunolabelled with FtsZ antibodies. The longitudinal section gold labelling pattern indicated that FtsZ creates a ring-like structure at the point of division, and that the diameter of the ring shrank as septation began. This was the first *in vivo* visualization of ring like structure at the midcell where the cell divides (Figure 1.2A) (Bi and Lutkenhaus, 1991). The early hypothesis that the Z-ring could be made up of a single, long protofilament coiled around the cell waist numerous times was prompted by the smooth view of the Z-ring observed by epifluorescence microscopy (Erickson *et al.*, 2010).

The imaging of *Caulobacter crescentus* using cryo-electron tomography (Cryo-ECT) provided the first *in vivo* high-resolution structure of the Z-ring. Overexpression of a FtsZ GTPase mutant (FtsZ^{G105S} or FtsZ^{D212A}) resulted in a dramatic increase in the length of the arced filaments and bundling, likely as a result of increased lateral interactions between filaments, as opposed to overexpression of wild-type FtsZ, which increased the number of filaments but not their length (Li *et al.*, 2007; Szwedziak *et al.*, 2014) (Figure 1.2B). At the location of midcell constriction, it was found that distinct, arc-like FtsZ filaments were situated about 13 nm below the inner membrane. Early single-molecule localization microscopy (SMLM) analyses of the *E. coli* Z-ring showed that the FtsZ clusters were organised in a discontinuous, diverse manner (Fu *et al.*, 2010) (Figure 1.2C).

However, calculations based on the GTP hydrolysis activity of FtsZ and the quantity of FtsZ monomers at midcell imply that the Z-ring may really be comprised of brief protofilaments that overlap and interact laterally (Anderson *et al.*, 2004; Erickson *et al.*, 2010). According to the current model of Z-ring structure, the inner membrane is located 13–16 nm below the Z-ring, which is made up of discontinuous, heterogeneously distributed FtsZ filaments that are contained in a toroidal zone that is 80–100 nm wide (McQuillen and Xiao, 2020) (Figure 1.2D). It is likely that these single layered FtsZ filaments are connected to one another by protein factors. FtsZ's GTPase activity regulates this organisation, as seen in mutants of FtsZ with low GTPase activities creating more uniformly ordered Z-rings (Figure 1.2B,C). This organisation is largely independent on FtsZ's cellular concentration or assembly regulators.

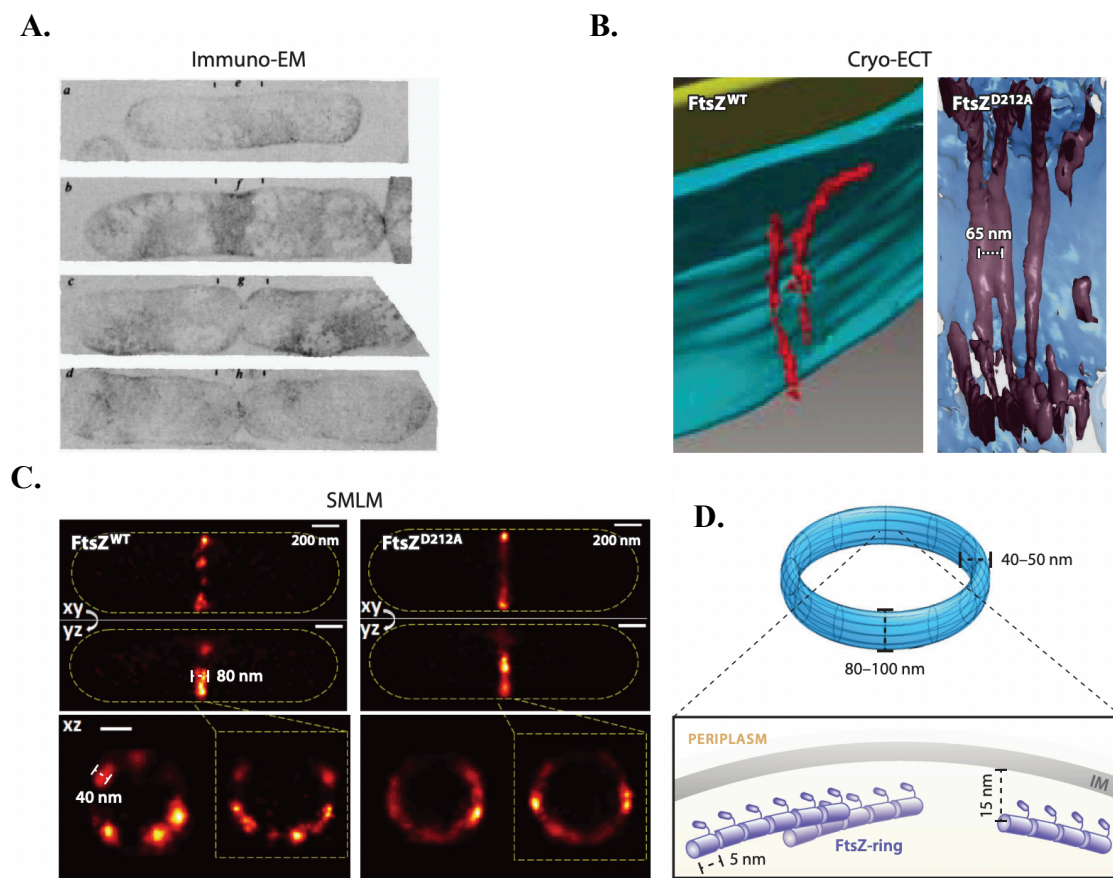


Figure 1.2 Structure of the Z-ring

A) FtsZ molecules are identified in immunogold EM images of contracting *Escherichia coli* cells at the leading edge of the invaginating septum, Bi and Lutkenhaus, 1991. B) *Caulobacter crescentus* FtsZ WT and FtsZ^{D212A} filaments in cryo-ECT images, Li *et al.*, 2007; Szwedziak *et al.*, 2014. C) WT FtsZ and FtsZ^{D212A} - rings captured in three-dimensional super-resolution fluorescence imaging, Fu *et al.*, 2010. D) The spatial and dimensional characteristics of the Z-ring are shown in the current model. Image adapted from McQuillen and Xiao, 2020.

1.3.1 Spatial regulation of Z-ring

The amount of FtsZ present in the cell is roughly 10 μM which is much higher than the critical concentration of 1-2 μM required for protofilament formation *in vitro*. This shows that FtsZ can only assemble at the appropriate time and location because of assembly inhibitors (Romberg and Levin, 2003). There are two majorly studied inhibitory systems to restrict the location of the Z-ring to the midcell. These are- Min system and nucleoid occlusion system which help in the precise positioning of the Z- ring (Figure 1.3).

The Min system consists of three proteins- MinC, MinD, and MinE which oscillate from one pole to the other (Raskin and de Boer, 1999). Upon ATP hydrolysis, the ATPase MinD is

released from the membrane after binding to the membrane in its ATP form (Suefuji *et al.*, 2002). By attaching to MinD and activating its ATP hydrolysis, MinE pushes MinD off the membrane. MinD-ATP rebinds to the membrane as a result of subsequent diffusion of MinD-ADP and nucleotide exchange. Due to the transient presence of MinE at the original site and the kinetics of nucleotide exchange, this new binding takes place away from the original site (Hu and Lutkenhaus, 2001). Only MinC, one of the three proteins, directly engages FtsZ to prevent it from forming a Z ring (Hu *et al.*, 1999) (Hernández-Rocamora *et al.*, 2013). The two domains of MinC work together *in vitro* to prevent the formation of higher order structures of FtsZ protofilaments. The strongest component, which shortens the existing FtsZ protofilaments, is the amino-terminal domain. The lateral contacts between protofilaments that lead to bundles appear to be repelled by the carboxy-terminal domain of MinC, which has a reduced inhibitory function against FtsZ (Dajkovic *et al.*, 2008a). It is at the cell poles that FtsZ assembly is impeded the greatest and at the midcell where it is least because MinC binds with the oscillating MinD protein. As a result, a FtsZ disassembly wave oscillates from pole to pole (Thanedar and Margolin, 2004).

The other regulating system for the Z-ring positioning is the nucleoid occlusion (NO) system. The NO system inhibits the FtsZ assembly on the chromosomal DNA which is unreplicated, making sure that FtsZ assembles only when the DNA is replicated and segregated at the poles (Woldringh *et al.*, 1990; Yu and Margolin, 1999). Proteins like SlmA and Noc in *E. coli* and *B. subtilis*, respectively are known to be involved in the nucleoid occlusion system (Wu and Errington, 2004; Bernhardt and de Boer, 2005).

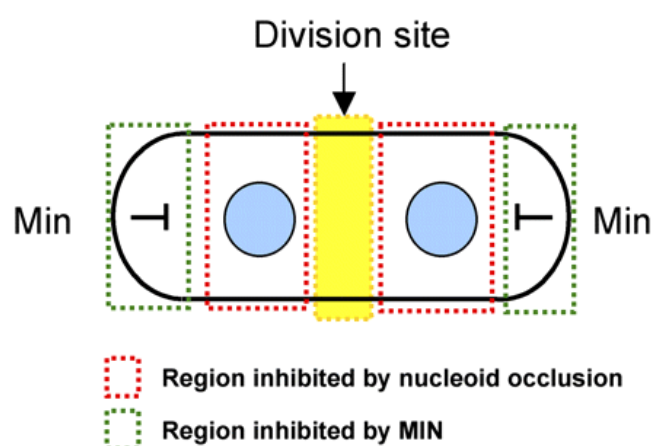


Figure 1.3 Spatial regulation of FtsZ in cell

The Min system and the nucleoid occlusion machinery both work to inhibit the formation of FtsZ rings at the cell ends and in close proximity to the nucleoids, respectively. Adapted from Oliferenko *et al.*, 2012.

1.3.2 Membrane tether of Z- ring

The FtsZ polymerizes at the midcell to form ring like structure, the Z-ring, which gets tether to the membrane via proteins like FtsA which is an actin homolog or ZipA (Ma *et al.*, 1996; Ma *et al.*, 1997; Yan *et al.*, 2000). In Gram positive bacteria, only FtsA is present whereas in Gram negative bacteria, both FtsA and ZipA are present. The C-terminal peptide of FtsZ has conserved residues to bind to the membrane anchor proteins (Figure 1.4A) (Rico *et al.*, 2013). FtsA is an actin homolog and its C-terminal has an amphipathic helix whereas ZipA has a transmembrane domain which binds to the membrane (Hale and de Boer, 1997; Krupka *et al.*, 2014) (Figure 1.4B)

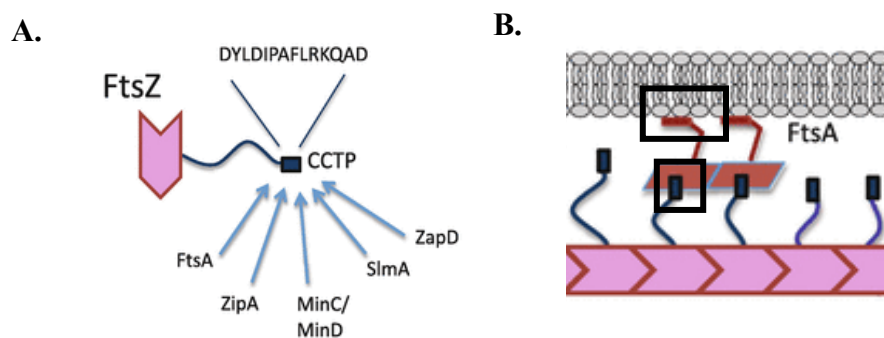


Figure 1.4 Membrane tether of FtsZ.

A) The C-terminal conserved peptide (CCTP) is shown to interact with proteins like FtsA, ZipA, MicC/D, SlmA, ZapD. B) Organization of FtsZ bound to FtsA which is further attached to the membrane via its amphipathic helix. Adapted from Löwe, 2017

1.3.3 Divisome complex

Upon attaching to the membrane via membrane anchors like ZipA or FtsA, the Z-ring recruits more than 30 proteins at the site of division to form a macromolecular complex known as divisome which is essential for cytokinesis (Weiss, 2004; Lutkenhaus *et al.*, 2012). More than half of these proteins are essentially responsible for the peptidoglycan (PG) synthesis, remodelling and its regulation (Margolin, 2005; Egan and Vollmer, 2013).

The macromolecular complex consists of proteins which- a) stabilizes the FtsZ assembly like FtsA, ZapA, Zip A, B, C, and D, (Hale and de Boer, 1997; Ma *et al.*, 1997; Durand-Heredia *et al.*, 2012; Buss *et al.*, 2017; Caldas *et al.*, 2019), b) functions as the septal peptidoglycan (sPG) synthases like FtsW and FtsI (Nguyen-Distèche *et al.*, 1998; Taguchi *et al.*, 2019). FtsI (also known as PBP3) plays a special role in the septal peptidoglycan's polymerization during cell division (Wientjes and Nanninga, 1991), c) DNA segregation like FtsK (Begg *et al.*, 1995; Grainge, 2010), d) that regulates sPG synthesis including FtsB, L, Q and N

(Buddelmeijer and Beckwith, 2004; Liu *et al.*, 2015). For active cell constriction to occur, *Escherichia coli* FtsN, a bitopic membrane protein, is required. FtsN's arrival at the division site causes the cell membrane to contract and the septal peptidoglycan to be synthesized. By interacting with FtsA in the cytoplasm and the FtsQLB complex in the periplasm, FtsN causes constriction. ZipA facilitates the interaction between FtsN and FtsA (Weiss, 2015; Söderström *et al.*, 2018; Pichoff *et al.*, 2018).

The recruitment of all the aforementioned proteins involved in the formation of divisome complex is dependent on the formation of Z- ring at the site of division (Margolin, 2005) (Figure 1.5). After the formation of the divisome complex, the Z-ring contracts and divides the cell into two halves.

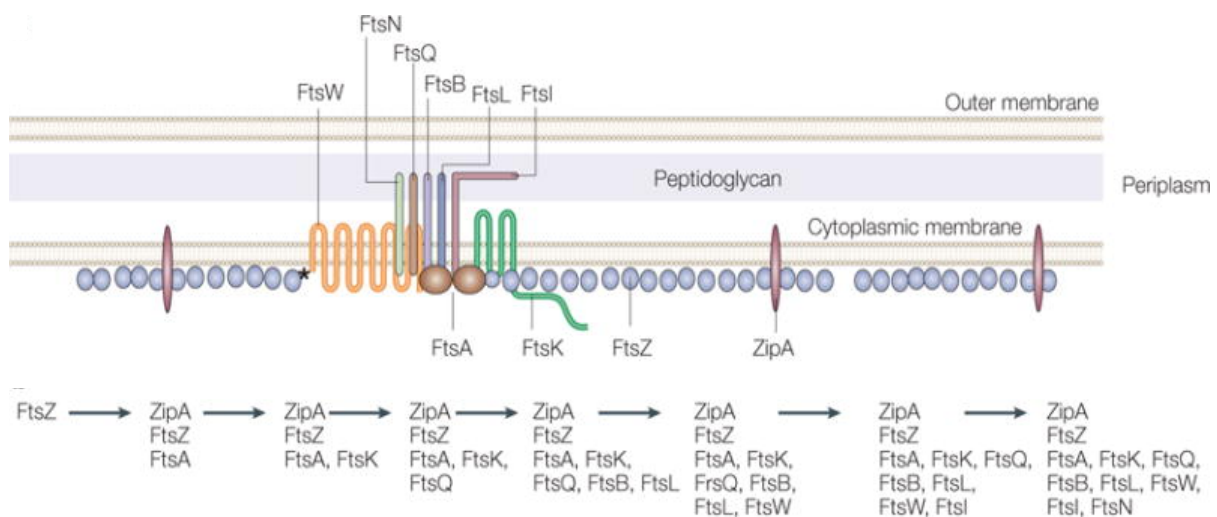


Figure 1.5 Overview of the proteins recruited in the divisome by Z-ring

The cross-sectional representation of the *Escherichia coli* Z ring and its necessary protein partners. Although the exact structure of FtsZ in the Z ring is unknown, it is represented as a collection of single protofilaments at the membrane. However, *Mycobacterium tuberculosis* (asterisk), which is deficient in ZipA and FtsA, directly interacts with FtsW. The order of recruitment of the proteins making the divisome is shown. Adapted from Margolin, 2005.

1.4 Z-ring constriction

In eukaryotes, cytokinesis involves contraction of an actomyosin based contractile ring (Balasubramanian *et al.*, 2012; Cheffings *et al.*, 2016). The anti-parallel bands of actin filaments are pulled together by the bipolar myosins. Myosin II dimers accumulate next to each other forming bipolar thick filament (BTF) forming a grip in the actin filaments and pulls it so that they slide and contracts (Sweeney and Houdusse, 2010; Pollard, 2010). However, the exact ring contraction mechanism is not fully understood in eukaryotes too.

There is no motor protein in case of bacterial cell division to contract the Z-ring or to walk over the filament to pull them closer to constrict the ring. So, it is only the Z-ring which generates the force to constrict but how performs this function is not known yet.

1.4.1 Z- centric model

Experiments on fluorescently labelled FtsZ reconstituted on liposome by C-terminal membrane targeting sequence (MTS) so that it tethers to the membrane reveals that FtsZ forms rings and produces a force in presence of GTP which constricts the liposome. Erickson and his group concluded that no other protein except FtsZ in presence of GTP is required for force generation (Osawa *et al.*, 2008). The above experiment led to the inference that FtsZ is sufficient to generate force for the ring constriction. Scientists came up with two hypotheses in order to prove that Z ring is able to pull the cytoplasmic membrane inward.

Hydrolysis and bend hypothesis-

By attaching the MTS in N terminal which is 180° from the C-terminal, the force of constriction reverses, i.e., the constriction occurs outwards in place of inwards as seen when the MTS was in C-terminal (Osawa *et al.*, 2009). This signified that protofilaments have fixed direction of curvature. FtsZ bound to GTP has a straight conformation with zero bend between the subunits, while GDP bound FtsZ has a curved conformation with 22 degree bend (Erickson, 2009). Also it has been seen that Z ring, coalescing into brighter ones, shows constriction (Osawa *et al.*, 2008). Upon hydrolysis of GTP, it cuts links which is formed by the gamma phosphate and the Mg^{2+} between adjacent monomers which yields filaments prone to depolymerization (Corbin and Erickson, 2020; Ruiz *et al.*, 2022). The FtsZ-GTP assembles into straight filaments and upon its hydrolysis to GDP, it forms a curved conformation which might generate the force for ring constriction (Allard and Cytrynbaum, 2009). Supporting this hypothesis, studies on *Mycobacterium tuberculosis* FtsZ have shown the presence of curved FtsZ filaments with GDP. It has been speculated that there might be a hinge opening mechanism associated with the FtsZ monomer which leads to such conformational switch (Li *et al.*, 2013).

Force generation by lateral bonding and sliding-

According to this hypothesis, the filaments have lateral interactions with each other and on hydrolysis of GTP, there is a conformational change due to which the lateral bonds are broken and filaments slide past and reconnect (Lan *et al.*, 2009). Contraction could be generated when protofilaments slide to increase the number of lateral bonds. This is the Hill sleeve-type mechanism (Allard and Cytrynbaum, 2009) in which the polymers slide relative

to one another, which reduces the free energy by increasing the number of lateral bonds. The major drawback about this hypothesis is that the model ignores the contribution of subunit entropy when adding bond energies. Taking the subunit entropy into consideration, the conclusion is that an association which involves 1 or 2 lateral bonds is so weak that it cannot achieve any significant effect, while an association involving 3 or more lateral bonds is so strong that it would not release in a time relevant to the cell cycle (Erickson, 2009).

1.4.2 Role of membrane targeting protein in Z ring constriction

The membrane tethering protein FtsA might play role in Z ring constriction. As seen in previous studies that Z-ring can provide the force for constriction of membrane. But FtsZ reconstitution in liposome did not provide a complete liposome division. In order to test whether FtsA, which tethers FtsZ to the membrane during Z-ring formation, has a role in complete liposome scission, experiments were performed by reconstituting FtsZ and FtsA on liposome. It is seen that liposomes was divided completely in some case (Osawa and Erickson, 2013). In the same year, Mitchison and his group experimentally proposed a hypothesis that monomeric FtsZ is not recruited by FtsA to tether to the membrane. Polymerized FtsZ binds to FtsA and then FtsA in turn tethers FtsZ to the membrane (Loose and Mitchison, 2014).

Thus it can be inferred that FtsA plays an important role in FtsZ reorganization and final cell scission. One more possibility is that the bond strength between FtsZ and FtsA is reported to be weaker, which implies that it is more readily reversible than the attachment of mts to FtsZ, facilitating the FtsZ to tether to the membrane. This weak interaction between FtsZ and FtsA may permit disassembly of Z-ring which is necessary in the final stage of cell division (Loose and Mitchison, 2014; Xiao and Goley, 2016).

1.4.3 Septal wall synthesis and Z- ring constriction

A recent model proposes that septal wall synthesis and Z ring constriction may work together to drive septum formation. The study by (Yang *et al.*, 2017b) shows that the rate of closure of septum remains unaffected by alterations to the Z ring. Also disassembly of Z ring does not drive constriction, it is seen to happen after the indentation of the cell wall. Septum closure rate significantly reduced when FtsI activity was compromised, which is the cell wall synthesizing machinery. The study supports a holistic view of constrictive force generation by the multicomponent divisome. Treadmilling of FtsZ which is powered by GTP guides the motion of synthesis of new peptidoglycan by FtsI in the septum. It is seen that reduction in

the treadmilling by FtsZ mutants alters septum synthesis as well as the molecular composition of the cell wall. Treadmilling of FtsZ has also been shown to deform membranes by generating torsional stress upon hydrolysis of GTP (Ramirez-Diaz *et al.*, 2021) which can guide the cell wall synthesis machinery along the circumference of the inner membrane to insert new peptidoglycan at the septum (Bisson-Filho *et al.*, 2017). Cell wall synthesis is also thought to be the primary force generator for constriction and the rate-limiting process in cytokinesis or septum closure (Coltharp *et al.*, 2016).

This leads us to an open question of how septum closure and cell division could occur in cell wall-less bacteria without a cell wall synthesis machinery. Thus, cell wall-less bacterial FtsZ can serve as a model to study fundamental features of FtsZ in ring constriction and cell division independent of cell wall synthesis.

1.5 *Spiroplasma* as model organism to study cell division driven by FtsZ

Spiroplasmas are cell-wall-deficient bacteria that belong to the Mollicutes class and are distinguished by their helical cell structure which are pleomorphic (Razin *et al.*, 1973). They were identified as helical organisms that resembled mycoplasmas and were linked to the citrus stubborn and corn stunt diseases (Trachtenberg, 2004; Stülke *et al.*, 2009).

Only 4 (*mraZ*, *mraW*, *ftsA*, and *ftsZ*) of the over 30 genes coded by the division and cell wall (*dew*) cluster in cell-walled bacteria are present in the majority of Mollicutes (Zhao *et al.*, 2004; Harne *et al.*, 2020). This shows that these four proteins make up the necessary machinery for cell division in bacteria lacking a cell wall, given the lower genome sizes of these bacteria. It is unclear, though, whether any of these proteins is essential for *Spiroplasma* cell division.

For *Spiroplasma*, two cell division strategies have been proposed. Based on the observation of the growth of *S. citri*, it is inferred that *Spiroplasma* cells split by one or more constrictions along the short axis of the cell and develop at one of the two poles by insertion of additional membrane components (Garnier *et al.*, 1981; Garnier *et al.*, 1984). Another mode of division which is proposed is the longitudinal fission or the Y shaped fission. The immunogold labelling by anti-FtsZ confirmed the presence of FtsZ in the branching area of the Y shaped cell, indicating that the division is dependent on FtsZ (Ramond *et al.*, 2016).

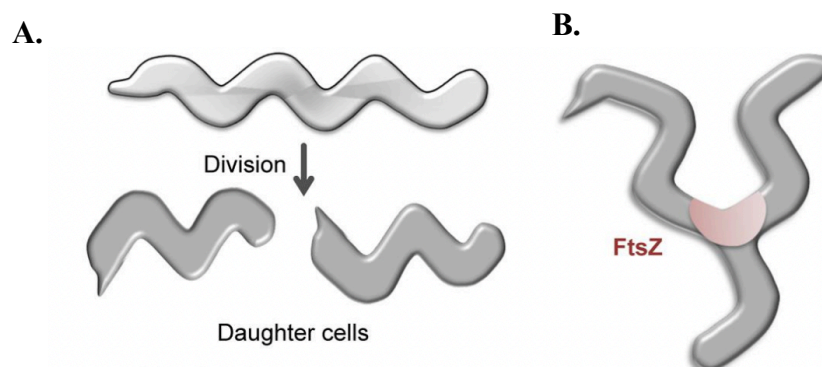


Figure 1.6 Cell division in *Spiroplasma*

A) Cell division in *Spiroplasma* along the shortest axis. B) Y-shaped cell division along the long axis. Adapted from Harne *et al.*, 2020

Both of these mechanisms may occur in some *Spiroplasma* species, but it is unknown whether a particular situation or environment will dictate which one is used (Figure 1.6). The *Spiroplasma* cells should have a set length if Y-shaped division was the major mechanism. However, growing cultures always contain cells of various lengths. This implies that depending on the species, the Y-shaped division can be a different type of cytokinesis that happens more or less frequently. It is necessary to look into the components of the *Spiroplasma* cell division machinery and the mechanisms governing their positioning at the proper site (Harne *et al.*, 2020).

As a result, in the absence of a cell wall and cell wall synthesizing machinery, *Spiroplasmas* serve as model organisms for understanding the function of FtsZ in fundamental mechanisms of cytokinesis in bacteria.

1.6 FtsZ: Homolog of eukaryotic tubulin

FtsZ is the homologue of the eukaryotic tubulin. Both tubulin and FtsZ are GTPases, having conserved residues for GTP binding which is GGGTG(T/S)G and GTP hydrolysis which is NxDxx(D/E) (Figure 1.7) (Erickson, 1995; Nogales *et al.*, 1998; van den Ent *et al.*, 2001). Both the proteins are homologous structurally and they form long filament structures which are oriented in a head to tail fashion (Mingorance *et al.*, 2010; Erickson *et al.*, 2010).

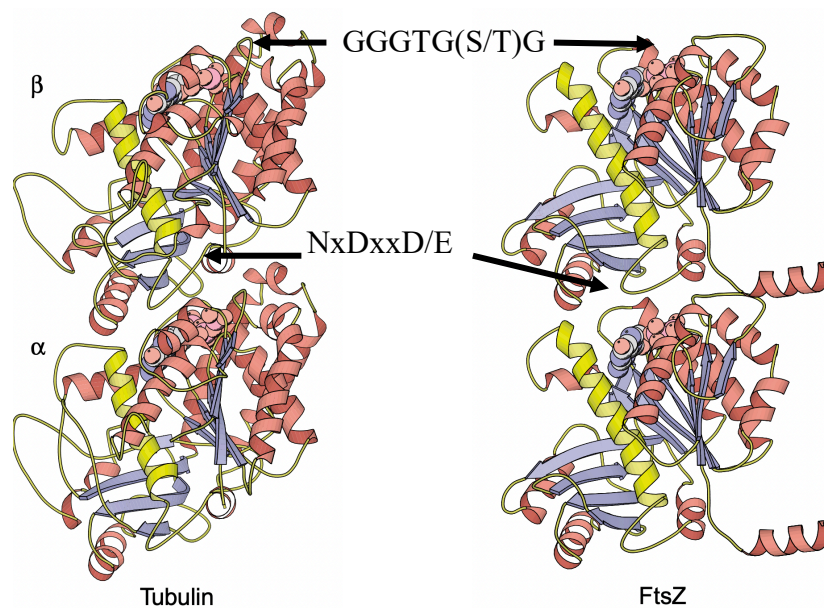


Figure 1.7 Structure of tubulin heterodimer and FtsZ

The structures of tubulin and FtsZ mark the conserved motifs in both the homologous proteins. Adapted from van den Ent *et al.*, 2001.

This association is regulated by GTP which is bound in the cleft between the adjacent monomers. Tubulin filaments are made up of an alpha and beta tubulin polypeptide interacting together head to tail. The tubulin protofilaments interact together to form a more complicated structure rather than existing independently. Common microtubules have 13 protofilaments and are aligned parallel to one another to form a tube (Li *et al.*, 2013). GTP is bound to both the alpha and beta subunits. Alpha tubulin-bound GTP does not undergo hydrolysis. Tubulin polymerization is only mediated by the beta subunit of the alpha and beta heterodimer, and growth occurs at the positive end (Cooper, 2000). Catastrophe happens when the production rate of new tubulin dimers slows, the GTP in the GTP cap ultimately hydrolyzes to GDP, and the microtubules disintegrate. FtsZ is known to polymerize in presence of GTP and forms homopolymers (Bramhill and Thompson, 1994; Erickson, 1995).

1.6.1 Domain organization of FtsZ

The crystal structure of FtsZ was first solved in 1998 from the hyperthermophilic methogen, *Methonococcus jannnaschii* (Löwe and Amos, 1998). FtsZ consists of two domains- one is the N-terminal domain (NTD) which harbours the nucleotide binding pocket and the other is C-terminal domain (CTD). Both these domains are separated by a 23-residue long H7 helix, the end of which harbours the catalytic T7 loop which aids in GTP hydrolysis (Figure 1.8).

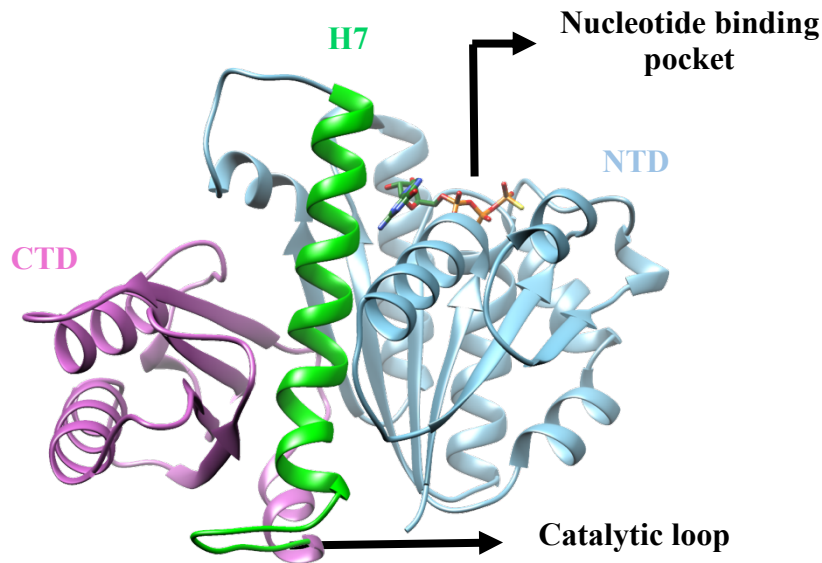


Figure 1.8 Overall structure of FtsZ.

The N-terminal domain (NTD) is shown in sky blue, the H7 helix is shown in green, and the C-terminal domain (CTD) is shown in pink, making up the FtsZ core domain. (PDB ID: 2RHO).

The core of FtsZ consisting of NTD and CTD is followed by a variable linker whose length and residues vary across bacterial species (CTL). The linker is followed by a conserved 17 residues, conserved C- terminal peptide (CCTP) which helps FtsZ to bind to membrane binding proteins like ZipA or FtsA and finally a C- terminal variable region (CTV) (Figure 1.9).

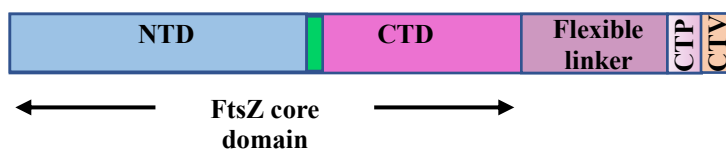


Figure 1.9 Schematic determining the domain architecture of FtsZ. The N-terminal domain (NTD) is shown in sky blue, the H7 helix is shown in green, and the C-terminal domain (CTD) is shown in pink, making up the FtsZ core domain. This is followed by a flexible linker (light pink), the conserved C-terminal peptide (baby pink) and C-terminal variable region (light orange).

1.6.2 Treadmilling of FtsZ and its mechanism

FtsZ filaments exhibit treadmilling, as opposed to the typical dynamic instability observed for microtubules. The basis of treadmilling of FtsZ protofilament (PF) is polymerization from

one end and depolymerization from the other end. FtsZ polymerization is a cooperative process that requires a critical concentration of the monomers for filament assembly (Mukherjee, 1998; Huecas and Andreu, 2003; Huecas *et al.*, 2008) (Surovtsev *et al.*, 2008). A conformational switch of each monomer from its low affinity state (R state; relaxed state favoured by monomers) to a high affinity state (T state; tense state adopted by monomers within a filament) favors cooperative assembly of FtsZ filaments (Huecas *et al.*, 2008; Dajkovic *et al.*, 2008; Wagstaff *et al.*, 2017). Earlier studies have reported that FtsZ can exist in two states: the R state and T state based on the crystal structures of FtsZ (Huecas *et al.*, 2008; Dajkovic *et al.*, 2008; Wagstaff *et al.*, 2017). In R state, the inter-domain cleft between the N-terminal domain (NTD) and the C-terminal domain (CTD) is closed whereas in T state, it is open (Fujita *et al.*, 2017). The transition from R to T state in monomeric subunit is thermodynamically less favorable as opening of the inter-domain cleft will require a positive free energy. However, within protofilaments, the interactions between adjacent subunits provide the required energy for opening the cleft and favors R to T state transitions to form a protofilament (Figure 1.10) (Corbin and Erickson, 2020).

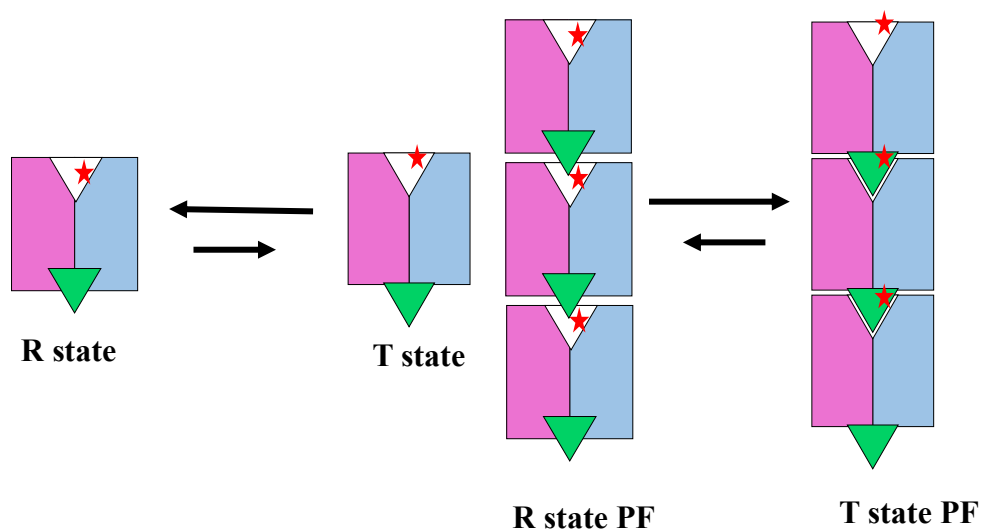


Figure 1.10 Monomer versus Filament state of FtsZ

The color code for the schematic of FtsZ is- blue (NTD), green (H7 helix and T7 loop), and pink (CTD). The star denotes the nucleotide. Adapted from Corbin and Erickson, 2020.

Another important feature of FtsZ filament dynamics is kinetic polarity, which is interestingly opposite to what is known for microtubules. Microtubules elongate from the top end, at which the N-terminal domain (NTD) of beta-tubulin is exposed, whereas FtsZ grows from the bottom end, the end with an exposed C-terminal domain (CTD) (Du *et al.*, 2018; Knossow *et al.*, 2020). It has been shown that how a bottom-face mutation prevents division

in living cells by integrating into the Z ring and causing it to dissolve, but in vitro, this mutation prevents FtsZ assembly without affecting its GTPase activity. However, a top-face mutant is not poisonous and has no impact on the assembly of FtsZ (Du *et al.*, 2018) (Figure 1.11).

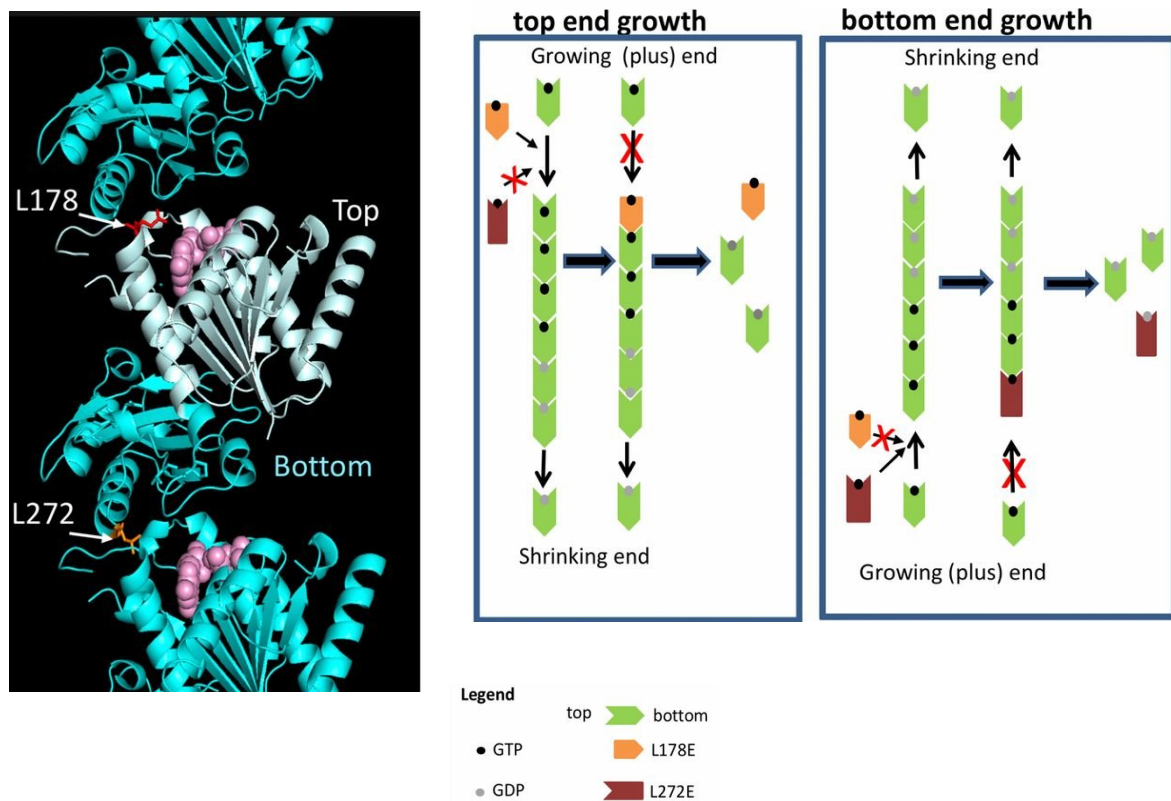


Figure 1.11 Determination of the direction of kinetic polarity of FtsZ

A) Top (L178) and bottom (L272) polymerization interface residue of FtsZ shown in PDB ID 3VOB (in filament structure) to mutate for the study. B) Two outcomes for the mutation in top or bottom interface of FtsZ: If the top end is the plus or growing end, then top interface mutant would attach the filament and act as a cap and prevent the other monomers to attach. This will lead to cell division defect due to effect in FtsZ assembly. In the similar way, if the bottom interface end is the growing end, it would attach to the filament and cap it, making cells carrying the mutation to be division defective. Image adapted from Du *et al.*, 2018

However, the structural basis of this strikingly opposite directionality of growth for the two homologous proteins is unknown till date.

1.7 FtsZ as a drug target

The emergence of new classes of antibiotics is urgently required due to the increase of bacterial antibiotic resistance. The DNA replication and cell wall synthesis of the bacterial

cell are the targets of the extensively used antibiotics today. Antimicrobials that have been created for novel targets and are in the pipeline can combat the problems caused by antibiotic resistance. As a crucial protein for bacterial cell division, the tubulin homolog of bacteria FtsZ is one such target. The altered FtsZ dynamics cause abnormalities in cell division in the bacterial cells, which ultimately leads to cell death.

After examining more than 500 benzamide analogues, PC190723 was selected as antimicrobial because it was more effective than the others. An analogue of 3-methoxybenzamide 2,6-difluorinated is PC190723 (Haydon *et al.*, 2008). All *Staphylococcus* species and strains, including the methicillin- and multidrug resistant *Staphylococcus aureus*, are susceptible to the drug, PC190723. *In vivo* testing on mice exposed to a fatal dosage of *Staphylococcus aureus* demonstrated its effectiveness (Haydon *et al.*, 2008). There is evidence that this drug works well against some *Bacillus* strains (Haydon *et al.*, 2008; Andreu *et al.*, 2010).

Nevertheless, FtsZs from bacteria like *Streptococcus*, *E. coli*, and *Enterococcus* were found to be resistant to this drug. It has been demonstrated that PC190723 does not cause FtsZ filaments from *E. coli* to condense or bundle (Haydon *et al.*, 2008; Kaul *et al.*, 2013; Silber *et al.*, 2020). The mechanism of mode of action of PC190723 in FtsZs, especially why some organisms are resistant to it, is not clear.

1.8 Objectives

FtsZ is known to treadmill at the site of division wherein one end polymerizes and the other end depolymerizes (Bisson-Filho *et al.*, 2017; Yang *et al.*, 2017a). The difference in the rate of assembly and disassembly between two ends of filaments, the basis for which is unknown, defines the kinetic polarity. FtsZ would be the excellent target for drug against bacteria as it is an essential protein for cell division in bacteria.

The main aim of my thesis was to unravel the not-known structural mechanisms by the help of which the kinetic polarity of FtsZ takes place, using *Spiroplasma* FtsZ as the model system. Also, my thesis aims to decipher the mode of action of inhibitor binding of FtsZ.

In order to address this, I proceeded with the following objectives-

- 1) Biochemical characterization of FtsZ from *Spiroplasma melliferum*, a cell wall less bacteria (SmFtsZ) and *Escherichia coli*, a cell walled bacteria (EcFtsZ)
- 2) Determination of crystal structures of conformational states of SmFtsZ and their structural analysis

- 3) Identification, biochemical and structural characterization of inter-domain cleft residue of FtsZ important for R to T state transition
- 4) Determination of mechanistic insights on binding of the drug- PC190723 to FtsZ

1.9 References

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Chapter 2

Biochemical characterization of FtsZs from *Spiroplasma melliferum* and *Escherichia coli*

2.1 Introduction

Reported literature on characterization of FtsZs mainly focuses on FtsZs from cell walled organisms. These studies have led to the recent model that the force generated to constrict the cell and divide it into two halves is contributed by the cell wall synthesis machinery for the synthesis of septum which is guided by the treadmilling of filaments of FtsZ (Bisson-Filho *et al.*, 2017; Whitley *et al.*, 2021). This raises the question of how constriction and division takes place in a cell wall-less bacterium. Hence, FtsZ from cell-wall less bacteria can serve as a model to study fundamental features of FtsZ in ring constriction and cell division independent of cell wall synthesis machinery. FtsZ molecule comprises two domains - the N-terminal domain (NTD) and the C-terminal domain (CTD) separated by a central helix which is the H7 helix (Löwe and Amos, 1998). FtsZ polymerizes in presence of GTP and hydrolyses GTP during depolymerization (Bramhill and Thompson, 1994, Margolin, 2005) . This chapter describes the cloning, purification and comparative biochemical analysis of FtsZ from *Spiroplasma melliferum* which is a cell wall-less bacterium (SmFtsZ) and FtsZ from *Escherichia coli*, a cell walled bacterium (EcFtsZ). We compared the GTPase activities and the critical concentrations of filament formation of FtsZs from these two organisms. Formation of SmFtsZ filaments in presence of GTP was confirmed by pelleting assay and visualizing using TEM. Comparison of the FtsZs from cell wall less and cell walled organisms will help in identification of the basic mechanism and conserved signatures for FtsZ function that operates independently of the cell wall synthesizing machinery.

2.2 Materials and methods

2.2.1 Cloning of FtsZs and their mutants from *Spiroplasma melliferum* and *Escherichia coli*

ftsZ genes from *Spiroplasma melliferum* (strain KC3) (SmFtsZ) and *Escherichia coli* (strain K12) (EcFtsZ) with UniProt IDs - A0A037UPJ1 and P0A9A6, respectively, were amplified from the genomic DNA of the respective organisms (*S. melliferum*: obtained from DSMZ, Germany, Catalogue number 21833; *E. coli*: genomic DNA extraction from the bacterial culture). The lengths of the full-length proteins used in this study of *ftsZ* from *E. coli* (EcFtsZ) and *S. melliferum* (SmFtsZ) are 1173 bp (391 amino acids) and 1257 bp (419 amino acids). In *Spiroplasma*, the codon TGA codes for tryptophan whereas in *E.coli*, TGA is a stop codon. Therefore, for using *E.coli* as the heterologous expression system, all TGA codons in between

the gene were changed to TGG (the codon which codes for tryptophan in *E.coli*). *ftsZ* gene from *Spiroplasma melliferum* has two tryptophans which are coded by TGA, at the amino acid positions 384 and 411. These two tryptophan codons were changed according to *E.coli* codon (TGG) using primers for site-directed mutagenesis. Using restriction free cloning (van den Ent and Löwe, 2006), the full length wild type genes were cloned into pHIS17 vector (obtained from Löwe lab, MRC LMB, Cambridge; refer Addgene plasmid #78201 for vector backbone) at the position between the restriction sites *NdeI* and *BamHI* using appropriate primers (Table 2.1). In a nutshell, the 3' end of the primers had sequences from the gene of interest, and the 5' end had sequences from the target vector. These primers were used in the initial PCR reaction to amplify the target gene. The target vector in this case, pHIS17, was used as the template in the subsequent step and the amplified gene containing regions from the vector at the 5' and 3' ends was used as a megaprimer to create a plasmid containing the gene of the insert. The negative control was a reaction with template plasmid but no primers. This was followed by *DpnI* digestion to digest the methylated parental plasmid. The *DpnI*-digested product was transformed by electroporation into NEB TurboTM electro-competent cells and was plated onto LB agar plates containing ampicillin (100 µg/ml) as selection marker. The colonies were inoculated in LB broth with 100 µg/ml ampicillin, incubated for 12 hours and followed by isolation of the plasmid DNA using QIAGEN miniprep kit. The screening for the positive clones was done by double digestion by restriction enzymes *NdeI* and *BamHI*, followed by DNA sequencing. For crystallization purposes, a truncated construct (containing amino acids from 1 to 314) of SmFtsZ (SmFtsZ^{ACT}) was cloned, wherein the flexible linker and the CCTP (conserved C- terminal peptide) was deleted and the length is 942 bp. This was done using wild type SmFtsZ as template and suitable reverse primer. A GTPase dead mutant, SmFtsZ^{D211G}, was generated by site directed mutagenesis using the wild type SmFtsZ plasmid as template. The EcFtsZ full length clone was generated by amplifying the FtsZ gene from the genomic DNA of *E. coli*, and the clone was generated using RF cloning. The list of primers used for cloning of the above genes and the relevant constructs is given in Table 2.1.

Table 2.1 List of primers used in generating the FtsZ constructs for the study.

Primer name	Sequence (5' to 3')	Purpose
EcFtsZ_F	ATGTTTGAACCAATGGAACCTTAC	EcFtsZ gene-specific amplification
EcFtsZ_R	ATCAGCTTGCTTACGCAGGAATG	EcFtsZ gene-specific amplification
EcFtsZ_vF	GTTTAACTTTAAGAAGGAGATATAC ATATGTTTGAACCAAT GGAACCTTACC	EcFtsZ clone; primer with vector overhang for incorporation into plasmid
EcFtsZ_vR	GCTTTTAGTGGTGATGGTGATGATGGG ATCCATCAGCTTGCTTACGC AGGAATGC	EcFtsZ clone; primer with vector overhang for incorporation into plasmid
SmFtsZ_F	ATGGACAATTTTGATAATTATGAAC AAGTCGCG	SmFtsZ gene-specific amplification
SmFtsZ_R	CAACTACGACGAACAAATGGTGGTAA ATCATC	SmFtsZ gene-specific amplification
SmFtsZ_vF	GTTTAACTTTAAGAAGGAGATATACATAT GGACAATTTTGATAATTATG	SmFtsZ clone; primer with vector overhang for incorporation into plasmid
SmFtsZ_vR	CTTTTAATGATGATGATGATGATGGGAT CCCCAACTACGACGAACAAATG	SmFtsZ clone; primer with vector overhang for incorporation into plasmid
SmFtsZ_384F	CGAATTAATGCGTGGCGAGAGCATG TTAATAATAATC	SmFtsZ clone; To replace TGA codon
SmFtsZ ^{ACT} _R	GCTTTTAATGATGATGATGATGATGG GATCCATCAAACCCAGTCGC	To generate SmFtsZ ^{ACT}
SmFtsZ ^{D211G} _F	CCTTAATTAATTTAGACTTTGCAGGTA TTAAGACTGTTATG	To generate SmFtsZ ^{D211G} in SmFtsZ ^{ACT} background

2.2.2 Expression and purification of FtsZ constructs

The plasmids incorporated with *ftsZ* genes were overexpressed in the BL21(AI) (Stratagene) strain of *E.coli*. The gene encoding for the T7 polymerase in the expression strain is under the tight regulation of *araBAD* operon. The gene of interest to be overexpressed is under T7 promoter. Therefore, upon addition of L-arabinose, the gene of interest will get overexpressed. The plasmids of wild type FtsZ from *S. melliferum* and *E. coli* were transformed into BL21 (AI) by heat shock method and spread on LB agar plates containing ampicillin. After 12 hours of incubation at 37 °C, 10-15 colonies were inoculated in 10 ml LB broth containing 100 µg/ml of ampicillin and incubated at 37 °C under shaking

conditions. The parameters for overexpression are as follows: optical density (OD) of the culture at which the culture was induced, OD₆₀₀ was 0.6-0.7; post induction temperature was 30 °C and incubated for 5 hours post induction. These were optimized to achieve maximum expression as well as solubility of the protein. For checking the expression and solubility of the protein, 5 ml culture (rest 5 ml kept as uninduced control) was induced with 0.2% L-arabinose at OD₆₀₀ = 0.6-0.7 and kept at 30 °C for 5 hours. The culture was pelleted, and resuspended with 500 µl lysis buffer (50 mM Tris pH 8, 200 mM NaCl, 10% glycerol). The cells were lysed by sonication using a probe sonicator (Time: 3 minutes, pulse: 1 second ON and 3 second OFF, amplitude: 60 %). The lysate was spun at 21,000 xg at 4 °C for 10 minutes, the supernatant of which is the soluble fraction. The total and soluble fractions of uninduced and induced were visualized on 12% SDS PAGE gel. The overexpressed band at the corresponding sizes was checked.

For purification purposes, 1 L of culture was grown similarly as mentioned above. The pellet was resuspended with an 80 ml lysis buffer, sonicated and spun in Beckman Coulter floor model centrifuge (Optima^M L-100k ultracentrifuge) at 100,000 xg at 4 °C for 45 minutes. The supernatant was loaded onto pre-equilibrated (with Buffer A- 50 mM Tris pH 8, 200 mM NaCl) 5ml His-Trap (GE Lifesciences) column. The hexahistidine-tagged protein was eluted with a step gradient of imidazole containing buffer B (50 mM Tris pH 8, 200 mM NaCl, 500 mM imidazole). The purest fractions of the protein were identified using 12% SDS PAGE gel. The fractions were pooled, concentrated till 1 ml in 10 kDa centricon and injected into the gel filtration column Superdex200 10/300 (GE Lifesciences) pre-equilibrated with the final buffer (HK50: 50 mM HEPES, pH 6.5, 50 mM KCl). The fractions corresponding to the peak observed (according to the absorbance monitored at 280 nm) were checked on 12% SDS PAGE gel. The purest fractions were pooled, concentrated, flash-frozen in aliquots, and stored in -80 °C till further use. The same protocol was followed for purification of all other constructs of FtsZs with histidine tag.

2.2.3 GTPase assay

Since FtsZ is a GTP hydrolysing protein, GTPase activity was measured by monitoring the released inorganic phosphate using malachite green assay (Feng *et al.*, 2011). The malachite green assay is a colorimetric assay in which the brown colour of the malachite green molybdate turns into green in presence of inorganic phosphate under acidic conditions. FtsZ

protein was spun at 21,000 xg at 4 °C for 15 minutes and supernatant was used to determine the concentration of the proteins by Bradford reagent, using 0 to 1 mg/ml BSA as standards.

2.2.3.1 GTPase assay for determining the Michaelis Menten constants

A protein concentration of 5 μ M was taken for the GTPase reaction. The GTPase reaction mixture with volume of 20 μ l contained 5 μ M of EcFtsZ, varying GTP concentrations from 0 to 2 mM (0, 0.002, 0.006, 0.008, 0.010, 0.012, 0.015, 0.020, 0.040, 0.050, 0.060, 0.080, 0.1, 0.15, 0.2, 0.25, 0.5, 0.75, 1, 2 mM), $MgCl_2$ at 5 times the GTP concentration and the volume made up with the reaction buffer (HK50). The reaction mixture was incubated for 15 minutes at 30 °C. The reaction was stopped by addition of 100 mM EDTA (5 μ l of 0.5 M). The reaction mixture (25 μ l) and phosphate standards (25 μ l of 0 to 400 μ M) were added to 96 well Tarsons plate (catalogue number 941196) and 100 μ l of malachite green mix (800 μ l of malachite green, 200 μ l of ammonium molybdate, and 16 μ l of 10% tween20) was added into it. Absorbance at 630 nm was recorded after 10 minutes incubation using a plate reader Varioskan Flash (Thermo Scientific).

The absorbance of mixture containing buffer and GTP was subtracted with the respective absorbances of buffer, GTP and protein mixture. The concentration of phosphate released was calculated by dividing the slope obtained from the phosphate standard. The Michaelis Menten plot was generated with the y-axis as the rate of reaction that is phosphate released per unit time (μ M/min) and the x-axis as different concentrations of GTP from 0 - 2 mM for EcFtsZ. The curve was fitted with the Michaelis Menten equation, $V_0 = V_{max} ([S]/([S] + K_M))$. Similarly, Michaelis Menten plot was generated for SmFtsZ, but with 10 μ M protein concentration instead of 5 μ M as no visible colour change was observed with the latter. The GTP concentrations used for SmFtsZ were 0.002, 0.008, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 mM.

The plots were generated using GraphPad Software, San Diego California USA, version 9.00 for MacOS.

2.2.3.2 GTPase assay to measure the specific activity of FtsZ constructs

To compare the apparent specific activities of EcFtsZ and SmFtsZ, 5 μ M EcFtsZ, 10 μ M SmFtsZ, 10 μ M SmFtsZ^{ACT}, and 10 μ M SmFtsZ^{D211G} were taken. The reactions were performed at saturating concentrations of GTP (estimated based on the Michaelis Menten plot) which was 1 mM for EcFtsZ and 2.5 mM for SmFtsZ constructs. GTPase reaction was set up

similarly as described in section 2.2.3a. k_{obs} (min^{-1}) of the reactions were calculated by dividing the concentration of phosphate released by time and the concentration of the protein.

2.2.3.3 GTPase assay to measure critical concentration

To measure the critical concentration of FtsZs from *E. coli* and *S. melliferum*, GTPase assay was performed at saturated concentrations of GTP (1 mM for EcFtsZ and 2.5 mM for SmFtsZ), 5 mM of MgCl_2 and different concentrations of protein (SmFtsZ - 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14 μM and EcFtsZ - 0, 0.25, 0.5, 0.75, 1, 2, 5, 8, 10 μM). The assay was set similarly as described in section 2.2.3.1. k_{obs} (min^{-1}) of the protein was plotted along y-axis and concentration of the protein along the x-axis.

2.2.4 Light scattering assay

The polymerization of FtsZ was measured by 90° angle light scattering in Fluoromax-4 (Horiba). The reaction volume used for the assay was 200 μl in a cuvette, with both the excitation and emission wavelengths set at 350 nm and a slit width of 1.5 nm.

A fluorometer cuvette with a 1 cm path length was filled with the appropriate buffer (HK50), 1 mM of MgCl_2 and different concentrations of FtsZ ranging from 0 to 12.5 μM . After that, the cuvette was put in a cuvette chamber that was kept at 30 °C, and data were collected for 300 seconds to create a baseline. After 300 seconds, 250 μM of GTP was added and reading was taken. The initial intensity is normalized with that of buffer (meaning 1-300 seconds taken as buffer intensity and divided the average of buffer intensity (1-300 seconds) to the protein intensity after 300 s.

2.2.5 Pelleting assay of SmFtsZ

Protein was spun at 100,000 xg at 4 °C in order to remove any precipitate. 10 μM of the protein was taken from the supernatant to set up 50 μl of reaction. The following reaction conditions were prepared- a) only protein, b) protein and MgCl_2 , c) protein, MgCl_2 and GDP, and d) protein, MgCl_2 and GTP. A concentration of 3 mM of nucleotide and 5 mM of MgCl_2 was taken for the reaction. The reaction mixtures were spun at 100,000 xg at 30 °C for 15 minutes. The supernatant was taken out and the pellet was resuspended in buffer at the same

amount as the initial reaction volume. The supernatant and pellet fractions were mixed with 2X SDS buffer and loaded onto 12% SDS-PAGE gel in equal amounts.

2.2.6 Sample preparation for Transmission Electron Microscopy (TEM)

In order to visualize the filaments of SmFtsZ (wild type and mutants), transmission electron microscopy was carried out. The protein was spun at 100,000 xg at 4 °C for 15 minutes and a reaction of 20 µl was set with a final protein concentration of 10 µM and GTP concentration of 2 mM at 30 °C for 15 mins. Carbon formvar grids (TED-PELLA, INC) were glow discharged with one round of 15 mA current for 25 seconds using plasma cleaner (Quorum Technologies). 5 µl of protein was added on a glow discharged Carbon formvar grid and incubated for 2 minutes before blotting out the protein with Whatman paper. After that, 5 µl of MQ was added, kept for 30 seconds and blotted out. Finally, 5 µl of 2% uranyl acetate was added and incubated for 30 sec and blotted out. The grids were dried before imaging using TEM (JEM- 2200FS Joel Ltd.). The images of the filaments were analysed using ImageJ (Schneider *et al.*, 2012).

2.3 Results

2.3.1 Purification of FtsZ constructs

All constructs of SmFtsZ were purified using two steps of purification - affinity chromatography and size exclusion chromatography (Figure 2.1A). In affinity chromatography, the protein eluted in fractions of 10 - 35% of buffer B (Figure 2.2B). In size exclusion chromatography, the protein was eluted in elution volume corresponding to 12 - 17 ml corresponding to a peak centred at 13.4 ml, as observed from the chromatography profile and the corresponding gel (Figure 2.2C). Analytical size exclusion chromatography runs for SmFtsZ, SmFtsZ^{ΔCT}, and SmFtsZ^{D211GΔCT} show one major peak at 15 ml corresponding to monomer size and a minor peak at 13.5 ml corresponding to dimer size (Figure 2.2D). The molecular weight of the protein was also confirmed using mass spectrometry.

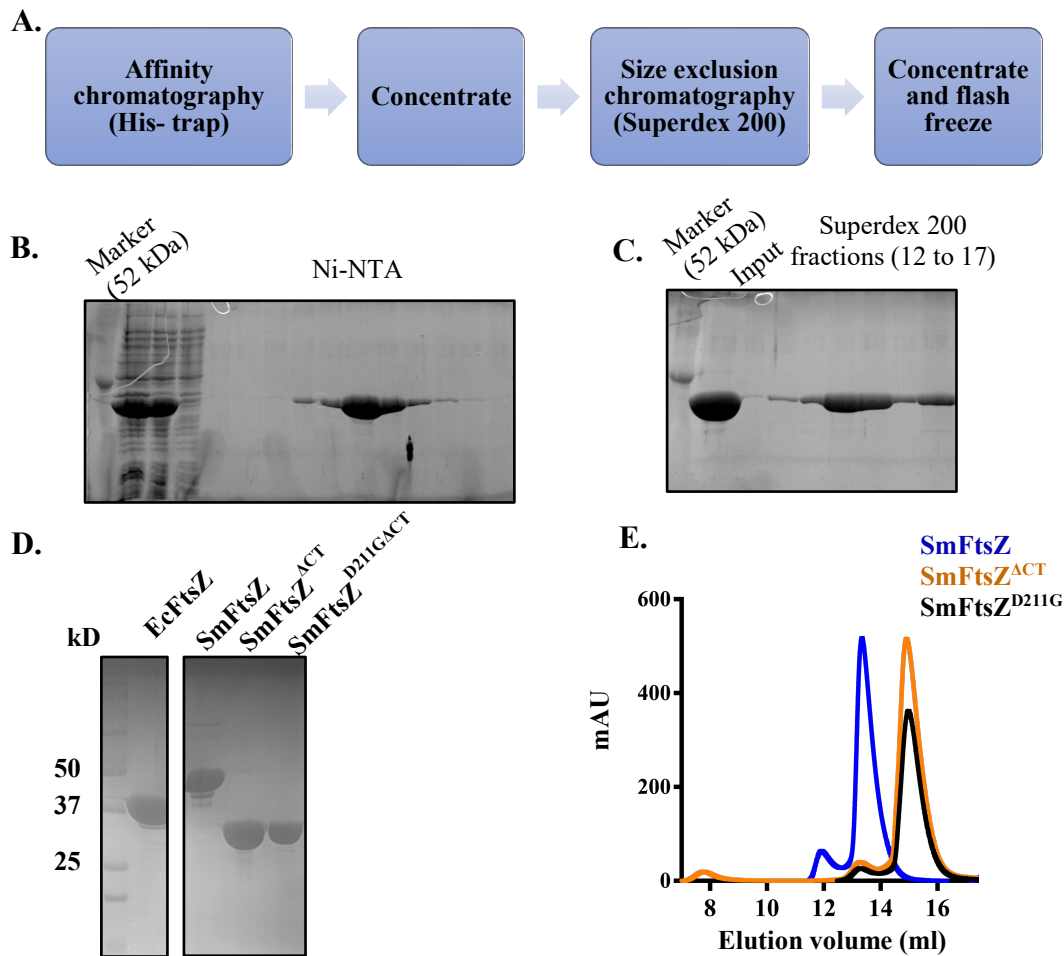


Figure 2.1 Purification of SmFtsZs.

A) Flow chart showing the purification steps of SmFtsZ and mutants. B) SDS PAGE gel showing the fractions after Ni-NTA column (affinity chromatography) for SmFtsZ^{ACT}. C) 12 % SDS-PAGE gel showing the fractions of Superdex 200 showing band of interest corresponding to elution volume 12 to 17ml for SmFtsZ^{ACT}. D) SDS-PAGE gel showing the final proteins- EcFtsZ, SmFtsZ, SmFtsZ^{ACT}, and SmFtsZ^{D211G}. E) Analytical run in Superdex 200TM 10/300 column showing the oligomeric status of the protein. For SmFtsZ, the elution volume is 12 ml (minor peak- dimer) and 13.4 ml (major peak- monomer). For SmFtsZ^{ACT} and SmFtsZ^{D211G}, the elution volume is 13.5 ml (minor peak- dimer) and 15 ml (major peak- monomer).

For wild type EcFtsZ, only affinity chromatography was carried out (Figure 2.2A, B) because size exclusion run gave a spread peak from the void till the monomeric size, indicating a heterogenous oligomeric state of the protein (Figure 2.2C).

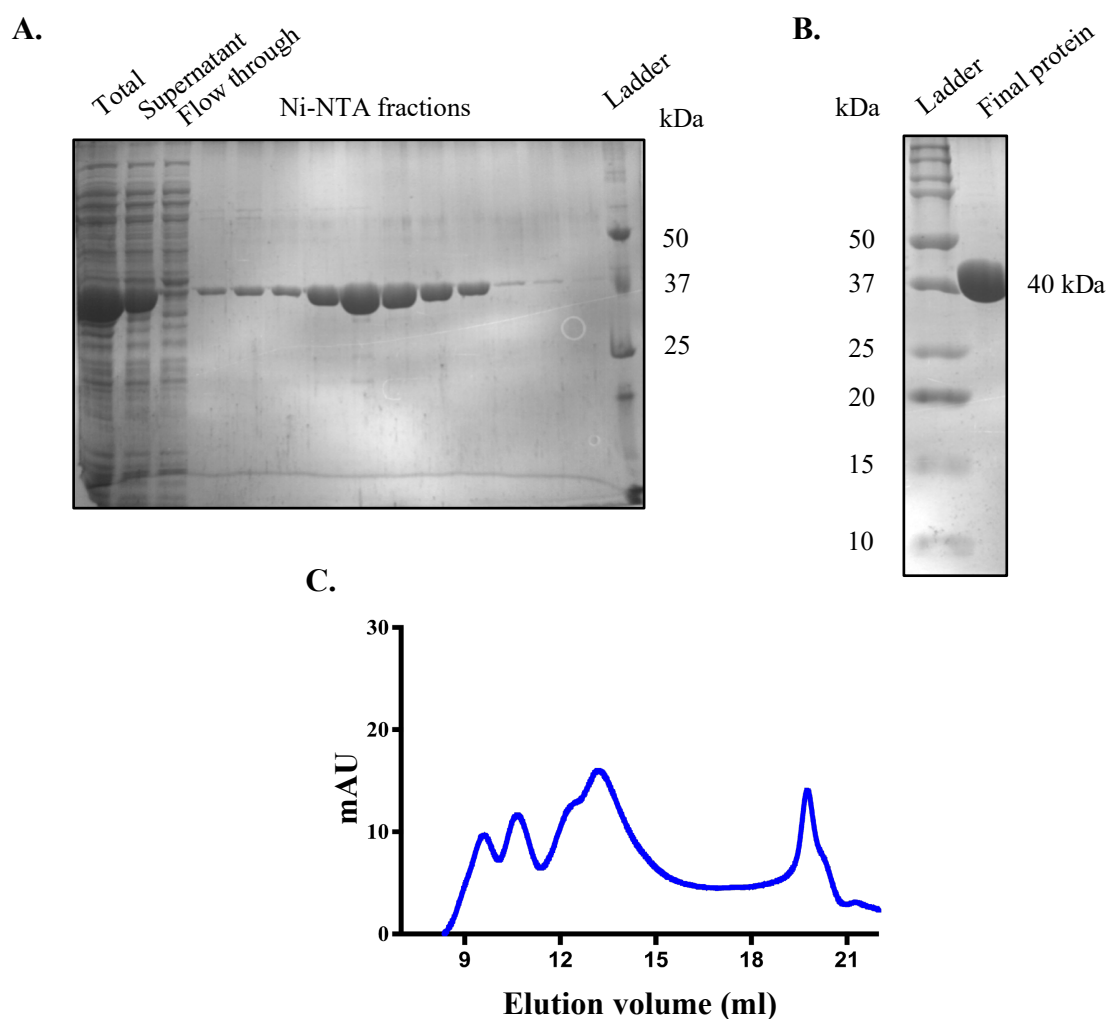


Figure 2.2 Purification of EcFtsZ.

A) 12 % SDS-PAGE gel showing the affinity chromatography purification profile by His-Trap column. B) 12 % SDS-PAGE gel showing the final protein. The molecular weight of EcFtsZ is 40 kDa. C) Analytical run in Superdex200 column showing the heterogenous oligomeric status of the protein.

2.3.2 Kinetic parameters of SmFtsZ and EcFtsZ

GTPase assay of SmFtsZ and EcFtsZ was performed with different concentrations of the substrate, which in this case is GTP. In order to determine the kinetic parameters of both the proteins, Michelis Menten plot was obtained from rate of reaction (y-axis) and different concentrations of the substrate GTP (x-axis) (Figure 2.3).

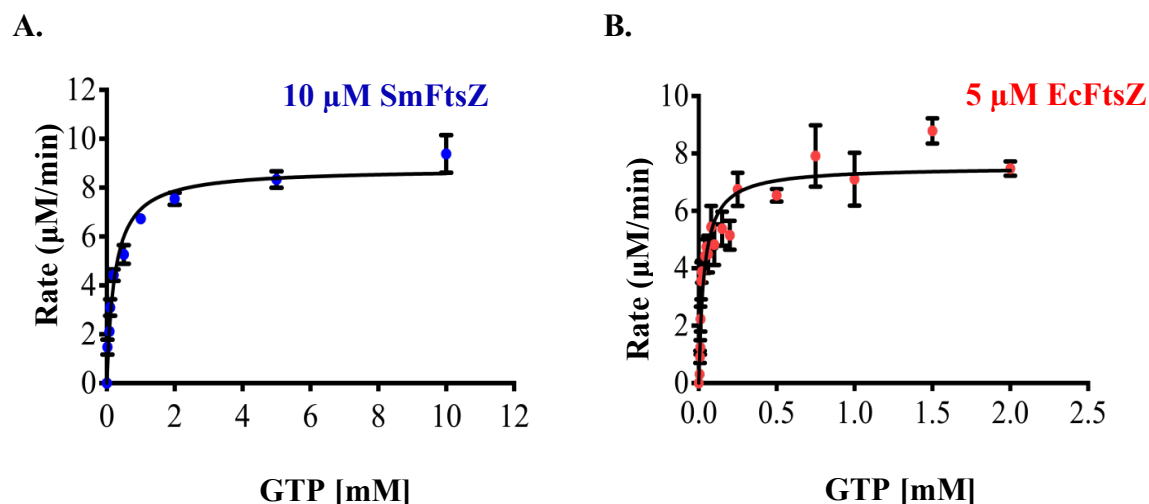


Figure 2.3 GTPase assay of SmFtsZ and EcFtsZ with varying substrate concentrations. A) GTPase activity of 10 μM SmFtsZ was measured with different concentrations of GTP ranging from 0 to 10,000 μM (purple). The curve was fitted with Michaelis Menten equation, $V_0 = V_{\max} ([S]/([S] + K_M))$, ($N = 3$; $n = 3$). B) GTPase activity of 5 uM EcFtsZ was measured with different concentrations of GTP ranging from 0 to 2000 μM (red) ($N = 3$; $n = 3$; $N =$ number of independent purification batches and $n =$ number of repeats).

Fitting the plot to the equation, $V_0 = V_{\max} ([S]/([S] + K_M))$, we obtained the kinetic parameters $V_{\max}$, K_M , k_{cat} , and catalytic efficiency which are listed in the following table (Table 2.2).

Table 2.2. Kinetic parameters obtained from Michelis Menten plot of EcFtsZ and SmFtsZ

Parameters	EcFtsZ	SmFtsZ
E_t (μM)	5	10
$V_{\max}$ (μM/min)	7.53 ± 0.28	8.78 ± 0.23
k_{cat} (min^{-1})	1.506 ± 0.05	0.87 ± 0.02
K_M (μM)	34	234
Catalytic efficiency (k_{cat} / K_M) ($\text{min}^{-1} \mu\text{M}^{-1}$)	44×10^{-3}	3.7×10^{-3}
Approximate critical concentration (μM)	1	8

It is observed from the values that the catalytic efficiency (k_{cat}/K_m) of SmFtsZ was 12-fold lower than that of EcFtsZ. Also the specific activity of SmFtsZ was 2-fold lower than that of EcFtsZ.

2.3.3 GTPase activity of SmFtsZ and EcFtsZ

In order to determine the apparent specific activity (k_{obs}) of the proteins, GTPase activity was measured for 10 μ M of SmFtsZ constructs and 5 μ M of EcFtsZ with saturating concentrations of GTP which were estimated based on the Michaelis Menten kinetics. The specific activity (k_{obs}) was measured as $0.66 \pm 0.007 \text{ min}^{-1}$ and $1.82 \pm 0.06 \text{ min}^{-1}$ (value $\pm$ after denotes standard error of mean, SEM) for SmFtsZ and EcFtsZ, respectively, comparable to the k_{cat} estimation through Michaelis Menten kinetics. The apparent k_{cat} values (k_{obs}) indicated that k_{obs} of SmFtsZ is threefold lower than that of EcFtsZ (Table 2.3).

Table 2.3 Mean specific activity (k_{obs}) values for different FtsZ constructs of EcFtsZ and SmFtsZ constructs.

Constructs	$k_{obs} (\text{min}^{-1})$
EcFtsZ	1.82 ± 0.05
SmFtsZ	0.67 ± 0.03
SmFtsZ ^{ACT}	0.55 ± 0.11
SmFtsZ ^{D211G}	0.04 ± 0.01

SmFtsZ and SmFtsZ^{ACT} showed similar GTPase activities, having no significant difference. SmFtsZ^{D211G} was observed to be GTPase dead, as expected, and did not show any GTPase activity (Figure 2.3).

Thus our *in vitro* data of FtsZs from *S. melliferum* and *E. coli* suggests that SmFtsZ possessed a lower GTPase activity compared to that of EcFtsZ.

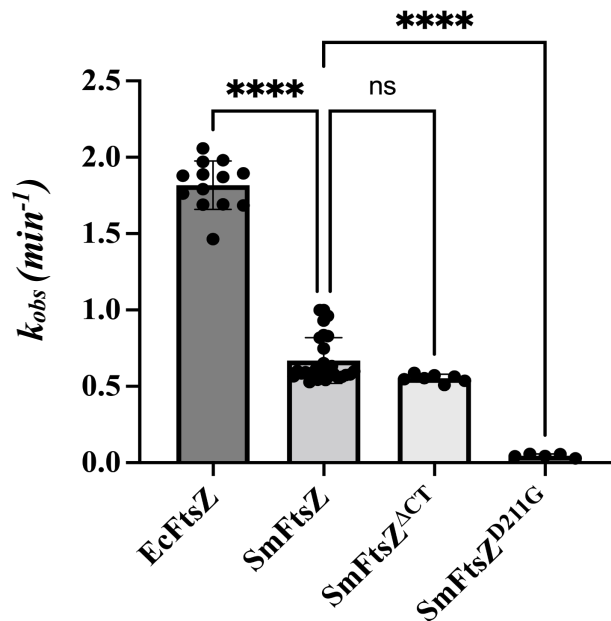


Figure 2.4 Comparison of GTPase activity (k_{obs}) across FtsZ constructs.

The plot shows comparison of GTPase activity with 5 μM of EcFtsZ and 10 μM of SmFtsZ constructs. SmFtsZ^{D211G}, a GTPase dead mutant, was taken as negative control. The experiments were done with saturating concentration of GTP. (EcFtsZ [$N = 4$; $n = 5$], SmFtsZ [$N = 3$; $n = 26$], and SmFtsZ^{D211G} [$N = 1$; $n = 5$]; N = number of independent purification batches and n = number of repeats). The mean with SEM and 95% confidence intervals are shown in the long and short horizontal lines in panel F; **** $p < 0.0001$; ns, non-significant.

2.3.4 Critical concentration of SmFtsZ and EcFtsZ

The active site of FtsZ is formed between the interface of two monomers, thereby GTPase assay was used to estimate the approximate critical concentration of the protein (Figure 2.5).

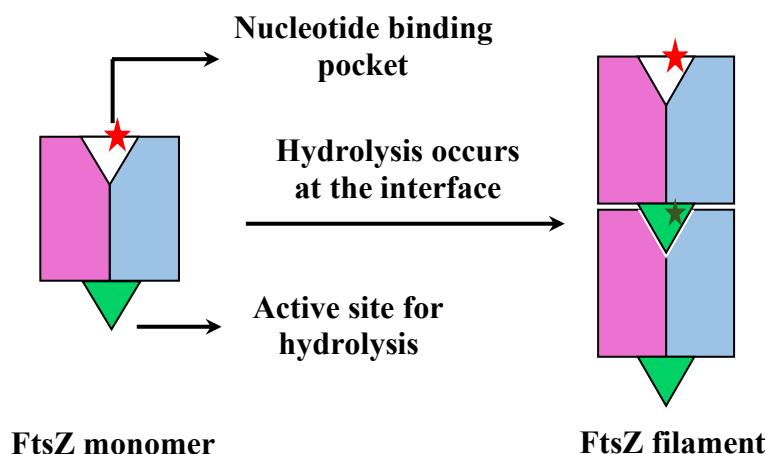


Figure 2.5 Schematic of nucleotide hydrolysis of FtsZ.

Schematic showing nucleotide hydrolysis at the interface, which in turn can be a read-out for critical concentration of FtsZ. The NTD is in sky blue; the H7 helix and the T7 loop are denoted in green; the CTD is in pink; the red star is denoted GTP.

The concentration of the protein from where the apparent specific activity of the protein becomes constant was considered as the approximate critical concentration of filament formation of FtsZ. GTPase activity of different concentrations of the protein with saturated amount of GTP was measured. The apparent specific activity (k_{obs}) and different concentrations of the protein was plotted along y- and x- axes respectively. We observed that the k_{obs} for SmFtsZ became constant from a protein concentration of approximately 8 μM (Figure 2.6A) whereas the k_{obs} for EcFtsZ became constant from the concentration of the protein of 1 μM (Figure 2.6B).

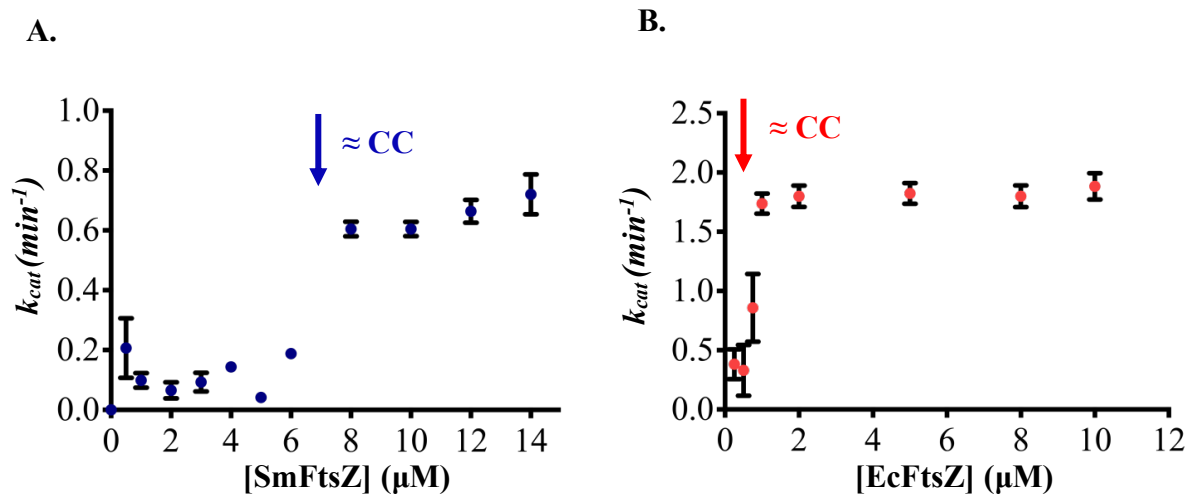


Figure 2.6 GTPase activity to determine the critical concentration.

A) GTPase activity of SmFtsZ was measured with different concentrations of the protein ranging from 0 to 14 μM , [WT; $N = 3$; $n = 4$]. B) GTPase activity of EcFtsZ with different concentrations of the protein from 0 to 10 μM , [WT; $N = 3$; $n = 3$].

Therefore, we conclude from our data that the critical concentration of SmFtsZ is around 8 μM and that of EcFtsZ is around 1 μM . This suggests that the critical concentration to form filaments is higher for SmFtsZ than that of EcFtsZ under the same *in vitro* conditions.

2.3.5 Light scattering assay to determine critical concentration

In order to determine if the critical concentration obtained from the GTPase assay is comparable, we went ahead to do 90° angle light scattering. For EcFtsZ, we indeed see an increase in the intensity after the addition of GTP as shown in (Figure 2.7A). We observe that as the time increases, the intensity starts to drop. As a result, the quick reduction in light scattering signals the end of the steady-state phase, during which only depolymerization takes place. This is because polymerization is being inhibited by the lack of GTP.

We plotted the maximum intensity with the different concentrations of EcFtsZ and the x-intercept of which gives the critical concentration of the protein (Figure 2.7B). The x-intercept obtained was 0.68 μM , which is close to the critical concentration of EcFtsZ obtained from the GTPase assay which was 1 μM .

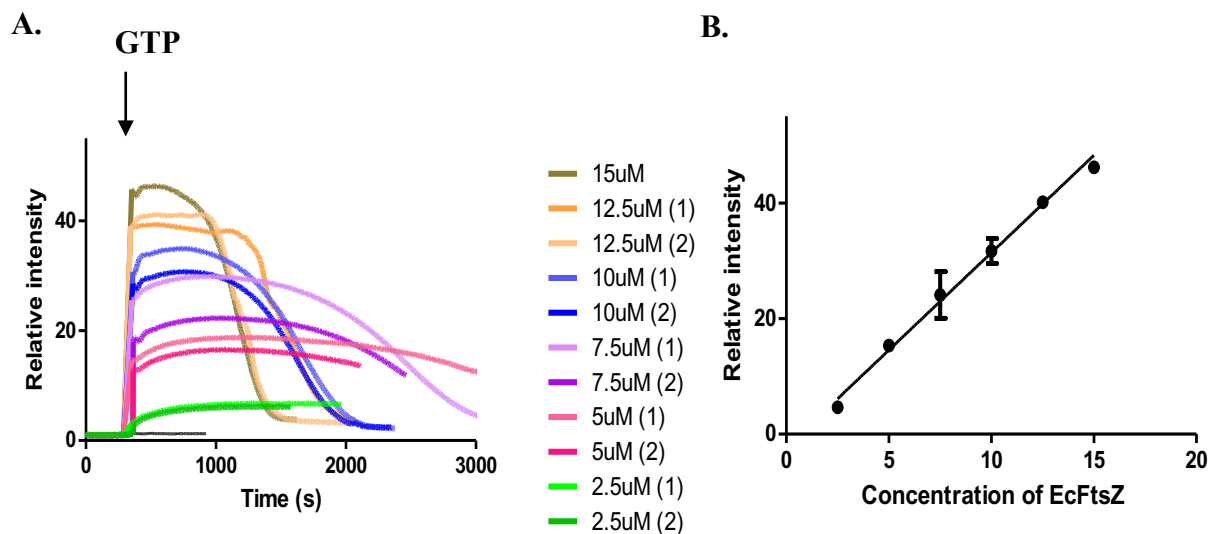


Figure 2.7 Light scattering assay of EcFtsZ to determine the critical concentration.

A) 90 light scattering assay of EcFtsZ was measured with different concentrations of the protein ranging from 0 to 12.5 μ M and GTP concentration of 250 μ M. B) The maximum relative intensity obtained from A was plotted against the concentration of EcFtsZ. [WT; $N = 2$; $n = 2$]

For SmFtsZ, we tried the same as above but there was no increase in the light scattering signal upon the addition of GTP.

2.3.6 Pelleting assay of SmFtsZ and EcFtsZ

In order to find out the condition in which SmFtsZ forms filaments, we performed pelleting assay of SmFtsZ. Previous reports have shown that FtsZ filaments pellets upon centrifugation at 100,000 xg. Thereby we sought to perform the same to find out filament formation of SmFtsZ. The 12 % SDS-PAGE gel shows that the fraction with GTP has protein in both supernatant and pellet. Other conditions do not show any protein in the pellet fraction (Figure 2.8A).

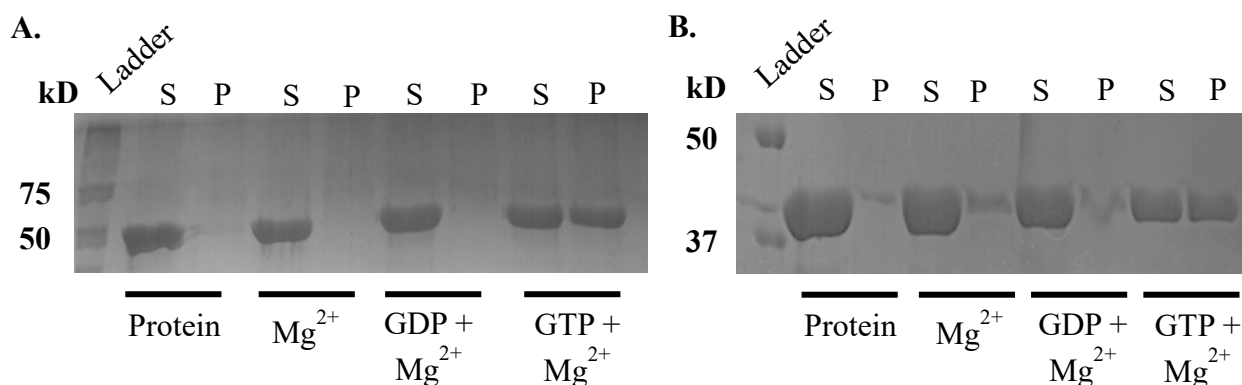


Figure 2.8 Pelleting assay gel for SmFtsZ and EcFtsZ.

A) A representative 12% SDS-PAGE gel for pelleting assay of SmFtsZ to determine the condition at which SmFtsZ forms filaments. B) 12 % SDS-PAGE gel image for pelleting assay of EcFtsZ. The protein concentration of 10 μ M for SmFtsZ and 12.5 μ M for EcFtsZ was taken and GTP/GDP concentration of 3 mM and $MgCl_2$ of 5 mM were taken. S and P represent fractions in the supernatant and pellet of the reaction.

This shows that SmFtsZ forms filaments in presence of GTP and not with GDP or $MgCl_2$.

The same experiment was performed with EcFtsZ to check the conditions in which EcFtsZ forms filaments. It was observed that majority of the protein comes in pellet in presence of GTP, which means that EcFtsZ forms filaments in presence of GTP (Figure 2.8B), consistent with reported literature (Król and Scheffers, 2013).

2.3.7 Visualization of FtsZ filaments under TEM

In order to confirm that SmFtsZ indeed forms filaments in presence of GTP and not aggregates, we went ahead to visualize the filaments under TEM by negative staining. The TEM images of SmFtsZ with GTP show that SmFtsZ formed filaments (elongated assemblies). The filaments were seen all across the grid (Figure 2.9). The length of the filaments were calculated using ImageJ (Schneider *et al.*, 2012). The approximate average length of filaments obtained was about 150 nm. The filaments were observed to be bundles of a few protofilaments, based on the estimated width.

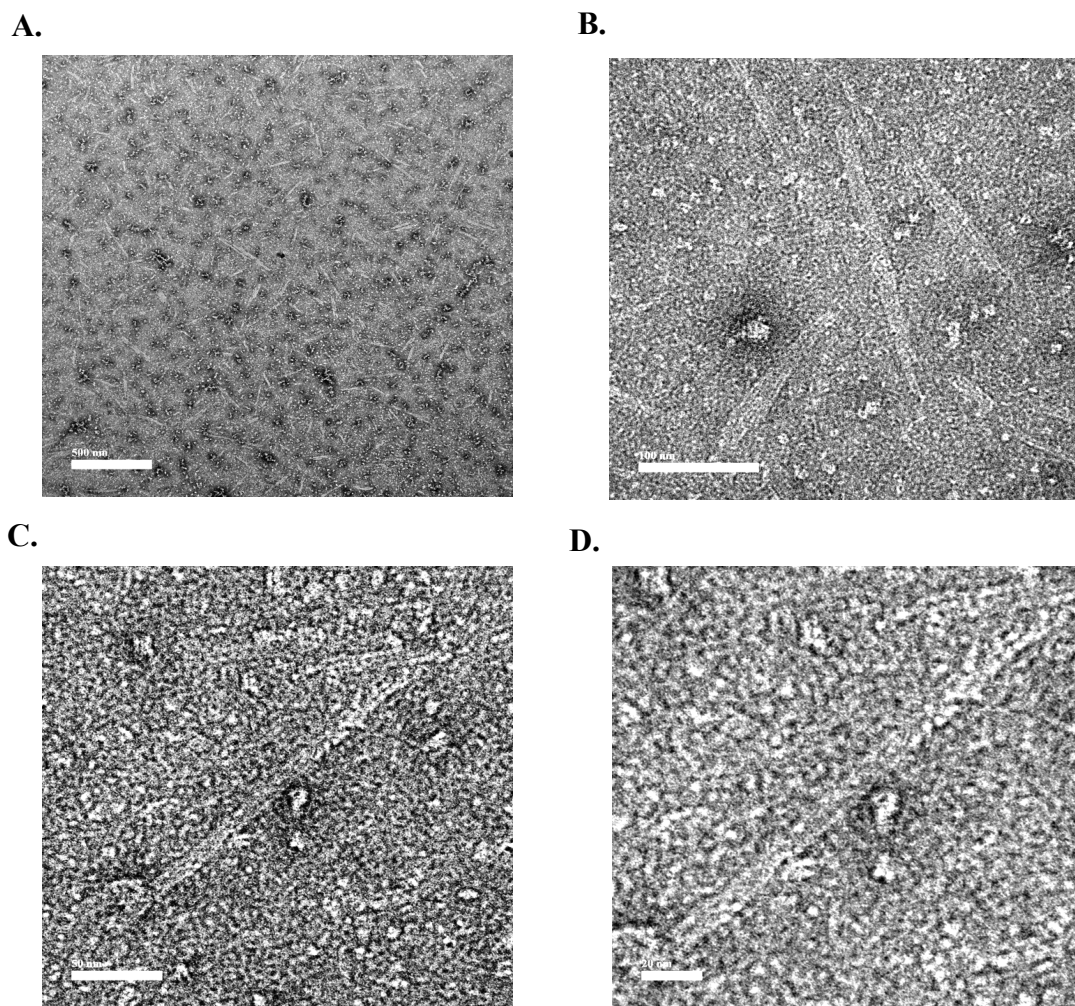


Figure 2.9 Filaments of SmFtsZ under TEM.

A) Filaments of SmFtsZ all over the grid square, scale bar = 500 nm. B) to D) Magnified images of the filaments with scale bars- 200, 50, 20 nm, respectively ($N = 2$, $n = 27$; N is the number of grids, n is the number of filaments taken for measurement).

2.4 Discussion

In this chapter, we characterized the bacterial cell division protein, FtsZ, from two bacteria- one is a cell wall less- *Spiroplasma melliferum* and the other is a cell walled organism- *Escherichia coli*. Cloning and purification of the FtsZ constructs were standardized to yield pure protein. The biochemical characterization of both the proteins indicate that the GTPase activity of EcFtsZ is 2 fold higher than that of SmFtsZ under similar *in vitro* conditions. We obtained Michelis Menten kinetics plot for both the proteins which signify that the catalytic

efficiency of EcFtsZ is 12 fold higher than that of SmFtsZ. The critical concentration of filament formation of FtsZ was obtained by the GTPase activity of different concentrations of the protein.

Table 2.4 Previous reports of GTPase activity of EcFtsZ

S.no.	Buffer	Method	Temperature (° C)	k _{cat} (/min)	References
1	5 mM [α - ³² P]GTP (40 mCi mmol ⁻¹), 15 mM magnesium acetate, reaction buffer (40 mM Tris-acetate (pH 7), 200 mM potassium acetate, 2 mM EDTA, 1 mM DTT and 0.5% Triton X-100	Radioactivity based	37	0.6	(de Boer <i>et al.</i> , 1992)
2	50 mM HEPES (pH 7.2), 10 mM MgCl ₂ , and 0.5 mM [γ - ³² P]GTP, 200 mM KCl	Radioactivity based	37	0.26	(Mukherjee <i>et al.</i> , 1993)
3	50 mM HEPES (pH 7.2), 15 mM MgCl ₂ , and 5 mM [γ - ³² P]GTP, 200 mM KCl	Radioactivity based	37	0.2	(Dai <i>et al.</i> , 1994)
4	50 mM HEPES (pH 7.2), 10 mM MgCl ₂ , 200 mM KCl, and 0.5 mM [γ - ³² P]GTP	Radioactivity based	30	0.4	(Wang <i>et al.</i> , 1997)
5	50 mM MES (pH 6.5), 50 mM KCl, 10 mM MgCl ₂ and 1 mM [γ - ³² P]GTP	Radioactivity based	30	1.55	(Mukherjee, 1998)
6	50mM PB, pH 7.5, 50 mM KCl, 5 mM Mg acetate, 0.5 M GTP	Radioactivity based	37	1.6	(Lu <i>et al.</i> , 1998)
7	MEMK6.5 (100 mM MES, pH 6.5, adjusted with KOH, 1 mM EGTA, 5 mM magnesium acetate), 0.5 M GTP	Radioactivity based	37	1.08	(Lu <i>et al.</i> , 1998)
8	MEMK6.5 (100 mM MES, pH 6.5, adjusted with KOH, 1 mM EGTA, 5 mM magnesium acetate), 2 mM GTP	Radioactivity based	37	6.5	(Lu <i>et al.</i> , 2001)
9	50 mM MES/NaOH, pH 6.5, and 50 mM KCl, 1 mM GTP, 1 mM MgCl ₂	Malachite green	30	3.8	(Scheffers <i>et al.</i> , 2002)

10	50 mM MES/NaOH, pH 6.5, and 50 mM KCl, 1 mM GTP, 5 mM MgCl ₂	Malachite green	30	4	(Scheffers <i>et al.</i> , 2002)
11	50 mM MES/NaOH, pH 6.5, and 50 mM KCl, 1 mM GTP, 10 mM MgCl ₂	Malachite green	30	2.7	(Scheffers <i>et al.</i> , 2002)
12	50 mM morpholineethanesulfonic acid [pH 6.5], 10 mM MgCl ₂ , 200 mM KCl	Radioactivity based	25	1.8	(Dajkovic <i>et al.</i> , 2008)
13	MES buffer, pH 6.5, 50 mM KCl, 5 mM MgCl ₂ , 0.5 M GTP	Fluorescent marker	30	2.5	(Mohammadi <i>et al.</i> , 2009)
14	MES buffer, pH 6.5, 50 mM KCl, 10 mM MgCl ₂ , 0.5 M GTP	Fluorescent marker	30	1.8	(Mohammadi <i>et al.</i> , 2009)
15	HEPES buffer, pH 7.5, 50 mM KCl, 5 mM MgCl ₂ , 0.5 M GTP	Fluorescent marker	30	2.9	(Mohammadi <i>et al.</i> , 2009)
13	HEPES buffer, pH 7.5, 50 mM KCl, 10 mM MgCl ₂ , 0.5 M GTP	Fluorescent marker	30	1.8	(Mohammadi <i>et al.</i> , 2009)
14	pH 6.5, 50 mM MES, 50 mM KCl, 2.5 mM MgCl ₂ , 1 mM EGTA, 1% sucrose	NADH coupled	23	0.66	(Yang <i>et al.</i> , 2017)

The value of the specific activity of EcFtsZ measured was comparable with the previously reported value (Mukherjee, 1998; Dajkovic *et al.*, 2008; Mohammadi *et al.*, 2009), but higher than the values reported in (de Boer *et al.*, 1992; Mukherjee *et al.*, 1993; Dai *et al.*, 1994; Wang *et al.*, 1997; Yang *et al.*, 2017) (Table 2.4). The differences in the values of the specific activity of EcFtsZ might be due to differences in purification protocol, differences in method for determining phosphate release, differences in reaction buffer, and temperature. Thus it is indeed important to note that identical conditions were used to compare the GTPase activity of the two FtsZs in this study.

We observed that the critical concentration to form filaments is higher in SmFtsZ than that in EcFtsZ. The light scattering assay also provided a comparable value of critical concentration for EcFtsZ as obtained by GTPase activity based estimation strategy and also comparable with the previously reported value (Mukherjee and Lutkenhaus, 1999), but we could not observe any increase in light scattering signal for SmFtsZ. We observed that SmFtsZ forms filaments only in presence of GTP as observed from the pelleting assay and we visualized the

filaments of SmFtsZ in presence of GTP by negative staining in TEM. We observed that the filaments of SmFtsZ were comparatively smaller than the previously reported EcFtsZ filaments.

Even though the residues for GTP binding and hydrolysis are conserved, we observe significant difference in the activity of FtsZ from the two organisms under identical *in vitro* conditions. Slower GTPase and high critical concentration could mean that the transition of R to T state of the FtsZ molecule is slower.

In order to gain molecular insights on why FtsZ from *Spiroplasma melliferum* is less active than that of *Escherichia coli*, we sought to do structural studies which is explained in the next chapter.

2.5 References

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Chapter 3

Structure determination of conformational states of SmFtsZ and their structural analysis

3.1 Introduction

The basis of treadmilling of FtsZ protofilament (PF) is the polymerization from one end and depolymerization from the other end. There is a conformational switch of each monomer from its low affinity state to a high affinity state that favors cooperative assembly of the FtsZ filaments (Huecas *et al.*, 2008; Dajkovic *et al.*, 2008). Previous reports have shown that FtsZ forms single protofilaments and the assembly is cooperative (Mukherjee, 1998; Huecas and Andreu, 2003). This implies that there should be conformational differences between the monomeric state of FtsZ and the filament state of FtsZ. Existing crystal structures show that there are two conformations of FtsZ. One is the R state which is predominantly in the monomeric form and the other one is the T state which is a filament compatible state (Wagstaff *et al.*, 2017). The two states are distinguished based on the nucleotide orientation in the N-terminal domain (NTD) and the relative orientation between the N-terminal domain (NTD) and the C-terminal domain (CTD) (Fujita *et al.*, 2017). In the R state of the protein, the guanine ring is tilted approximately 30 degrees with respect to that in the T state structure. The interdomain cleft is closed in the R state structure whereas it is opened in the T state structure of FtsZs (Figure 3.1).

In the previous chapter, we compared biochemical properties of FtsZ from a cell wall-less bacterium, *Spiroplasma melliferum* with that of cell walled bacterium, *Escherichia coli*. We observe that the FtsZ from *S. melliferum* is less active than that of *E. coli* under similar *in vitro* conditions. In order to gain mechanistic insights on SmFtsZ activity, we sought to find out the molecular details of FtsZ from *S. melliferum*. In this chapter, I discuss about the crystallization of the wild type SmFtsZ with different nucleotides, their structure determination and comparison of the structures we obtained with the existing structures of FtsZ. The insights from the structures led us to propose a mechanistic basis of kinetic polarity of FtsZ.

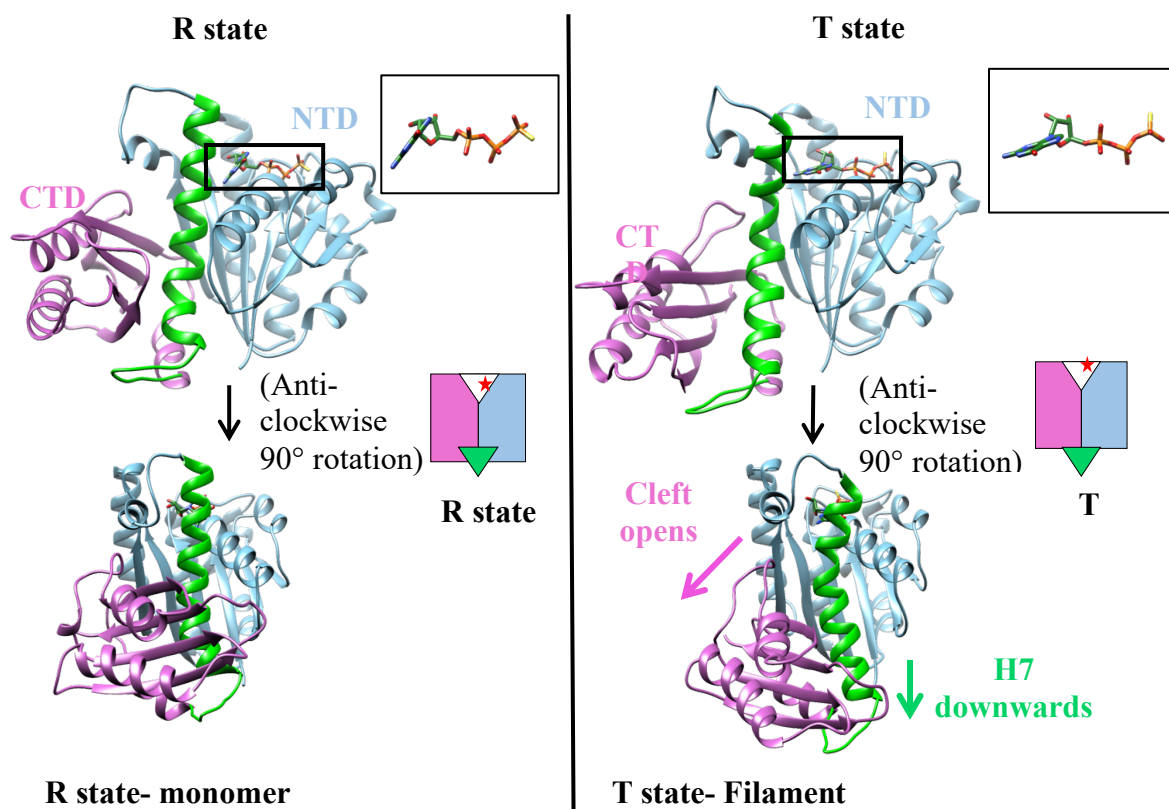


Figure 3.1 R-state versus T-state conformations of FtsZ

A) FtsZ monomer structure in R state (PDB ID: 2RHO). B) FtsZ monomer structure in T state (PDB ID: 3WGM). The insets show the N-terminal domain with nucleotide orientation and the C-terminal domain showing the interdomain cleft orientation.

3.2 Materials and methods

3.2.1 Analysis of FtsZ structures

The available structures of FtsZ from PDB are listed in Table 3.1. FtsZ structure from *Staphylococcus aureus* (SaFtsZ; PDB ID: 5H5G) was taken as reference for the R- and T-states. Chain A of 5H5G is in T-state while chain B is in R-state. All the available structures of FtsZ were classified into R- and T- states based on the nucleotide orientation in NTD and the interdomain cleft in the CTD (Figure 3.1). For measuring the angle in the guanine tilt, the T-state structure (PDB: 5H5G, chain A) was considered as reference. The angle was calculated by estimating the angle between the lines defined by the ‘define axis’ tool using three atoms (C2, C8, N1) of the guanine ring for each structure. Similarly, the movement of the CTD was calculated by measuring the RMSD between the C-alpha of the reference structure and the sample structure. For all the superimpositions, the NTD (residue range 1 to

175- according to SaFtsZ) of the molecules was superimposed using ‘matchmaker’ command in Chimera. All analyses were done using UCSF Chimera version 1.13.1 (Pettersen *et al.*, 2004). The structures used in the study are listed in Table 3.1.

Table 3.1 List of structures used in the study

S.no.	PDB ID	Organism	Resolution (Å)	Ligand bound	Mutation	State
Globular						
1	5H5G	<i>S. aureus</i>	2.2	Chain A- GDP, Ca ²⁺	No	T
				Chain B- GDP		R
2	3WGJ	<i>S. aureus</i>	2.17	Ca ²⁺	T7 loop sequence (VSGEV) replaced by BsFtsZ (TPGLI)	-
3	4DXD	<i>S. aureus</i>	2.01	GDP, inhibitor	No	T
4	5H5H	<i>S. aureus</i>	1.7	GDP, Ca ²⁺	R29A	T
5	3VO8	<i>S. aureus</i>	2.2	GDP, Ca ²⁺	No	T
6	3VOA	<i>S. aureus</i>	1.7	GDP, Ca ²⁺	No	T
7	5MN4	<i>S. aureus</i>	1.5	GDP, (4S)-2-Methyl-2,4-pentanediol (MPD)	F138A	T
8	3VOB	<i>S. aureus</i>	2.7	GDP, Ca ²⁺ , inhibitor	No	T
9	3WGN	<i>S. aureus</i>	2.6	GTPγS	No	T
10	5XDT	<i>S. aureus</i>	1.3	GDP, Ca ²⁺ , MB3, ZI7(inhibitor)	No	T
11	5XDU	<i>S. aureus</i>	2	GDP, Ca ²⁺ , ZI6 (inhibitor)	No	T
12	5XDW	<i>S. aureus</i>	2	GDP, Ca ²⁺	G196S	
13	3WGM	<i>S. aureus</i>	2.09	GTP, Mg ²⁺	T7 loop substituted with GAN	T
14	7OHH	<i>S. aureus</i>	1.45	GDP, BeF ₃ ⁻ , K ⁺	No	T
15	7OHK	<i>S. aureus</i>	1.75	GDP, BeF ₃ ⁻ , K ⁺ , Mg ²⁺	No	T
16	7OHL	<i>S. aureus</i>	1.75	GDP, BeF ₃ ⁻ , K ⁺ , Mn ²⁺	No	T
17	7OHN	<i>S. aureus</i>	1.62	GDP, AlF ₄ ⁻ , Mg ²⁺	No	T
18	7OMJ	<i>S. aureus</i>	1.57	GMPPCP, K ⁺	No	T
19	7OMP	<i>S. aureus</i>	1.52	GMPPCP, Mg ²⁺ , EDO	No	T
20	7OMQ	<i>S. aureus</i>	1.45	GMPPCP, Mn ²⁺ , EDO	No	T
21	7OJZ	<i>S. aureus</i>	1.65	GMPCP, EDO, K ⁺	No	T
22	7OI2	<i>S. aureus</i>	1.9	GDP, EDO, Na ⁺	No	T
23	7ON2	<i>S. aureus</i>	1.69	GDP, EDO, K ⁺	No	T

24	7ON3	<i>S. aureus</i>	2.32	GDP, EDO, K ⁺ , soak 10 mM EGTA	No	T
25	7ON4	<i>S. aureus</i>	1.79	GDP, EDO, K ⁺ , 1 mM EDTA	No	T
26	7OJA	<i>S. aureus</i>	2.22	GDP, EDO, SO ₄ ⁻	D210N	T
27	7OJB	<i>S. aureus</i>	1.7	GDP, EDO, K ⁺	R143K	T
28	7OJC	<i>S. aureus</i>	1.95	GDP, EDO, K ⁺	Q48A	T
29	7OJD	<i>S. aureus</i>	1.82	GDP, EDO, K ⁺	D46A	T
30	5XDV	<i>S. aureus</i>	1.7	GDP (not added externally), Ca ²⁺ , inhibitor	No	T
31	6KVP	<i>S. aureus</i>	1.4	Zl1, GDP, Ca ²⁺	No	T
32	6KVQ	<i>S. aureus</i>	1.6	DVX, GDP, Ca ²⁺	No	T
33	6SI9	<i>S. aureus</i>	1.9	GDP, Ca ²⁺ , EDO	No	T
34	5MN5	<i>S. aureus</i>	2.8	GTP	T66W	R
35	3WGL	<i>S. aureus</i>	3	GDP	T7 loop substituted with GAN	R
36	3WGK	<i>S. aureus</i>	2.7	GDP	T7 loop substituted with GAG	R
37	5H5I	<i>S. aureus</i>	1.9	GDP	R29A	R
38	5MN6	<i>S. aureus</i>	3.2	GDP	F138A	R
39	5MN8	<i>S. aureus</i>	3.5	GTP	F138A	R
40	4U39	<i>B. subtilis</i>	3.19	PO ₄ ⁻	No	-
41	2VXY	<i>B. subtilis</i>	1.7	Citric acid, K ⁺	No	-
42	2RHL	<i>B. subtilis</i>	2.45	GDP	No	R
43	2RHJ	<i>B. subtilis</i>	1.76	SO ₄ ⁻ , acetate ion, tetraethylene glycol, Na ⁺	No	-
44	2RHO	<i>B. subtilis</i>	2.45	Chain A- GDP, Chain B- GSP	No	R
45	2VAM	<i>B. subtilis</i>	2.5	SO ₄ ⁻	No	-
46	2RHH	<i>B. subtilis</i>	2.01	SO ₄	No	-
47	4KWE	<i>M. tuberculosis</i>	2.9	GDP	No	R
48	1RQ7	<i>M. tuberculosis</i>	2.6	GDP	No	R
49	1RLU	<i>M. tuberculosis</i>	2	Chain A- GSP, Chain B- No	No	R
50	2Q1Y	<i>M. tuberculosis</i>	2.3	Chain A- GSP, Chain B- No	No	R
51	5V68	<i>M. tuberculosis</i>	3.4	GDP	No	R
52	5ZUE	<i>M. tuberculosis</i>	2.7	GTP	No	R
53	2Q1X	<i>M. tuberculosis</i>	2.35	Chain A- Citrate, Chain B- No	No	-
54	1RQ2	<i>M. tuberculosis</i>	1.86	Chain A- Citrate, Chain B- No	No	-
55	1W5A	<i>M. jannaschii</i>	2.4	GTP	No	R

56	1W5B	<i>M. jannaschii</i>	2.2	GTP	No	R
57	1W5E	<i>M. jannaschii</i>	3	GTP	W319Y	R
58	1W58	<i>M. jannaschii</i>	2.5	Phosphomethyl phosphonic acid guanylate ester (G2P), Mg^{2+}	No	R
59	1FSZ	<i>M. jannaschii</i>	2.8	GDP	No	R
60	2VAP	<i>M. jannaschii</i>	1.7	GDP	No	R
61	1W59	<i>M. jannaschii</i>	2.7	SO_4^{4-}	No	-
62	2R6R	<i>A. aeolicus</i>	1.7	GDP	No	R
63	2R75	<i>A. aeolicus</i>	1.4	8-morpholin-4-ylguanosine 5'-(tetrahydrogen triphosphate), Mg^{2+}	No	R
64	6UNX	<i>E. coli</i>	1.4	GTP	L178E	R
65	6UMK	<i>E. coli</i>	1.3	GDP	L178E	R
66	6LL6	<i>E. coli</i>	2.5	GDP	No	R
67	6LL5	<i>K. Pneumoniae</i>	1.7	GDP, glycerol	No	R
68	4E6E	<i>T. fusca</i>	2.12	SO_4^{4-} , Cl^- , Mg^{2+}	No	-
69	1FOU	<i>P. aeruginosa</i>	2.1	GDP, SulA	No	R
70	2VAW	<i>P. aeruginosa</i>	2.9	GDP	No	R
Apo- SaFtsZ (Domains not separated)						
71	3VO9	<i>S. aureus</i>	2.7	No	No	-
72	3VPA	<i>S. aureus</i>	2.4	No	No	-
Domain swapped						
73	5MN7	<i>S. aureus</i>	2.3	GTP, Mg^{2+}	F138A	R (NTD)
74	1W5F	<i>T. maritima</i>	2	Phosphomethyl phosphonic acid guanylate ester, Mg^{2+}	T7 loop mutated	R (NTD)

3.2.2 Crystallization and data collection of SmFtsZ with and without GMPPNP

Crystallization screens of SmFtsZ were set up using 100 nl of 10 mg/ml protein (SmFtsZ^{ACT} – SmFtsZ of residues 1-314) and 100 nl of conditions from the 500 commercially available screens (Molecular dimensions, Hampton Research) by sitting drop vapour diffusion method. The crystallization screens were attempted simultaneously for SmFtsZ (without addition of nucleotide externally) and SmFtsZ-GMPPNP state (protein concentration of 10 mg/ml, 2 mM GMPPNP, and 5 mM $MgCl_2$) using sitting drop 2 drop 96 well plates (Swiss Sci). In order to obtain bigger crystals, 1 μ l drop was set up 48-well plates for the same. The conditions where we obtained SmFtsZ crystals are listed in Table 3.2. The crystals were frozen with 20% ethylene glycol as cryoprotectant with the respective parent condition in

which the crystals were obtained. The crystals were diffracted and data were collected at the beamlines at Diamond Light Source, Harwell, UK. The diffraction data obtained were indexed using XDS (Kabsch, 1988) and scaled using AIMLESS present in the CCP4 package (Evans and Murshudov, 2013), (Potterton *et al.*, 2018).

Table 3.2: Crystallization conditions for SmFtsZ without and with GMPPNP

	SmFtsZ	SmFtsZ- GMPPNP
Method	Sitting drop vapour diffusion	Sitting drop vapour diffusion
Plate type	48-well	48-well
Temperature (K)	291	291
Construct and protein concentration (mg/ml)	SmFtsZ ^{ΔCT} -10 mg/ml	SmFtsZ ^{ΔCT} – 10 mg/ml
Buffer composition of protein solution	50 mM HEPES pH 6.5, 50 mM KCl	50 mM HEPES pH 6.5, 50 mM KCl, 2 mM GMPPNP, 5 mM MgCl ₂
Composition of reservoir solution	1.5 M ammonium sulphate, 0.1 M Tris pH 8.5	0.3 M sodium acetate, 0.1 M Tris pH 8.5, 15% w/v PEG 4000
Volume and ratio of drop	500 nl: 500 nl	500 nl: 500 nl
Volume of reservoir (ml)	0.1	0.1

3.2.3 Structure determination of SmFtsZ with and without GMPPNP

The protein sequence of SmFtsZ was given in BLAST and searched for homologous structures in the Protein Data Bank (PDB). FtsZ from *Bacillus subtilis* (PDB ID: 2VXY) was chosen as the homologous structure having a sequence identity of 60% with SmFtsZ. A monomeric subunit of BsFtsZ structure (PDB ID: 2VXY) was hence taken for molecular replacement in order to solve the structure of SmFtsZ. The molecular replacement was done using PHASER and the model building was done using COOT (McCoy *et al.*, 2007) (Emsley *et al.*, 2010). The model was refined using the PHENIX software package (Adams *et al.*, 2010).

3.2.4 HPLC run to determine the nucleotide bound to the protein

50 mmol of SmFtsZ^{ACT} was denatured at 75 °C, spun at 21,000 g at 4 °C and the supernatant was filtered using 0.22 µm cellulose acetate filter (Corning). The filtrate was loaded onto DNA-PAC PA 200 ion exchange column, which was pre-equilibrated by buffer A (2 mM Tris, pH 8). The runs were performed using a linear gradient of 0-20% buffer B (2 mM Tris, pH 8, and 1.25 M NaCl) for 3 CV, 20-40% buffer B for 3 CV and 100% buffer B for 2 CV. The standards used for the run were GDP and GTP of 100 mmol each. The graph was plotted with absorbance at 255 nm against the conductivity using GraphPad Software version 9.00 for MacOS, San Diego California USA.

3.3 Results

3.3.1 Summary of crystal structures of FtsZs

There are 74 FtsZ structures reported in PDB from ten bacteria, which are *Staphylococcus aureus* (Sa), *Bacillus subtilis* (Bs), *Mycobacterium tuberculosis* (Mt), *Methanococcus jannaschii* (Mj), *Thermobifida fusca* (Tf), *Aquifex aeolicus* (Aa), *Pseudomonas aeruginosa* (Pa), *Escherichia coli* (Ec), *Thermotoga maritima* (Tm) and *Klebsiella pneumonia* (Kp). The nucleotide bound structures are further classified into T- or R- state conformations of FtsZ. The difference in the two states of FtsZ lies in the- a) difference in nucleotide orientation and residues interacting with nucleotide, b) difference in the cleft between N- terminal domain (NTD) and C- terminal domain (CTD) (Figure 3.1). The existing structures were classified by superimposing with the representative R- and T-state structures of FtsZ from *S. aureus* (PDB ID: 5H5G) (Figure 3.2A, B, Table 3.1). We observed that the existing structures of FtsZs were captured in R-state for organisms other than *S. aureus* (Figure 3.2C). FtsZ from *S. aureus* was crystallized in T state as well as R state. Also, there are two domain swapped structures of FtsZ from *S. aureus* and *T. maritima*. One is SaFtsZ^{F138A} mutant (PDB ID: 5MN5) where the mutation is at the upper polymerization interface and another one is the TmFtsZ structure with T7 loop mutated (PDB ID; 1W5F). The pie charts and the bar graph depict a summary of all available structures of FtsZ from the PDB from different organisms (Figure 3.2). It is indeed seen that the T state structure of FtsZ is captured only from *S. aureus* whereas FtsZs from other organisms are captured in R state (Figure 3.2C).

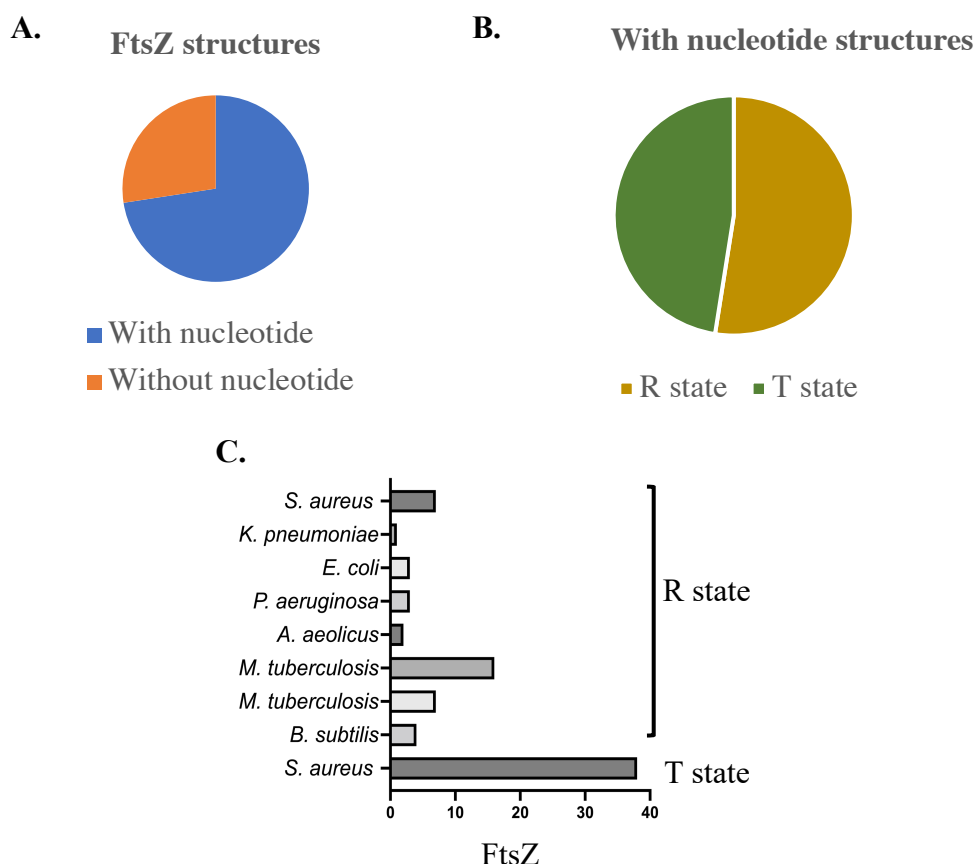


Figure 3.2 Summary of existing structures of FtsZ

A) Pie chart showing the FtsZ structures without and with nucleotide. B) Pie chart showing the FtsZ structures in R state and T state. C) Organism wise distribution of FtsZ structures.

3.3.2 Crystal structures of SmFtsZ

We then sought to find out the molecular details of SmFtsZ by crystallizing the protein without and with excess of GMPPNP. The crystals of SmFtsZ for both the conditions were needle shaped (Figure 3.3). The crystals diffracted to 2.3 Å for SmFtsZ and 2.2 Å for SmFtsZ-GMPPNP (refer Table 3.3 for data collection statistics). The space group was identified as P4₁2₁2. Calculation of Mathew's coefficient indicated two molecules of FtsZ in the asymmetric unit.

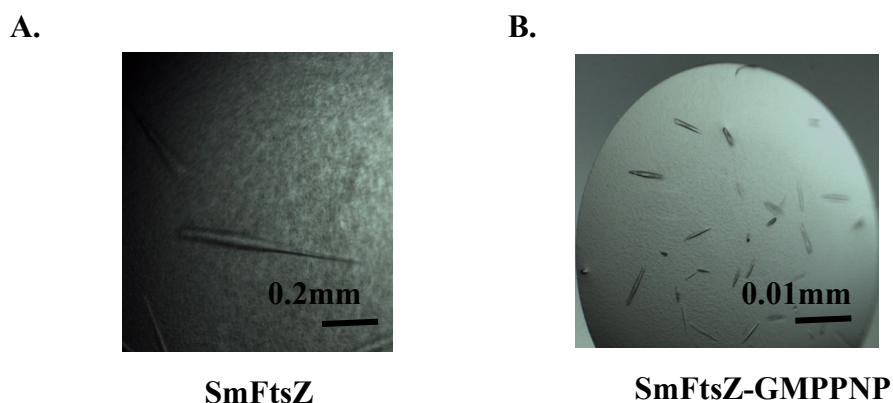


Figure 3.3 Crystals of SmFtsZ without and with GMPPNP

A) Needle-like crystals of SmFtsZ without adding nucleotide externally. B) Needle-like crystals of SmFtsZ with GMPPNP.

Table 3.3: Data collection statistics for SmFtsZ without and with GMPPNP

	SmFtsZ	SmFtsZ-GMPPNP
Collected at	DLS, UK	DLS, UK
Wavelength (Å)	0.976	0.976
Space Group	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2
a, b, c (Å)	106.59, 106.59, 128.61	106.55, 106.55, 127.60
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)*	49.16-2.3 (2.38-2.3)	49.24-2.2 (2.27-2.2)
Number of unique reflections*	33121 (3217)	38294 (3238)
R _{merge} (%) *	8.4 (69.1)	9 (64)
R _{pim} (%) *	4.3 (35.6)	4.2 (28)
CC _{half} *	0.998 (0.794)	0.998 (0.894)
Mean I/ σ I	14.9 (3.1)	17.0 (4.3)
Redundancy*	8.7 (8.8)	7.5 (7.1)
Completeness (%) *	99.7 (100)	99.9 (98.3)

*Values in parantheses denote last resolution shell

After scaling the diffraction data, the structure solution of SmFtsZ structure was obtained by molecular replacement using PHASER using FtsZ structure from *Bacillus subtilis* (PDB ID: 2VXY). The refinement statistics for both the structures is listed in Table 3.4.

Table 3.4: Refinement statistics of SmFtsZ without and with GMPPNP

	SmFtsZ	SmFtsZ-GMPPNP
PDB ID	7YSZ	7YOP
Resolution (Å)	49.16-2.3	49.24-2.2
Number of unique reflections (test set)	33090 (1657)	38229 (1915)
R _{work} / R _{free} (%)	0.179/0.235	0.173/0.221
Average B-factor (Å ²)	50	43
Wilson B-factor (Å ²)	41.8	33.4
RMS deviations		
Bond lengths (Å)	0.01	0.011
Bond angles (°)	1.079	1.148
Ramachandran map statistics		
Favoured (%)	96.9	98.30
Allowed (%)	3.1	1.70
Outliers (%)	0	0

3.3.3 Domain swapped packing of SmFtsZ structures

We then analyzed the SmFtsZ structures in different nucleotide states. Both the crystal structures of SmFtsZ were obtained in the same unit cell and space group. This implies that they have a similar crystal packing. The stretch of residues in N-terminal domain (NTD)- 1 to 176. The stretch of residues in H7 helix- 177 to 200. The stretch of residues in C-terminal domain (CTD)- 201 to 314. The numbering is based on FtsZ from *Spiroplasma melliferum* and is of the core of FtsZ.

After superposing the C-alpha NTD of BsFtsZ (PDB ID: 2RHL) with the NTD of SmFtsZ, we observed that the two molecules in the asymmetric unit exist as a domain-swapped dimer (Figure 3.4A). Interestingly, the domain-swapped structure of SmFtsZ has NTD of one chain packed against the CTD of the other chain, which resembles the globular structure of FtsZ (Figure 3.4B).

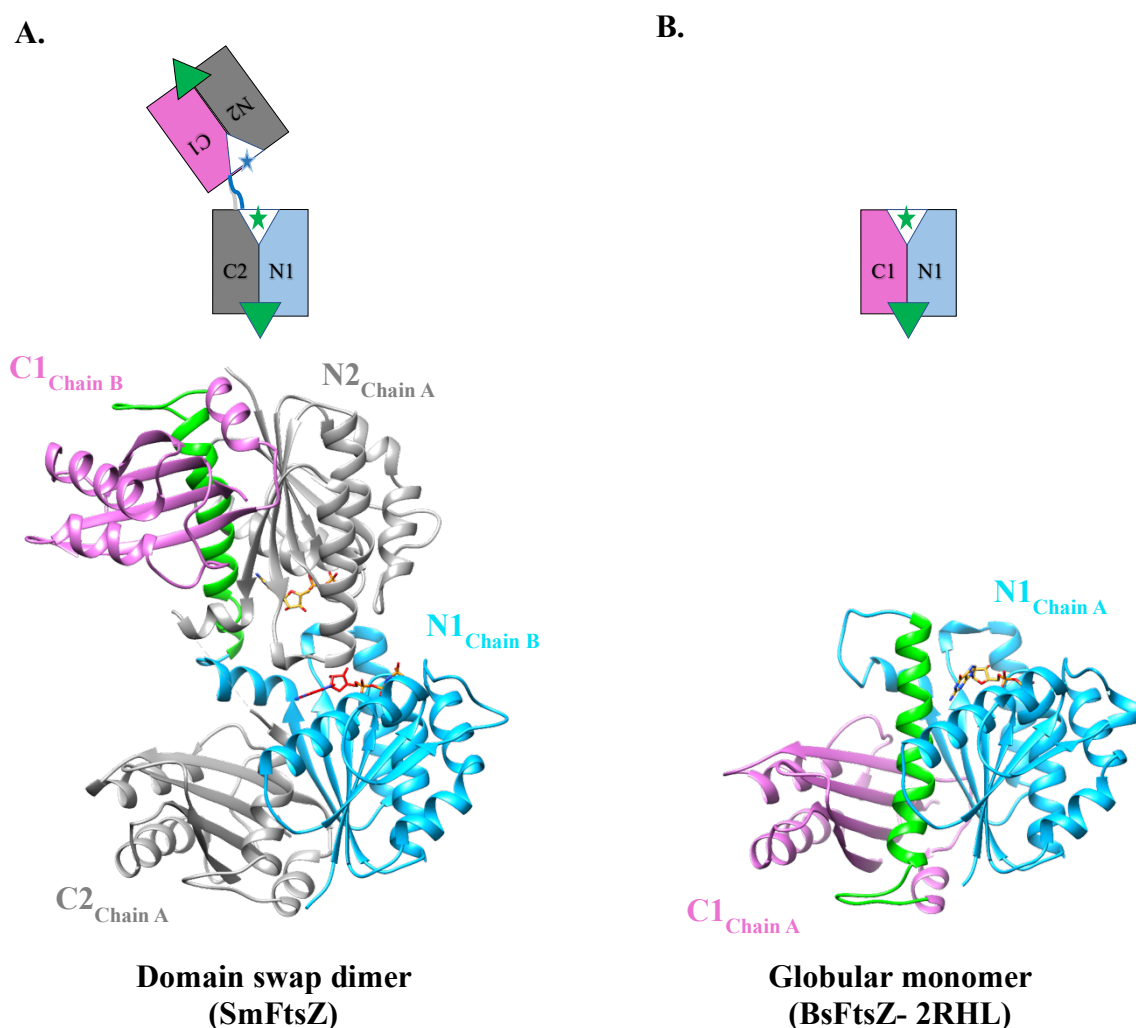


Figure 3.4 Domain organization of SmFtsZ crystal structure.

A) Overall structure of SmFtsZ, along with the schematic, containing both the chains forming domain swap dimers. The domains and the chains are denoted with their respective colours. B) Overall structure of globular FtsZ from *B. subtilis* (PDB: 2RHL).

In the extended conformation of one molecule of the domain-swapped dimer, the NTD and the CTD are separated at the T6 loop which is the start of the H7 helix. A clear and continuous electron density in the T6 loop region is observed in this region for chain B where the two domains (NTD and CTD) separated (Figure 3.5), confirming the domain swapped conformation of SmFtsZ in crystal structure. For chain A, the corresponding loop was disordered and hence the residue stretch of 170 – 185 has not been modelled. The relative orientations of the NTD and CTD in the extended conformation of each chain (A and B) differ, as observed from (Figure 3.5).

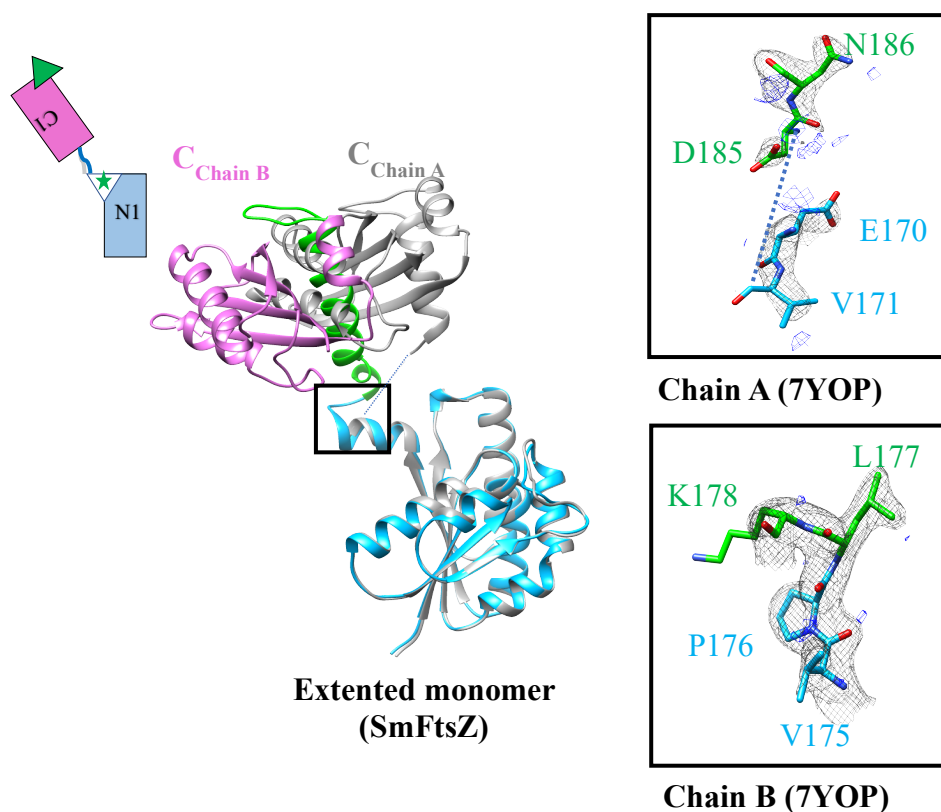


Figure 3.5 Extended monomer of SmFtsZ.

Relative orientations of the CTD in the extended conformation of each chain, fixing C-alpha of NTD of both the chains. Electron density map of the region shown in the inset where the two domains separate at T6 loop (map $F_o - F_c$ shown at 2.5σ , blue and $2F_o - F_c$ at 1.2σ , black).

3.3.4 Molecular details of SmFtsZ structures bound to different nucleotide states

One of the structures (SmFtsZ 1; PDB ID: 7YSZ) has GDP bound in the nucleotide pocket in both the molecules, GDP from the *E. coli* cell being bound to FtsZ during overexpression and purification of the protein (Figure 3.6A, B). In the second structure (SmFtsZ 2; PDB ID: 7YOP) where GMPPNP was added externally, GDP and GMPPNP were observed in the nucleotide-binding pockets of chain A and chain B, respectively (Figure 3.6C).

Despite the presence of a domain-swapped dimer in the crystal structure, the size exclusion chromatography coupled with multi angle light scattering (SEC-MALS) profile indicates predominantly a monomeric nature for SmFtsZ in solution at lower concentrations of the protein (Figure 3.6D). At a higher concentration of the protein, the SEC profile shows two peaks - one major peak corresponding to monomer and a minor peak at dimer (Figure 3.6E).

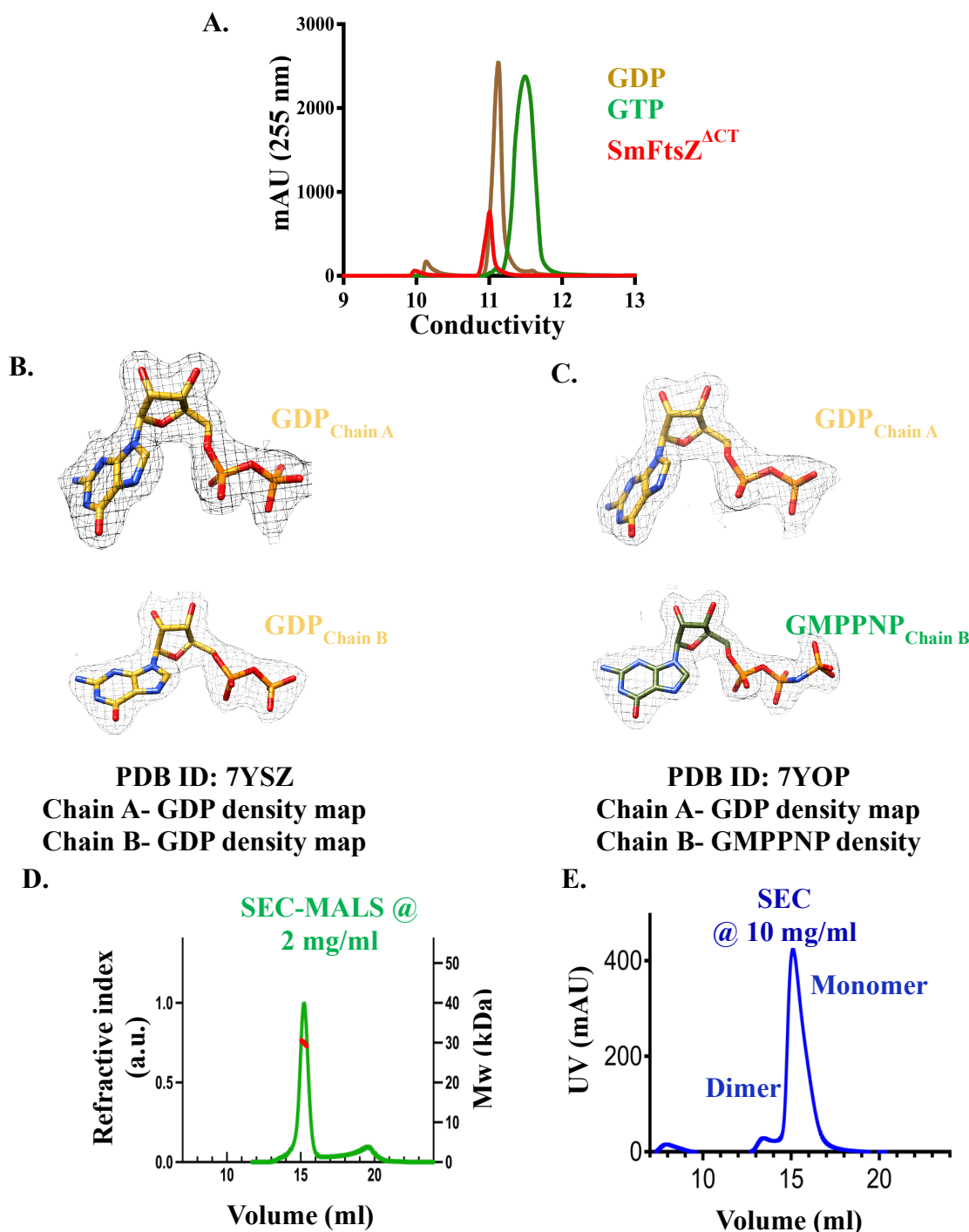


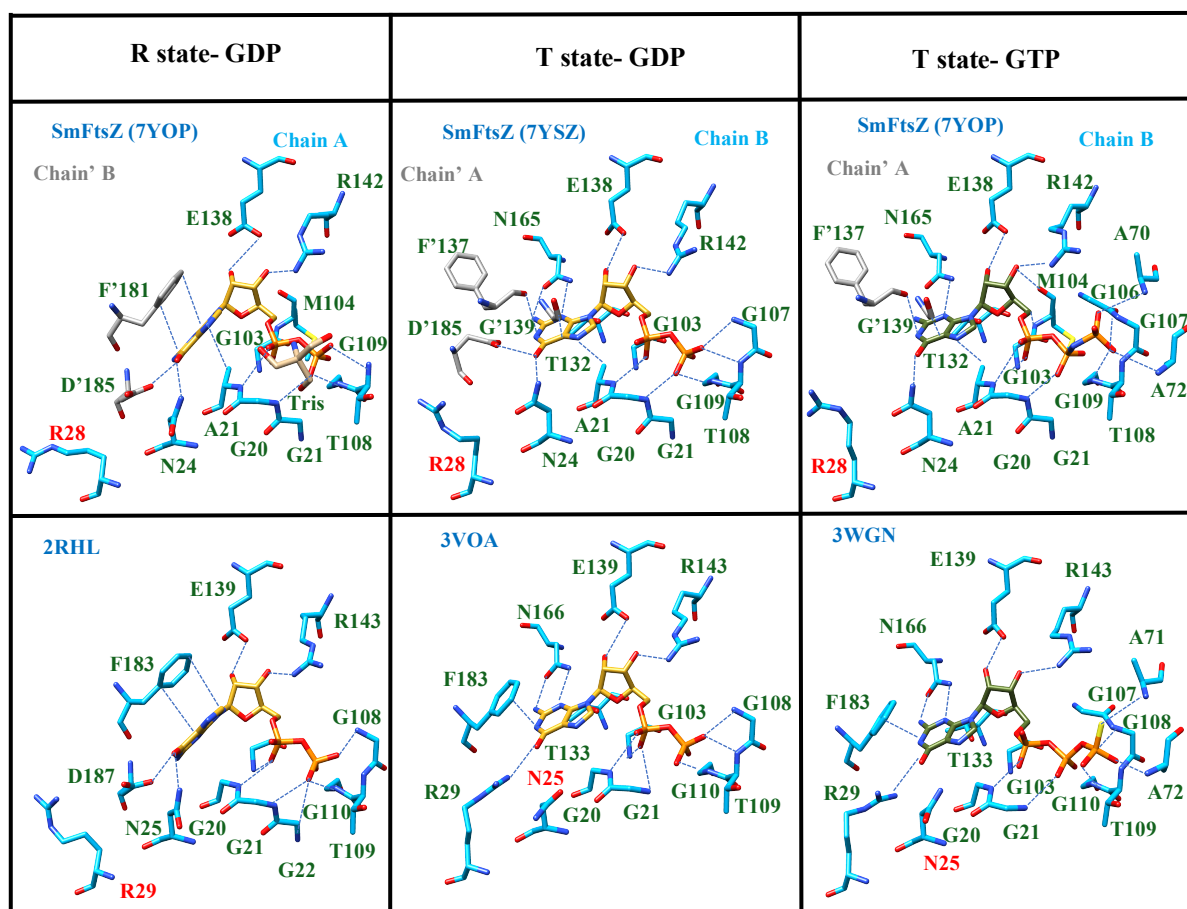
Figure 3.6 Density map for the nucleotides and oligomeric status of SmFtsZ

A) HPLC run to determine the nucleotide bound to the protein- SmFtsZ. 50 mmol of denatured SmFtsZ^{ACT} was run in DNAPAC PA 200 column along with 50 mmol of GTP and GDP, as shown in the plot. B) Density map showing GDP in chain A and chain B for PDB ID: 7YSZ. C) Density map showing GDP in chain A and GMPPNP in chain B for PDB ID: 7YOP (map $F_o - F_c$ shown at 2.5σ and $2F_o - F_c$ at 1.2σ). D) SEC-MALS profile for low concentration of SmFtsZ^{ACT} (2 mg/ml). E) SEC profile for high concentration of SmFtsZ^{ACT} (10 mg/ml). The column used for the runs is Superdex200 for D) and E).

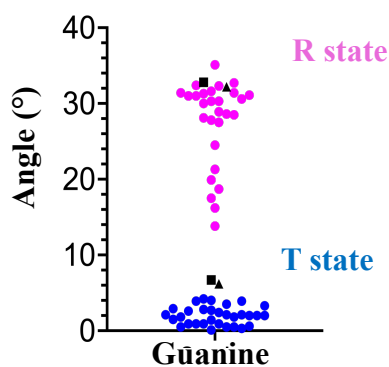
3.3.4.1 Nucleotide states in SmFtsZ

In order to investigate the structural details of the unique conformations of SmFtsZ, we compared the structures with R-state GDP bound (PDB ID: 2RHL), T-state GDP bound (PDB ID: 3VOA), and T-state GTP bound (PDB ID: 3WGN) conformations of FtsZ, respectively. In chain A of SmFtsZ 1 and SmFtsZ 2 (PDB ID: 7YSZ and 7YOP) structures, the orientation of the guanine ring of the nucleotide matches that of the R state nucleotide orientation, while in chain B of SmFtsZ 1 (GDP bound; 7YSZ) and SmFtsZ 2 (GMPPNP bound; 7YOP), the orientation of the nucleotide base is the same as that of a T state structure (Figure 3.7A, Table 3.5). The conformation of the nucleotide is held by both the chains of the domain swapped dimers (one chain is blue and the other chain is grey) in SmFtsZ. Hence, the structures captured conformations of GDP in both R and T state orientations and GMPPNP in the T state orientation, as observed from the distribution of angles between the plane of the guanine ring with respect to T state nucleotide orientation (Figure 3.7B). Interestingly, the domain swapped dimers exist as two globular FtsZ molecules, with the NTD of one chain packed against the CTD of the other (Figure 3.4). Superposition of the NTD with that of globular FtsZ structures and subsequent calculation of RMSD of the CTD shows that the relative orientations of NTD and CTD of the globular domain-swapped FtsZ subunit resembles that of an R-state conformation (Figure 3.7C). Hence, we conclude from the SmFtsZ structures that for chain B, the NTD is in T state nucleotide orientation whereas the relative orientation between NTD and CTD corresponds to the R state orientation, and hence we refer to it as an intermediate state.

A.



B.



C.

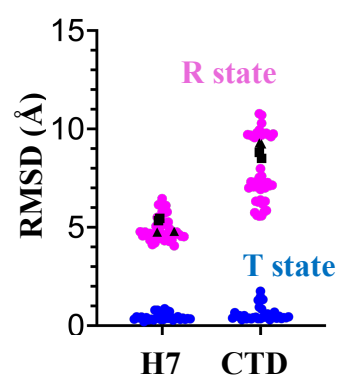


Figure 3.7 NTD and CTD orientation of SmFtsZ.

A) Nucleotide conformation of R state in GDP (2RHL), T state in GDP (3VOA), GTP (3WGN) along with that of SmFtsZ are shown (GDP- brown, GTP- green). B) Quantification of the guanine ring tilt angle keeping a T state structure (PDB ID- 5H5G) as reference. C) Quantification of the CTD movement, keeping T state structure (PDB ID- 5H5G) as reference. The pink cluster shows the structures in R state and the blue cluster shows the structures in T state. Triangle represents SmFtsZ 1 (7YOP) and square represents SmFtsZ 2 (7YSZ).

Table 3.5: Residues interacting with the nucleotide

	T state	T state	R state	R state	Chain A (7YOP)	Chain B (7YOP)	SmFtsZ (7YSZ)	SmFtsZ (7YSZ)
	GDP	GTP	GDP	GTP	GDP	GTP	GDP	GDP
Guanine								
	G22	G22			A21	A21	A21	A21
			N25	N25	N24	N24	N24	N24
	R29	R29						
						F137 (A) mc		F137 (A) mc
						G139 (A) mc		G139 (A) mc
	N166	N166				N165		N165
	F183	F183	F183	F183	F181 (B)		F181 (B)	
			D187	D187	D185 (B)		D185 (B)	D185 (A)
Ribose								
	G104	G104	G104	G104	G103	G103	G103	G103
					M104 (mc)	M104 (mc)	M104 (mc)	
						T132		
	E139	E139	E139	E139	E138	E138	E138	E138
	R143	R143	R143	R143	R142	R142	R142	R142
	N166	N166				N165		N165
Phosphate								
			G20	G20	G19	G19	G19	G19
	G21	G21	G21	G21	G20	G20	G20	G20
	G22	G22	G22	G22	A21	A21	A21	A21
		A71		A71		A70		
		A73		A73		A72		
		G107				G106		
	G108	G108	G108	G108		G107		G107
	T109	T109	T109	T109	T108	T108	T108	T108
	G110	G110	G110	G110	G109	G109	G109	G109
					Tris		Tris	

mc= main chain

3.3.4.2 Conformational difference between GDP versus GMPPNP states

To observe the conformational changes upon GMPPNP binding compared to that of GDP, the C-alpha of NTD of GDP (chain A, chain B of 7YSZ) and GMPPNP bound (chain B of 7YOP) structures were superposed. The T3 loop of FtsZ surrounds the gamma phosphate and is also involved in interface interaction (Yoshizawa *et al.*, 2020). We observed that when GDP is bound to SmFtsZ in the R state, the loop is ordered and has a water mediated interaction between carbonyl oxygen of Gly71 and with the beta phosphate of GDP in chain A of both the structures (Figure 3.8A,B). In chain B of the GDP bound T-state structure (PDB ID: 7YSZ), the loop is disordered and is only partially modelled due to poor electron density (Figure 3.8C). In chain B of the GMPPNP bound T-state structure (PDB ID: 7YOP), the T3 loop is partially ordered (Figure 3.8D). This suggests that the gamma phosphate indeed plays a role in stabilising T3 loop. We also observe a difference in the orientation of Y145 side chain in the R and T states. The Y145 residue is free to move when the loop is disordered in presence of T-state GDP and GMPPNP bound structures. Though the electron density indicated the presence of alternative conformations, the two states could not be modeled as alternative conformations during refinement. Hence, only one of the orientations has been modeled in the crystal structures (Figure 3.8).

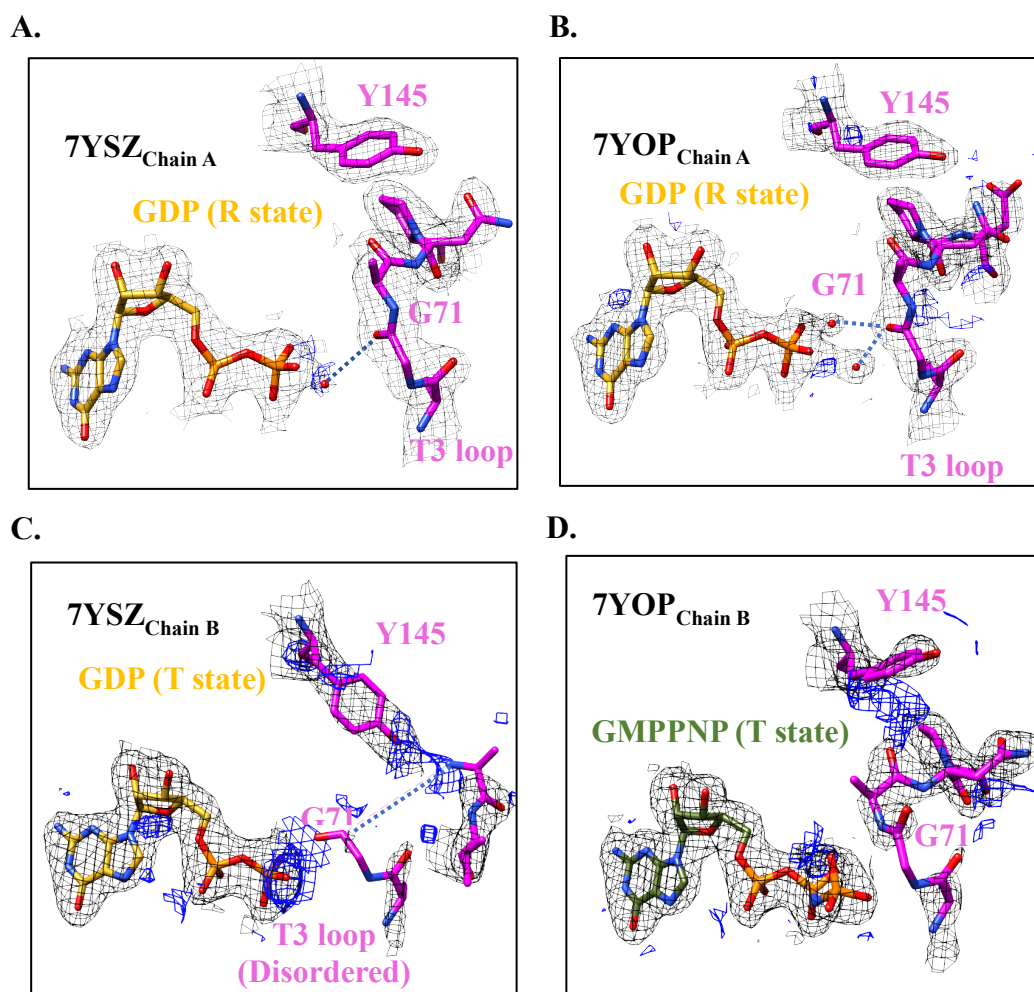


Figure 3.8 T3 loop orientation of SmFtsZ.

A), B) T3 loop orientation in chain A for PDB ID: 7YSZ and 7YOP. Both are in R state GDP bound state. C), D) T3 loop orientation of chain B of PDB ID: 7YSZ and 7YOP. PDB ID: 7YSZ, chain B is bound to GDP and chain B of PDB ID: 7YOP is bound to GMPPNP. Both are in T state nucleotide orientation (map $F_o - F_c$ shown at 2.5σ and $2F_o - F_c$ at 1.2σ).

In the GMPPNP-bound state (chain B of PDB ID: 7YOP), the peptide of Gly71 of the loop flips out of the pocket, (Figure 3.9A). The gamma phosphate possibly causes steric hindrance with the carbonyl oxygen of Gly71 facing towards the nucleotide pocket, and hence it is essential that the peptide flips to accommodate the gamma phosphate. In addition to flipping of the peptide of T3 loop upon GTP binding, the flipped conformation of the loop facilitates the interaction with the next subunit via T3 loop (Figure 3.9B) as seen in FtsZ filament. Based on these observations, we propose that the flipping of Gly71 is a trigger for GTP-induced polymerization of FtsZ.

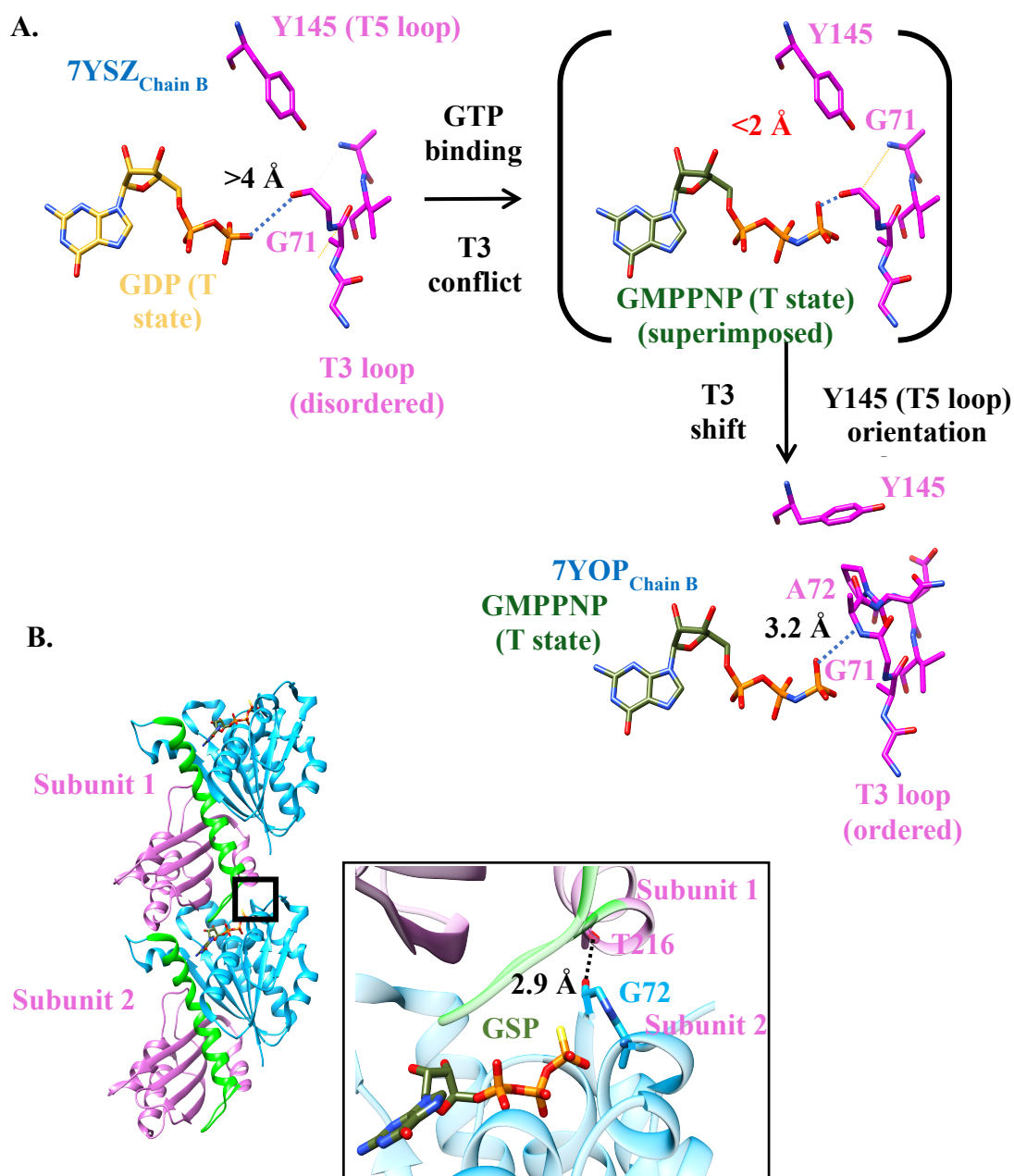


Figure 3.9 T3 loop flipping in presence of GMPPNP

A) Chain B of 7YSZ is GDP bound in R state, the T3 loop is disordered. When GMPPNP is present, the gamma phosphate clashes with Gly71 if it faces towards the nucleotide pocket. The Gly71 of T3 loop flips in order to accommodate the gamma phosphate of GMPPNP. B) Overall filament structure of SaFtsZ (PDB ID: 3WGN). The inset of filament interface shows the T3 loop interaction with the next subunit.

3.4 Discussion

The study described in this chapter shows that the structures of SmFtsZ are obtained in domain swapped dimer where the NTD is in T state nucleotide orientation and the CTD of one chain is packed against NTD of another chain in the R state conformation. Previously reported domain swapped structures are mutants in the polymerization interface. One is SaFtsZ^{F138A} mutant (PDB ID: 5MN5) where the mutation is at the upper polymerization interface and the domain swap is observed in the beginning of H7 helix of the protein. Another one is the TmFtsZ structure with T7 loop mutated (PDB ID; 1W5F). In the TmFtsZ structure, the mutation is at the lower interface and the domain swap is observed at the end of the H7 helix of FtsZ. This signifies that FtsZ molecule might go to an extended conformation when not stabilised by the next subunit.

Our crystal structures illustrate that upon binding to GTP, the peptide of the T3 loop flips in order to accommodate the third phosphate. This leads to the hydrogen bonding between the gamma phosphate and the N-H group of Gly71, and between the flipped carbonyl oxygen and the adjacent subunit in the filament (Figure 3.9B). The loop is disordered when GDP is present as there is no third phosphate to form hydrogen bonds with the N-H of Gly71 in the loop. This is in accordance with previous reports which state that GTP binding stabilises the T3 loop of FtsZ (Díaz *et al.*, 2001; Yoshizawa *et al.*, 2020; Ruiz *et al.*, 2022). The flipping of the peptide also favors the interaction with the next subunit as seen in filament structure of FtsZ from *S.aureus* as well as the DNP NMR study of GTP bound FtsZ filaments of *E.coli* (McCoy and McDermott, 2022). Our structures represent conformations of the T3 loop captured in the same crystal packing environment differing only in an ordered T3 conformation with a flipped Gly71 peptide. The observation of a flipped peptide state of the T3 loop in response to GMPPNP-binding demonstrates that GMPPNP (or GTP) binding stabilises the favourable conformation of T3 loop that assists in polymerization, despite the absence of the interacting subunit of the polymerised state.

Furthermore, it is observed from our structures that the NTD is in T state nucleotide orientation whereas the cleft remains in the R state orientation. This can be referred to as an intermediate state or T/R state. The capture of an intermediate state shows that GTP binding is sufficient for the transition of the NTD to a T state. In an FtsZ filament, the T state of NTD will further be stabilised by interactions with the adjacent subunit within the filament and is independent of the conformational changes in CTD, while in our crystal structure, interactions with the other molecule in the crystal packing stabilised the T state conformation of NTD. Thereby, we hypothesize that it could be possible for the T-state GTP bound NTD to

attach to the bottom interface, comprising of the CTD, of the FtsZ filament. This indeed imply that the bottom addition is more feasible than the top as seen in earlier reports (Du *et al.*, 2018).

It is possible that there is an inherent tendency for the NTD and CTD to go into an extended conformation if the addition of subsequent monomers do not stabilize the T-state conformation. The extended conformation is not stable and thereby interacts with another extended conformation to form a domain swap dimer. We observe the same from our crystal structures of wild-type SmFtsZ. Previous reports have indeed shown that the NTD and the CTD are independent of each other which can reconstitute non-covalently to form the functional FtsZ (Oliva *et al.*, 2004). The extended conformation of FtsZ might be an intermediate state during the transition from R to T state which requires the opening of the inter-domain cleft.

In the next chapter, we sought to study about the residues which are involved in the inter-domain cleft opening for R to T state transition.

3.5 References

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Chapter 4

Identification of an interdomain cleft residue in FtsZ important for R to T state transition

4.1 Introduction

The assembly dynamics of FtsZ has recently been attributed to treadmilling at the site of bacterial cell division, in which subunits add to the bottom, comprising the C-terminal domain (CTD) interface, and detach from the top, comprising the N-terminal domain (NTD) interface, of protofilaments (Du *et al.*, 2018). The molecular determinants of treadmilling, however, have not yet been understood mechanistically. An important step for the polymerization of FtsZ monomers is the opening of the inter domain cleft between the N-terminal domain (NTD) and the C-terminal domain (CTD). A sharp critical concentration (cc) in the polymerization of FtsZ indicates cooperative assembly (Mukherjee, 1998; Caplan and Erickson, 2003; González *et al.*, 2003). The cooperative assembly of FtsZ filaments is favored by a conformational switch of each monomer from its low affinity state (R-state; relaxed state favoured by monomers) to a high affinity state (T-state; tense state adopted by monomers within a filament) (Huecas *et al.*, 2008; Dajkovic *et al.*, 2008; Wagstaff *et al.*, 2017). In the R-state of FtsZ structure, the inter-domain cleft between the NTD and the CTD is closed whereas in the T-state, it is opened (Fujita *et al.*, 2017; Wagstaff *et al.*, 2017). The transition from R- to T-state within a monomeric subunit is thermodynamically less favorable as opening of the inter-domain cleft will require a positive free energy. Whereas, within protofilaments, the interactions between adjacent subunits provide the required energy for opening the cleft and favors R- to T- state transitions to form a protofilament (Corbin and Erickson, 2020).

In the previous chapters, we have shown the biochemical characterization and crystal structures of SmFtsZ with different nucleotide states. It is observed that SmFtsZ has a higher critical concentration and a tendency to form a domain swapped dimer probably during the transition from R- to T- state. In this chapter, we sought to look for the residues which determine the opening of the cleft that is crucial for the R- to T- state transition and in turn for FtsZ filament formation. We found out a residue which might play an important role in cleft opening, and performed biochemical and structural characterization of the cleft mutant. Our results provide a structural basis for the comparatively inefficient polymerization of SmFtsZ compared to that of *E. coli* and highlight a probable rate-limiting step in the transition from R to state in FtsZ.

4.2 Materials and methods

4.2.1 Sequence and structural analysis for residues involved in cleft interaction

In order to find out the mechanism involved in the opening and closing of the interdomain cleft, we selected the residues of the H7 helix from one of the structures of FtsZ (PDB ID: 5H5G) and listed the residues in the CTD which within 4 Å. The residues were selected from the structure using UCSF Chimera version 1.13.1 (Pettersen *et al.*, 2004). We performed multiple sequence alignment using Clustal Omega (Madeira *et al.*, 2022) for the aforementioned residues and checked for the conservation in other organisms.

4.2.2 Cloning and purification of the cleft mutant

The residue which was hypothesized to be involved in the cleft movement was mutated by site directed mutagenesis (SDM) in SmFtsZ full length as well as in SmFtsZ^{ΔCT}. The primers used for the cloning of the cleft mutant are listed below in Table 4.1.

Table 4.1: List of primers for generation of cleft mutant

Primer name	Sequence (5'-3')	Clone generated
SmFtsZ ^{F224M} _F	GGAACGCTTTAATGGGGATCGGTATTGG	SmFtsZ ^{F224M}
SmFtsZ_vR	CTTTTAATGATGATGATGATGATGGGATCC CCAACACTACGACGAACAAATG	
SmFtsZ ^{ΔCT} _R	GCTTTTAATGATGATGATGATGATGGGAT CCATCAAACCCAGTCGC	SmFtsZ ^{F224MΔCT}

The full-length as well as the truncated SmFtsZ cleft mutant genes were cloned into pHis17 vector (obtained from Lowe lab, MRC LMB, Cambridge; refer Addgene plasmid #78201 for vector backbone) between the restriction sites NdeI and BamHI using restriction free cloning (van den Ent and Löwe, 2006). The methylated parental plasmid was then digested with *DpnI*. The *DpnI*-digested product was transformed into NEB Turbo electro-competent cells through electroporation before being plated onto LB agar plates with ampicillin (100 g/ml) as a selection marker. The colonies were infected with 100 g/ml of ampicillin in LB broth, allowed to grow for 12 hours, and then the plasmid DNA was isolated using a QIAGEN miniprep kit. The plasmids were sent for sequencing for screening for the positive clones. After obtaining the constructs, the mutant was purified by affinity and size exclusion chromatography using the same protocol mentioned in chapter 2, section 2.2.2.

4.2.3 GTPase assay of the cleft mutant

The GTPase assay of the cleft mutant was performed using malachite green assay to measure the release of the inorganic phosphate (Feng *et al.*, 2011). The GTPase activity of the mutant was measured with different concentrations of the substrate which in this case is GTP. The concentration of GTP used is from 0 to 10 mM. The data was plotted using Michaelis-Menten equation and the kinetic parameters of the mutant were obtained. Similarly, the specific activity of the mutant was obtained using the saturated concentrations of GTP and compared with that of the wild type. We also calculated the critical concentration for the filament formation of the mutant using GTPase activity as described in chapter 2, section 2.2.3.3.

4.2.4 Crystallization and structure determination of the cleft mutant

10 mg/ml of the protein was used to set up crystallization by hanging drop vapor diffusion method using the same condition used for the wild type. The drops for without and with GMPPNP were set up (Table 4.2). The crystals diffracted to 2.4 Å and data was collected at ELETTRA, Trieste. The diffraction data obtained were indexed using XDS and scaled using AIMLESS present in the CCP4 package (Evans and Murshudov, 2013), (Potterton *et al.*, 2018). The molecular replacement was done using PHASER and the model building was done using COOT (McCoy *et al.*, 2007) (Emsley *et al.*, 2010). The model was refined using the PHENIX software package (Adams *et al.*, 2010). All analyses were done using UCSF Chimera version 1.13.1 (Pettersen *et al.*, 2004).

Table 4.2 Crystallization condition for SmFtsZ^{F224M}

	SmFtsZ ^{F224M}
Method	Hanging drop vapour diffusion
Plate type	24-well
Temperature (K)	291
Construct and protein concentration (mg/ml)	SmFtsZ ^{F224MACT} -10 mg/ml
Buffer composition of protein solution	50 mM HEPES pH 6.5, 50 mM KCl
Composition of reservoir solution	0.3 M sodium acetate, 0.1 M Tris pH 8.5, 15% w/v

	PEG 4000
Volume and ratio of drop	2.5 μ l: 2.5 μ l
Volume of reservoir (ml)	750 μ l

4.2.5 Pelleting assay and TEM for filaments of the cleft mutant

The protein of the cleft mutant was thawed and spun at 100,000 g at 4 °C for 15 minutes.

For pelleting assay, 10 μ M of the protein was taken from the supernatant to set up 50 μ l of reaction. The following reaction conditions were prepared- a) only protein, b) protein and MgCl₂, c) protein, MgCl₂ and GDP, and d) protein, MgCl₂ and GTP. A concentration of 3 mM of nucleotide and 5 mM of MgCl₂ was taken for the reaction. The reaction mixtures were spun at 100,000 xg at 30 °C for 15 minutes. The supernatant was taken out and the pellet was resuspended in buffer at the same amount as the initial reaction volume. The supernatant and pellet fractions were mixed with 2X SDS buffer and loaded onto 12% SDS-PAGE gel in equal amounts.

For preparation of TEM samples, a reaction of 20 μ l was set with a final protein concentration of 10 μ M, a GTP concentration of 3 mM and MgCl₂ concentration of 5 mM. The reaction mixture was incubated at 30 °C for 15 minutes. After which 5 μ l of sample was taken for grid preparation. The reaction mixture was again spun at 100,000 g at 30 °C for 15 minutes. The supernatant was taken out and pellet was resuspended with same volume of buffer as reaction. 5 μ l of resuspended pellet was taken for grid preparation. Carbon formvar grids (TED-PELLA, INC) were glow discharged with one round of 15 mA current for 25 seconds using plasma cleaner (Quorum Technologies). 5 μ l of the protein sample was added onto the glow discharged grid and incubated for 2 minutes before blotting out the protein with Whatman paper. Following this, 5 μ l of 2% uranyl acetate was added and incubated for 30 sec and blotted out. The grids were dried before imaging under TEM (JEM- 2200FS Joel Ltd.). The images of the filaments were analysed using ImageJ (Schneider *et al.*, 2012).

4.3 Results

4.3.1 Identification of cleft residue and purification of cleft mutant

A key step for the R- state to T- state transition, which will determine the critical concentration and kinetics of polymerization, is the relative rotation between NTD and CTD leading to the opening of the cleft between them. To identify the residues involved in cleft

opening, we listed the interacting residues for the cleft formation and analysed their conservation across FtsZ sequences from different organisms (Figure 4.2). The multiple sequence alignment of FtsZs indicated that FtsZ from *Spiroplasma* have phenylalanine (F224) in the S7 sheet located at the interdomain cleft. However, FtsZs from other organisms, such as *E. coli* or *S. aureus*, have methionine in the aforementioned position (Figure 4.1).

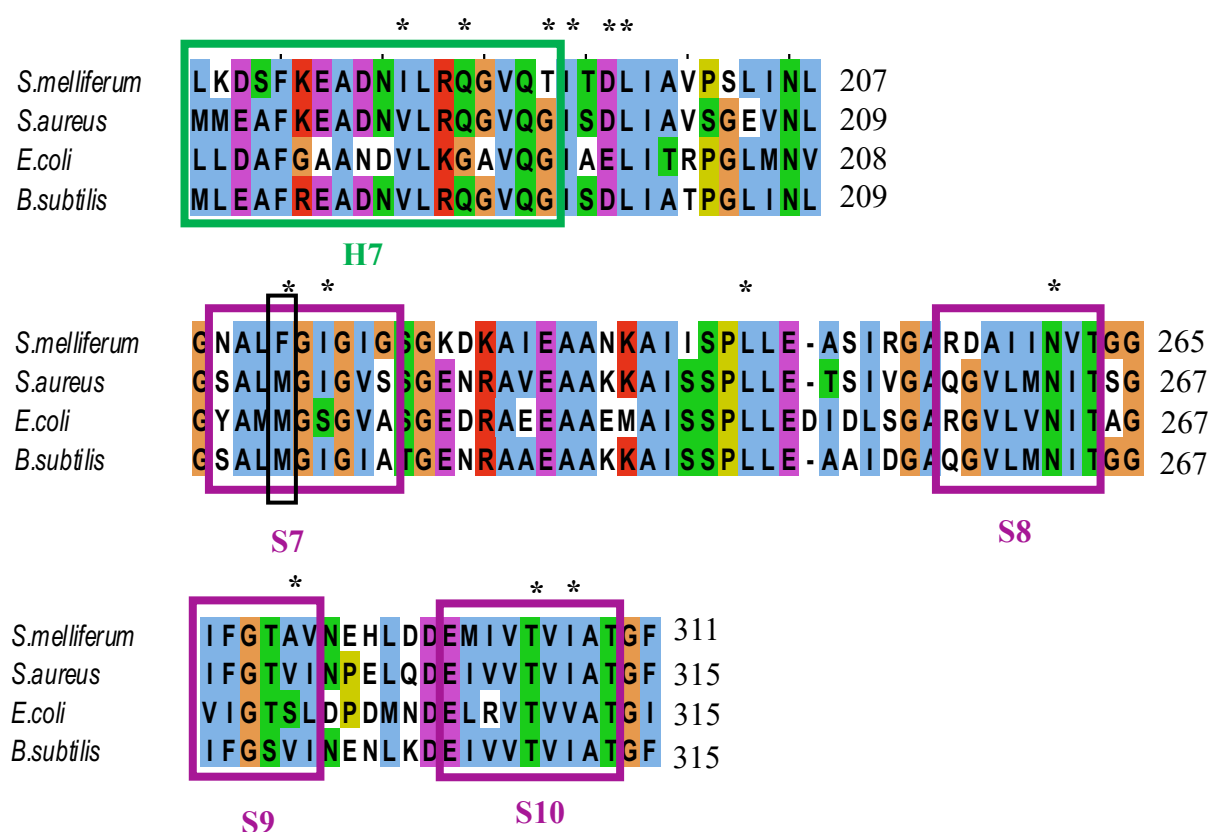


Figure 4.1 Residues of FtsZ involved in the cleft interaction

Multiple sequence alignment of FtsZs highlighting the interacting residues in the interdomain cleft of three organisms as representation- *S. melliferum*, *S. aureus*, *E. coli*, and *B. subtilis*.

There is movement of the H7 helix downwards when FtsZ transits from R to T state. Also, the S7, S8, S9, S10 move along with the H7 helix, due to which the methionine is seen to move in the structures from R to T state conformation (Figure 4.2).

We hypothesized that the flexibility of methionine might have a lubricating effect on the state transition (eg. EcFtsZ) whereas when phenylalanine, a rigid residue is present, the movement

might be restricted due to which the state transition is slower in the case of SmFtsZ. This might result in the higher CC and lower GTPase activity, as seen from our biochemical studies of wild type SmFtsZ.

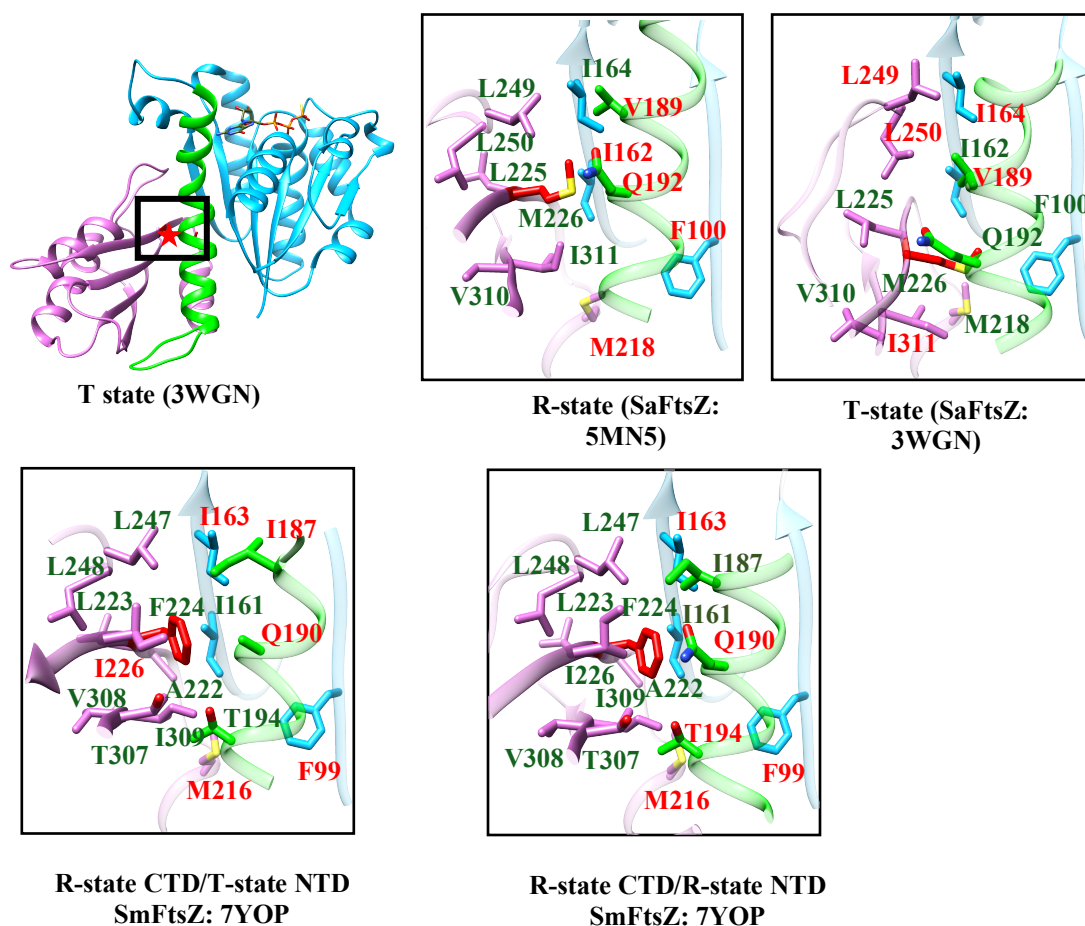


Figure 4.2 Movement of the flexible methionine in R vs T state.

Overall structure of SaFtsZ, the star highlights the position of the methionine, located at the pivot of the interdomain cleft. The insets show the position of M226 in SaFtsZ and SmFtsZ in S7 strand comparing R-state (SaFtsZ; PDB ID: 5MN5), T-state (SaFtsZ; PDB ID: 3WGN), chain A and chain B of SmFtsZ (PDB ID: 7YOP). The residues colored ingreen are interacting with Met/Phe and the residues in red are not interacting.

We mutated F224 in SmFtsZ to methionine in full length as well as the truncated background of SmFtsZ and purified the proteins (Figure 4.3A). Both the proteins came as monomer in the size exclusion chromatography with a minor peak corresponding to a dimer (Figure 4.3B).

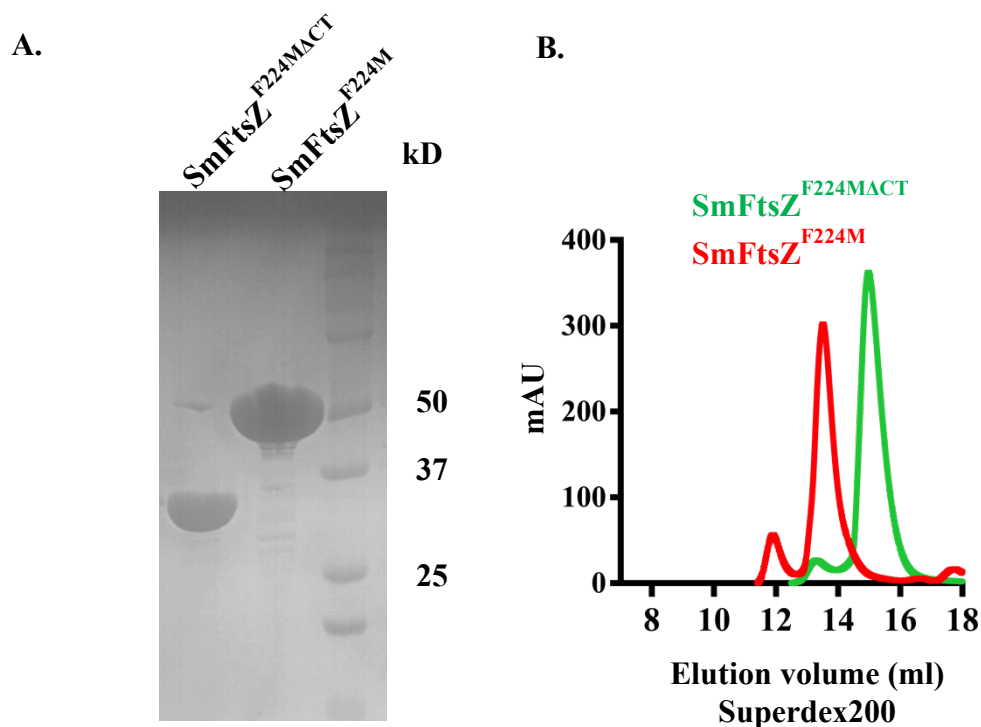


Figure 4.3 Purification of SmFtsZ^{F224MACT} and SmFtsZ^{F224M}.

A) 12% SDS gel representing final proteins of the interdomain cleft mutants- SmFtsZ^{F224MACT} and SmFtsZ^{F224M}. B) Gel filtration (Superdex200) profile of the interdomain cleft mutants.

4.3.2 GTPase activity of the cleft mutant compared to wild type

To check whether the mutation confers any change in the process of nucleation (by comparison of critical concentrations), GTPase assay with different concentrations of SmFtsZ^{F224M} was performed. It is observed that the k_{obs} was constant from protein concentration of 3 μ M for SmFtsZ^{F224M} (Figure 4.4A). This indicated that the critical concentration is lower in the mutant than that of the wild type. We observed from the Michaelis Menten plot that the catalytic efficiency of this mutant is five times higher (Figure 4.4B, Table 4.3) and the GTPase activity is three times higher than that of the wild type (Figure 4.4C, Table 4.4).

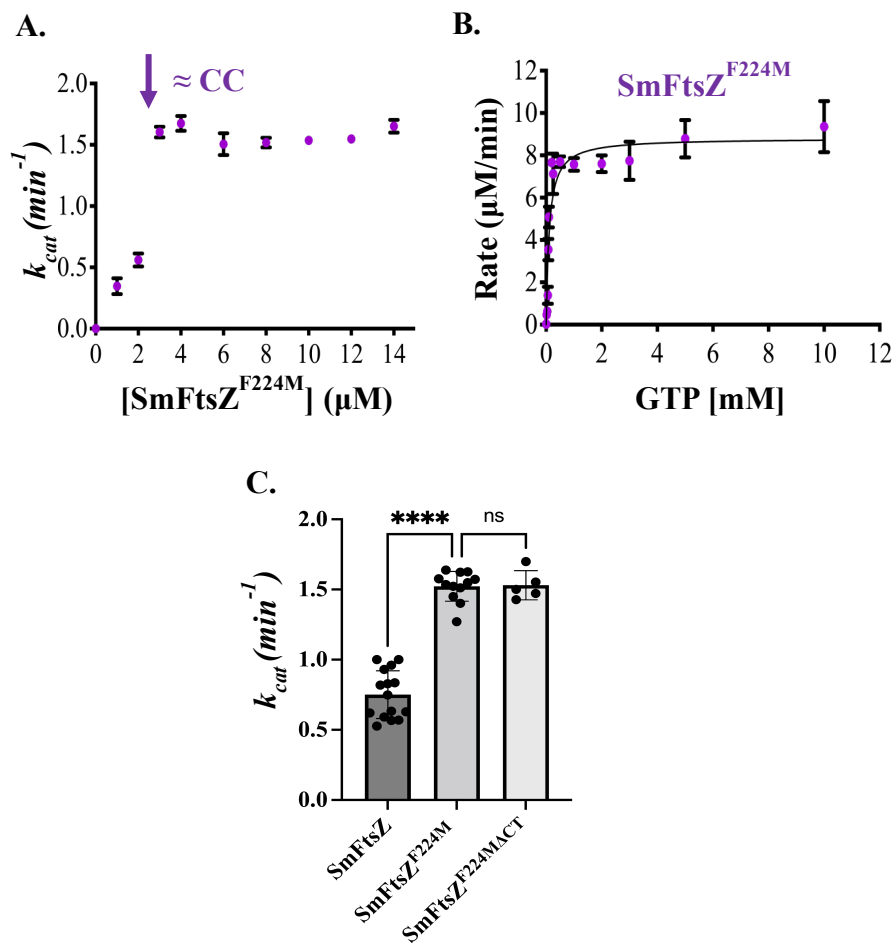


Figure 4.4 GTPase activity of the interdomain cleft mutant of SmFtsZ.

A) GTPase activity of $\text{SmFtsZ}^{\text{F224M}}$ with different protein concentrations ranging from 0 to 14 μM . B) GTPase activity of $\text{SmFtsZ}^{\text{F224M}}$ was measured with different concentrations of GTP ranging from 0 to 10,000 μM (pink). The curve was fitted with Michaelis Menten equation, $V_0 = V_{\text{max}} ([S]/([S] + K_M))$, [$N = 2$; $n = 3$]. C) GTPase activity comparison of $\text{SmFtsZ}^{\text{F224M}}$ mutant with the wild type construct ($\text{SmFtsZ}^{\text{F224M}}$ [$N = 2$; $n = 12$], $\text{SmFtsZ}^{\text{F224M}\Delta\text{CT}}$ [$N = 2$, $n = 5$] and SmFtsZ [$N = 3$; $n = 11$])

The cleft mutant, $\text{SmFtsZ}^{\text{F224M}}$, indicates that a flexible residue at position 224 (corresponding to SmFtsZ) in the inter domain cleft might be important for a quicker transition from R to T state.

Table 4.3 Kinetic parameters of the interdomain cleft mutant of SmFtsZ

Parameters	SmFtsZ	SmFtsZ ^{F224M}
E _t (μM)	10	5
V _{max} (μM/min)	8.78 ± 0.27	8.808 ± 0.34
k _{cat} (min ⁻¹)	0.87 ± 0.02	1.76 ± 0.068
K _M (μM)	234	108
Catalytic efficiency (k _{cat} / K _M) (min ⁻¹ μM ⁻¹)	0.0037	0.016
Approximate critical concentration (μM)	8	3

Table 4.4 *k_{obs}* comparison for wild type and interdomain cleft mutant of SmFtsZ

Constructs	<i>k_{obs}</i> (min ⁻¹)
SmFtsZ	0.58 ± 0.03
SmFtsZ ^{F224MACT}	1.74 ± 0.07
SmFtsZ ^{F224M}	1.52 ± 0.02

4.3.3 Structural features of the cleft mutant

Next, we went ahead to crystallize the truncated construct of the cleft mutant of SmFtsZ, SmFtsZ^{F224MACT}. Needle like crystals were obtained in the same condition without any nucleotide as obtained for the wild type. However, no crystals were obtained in presence of GMPPNP (a non-hydrolysable analog of GTP).

The mutant crystallized in the same space group with similar cell parameters as the wild type SmFtsZ. Molecular replacement was done with PDB ID: 7YSZ as template. The data collection and refinement statistics are given in Table 4.5.

Table 4.5 Data collection and refinement statistics of cleft mutant

PDB ID	8GRW
Collected at	ELETTRA, Trieste
Wavelength (Å)	1
Data collection Statistics	
Space Group	P4 ₁ 2 ₁ 2
a, b, c (Å)	106.68, 106.68, 128.78
α, β, γ (°)	90, 90, 90
Resolution (Å)*	106.67-2.4 (2.49-2.4)
Number of unique reflections*	29504 (3038)

R _{merge} (%) *	9.6 (68.4)
R _{pim} (%) *	5.7 (41.2)
CC _{half} *	0.998 (0.785)
Mean I/σI	15.1 (2.8)
Completeness (%) *	99.5 (99.3)
Redundancy *	7.0 (7.0)
Refinement Statistics	
Resolution (Å)	49.28- 2.4
Number of unique reflections (test set)	29477 (1503)
R _{work} / R _{free} (%)	0.192/0.236
Average B-factor (Å ²)	46
Wilson B-factor (Å ²)	38.7
RMS deviations	
Bond lengths (Å)	0.01
Bond angles (°)	0.972
Ramachandran map statistics	
Favoured (%)	97.92
Allowed (%)	2.08
Outliers (%)	0

*Values in parentheses denote last resolution shell

Upon superposing the NTD of the cleft mutant and the wild type structure of SmFtsZ, it is observed that the two molecules are arranged as domain swap dimers as we observed for the wild type SmFtsZ structures (Figure 4.5A). Both the molecules contain GDP in the nucleotide pocket (Figure 4.5B). The nucleotide conformation of chain A of PDB ID: 8GRW is same as R state and that of chain B is same as T state as calculated from the guanine tilt angle (Figure 4.5C). The CTD is in the R state conformation as seen in the wild type structures as calculated from the RMSD of CTD from the reference structure (chain A, PDB ID: 5H5G, T state) (Figure 4.5D). The methionine at position 224 interacts with fewer residues than what is seen with phenylalanine in the wild type structures of SmFtsZ (Figure 4.5E, F).

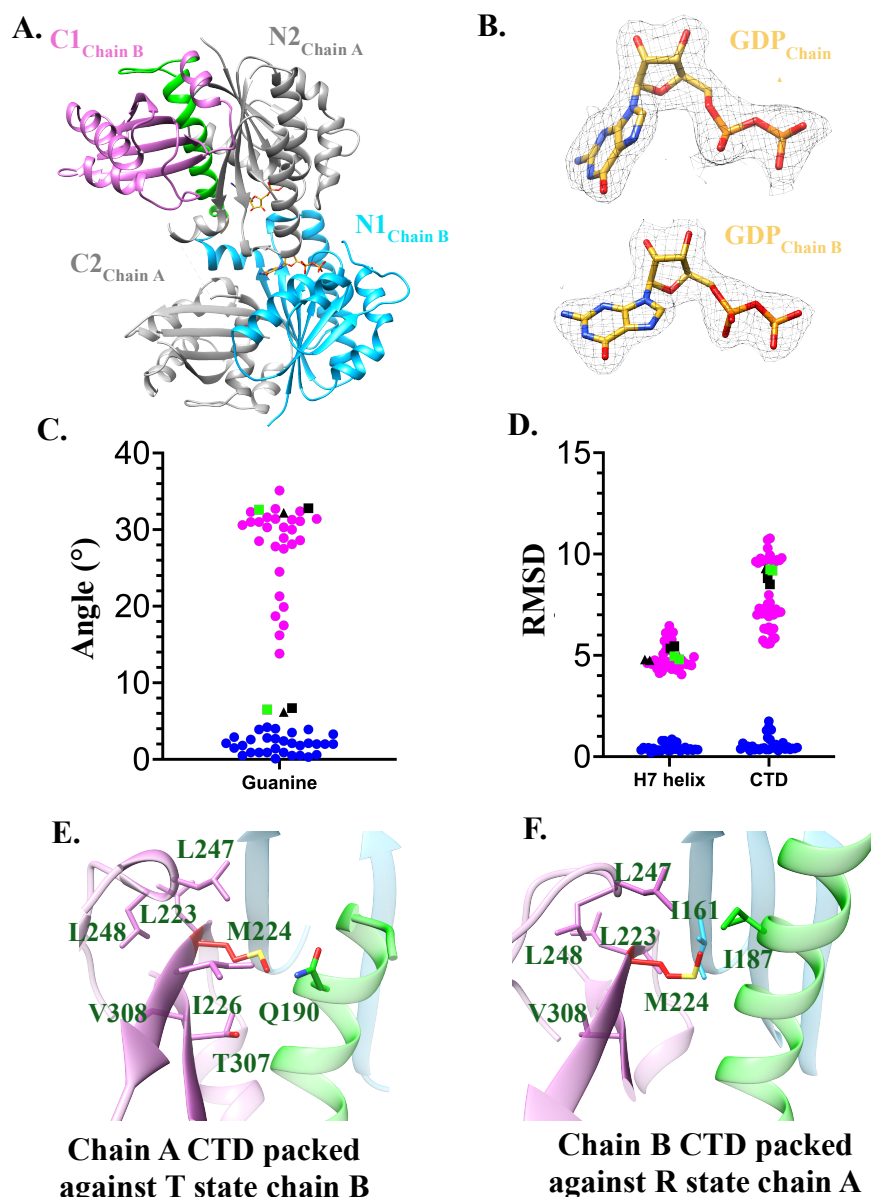


Figure 4.5 Structural features of the cleft mutant, SmFtsZ^{F224MACT}.

A) Domain swapped dimer of the cleft mutant (PDB ID: 8GRW), the respective colors of the chains are labelled in the figure. B) Electron density map for the nucleotide, (map $F_o - F_c$ shown at 2.5σ and $2F_o - F_c$ at 1.2σ). C), D) Guanine angle tilt for the nucleotide and RMSD for the CTD. Blue represents the T state structures, magenta represents the R state structures, black square represents SmFtsZ (7YOP), black triangle represents SmFtsZ (7YSZ) and green square represents the cleft mutant of SmFtsZ (8GRW). E) and F) shows the residues around F224M in 8GRW.

4.3.4 Pelleting assay and TEM of the cleft mutant of SmFtsZ

The pelleting assay was performed for the cleft mutant to know the condition in which it forms pellets which in turn signifies the condition in which it forms filaments. The 12 %

SDS-PAGE gel shows that the fraction with GTP has protein in both supernatant and pellet (Figure 4.6A). This shows that the cleft mutant forms filaments in presence of GTP.

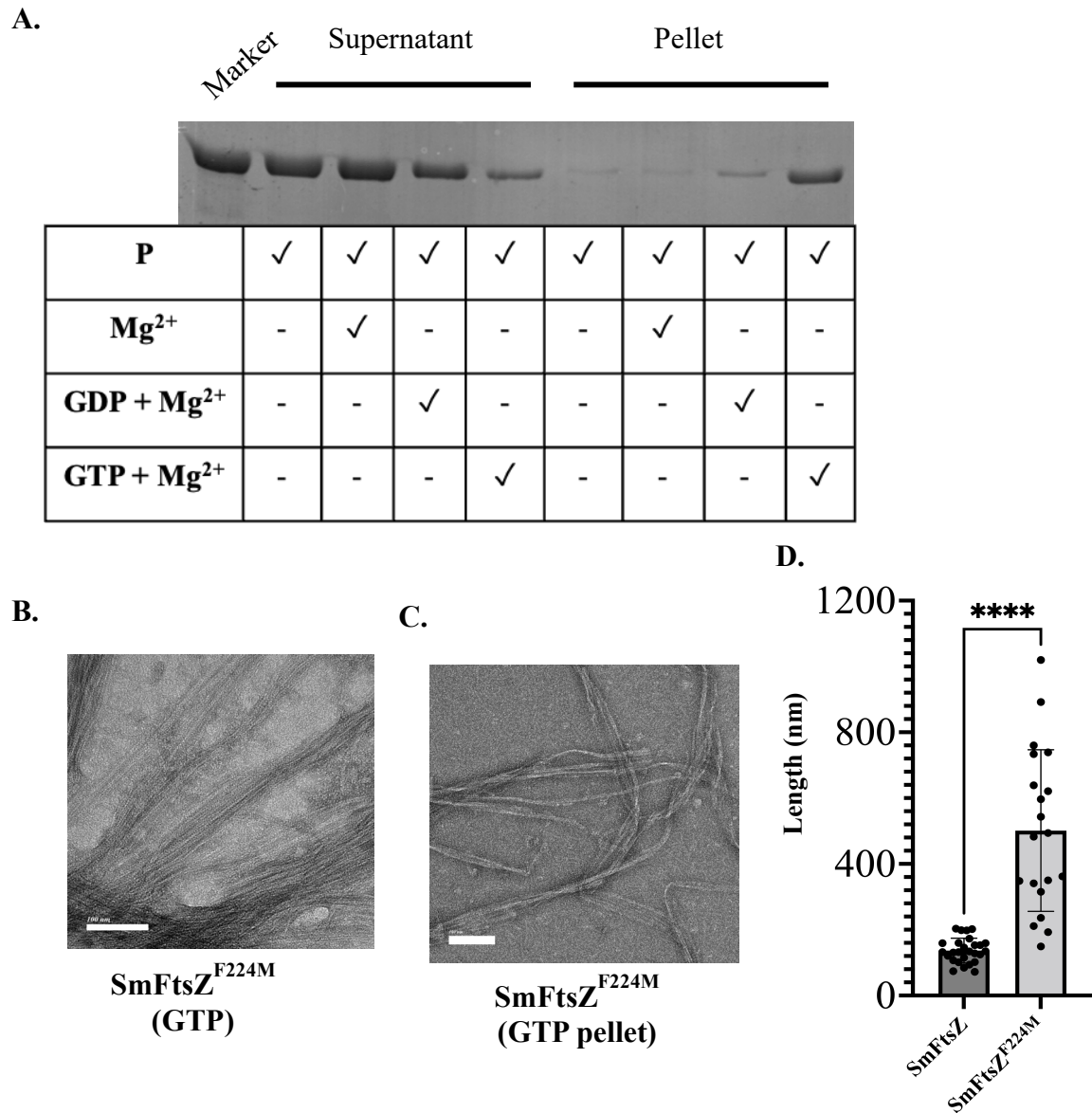


Figure 4.6 Pelleting assay and negative staining of filaments of SmFtsZ^{F224M}.

A) A 12 % SDS- PAGE gel for pelleting assay of SmFtsZ^{F224M} to determine the condition at which SmFtsZ forms filaments. B) Filaments of SmFtsZ^{F224M} with GTP, without 100,000 g spin. Scale 100 nm. C) Filaments of SmFtsZ^{F224M} with GTP, with 100,000 g, resuspending the pellet fraction. Scale 100 nm. D) Comparison of lengths of filaments of wild type SmFtsZ and SmFtsZ^{F224M}, under similar conditions. [SmFtsZ, $N = 2$, $n = 27$; SmFtsZ^{F224M}, $N = 2$, $n = 20$], N is number of grids, n represents number of filaments.

Upon observation of electron microscopy images of the filaments of the cleft mutant, it was seen that the average length of SmFtsZ^{F224M} filaments was higher than that of SmFtsZ (Figure 4.6B). We also imaged the filaments upon pelleting the protein with GTP and GMPPNP at 100,000 g spin (Figure 4.6C). We quantified the length of the filaments using ImageJ (Schneider *et al.*, 2012) for both SmFtsZ and SmFtsZ^{F224M} in presence of GTP (Figure 4.6D) and confirmed that the length of the filament of cleft mutant is longer than that of the wild type.

4.4 Discussion

In the previous chapter, we see that the FtsZ monomer can attach to the bottom interface of the filament via the NTD, which is in the T state, because the NTD transition from the R to the T state is independent of the CTD transition and GTP binding enhances subunit interactions. The top interface interactions might further cause the inter-domain cleft to open once the monomer attaches to the filament's base.

In this chapter, we discovered a residue in the interdomain cleft of SmFtsZ which is conserved. We hypothesised that this residue might be responsible for the lower GTPase activity of SmFtsZ as it is not conserved in SmFtsZ. We cloned and purified the mutant wherein the bulky residue Phe224 is replaced by methionine which is conserved and flexible at S7 sheet of FtsZ. We observed that the cleft mutant, SmFtsZ^{F224M} has higher activity than that of wild type. Also the mutant shows longer filaments as compared to wild type under TEM. We infer that methionine at position 224 of SMFtsZ helps in the opening of the cleft to ease the transition of R to T state. A flexible residue (Met224) in substitution of a bulky residue (Phe224) may act as a lubricant facilitating smoother transitions between states because it is located at the pivot where the interdomain cleft opens. We indeed see a higher GTPase activity and a lower critical concentration for the cleft mutant as compared to the wild type SmFtsZ. Therefore, the slower cleft opening in wild type SmFtsZ explains why it exhibits lower GTPase activity than EcFtsZ under identical *in vitro* conditions.

Although the structure of the cleft mutant was similar to that of the wild type structures, the methionine at position 224 in place of phenylalanine interacts with fewer residues than that of the latter. We speculate that the methionine confers more flexibility than that of phenylalanine located at the hinge of the cleft opening which is essential for the R to T transition and the opening of the cleft is rate limiting.

4.5 References

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Chapter 5

Mechanistic insights on binding of the drug PC190723 to FtsZ

5.1 Introduction

The spread of antibiotic resistance in bacteria has resulted in the urgent need for the development of new classes of antibiotics. The existing antibiotics that are widely in use target the cell wall synthesis, and DNA replication of the bacterial cell (Butler and Paterson, 2020). There are antimicrobials in the pipeline which have been developed for new targets to overcome the challenges of antibiotic resistance. One such target is the tubulin homolog of bacteria FtsZ, as it is an essential bacterial cell division protein (Bi and Lutkenhaus, 1991; Carro, 2019). The altered FtsZ dynamics leads to cell division defects in the bacterial cells and hence results in cell death.

Many drugs have been identified to target FtsZ in previous studies. There are three druggable regions in FtsZ protein which can bind to small molecules like alkaloids (berberine, sanguinarine), benzamides (PC190723, trisubstituted benzamidazols, guanidinomethyl biaryl compounds), arene diol digallates (UCM05, UCM44 and UCM64) (Haydon *et al.*, 2008; Kumar *et al.*, 2011; Kaul *et al.*, 2012). These regions correspond to the nucleotide binding domain, T7 loop and the interdomain cleft present between H7 helix and the C-terminal domain (Figure 5.1). But the binding of these drugs to FtsZs in different species could vary as some are susceptible and some are resistant. The most promising drug against FtsZ among the above mentioned is PC190723 (Haydon *et al.*, 2008). 3-Methoxybenzamide (3-MBA) was used as a starting point for a fragment based approach to select for molecules which have more antibacterial potency (Haydon *et al.*, 2008). 3-MBA was previously reported to have antimicrobial activity but with weaker potency against *Bacillus subtilis* (Haydon *et al.*, 2008). On analysing more than 500 analogs of benzamide, PC190723 was chosen as it was more potent than the rest. PC190723 is a 2,6-difluorinated 3-methoxybenzamide derivative.

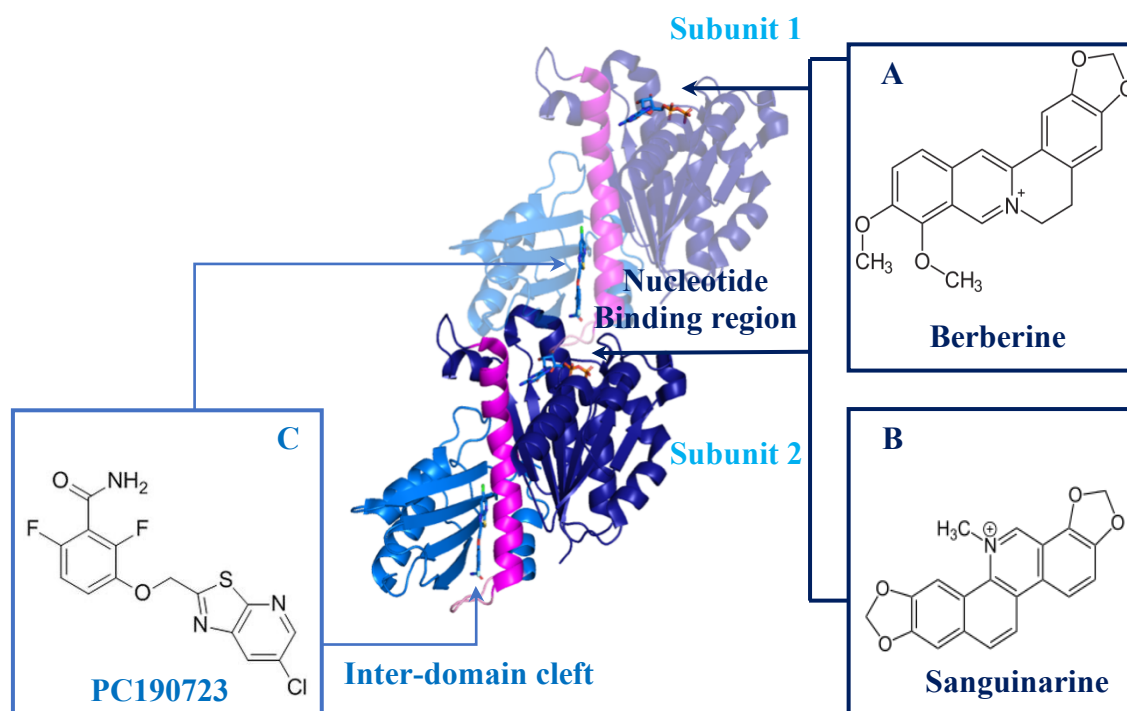


Figure 5.1. Druggable regions in FtsZ filament structure. A) Berberine binding site. B) Sanguinarine binding site. C) PC190723 binding site.

It is effective against all strains and species of *Staphylococcus* including the methicillin- and multi drug resistant *Staphylococcus aureus*. It has been proven to be efficacious in *in vivo* model of mice infected with lethal dose of *Staphylococcus aureus* (Haydon *et al.*, 2008). PC190723 is also shown to be effective against strains of *Bacillus*. According to the crystal structure of SaFtsZ bound to PC190723, the drug binds to the interdomain cleft of FtsZ which is in between the H7 helix and the C- terminal domain (CTD) (Matsui *et al.*, 2012). PC190723 stabilizes the filaments of FtsZ by preferentially binding to the T state of the protein, reduces the GTPase activity and decreases the critical concentration of filament formation (Andreu *et al.*, 2010; Matsui *et al.*, 2012; Fujita *et al.*, 2017). FtsZ tagged with GFP is shown to form a well-defined Z-ring in *B. subtilis*, but in presence of PC190723, the filaments are formed all over the cell. Also it disrupts the cell division, as the cells are filamentous in this case (Haydon *et al.*, 2008).

However, FtsZs from bacteria such as *E. coli*, *Streptococcus*, *Enterococcus* were shown to be resistant to PC190723. It has been shown that PC190723 does not induce condensation or bundling in EcFtsZ filaments (Haydon *et al.*, 2008; Kaul *et al.*, 2013). From these

observations, we were curious to know the mechanism of resistance of PC190723 to FtsZ. With this goal, we carried out sequence and structural analysis of various FtsZs which have been reported to be susceptible and resistant to the drug- PC190723. Based on the sequence and structural analysis of FtsZs from different organisms, we demonstrate that the presence of salt-bridge interactions between the central H7 helix and beta-sheet S10 and S7 in FtsZ protein mediate resistance to PC190723 in FtsZ.

5.2 Materials and methods

5.2.1 Sequence analysis for PC190723 binding to FtsZ

FtsZ sequences from various organisms were taken from UniProt (The UniProt Consortium, 2021). The sequences were aligned using Clustal Omega and represented using Jalview (Waterhouse *et al.*, 2009; Madeira *et al.*, 2022). A reference structure of FtsZ bound to PC190723 was taken from PDB (PDB ID: 4DXD). The residues which are within 4 Å from PC190723 were listed down using UCSF Chimera version 1.13.1 (Pettersen *et al.*, 2004). The listed residues were marked in the sequence alignment of FtsZs to identify the corresponding residues in other organisms.

5.2.2 Structural analysis for PC190723 binding to FtsZ

The reference structure (PDB ID: 4DXD) which was taken is in the T state. FtsZ structure with R state conformation (PDB ID: 5H5I) was chosen to check whether PC190723 can be accommodated in the inter-domain cleft region in the R state conformation. The C-alpha of NTDs of both the T state (PDB ID: 4DXD) and R state structures (PDB ID: 5H5I) were superposed and checked whether PC190723 can bind. Structures of FtsZ from *E. coli* and *S. melliferum* were modelled in T state using 4DXD as template in SWISS-MODEL (Waterhouse *et al.*, 2018). Similarly the C-alpha of the NTD of EcFtsZ (T state) structure was superposed with 4DXD, and checked whether the drug can be accommodated in the interdomain cleft. All the structural analyses were done using UCSF Chimera version 1.13.1 (Pettersen *et al.*, 2004).

The residues involved in salt bridge interactions seen in FtsZ from *E.coli* were marked in the sequence alignment using Clustal Omega (Madeira *et al.*, 2022) and represented by Jalview (Waterhouse *et al.*, 2009) in order to look for residues in other organisms. A table was made for different organisms listing the corresponding residues involved in salt bridge interactions in *E.coli* FtsZ as well as the residues which are conserved in PC190723 binding.

5.3 Results

5.3.1 Binding of PC190723 to FtsZ

Crystal structures of SaFtsZ with the drug PC190723 (PDB ID: 4DXD) show that the drug binds at the inter-domain cleft of FtsZ (Huecas *et al.*, 2021) (Figure 5.2A). The inter-domain cleft is open in the T state of the protein, thereby the drug can be accommodated in the cleft (Figure 5.2B). Upon superposing the C-alpha atoms of NTD of 4DXD (T state with PC190723) and 5H5I (R state structure), we observed that the drug clashes with residues of the inter-domain cleft in the R state of the protein (Figure 5.2C). This is because the inter-domain cleft is closed in the R state conformation of the protein and thereby it cannot accommodate PC190723 in the inter-domain cleft.

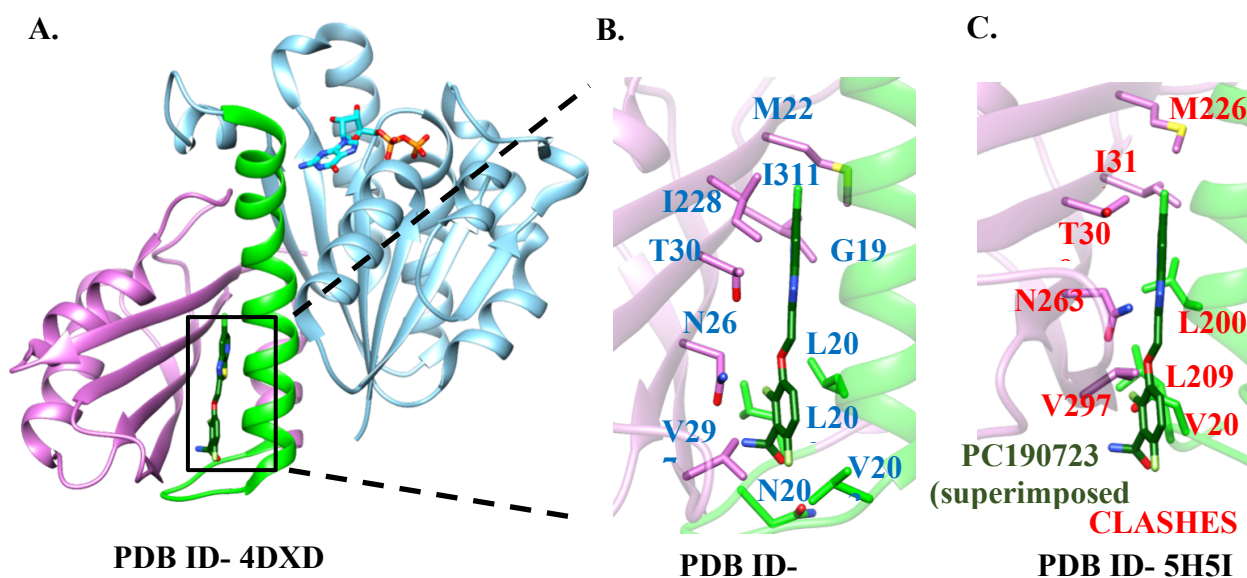


Figure 5.2 PC190723 binding to FtsZ. A) Overall structure of SaFtsZ bound to PC190723 (PDB ID- 4DXD; NTD- Light blue, H7 helix- green, CTD- pink). B) Zoomed-in image of PC190723 bound to T state structure of SaFtsZ (PDB ID- 4DXD). C) R state structure (PDB ID- 5H5I) superposed to 4DXD to check for PC190723 binding. The residues colored in red clash with the interdomain cleft.

These observations led to the hypothesis that PC190723 can bind only to the T state conformation of FtsZ where the inter-domain cleft is in an open conformation.

5.3.2 Conservation of residues in FtsZ for PC190723 binding

While analyzing the FtsZ structure bound to PC190723, we checked for residues in FtsZ which are involved in interactions with the drug. It is seen from the structure that the residues G196, L200, V203, N208, L209, M226, I228, N263, V297, T309 and I311 interact with the drug (Figure 5.2B). From the multiple sequence alignment of FtsZs from various organisms, it was observed that the aforementioned residues are conserved in *E. coli* (Figure 5.3). This means that the drug could potentially bind to EcFtsZ in the T state as the residues for binding are conserved.

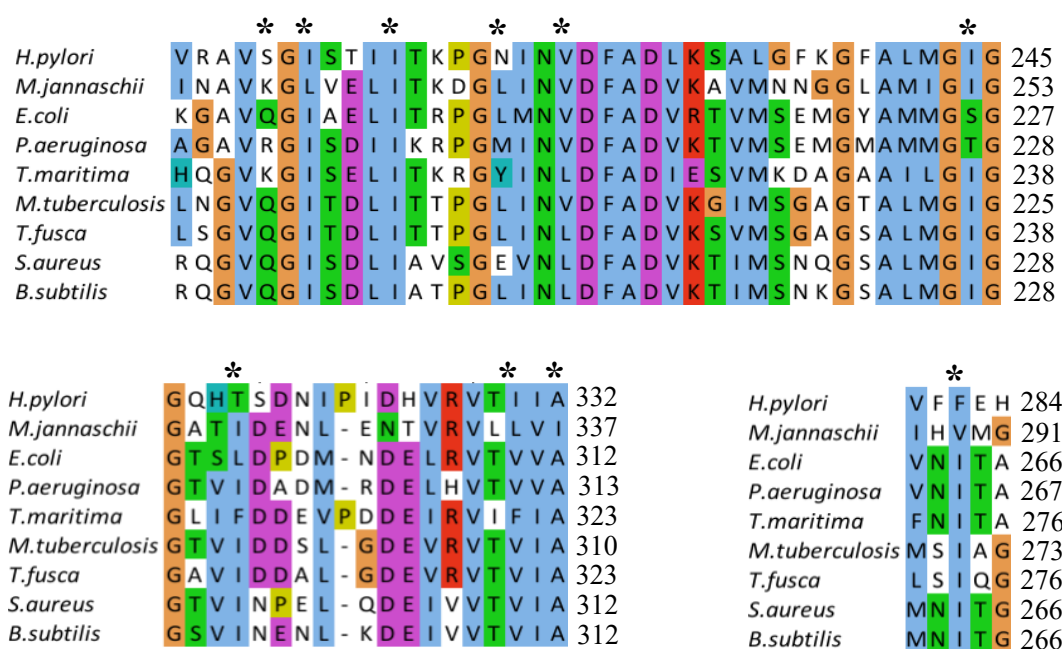


Figure 5.3 Conservation of PC190723 binding residues across FtsZs. Multiple sequence alignment of FtsZs showing residues which interact with PC190723, marked with stars.

5.3.3 Salt bridges across the interdomain cleft might render PC190723 resistance to some FtsZs

Previous reports have shown that PC190723 cannot bind to EcFtsZ (Haydon *et al.*, 2008; Kaul *et al.*, 2013). However, our sequence analysis suggested that the potential interacting residues are conserved. In order to probe this apparent discrepancy, a thorough sequence and structural analysis was done of FtsZs from susceptible and resistant species. The residues in the cleft neighbourhood in the EcFtsZ T-state model structure were identified. We found that there are two salt bridge interactions across the inter domain cleft in case of EcFtsZ (Figure 5.4A). It was observed that the two salt bridge interactions are present in EcFtsZ but not in

SaFtsZ or BsFtsZ from the multiple sequence alignment (Figure 5.4B). We hypothesize that these salt bridges might occlude the cleft for the drug binding.

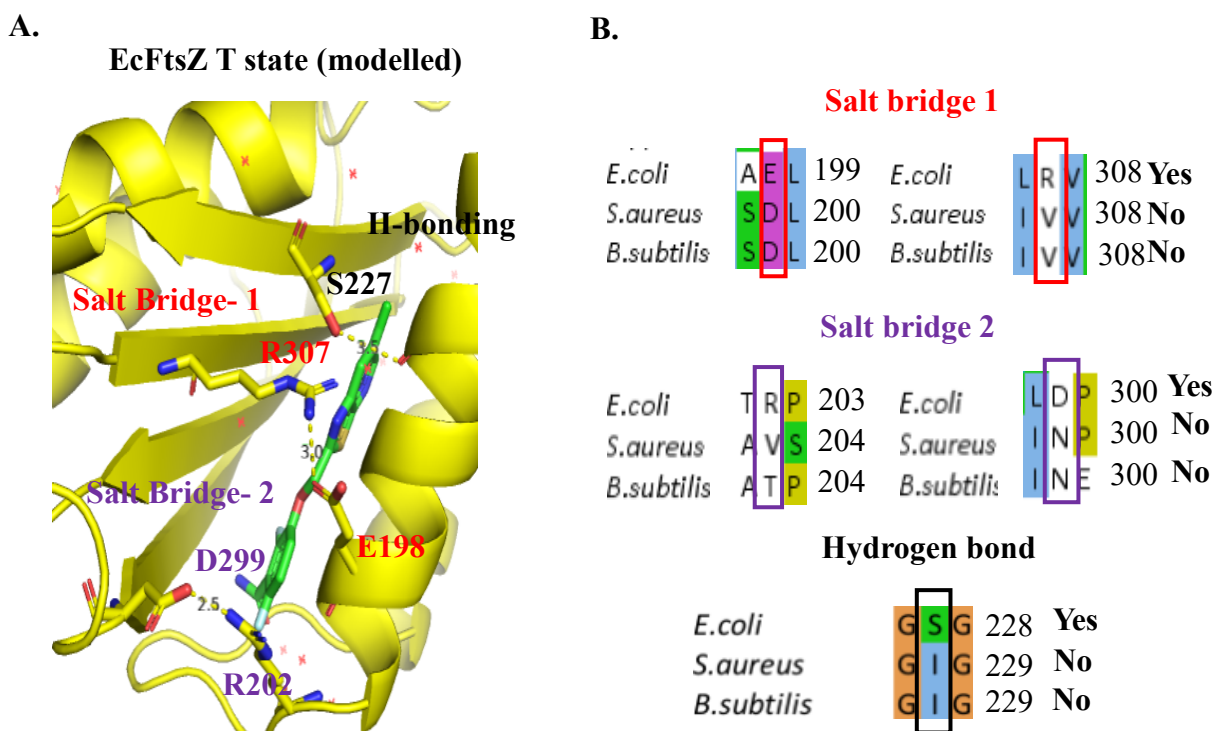


Figure 5.4 Salt bridge interactions occluding the cleft for PC190723 binding. A) FtsZ from *E.coli* is modelled in T state structure and the residues involved in forming salt bridges- R307 and E198; D299 and R202 and hydrogen bond between S227 and main chain of H7 helix residue are labeled around the interdomain cleft in red, purple and black, respectively. The dashed lines represent distances lower than 3.5 Å. B) Multiple sequence alignment of FtsZs from *E.coli*, *S.aureus* and *B.subtilis*. Residues involved in salt bridge formation are boxed.

5.3.4 Prediction for organisms whether they are PC190723 resistant or susceptible

The table with the organisms and their corresponding residues in their FtsZs show that the organisms whose FtsZs do not have salt bridge interactions around the interdomain cleft as well as have conserved binding residues are susceptible to the drug. Among the organisms included in our analysis (Table 5.1), *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdunensi*, *Staphylococcus saprophyticus*, *Staphylococcus warneri*, *Bacillus subtilis*, *Bacillus cereus* were previously experimentally reported to be susceptible to the drug (Haydon *et al.*, 2008). Earlier experiments show that organisms like *Escherichia coli*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus*

faecium, *Streptococcus agalactiae* are resistant to the drug (Kaul *et al.*, 2013). We indeed observe in our analysis that these organisms have FtsZs that have salt bridge interaction(s) around the inter domain cleft. We have also listed some other pathogens in our analysis which have not been reported to be resistant or susceptible to this drug. We have marked them susceptible or resistant based on our analysis of the FtsZ sequences (Table 5.1).

Table 5.1 Prediction of organisms resistant or susceptible to PC190723 based on FtsZ sequence analysis

Organisms	Salt bridge 1		Salt bridge 2		H-bond	Bridges	At G196	At M224	Resistant/Susceptible	Report
<i>Staphylococcus aureus</i>	D199	V307	N299	V203	I228	0	G196	M226	Susceptible	Yes
<i>Staphylococcus epidermidis</i>	D199	V307	N299	V203	I228	0	G196	M226	Susceptible	Yes
<i>Staphylococcus haemolyticus</i>	D199	V307	N299	V203	I228	0	G196	M226	Susceptible	Yes
<i>Staphylococcus hominis</i>	D199	V307	N299	V203	I228	0	G196	M226	Susceptible	Yes
<i>Staphylococcus lugdunensi</i>	D199	V307	N299	V203	I228	0	G196	M226	Susceptible	Yes
<i>Staphylococcus saprophyticus</i>	D199	V307	N299	V203	I228	0	G196	M226	Susceptible	Yes
<i>Staphylococcus warneri</i>	D199	V307	N299	V203	I228	0	G196	M226	Susceptible	Yes
<i>Bacillus cereus</i>	D199	V307	N299	T203	I228	0	G196	M226	Susceptible	Yes
<i>Bacillus subtilis</i>	D199	V307	N299	T203	I228	0	G196	M226	Susceptible	Yes
<i>Escherichia coli</i>	E198	R307	D299	R202	S227	3	G195	M225	Resistant	Yes
<i>Haemophilus influenzae</i>	D223	R335	V327	S227	F252	1	G220	I250	Resistant	Yes
<i>Pseudomonas aeruginosa</i>	D199	H308	D300	R203	T228	2	G196	M226	Resistant	Yes
<i>Streptococcus pneumoniae</i>	D200	R308	D300	N204	I229	1	G197	M227	Resistant	Yes

<i>Streptococcus pyogenes</i>	D200	R308	D300	S204	I229	1	G197	M227	Resistant	Yes
<i>Enterococcus faecium</i>	D200	R308	N300	A204	I229	1	G197	M227	Resistant	Yes
<i>Streptococcus agalactiae</i>	D200	R308	D300	N204	I229	1	G197	M227	Resistant	Yes
<i>Klebsiella pneumoniae</i>	E198	R307	D299	R202	S227	3	G195	M225	Predicted to be resistant	-
<i>Acinetobacter baumannii</i>	D204	R313	D306	N204	A232	1	S201	M231	Predicted to be resistant	-
<i>Enterobacter spp.</i>	E198	R307	D299	R202	S227	3	G195	M225	Predicted to be resistant	-
<i>Campylobacter jejuni</i>	S201	E311	D305	D206	V231	0	G199	M229	Predicted to be resistant	-
<i>Spiroplasma melliferum</i>	D197	I305	N297	V201	I226	0	T194	F224	Predicted to be resistant	-
<i>Spiroplasma citri</i>	D197	I305	N297	V201	I226	0	T194	F224	Predicted to be resistant	-
<i>Spiroplasma apis</i>	D197	I305	N297	V201	I226	0	T194	F224	Predicted to be resistant	-
<i>Clostridium innocuum</i>	E240	I348	D299	I205	V270	0	A236	I267	Predicted to be resistant	-
<i>Mycobacterium tuberculosis</i>	D196	R304	D296	T200	I225	1	G193	M223	Predicted to be resistant	-
<i>Mycobacterium tuberculosis</i>	D196	R304	D296	T200	I225	1	G193	M223	Predicted to be resistant	

<i>Salmonella enterica</i>	E198	R307	D299	R202	S227	3	G195	M225	Predicted to be resistant	-
<i>Methanococcus jannaschii</i>	E225	R332	D324	K229	I253	2	G222	I251	Predicted to be resistant	-
<i>Thermotoga maritima</i>	E209	R317	D309	K213	I238	2	G206	L236	Predicted to be resistant	-
<i>Helicobacter pylori</i>	T216	R326	S317	K220	I245	0	G213	M240	Predicted to be resistant	-
<i>Caulobacter vibrioides</i>	D202	R311	D303	L207	T231	2	S199	M229	Predicted to be resistant	-

5.3.5 Prediction for the effect of PC190723 on Spiroplasmas

Even though the majority of Spiroplasma species that have been studied so far have been found to be harmless commensals, some have developed pathogenicity towards their arthropod or plant hosts. The majority of pathogenic Spiroplasma species are members of the Citri-Chrysopicola-Mirum clade (Regassa and Gasparich, 2006). Notable examples include *S. kunkelii*, which causes Corn Stunt Disease (Whitcomb *et al.*, 1986), *S. phoeniceum* which infects periwinkle (Saillard *et al.*, 1987), *S. penaei* which infects Pacific White Shrimp, *S. eriocheiris* which infects Chinese mitten crab (Wang *et al.*, 2011), *S. citri* which causes Citrus Stubborn Disease (Saglio *et al.*, 1973), and *S. melliferum* which infects honeybee (Clark *et al.*, 1985).

The FtsZs in Spiroplasmas have sequence identity of more than 90 % in the core domain. We checked sequences of Spiroplasmas to check whether it can bind to PC190723. It is seen from the multiple sequence alignment that Spiroplasmas have two residues in the interdomain cleft- F224 and T194 which can potentially clash with PC190723 (Figure 5.5A, B). The G196 is a conserved residue (T194 in Spiroplasmas) and it has been shown in previous studies that when G196 is mutated to alanine in SaFtsZ/BsFtsZ, the protein becomes resistant to the inhibitor. (Haydon *et al.*, 2008; Sharma *et al.*, 2022).



Figure 5.5 Analysis for PC190723 binding to SmFtsZ. A) T-state SaFtsZ structure bound to PC190723 (green) superposed to the T-state model of SmFtsZ. The residues of SmFtsZ clashing with PC190723 are highlighted in red. B) Multiple sequence alignment of FtsZ from different organisms, showing the conservation of the residues highlighted in red in A.

5.4 Discussion

FtsZ is an essential protein in the bacterial cell division (Hale and de Boer, 1997). Altering the activity of this protein would inhibit the multiplication of bacteria and thereby it is a promising target for developing anti-microbial drugs. Many small molecules have been identified to target the FtsZ (Carro, 2019). Among those, PC190723 is the most potent till date because it has been shown to cure mice models infected with lethal dose of *Staphylococcus aureus* (Haydon *et al.*, 2008). This drug binds to the inter-domain cleft of FtsZ and stabilizes the filament state of the protein (Andreu *et al.*, 2010; Tan *et al.*, 2012). Thereby, PC190723 has been one of the most promising drugs against FtsZ and remains the only drug for which the binding site in FtsZ has been clearly established. While this drug is effective against species like *Staphylococcus aureus* and *Bacillus subtilis*, it has been shown to be ineffective against species like *E.coli*, *Enterococcus*. However, PC190723 predominantly affects Gram positive bacteria and the Gram negative bacteria have been shown or predicted to be resistant (Haydon *et al.*, 2008). The outer membrane in *E. coli* or Gram negative bacteria has been shown to be a permeability barrier for PC190723 (Khare *et al.*, 2019; Chai *et al.*, 2020).

From all these observations, we wanted to decipher the sequence determinants of resistance or susceptibility of PC190723 to FtsZ (Kaul *et al.*, 2013) and to indeed confirm if the resistance arises due to differences in specificity to FtsZ binding or due to other factors such as drug permeability into the bacterium. The sequence and structural analyses presented here indicate that the drug PC190723 binds to the T state conformation of the protein wherein the interdomain cleft is opened to accommodate the drug. Based on the analysis, we predicted that organisms whose FtsZs have the salt bridge interactions between the beta sheets (S7 and S10) and H7 helix (in case of *E. coli*) occlude the cleft for the binding of the drug. We correlated our hypothesis with the existing literature and listed the organisms which are susceptible and resistant to the drug (Haydon *et al.*, 2008; Stokes *et al.*, 2013; Kaul *et al.*, 2013) (Table 5.1). While host factors such as outer membrane permeability and efflux pumps might be contributing factors to resistance in Gram negative bacteria, further work is necessary to fully elucidate the action of PC190723 and its influence on Z-ring assembly dynamics. Nonetheless, it might be possible to target FtsZ in Gram negative bacteria which are predicted to be susceptible to PC190723 according to the FtsZ sequence, by increasing membrane permeability for the drug PC190723 to get inside the cell.

5.5 References

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Chapter 6

Conclusion and future prospects

6.1 Chapter-wise summary

The cell wall synthesis in bacteria is also thought to be the primary force generator for constriction and the rate-limiting process in the cytokinesis or septum closure (Coltharp *et al.*, 2016; Bisson-Filho *et al.*, 2017) which raises the question of how cell division could take place in bacteria that lack cell wall and cell wall synthesis machinery. Thus, FtsZ from a cell-wall less bacterium which works independent of cell wall synthesis can serve as a model to study fundamental features of FtsZ in ring constriction and cell division. In summary, the thesis provides the structural basis for GTP-dependent polymerization to one end of the single protofilament, and hence for the kinetic polarity exhibited by FtsZ. The results also describe the mechanistic insights into the inhibitor, PC190723, binding to FtsZ. Discussed below are the major conclusions obtained from the work and experiments that can be performed in the future.

Following the introduction to FtsZ and the current review of literature in the field in Chapter 1, chapter 2 describes the biochemical comparison of FtsZs from an organism devoid of cell wall synthesis machinery *S. melliferum* (SmFtsZ) and cell walled organism *E. coli* (EcFtsZ). It is observed that SmFtsZ has lower GTPase activity and higher critical concentration of polymerization than that of EcFtsZ under similar *in vitro* conditions. SmFtsZ formed filaments in the presence of GTP, similar to other characterized FtsZs from cell walled organisms. We observe that features like GTP binding, hydrolysis and filament formation is conserved for FtsZ from organism devoid of cell wall.

Chapter 3 describes the structures of available FtsZs and that of SmFtsZ. On determining the structures of SmFtsZ with different nucleotide states, it is observed that the nucleotide orientation in the NTD is in T-state, whereas the interdomain cleft between NTD and CTD is in the R state. Thereby, we classify the structures we obtained as an intermediate state or T/R state. The interactions between the adjacent subunit and the filament will further stabilise the T state of NTD in FtsZ filament, which could occur independent of CTD conformation. The T state conformation of NTD in our crystal structure was stabilised by interactions with the other molecule in the crystal packing. We propose that it might be possible for FtsZ to convert to an extended conformation if addition of subsequent monomers do not stabilize the T-state conformation. The extended conformation is not stable and thereby interacts with

another extended conformation to form a domain swap dimer, as observed in crystal structures of wild type SmFtsZ and non-polymerizing mutants of other FtsZs too.

Our crystal structures of SmFtsZ (PDB ID: 7YSZ and 7YOP) demonstrate how the carbonyl group of the Gly71 of T3 loop flips to make room for the third phosphate when it binds to GTP. According to the observation of a flipped peptide state of the T3 loop in response to GMPPNP binding, GMPPNP (or GTP) binding stabilises the T3 loop's conformation, which aids in polymerization. We suggest that this crucial step determines whether a monomer is capable of polymerizing in the presence of GTP rather than GDP.

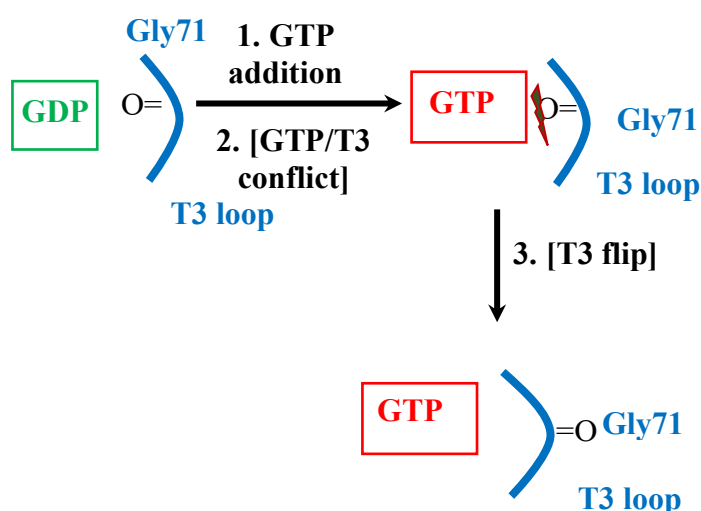


Figure 6.1 T3 loop conformation change.

Schematic of GDP versus GTP binding in the nucleotide pocket of FtsZ. The peptide of Gly71 in T3 loop has to flip out of the nucleotide pocket in order to accommodate the gamma phosphate.

The activation of tubulin for microtubule assembly by GTP binding was observed in tubulin structures similar to what we observe for FtsZ (Knossow *et al.*, 2020). Upon GTP binding to tubulin, there is a conformational change in the T3 loop in order to accommodate the gamma phosphate. The T3 loop movement destabilises the T5 loop, which causes the T3-T5 conflict and results in a conformational change in T5 loop. This conformation of the T5 loop interacts with the incoming heterodimer (Nawrotek *et al.*, 2011). Therefore, both FtsZ and tubulin share the property of conformational modifications following GTP binding and interaction with the next subunit in the equivalent regions of the tubulin fold. As the NTD transition

from R to T state is independent of the CTD transition and GTP binding facilitates subunit interactions, GTP-bound FtsZ monomers could, in principle, attach to the bottom interface of the filament via the NTD which is in T state. Upon attaching to the bottom of the filament, the top interface interactions could further trigger the opening of the inter-domain cleft. We propose this as a model for the structural basis of kinetic polarity.

Chapter 4 describes the biochemical and structural characterization of the residue in the interdomain cleft important for transition from R to T state. Upon replacing a rigid residue, phenylalanine, to a flexible residue, methionine, in the inter-domain cleft, we observe that the cleft mutant, SmFtsZ^{F224M} has higher GTPase activity than that of wild type. The filaments of the cleft mutant are longer and the critical concentration is lower than the wild type, indicating more nucleating events and increased addition of monomers to the filament owing to lower critical concentration. We infer that the mutation eases the transition of R to T state by facile opening of the cleft. The residue is located at the hinge where the inter domain cleft opens and a flexible residue (M224) in place of a rigid bulky residue (F224) might act as a lubricant for easier transition between states. Therefore, the slower cleft opening in wild type SmFtsZ could account for its lesser GTPase activity than EcFtsZ under the same *in vitro* conditions (Figure 6.2B).

In the *in vivo* study (done in collaboration with Dr. Ramanujam Srinivasan lab), when EcFtsZ is mutated to phenylalanine at M225, we observe filamentous morphology of cells indicative of aberrant cell division. Further, expression of EcFtsZ^{M225F} mutant in *E. coli* perturbs FtsZ assembly. The expression of EcFtsZ^{M225F} in an *E. coli* strain carrying an endogenous mNeonGreen tagged EcFtsZ (BW27783) affected ring assembly. It is likely that the slow R to T transition due to the bulky side chain of phenylalanine in the cleft affects the rate of subunit addition at the bottom end of the FtsZ filament. The *in vivo* results in *E. coli* further suggest that slower GTPase activity by slower cleft opening in SmFtsZ is probably due to the presence of bulkier phenylalanine at 224.

Chapter 5 describes the mechanism of inhibitor binding to FtsZ. In R state, the FtsZ inter-domain cleft is closed which cannot accommodate the drug (Figure 6.2A), whereas in T state, the FtsZ inter-domain cleft is open and can thereby accommodate the drug (Figure 6.2B). But

the exceptions where the cleft is occluded for the binding of the drug are sequences with potential salt bridge interactions across the inter- domain cleft (Figure 6.2C).

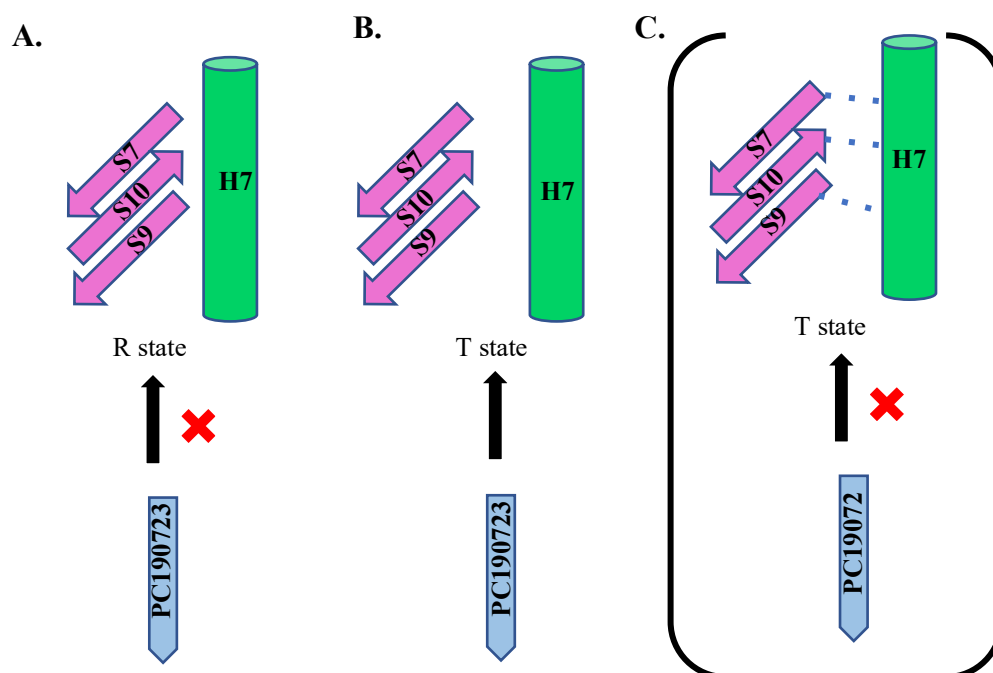


Figure 6.2 Model for the PC190723 binding to FtsZ. A) The schematic shows the inter-domain of FtsZ in the R state which is closed and PC190723 cannot get accommodated. B) The inter-domain cleft in the T state is opened and can accommodate PC190723. C) One of the circumstances where the cleft gets occluded to the drug binding is when there are salt bridge interactions across the cleft.

This hypothesis has been further experimentally validated by heterologously expressing *Helicobacter pylori* FtsZ (HpFtsZ) in fission yeast. When a salt bridge interaction was introduced in HpFtsZ, FtsZ assembly is unaffected in the presence of the drug (Sharma *et al.*, 2022). In conclusion, our model (Figure 6.2) shows the mechanism of PC190723 binding to FtsZ. Further work is necessary to fully elucidate the action of PC190723 and its influence on Z-ring assembly dynamics in Gram negative bacteria where the host factors such as outer membrane permeability and efflux pumps might be contributing factors to resistance. It might be possible to target FtsZ from Gram negative bacteria which are predicted to be susceptible to PC190723 according to the FtsZ sequence from our analysis, by increasing membrane permeability for the drug PC190723 to get inside the cell.

6.2 Future prospects

6.2.1 Mechanism of action of FtsA along with FtsZ in cell wall less bacterium

To decipher the mechanism of division in cell wall less organism, I have cloned and standardized the purification FtsA, which helps the Z-ring to tether to the membrane, from *S. melliferum*. The biochemical and structural characterization of FtsA and its binding with membrane and FtsZ can help us gain insights into the mechanism of action of these proteins independent of cell wall synthesis machinery. The question is whether FtsZ and FtsA are sufficient for the division of cell for organisms devoid of cell wall.

6.2.2 SAXS study to elucidate the extended state of FtsZ during state transition in solution

It is shown in previous reports that the NTD and the CTD can reconstitute non covalently to form the functional FtsZ (Oliva *et al.*, 2004). This signifies that they can fold independent of each other. On the other hand, tubulin is composed of a heterodimer which polymerizes to form microtubules (Nogales *et al.*, 1998). It is interesting to speculate that the existence of tubulin as a heterodimer has probably evolved to prevent a non-productive extended conformation which is seen in case of the monomeric FtsZ from our crystal structures and in other non-polymerizing mutants of FtsZ as well.

This hypothesis has to be further verified by solution studies of the polymerization intermediates by using SAXS. The study is in progress.

6.2.3 Structural comparison of kinetic polarity of FtsZ and its homolog tubulin

Our results on the SmFtsZ structures suggest that GTP binding is sufficient for addition of FtsZ to the bottom of the filament, while the cleft opening between NTD and CTD is possibly a rate-limiting step for polymerization. Since addition to the top of the filament requires nucleotide binding and the relative rotation between NTD and CTD, filament growth at the top is expected to be comparatively slower than that at the bottom (Figure 6.3).

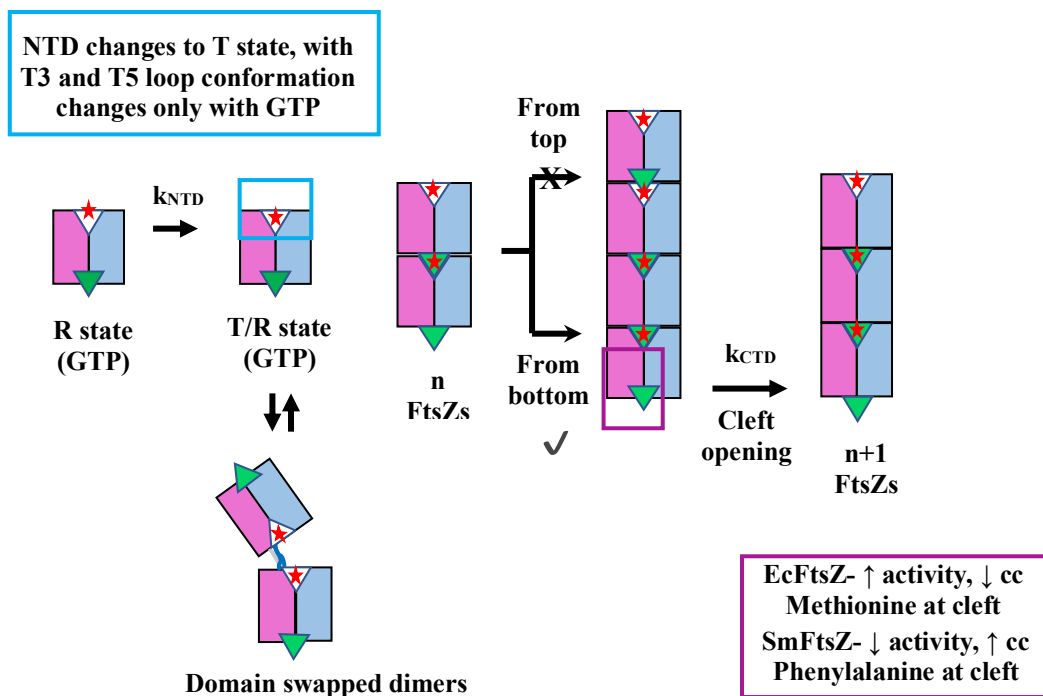


Figure 6.3 Model for FtsZ kinetic polarity.

According to our model, A) GTP binding triggers the conformational changes in T3 loop which plays an important role in the interaction with the bottom interface of the upper FtsZ subunit in filament. This further assist the T state conversion of the NTD, independent of CTD conformational change. This T-state of NTD is stabilized by binding to the bottom of the FtsZ filament. The top addition requires opening of the inter-domain cleft whereas the closed conformation does not fit in the top of the filament. B) After addition to the bottom interface, the cleft opening is determined by the flexibility of the residue in the cleft. GTPase activity is higher in EcFtsZ having methionine in the cleft than SmFtsZ having phenylalanine in the cleft. We propose that inter-domain cleft opening during R- to T-state transition is rate limiting and also there could be possibility of the monomer going to an extended conformation during the state transition. The NTD is in sky blue; the H7 helix and the T7 loop is denoted in green; the CTD is in pink; the red star denoted GTP.

Earlier reports show that FtsZ filaments and tubulin filaments have opposite kinetic polarities (Du *et al.*, 2018). The microtubules are composed of alpha and beta-tubulin heterodimers which are in the curved form in solution and straight in the filament form (Wang and Nogales, 2005). To obtain structural insights into the differences in kinetic polarity, we compared the conformations of straight and curved tubulin dimers. It is observed that there are clashes when the addition is from the bottom. This can be due to the fact that the CTD of beta has to change its conformation to switch from curved to straight. On the other hand, the

alpha subunit does not clash when it attaches on the top. As we see that the CTD of alpha-tubulin is preformed, thereby it can attach on the top of the microtubule whereas the conformation of the CTD of beta needs to change to adopt the straight conformation. The curved heterodimer, therefore, cannot sit at the bottom of the microtubule as it will clash. Thereby, plus end of the microtubule is the top of the filament. This analysis is currently in progress.

Based on the available data, we provide a model of filament formation of FtsZ from the bottom end whereas the filaments of its homolog, tubulin, grows from the top.

6.3 References

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