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OVEREXPRESSION, PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF THE BACTERIAL CELL DIVISION PROTEIN FtsZ OF *Mycobacterium smegmatis*

A PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

ertified that this project report "**OVEREXPRESSION, PURIFICATION AI**
IOCHEMICAL CHARACTERIZATION OF THE BACTERIAL CELL DIVISIO
ROTEIN FtsZ OF *Mycobacterium smegmatis*" is the bonafide work of **AISHWARYA**
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The project work was aimed at the overexpression, purification, and biochemical characterisation of the cytokinetic protein FtsZ of *Mycobacterium smegmatis*. The cloned *ftsZ* gene of *M. smegmatis* mc^2 155 (*MsftsZ*) was already available in pET15b in the laboratory. Competent *E. coli* JM109 cells were prepared and transformed with pET15b-*MsftsZ* plasmid vector. The pET15b-*MsftsZ* plasmid DNA was then isolated from *E. coli* JM109 and purified. Competent *E. coli* C41 cells were then transformed with pET15b-*MsftsZ* plasmid for expression of the gene product. Overexpression of the protein was achieved by induction of the gene expression with IPTG for 4 hrs. The protein was then affinity purifed using Ni^{2+}-NTA agarose column chromatography. The concentration of the eluted protein was judged by observing the intensity of the protein band on SDS-PAGE. The eluted protein was then dialysed And the concentration was again determined on SDS-PAGE. The protein was biochemically characterised for the GTP-dependent polymerisation and GTPase activities with the help of two assays: Light Scattering assay (LS assay) and GTPase assay. Light scattering assay involves 90° differential scattering of light by polymerised FtsZ (after addition of GTP). The unpolymerised FtsZ does not scatter light (without GTP addition). GTPase assay determines the GTPase activity of the protein, in terms of the quantity of phosphate released.

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LIST OF ABBREVIATIONS

ALS	Alkaline lysis solution
APS	Ammonium Persulphate
Amp	Ampicillin
BSA	Bovine serum albumin
DD	Double Distilled
DI	De-Ionised
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Fts	Filamenting temperature sensitive
GDP	Guanosinediphosphate
GTP	Guanosine triphosphate
HEPES	N-2-Hydroxyethylpiperazine- N'-2-ethanesulfonic acid
IPTG	Isopropylthio-D-galactoside
kDa	Kilo Dalton
LB	Luria-Bertani medium
LS	90° Light Scattering
MES	2 - (N-morpholino) ethane sulfonic acid
ml	Millilitre

mM	Millimolar
µl	Microlitre
µM	Micromolar
Ms	*Mycobacterium smegmatis*
NTA	Nitrilo-tri-acetic acid
ng	Nanogram
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
P_i	inorganic Phosphate
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
STE	Sucrose-Tris-EDTA
TE	Tris-EDTA
TEMED	Tetraethyl methylene diamine

INTRODUCTION

CHAPTER 1
INTRODUCTION

Bacterial cell division occurs by binary fission where a single bacterial cell divides into two daughter cells distributed with identical genetic material. The division process begins with Karyokinesis (nucleoid replication and segregation) followed by Cytokinesis (cytoplasmic division). A cell wall constriction occurs during cytokinesis, after chromosome segregation is completed, and the cytoplasm is divided into two equal parts through septum formation at the mid-cell site. The septum formation involves the participation of several cell division proteins, namely the Fts proteins. Conditional *fts* mutants of *E. coli*, fail to divide at non-permissive temperature, and instead, form filaments, eventually resulting in loss of viability. Therefore, those mutants were named *filamenting temperature sensitive (fts)*. The genes that complemented the filamentation defect were called *fts* genes, which include *ftsA*, *ftsQ*, *ftsH*, *ftsK*, *ftsI*, *ftsE* and *ftsZ*, of which *ftsZ* is of prime importance.

1.1 FtsZ

FtsZ is the first cell division protein to arrive at the future division site of bacteria (mid-cell site). It is responsible for recruiting other proteins (FtsA, FtsB, FtsE, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, FtsX, ZipA and AmiC) that are required for a complete cell division to occur. FtsZ monomers assemble at the mid-cell site and polymerise to form a circular ring structure called the Z-ring. The Z-ring provides a scaffold for other cell division proteins to assemble and brings about an invagination by building a septal peptidoglycan layer (Lutkenhaus and Addinall, 1997). FtsA and ZipA bind directly to FtsZ after polymerisation (Hale and de Boer, 1997). This stabilises FtsZ ring and facilitates binding of all other Fts proteins required for cytokinesis in the following order and form the multi-protein complex, called 'Divisome': FtsK, FtsQ, FtsL, FtsI and FtsN (Rothfield *et al.*, 1993). This complex facilitates the process of cell division.

1.2 Structure of FtsZ

a C-terminal conserved peptide. The core region consists of the tubulin signature motif, GGGTGTG, and GTP binding site that binds GTP for polymerisation, and a GTPase site that hydrolyses GTP into GDP and Pi, for polymerisation. These two activities are responsible for the polymerisation-dependent self-assembly of FtsZ and depolymerisation. The core region has N-terminal (Nt) core and C-terminal (Ct) core regions. The Nt core region binds the bottom portion of the adjacent monomer in the protofilament, whereas the Ct core binds the top portion. The C-terminal peptide is responsible for interactions with other membrane-associated cell division proteins (FtsA and ZipA). FtsZ function is blocked by deletion of this C-terminal peptide by preventing the interaction (Margolin, 2005).

FtsZ is often referred to as the bacterial homologue of the eukaryotic tubulin. The two proteins seem to share common ancestry. The GTP binding domains of both the proteins are very similar. The GTP binding domain in FtsZ has the sequence ^{105}GGGTGTG^{111} whereas the GTP binding domain in tubulin has the sequence ^{140}GGGTGSG^{147}, which shows high degree of similarity. Moreover, the three dimensional structure of the two proteins were also found to be similar. However, it is interesting to note that the two cell division proteins share only 10-18% sequence similarity on the entirety.

1.3 Regulation of Z-ring

The MinCDE system and nucleoid occlusion are two factors that are responsible for exact placement of FtsZ at the mid-cell site. The MinCDE system prevents FtsZ polymerisation near certain parts of the plasma membrane. MinD localises to the membrane only at cell poles. Once MinD is anchored in the membrane, it polymerises, forming clusters of MinD. These clusters bind and then activate another protein called MinC, which has activity only when bound by MinD. MinC serves as FtsZ inhibitor that prevents FtsZ binding at the poles and thereby polymerisation. The MinC and MinD proteins keep oscillating between the two poles of the bacterial cell so that they curb FtsZ binding at both the poles, unless otherwise, the cell may divide disproportionately (de Boer, *et al.*, 1991). High concentration of FtsZ polymerisation inhibitor at the poles prevents FtsZ from initiating division at anywhere but the mid-cell.

MinE prevents MinCD complex from binding at the mid-cell site, thus ceasing the effect of MinCD and enabling FtsZ assembly, polymerisation and thereby division at the

complex from binding at the centre, it allows MinCD to bind to the poles. Thus, regulation of the Min system is highly important for the generation of two daughter cells. Mutations that prevent the formation of MinE rings result in MinCD polymers extending well beyond the polar zones, preventing division site formation and FtsZ assembly at mid-cell site.

Nucleoid occlusion from the mid-cell site enables provision of the mid-cell site being vacant without the presence of nucleoids and thereby enables localisation of FtsZ. Nucleoid exerts a negative control on cell division wherever it occupies space in cell (Wu and Errington, 2004). Furthermore, chromosome segregation and diminished occlusion of the nucleoid at the cell centre allows a septum to form at the mid-cell site. Although there might be no DNA-free region in the entire gamut of the cell, lowered concentration of DNA at the mid region becomes sufficient for Z-ring formation. Also, Z-ring formation does not require complete segregation of nucleoids, in general, it is described that FtsZ assembly, ring formation, and septal invagination all occur after nucleoid segregation.

1.4 GTP binding and GTPase activity

GTP binding and GTP hydrolysis form the most important mechanism that influences cell division. FtsZ monomers assemble exactly at the mid-cell site where the septum formation should be initiated in order to give rise to two daughter cells. The monomers polymerise in a GTP-dependant manner (Mukherjee *et al.*, 1993). FtsZ has GTP binding activity and GTPase activity. A monomer of FtsZ binds GTP with the GTP binding activity, hydrolyses the GTP with the GTPase active site, and binds another monomer of FtsZ in a head-to-tail fashion. Thus, regulation and coordination of FtsZ and other cytokinetic proteins plays a vital role in septum formation and thereby cell division.

1.5 SeptalInvagination

Septal invagination occurs by the ingrowth of the rigid murein layer. This ingrowth provides the driving force for the invagination of inner (cytoplasmic) and outer layers. The cytoplasmic membrane is pushed inward and the outer layer is pulled inward ultimately resulting in septal invagination.

1.6 Function of others division proteins

The division proteins not only take care of septum formation but also ensure the coordination of septation with other cell cycle events. These events include chromosome replication and segregation. Also, a switch from elongation mode to septal mode is regulated by one or more of the cell division proteins.

The chromosome replication and segregation involves four steps: i) termination of chromosome replication, ii) decatenation of the linked daughter chromosomes that are the final product of replication of the circular genome, iii) resolution of the covalent chromosomal dimers that are frequently by homologous recombination between sites on the partially replicated chromosomes, and iv) movement of the resolved daughter chromosomes away from the mid-cell site.

If septation occurs before any of these processes' completion, the mother cell divides into two unequally sized, disproportionate daughter cells.

1.7 OBJECTIVES

- To express the MsFtsZ-stop protein in *E. coli* C41cells
- To purify the MsFtsZ-stop protein by Ni^{2+} NTA-Agarose column chromatography and dialysis
- To characterise the MsFtsZ-stop using *in vitro*90° light scattering and GTPase assays.

LITERATURE REVIEW

CHAPTER 2
LITERATURE REVIEW

2.1 Replication and segregation of bacterial DNA

The chromosome of bacteria is circular and replicates bi-directionally from a specific origin of replication. Chromosome is found as a compact mass of nucleic acid called the nucleoid. Asaresult of chromosome replication, the daughter chromosomes formed and segregate into opposite poles of the cell. The process of getting resolved or segregation occurs consequentially after the replication has terminated. The replication machinery consists of several proteins. These proteins help in the elongation phase for DNA synthesis. The process of DNA segregation does not require any separate system for its initiation and regulation. The replication of DNA serves as the driving force for resolving of DNA. An outward push of the replicated DNA towards the opposite halves occurs. A series of activities occur for segregation to occur: Push, Direct, Condense, Hold and Clear.

The push event characterizes the generation of force to push away the newly replicated DNA. As mentioned earlier, the replication partly forces the DNA to the corresponding directions. The replication forks play an important role in pushing away the DNA from the cell division plane. Each fork is responsible for one nucleoid formation and is believed that the forks drive their respective DNAs into appropriate directions.

The direct event leads the DNA through relatively long distance to the poles. Homologues of the Par proteins (ParA and Par B homologues) are essential for partition or segregation of DNA. The configuration of DNA polymerases during replication feeds the DNA strands into some channel that directs the DNA in the specific direction. The condense event involves condensing of the chromosome into a compact nucleoid. The proteins HU and H-NS that are displaced during replication rebind to DNA and topoisomerases initiate supercoiling. These activities result in chromosome condensation. The hold event emphasizes the necessity to hold the condensed DNA in order to prevent it from drifting back to the cell centre. So, the nascent membrane proteins anchor the condensed mass in an appropriate position. The clear event deals with cleaning up any left-over DNA at the division plane. It clears any errors that might have occurred during segregation. (Sawitzke, J. and Austin, S., 2001).

2.2 FtsZ, the principle cytokinetic protein

During septation, FtsZ is the first protein to get localised to the mid-cell division site and polymerise to form a ring-like structure (the Z-ring) at the site (Bi and Lutkenhaus, 1991). The Z-ring plays a key role in the constriction of the cell membrane as well as in the coordination of the whole process of division. The Z-ring recruits a dozen other Fts proteins to form a complete septal ring capable of carrying out cytokinesis (Bi and Lutkenhaus, 1991; Wang and Lutkenhaus, 1993). The *ftsZ* gene is a highly conserved cell division gene that is present in most bacteria. Thus, the Z-ring is likely to be the cytoskeletal element that drives cytokinesis in most of the prokaryotes.

2.3 MinCDE system and nucleoid occlusion

The two key factors involved in the selection of the correct mid-cell site for cell division are – nucleoid occlusion and the Min system (blocking the potential division site at the cell poles). In the cells that are about to divide, the correct mid-cell division site is selected by the combined action of these two negative regulatory systems. Placement of FtsZ at the mid-cell site of bacteria is shown to be regulated by MinCDE system (de Boer *et al.*, 1989) and mutation in MinCD leads to formation of mini cells (Adler *et al.*, 1967). A phenomenon called nucleoid occlusion (Woldringh *et al.*, 1991), allows Z-ring to be formed at the mid-cell site only after the nucleoid has replicated and segregated away from the mid-cell.

Nucleoid Occlusion: Nucleoid inhibits Z-ring formation at the mid-cell site prior to initiation of chromosome segregation (Woldringh *et al.*, 1991; Sun *et al.*, 1998; Harry *et al.*, 1999; Yu and Margolin, 1999). Non-specific DNA binding proteins like Noc in *Bacillus subtilis,* and SlmA in *Escherichia coli* were found to be responsible for nucleoid occlusion (Bernhardt and de Boer, 2005; Wu and Errington, 2004). Noc and SlmA topologically restrict the assembly of FtsZ, enhancing the cooperativity of the Z-ring assembly, and serve as a checkpoint to prevent guillotining of nucleoids.

Regulation of MinCD: The second process is regulated by MinCD complex (de Boer *et al.*, 1989), which consists of MinC, an inhibitor of FtsZ polymerisation,and MinD, a membrane-associated ATPase that recruits MinC to the membrane (Marston and Errington, 1999). MinD binds to the membrane in its ATP-bound form and is released from the membrane upon ATP hydrolysis. The topological specificity marker MinE forms a ring structure at the division site, independent of FtsZ, and marks the site for FtsZ localisation (Raskin and de Boer, 1997). MinE drives the MinD (and thus MinC) off the membrane by binding to MinD and stimulating its ATP hydrolysis. Subsequent diffusion of MinD-ADP and nucleotide exchange causes MinD-ADP to rebind to the membrane away from the original site because of the transient presence of MinE at the original site and the kinetics of nucleotide exchange (Hu and Lutkenhaus, 2001). This causes a MinC-mediated FtsZ disassembly wave to oscillate from pole to pole (Thanedar and Margolin, 2004).

2.4 Structure of FtsZ

The structural similarity between FtsZ and tubulin reveal that they form a unique family of GTPases and that FtsZ could be an ancestral form of eukaryotic tubulin (de Pereda *et al.*, 1996; Lowe and Amos, 1999). Like tubulin, purified FtsZ binds and hydrolyses GTP (RayChaudhuri and Park, 1992; de Boer *et al.*, 1992). FtsZ contains four main protein domains, as determined by the crystal structure of FtsZ from the thermophilic bacterium *Thermotoga maritima* (Lowe and Amos, 1998) and by phylogenetic analysis (Vaughan *et al.*, 2004). These domains comprise a variable N-terminal segment of unknown function, a highly conserved core region containing tubulin signature motif for GTP binding, a variable spacer of uncharacterised function, a C-terminal conserved peptide essential for interactions with other membrane-associated cell division proteins (Margolin, 2004).

2.5 FtsZ, a prokaryotic homolog of tubulin

The suggestion that FtsZ is a homolog of tubulin was confirmed by a short sequence of amino acids GGGTGTG which is very similar to the tubulin signature motif SGGTGTG, which is found in all α and β tubulins. Mukherjee and Lutkenhaus identified a dozen other

between tubulin and FtsZ. These evidences clearly indicate that FtsZ and tubulin are homologs. This strongly implies that the two proteins share a common ancestry. However it is questionable whether the two proteins perform the same function. The function of tubulin is to assemble into a cytoskeletal polymer, the microtubule and this is facilitated by formation of 2-D lattice of sub-units that build the microtubule wall. FtsZ polymerises to form protofilaments during the assembly *in vitro.* This provided the first suggestion that FtsZ has a similar cytoskeletal function to that of tubulin. Yet, the lattice of the assembly remained unsolved. Later, the lattice of two distinct FtsZ was resolved and proved to be similar to that of tubulin lattice. One of the two lattices resolved was a 2-D sheet of straight protofilaments with a lattice very similar to the microtubule wall. The second was a miniring which was identified as a protofilament in curved conformation. These minirings are structural homologs of tubulin rings. The assembly of FtsZ and tubulin into protofilaments demonstrates a conservation of function as a protofilament.

FtsZ binds to and hydrolyses GTP but unlike tubulin, where GTP hydrolysis is tightly coupled to assembly, the *in vitro* GTPase activity of FtsZ seems independent of its assembly into large polymers. FtsZ can hydrolyse GTP at a relatively very fast rate. (Erickson, H. P., 1997).

2.6 Coordination of septum assembly with nucleoid segregation

As already mentioned, nucleoid occlusion is one mechanism for linking cell division to chromosome segregation. Another mechanism involves FtsK. FtsK of *E. coli* is an enormous protein, 1329 aminoacids in length, and has several activities. The N-terminal domain (200 residues) alone is sufficient to support celldivision (Draper *et al.*. 1998; Wang and Lutkenhaus,1998). It contains four transmembrane helices (Dorazi andDewar, 2000) and localizes to the septal ring, where it is needed for recruitment of several additional essential division proteins (Wang and Lutkenhaus, 1998; Yu *et al.*,1998; Chen and Beckwith, 2001). The essential activity supplied by the N-terminal domain of FtsK is not entirely clear. Its role in recruitment of downstream division proteins implies an essential function in assembly of the septal ring before the onset of cytokinesis, but there is also evidence that the N-terminal domain of FtsK plays additional roles during septum closure. The remainder of FtsK is cytoplasmic. It consists of a proline- and glutamine-rich region (500 residues) that might

segregation (Yu *et al.*,1998b; Steiner *et al.*, 1999). The C-terminal domain belongs to the AAA family of ATPases, a set of proteins associated with a wide variety of cellular activities (Vale,2000). Some AAA domains catalyse the folding and unfolding of proteins, whereas others disassemble protein complexes or generate unidirectional movement along tracks.

To understand how FtsK facilitates chromosome segregation, it is helpful to know that newly replicated chromosomes are linked, and that these linkages must be resolved for partitioning to go to completion. One way in which chromosomes are linked is that they are catenated. Catenanes are resolved by TopIV. The C-terminal domain of FtsK interacts directly with Top IV, recruits it to the midcell and stimulates its decatenase activity (Espeli *et al.*, 2003). The other form of linkage is that chromosomes are sometimes dimeric. Chromosome dimers arise from *recA*-dependent (homologous) recombination between sister chromosomes, and are resolved by XerCD, a recombinase that acts at a 28 bp chromosomal site near the terminus named *dif*. The C-terminal domain of FtsK activates the XerCD recombinase and positions the *dif* sites so that they can form an appropriate synapse (Aussel *et al.*, 2002; Capiaux *et al.*, 2002).Finally, the cytosolic portion of FtsK is an ATP-dependent DNA translocase (Aussel *et al.*, 2002). There are several possibilities, none of which are mutually exclusive, for how the translocase activity might facilitate chromosome segregation. These include aligning *dif* sites, creating a domain of supercoiled DNA where TopIV acts and pumping DNA away from the closing septum. Depending on the growth conditions, the C-terminal domain of FtsK is only essential in the 10% of cells in a population that contain chromosome catenanes or dimmers that need to be resolved. Most of the cells in a culture (90%) divide well even if the C-terminal domain of FtsKis absent.

2.7 FtsZ, a potential GTPase

FtsZ has been studied from two perspectives: localisation *in vivo* and its ability to assemble *in vitro*. The Z-ring almost certainly consists of FtsZ polymers because these can readily be formed *in vitro*, in the presence of GTP, under a range of conditions. It is a GTP-binding protein with GTPase activity (Ray Chaudhuri and Park, 1992; de Boer *et al.*, 1992).GTP binding induces FtsZ self-assembly into protofilaments that consist of a head-to-tail linear polymer of FtsZ. However, unlike tubulin, FtsZ protofilaments do not assemble into microtubule-like structures. Instead, FtsZ protofilaments associate laterally to form

1999; White *et al.*, 2000) and tubes (de Boer *et al.*, 1992; Rothfield *et al.*, 1997). This FtsZ protofilament formation *in vitro* is probably driven by the affinity between GTP-bound FtsZ monomers. GTP is subsequently hydrolysed by an active site formed between the two associated monomers, as in microtubule assembly (Sossong *et al.*, 1999; Scheffers *et al.*, 2002). It is now widely accepted that GTP hydrolysis is the rate-limiting step in the polymerisation of FtsZ. The turnover of the Z-ring also appears to be dependent upon the hydrolysis of GTP (Stricker *et al.*, 2003). GTP hydrolysis was cooperative and was dependent upon the concentration of the protein and was suggestive of oligomerisation of the protein (de Boer *et al.*, 1992; Wang and Lutkenhaus, 1993; Sossong *et al.*, 1999). Cooperativity in GTP hydrolysis has however been questioned (Romberg *et al.*, 2001) and it was recently suggested that FtsZ initially assembles isodesmically as a curved protofilament, but after reaching a certain length the protofilament ends are able to contact each other. This process of cyclisation would cause the formation of additional lateral bonds, resulting in cooperativity (Gonzalez *et al.*, 2005).

2.8 Septal ring and cell division

Cell division proceeds through the concerted inward growth of all the three layers of the cell envelope – cytoplasmic membrane, peptidoglycan and outer membrane. Constriction of the Z-ring pulls the cytoplasmic membrane inward. Proteins that are responsible for the synthesis of peptidoglycan layer are embedded in the cytoplasmic membrane. As the membrane invaginates, the synthesis of peptidoglycan layer occurs. After formation, the Z-ring has been found to be extremely dynamic. FtsZ subunits exchange in ad out of the ring on a time scale of seconds even while the overall morphology of the cell appears to be static. The rate limiting step in the formation of FtsZ assembly is the hydrolysis of GTP.

Coordination of chromosome replication with the cell division is done by another protein FtsK which is a DNA translocase that facilitates decatenation of sister chromosomes by TopIV and resolution of chromosome dimers by Xer-CD recombinase. Finally, two murein hydrolases AmiC and EnvC localize to the septal ring and aid in the separation of daughter cells.

The Z-ring is a dynamic structure with constant exchange of monomers. The static

have revealed that Z-ring constriction begins after 20 minutes of its assembly, when the growth is slow *i.e.*,when the doubling time is high. The significant lag between the Z-ring formation and constriction probably reflects the time requires for the assembly or polymerization of the septal ring. However, it is suggested that the assembly and constriction of septal ring are regulated separately and differently. For the Z ring to exert force on the cell envelope, it needs a solid connection to the cytoplasmic membrane. There appear to be redundant mechanisms for this. ZipA is an integral membrane protein and binds directly to FtsZ (Hale and de Boer, 1997), but is only found in a subset of the proteobacteria and even there the requirement for ZipA can be bypassed by a mutation in *ftsA* (Geissler*et al.*, 2003).

Several studies indicated that the Z ring can assemble and disassemble rapidly, within 1 min or less (Addinall*et al.*, 1997; Sun and Margolin, 1998; Rueda *et al.*, 2003).Nevertheless, it came as a surprise when fluorescence recovery after photobleaching (FRAP) revealed that FtsZ molecules in the ring turn over rapidly (Stricker*et al.*, 2002). Briefly, a laser was used to bleach Z rings in cells that expressed *ftsZ-gfp*, and return of fluorescence to the ring, owing to exchange with unbleached FtsZ-GFP from the cytoplasmic pool, was monitored by time-lapse photography. The initial study found that the half-time for remodelling in *E. coli* was 30 s, but more recent work, under different experimental conditions, indicates a halftime of about 9 s in both *E. coli* and *B. subtilis* (Anderson*et al.*, 2004). Because all of the other division proteins require FtsZ for septal localization, if FtsZ subunits are turning over, the remaining proteins in the septal ring might be too. This expectation was confirmed for ZipA (Stricker *et al.*, 2002).

A number of additional interesting observations have come from these studies. First, the rate-limiting step in turnover is probably GTP hydrolysis. In support of this inference, the FtsZ84(Ts) mutant protein, which has a lesion in the GTP binding site and diminished GTPase activity, exchanges about threefold slower than wild-type FtsZ (Anderson *et al.*, 2004). Moreover, the half-time of 9 s for turnover of wild-type FtsZ as determined by FRAP means that, on average, each FtsZ molecule cycles into and out of the Z ring approximately five times per minute. This rate is strikingly similar to the rate of GTP hydrolysis – 5–10 GTP per FtsZ per minute – determined *in vitro* under conditions that support formation of protofilaments (Lu *et al.*, 1998; Romberg and Mitchison, 2004).If the rate-limiting step in FtsZ turnover is in fact GTP hydrolysis, the Z ring consists primarily of FtsZ-GTP and has a limited capacity to generate force. A second intriguing finding is that turnover does not

permissive temperature despite the diminished GTPase activity and slower turnover of the FtsZ84 protein. These observations caution against using the observed rapid turnover of FtsZ as support for the notion that depolymerization drives cytokinesis. A third remarkable finding is that elimination ofZapA, EzrA or MinCD, all of which are implicated in modulatingFtsZ assembly *in vivo*, had little or no effect on therate of Z ring remodelling (Anderson *et al.*, 2004). Finally,as noted by Romberg and Levin (2003), rapid turnover has implications for the regulation of cell division. Because the Z ring must be actively maintained, assembly of a Zring does not commit the cell to division at that site. Instead, it is easy for cells to disassemble an existing septal ring to abort division, as occurs during the SOS response to DNA damage in *E. coli* (Bi and Lutkenhaus,1993), or to redeploy FtsZ to another site, as occurs during the switch from medial to polar septation during Sporulation in *B. subtilis*. (Ben-Yehuda and Losick, 2002).

2.9 Z-ring contraction

In spite of the fact that the necessity of FtsZ for cytokinesis is acknowledged, it is unclear whether the Z-ring is obligate for the active mechanical membrane constriction or its function is passive, i.e., to recruit enzymes synthesizing cell wall components. Probably,the Z-ring exerts both functions. The most recent dataindicate that only one third of bulk FtsZ is incorporated into the protofilament ring. The two remaining thirds ofthe FtsZ molecules also partially build protofilaments outside the ring and are distributed in the cell nonrandomly. They exhibit rapid and wavy movements from pole to pole thus coiling the cell. It is thought that FtsZprotofilaments participate not only in cytokinesis but in support of bacterial shape (Thanedar, Margolin, 2004).

Several models of Z-ring constriction have been suggested. Two of them were described in detail in the review of Bramhill (1997). One model is based on FtsZprotofilaments sliding relative to each other with amotor protein such as myosin. In recent years this model has not been argued. The other, most supported, model supposes the gradual depolymerization of the Z-ring tightly anchored on the membrane with other accessory proteins which results in its contraction and mechanical force transfer to the membrane. A number of new observations have added some details to the model (Thanedar, Margolin, 2004). Z-ring contraction in *B. subtilis* is regulated by at least two other proteins,

FtsZ dimers, protofilaments and their parts. During a cell division individual FtsZ protofilaments of curved or coiled shape are separated from the contracting ring. These structures are preserved on the cytoplasmic membrane in daughter cells after the division is accomplished andthe Z-ring disintegrates.

According to the third model, contraction is realized through conformational alterations of the FtsZ protofilament curvature regulated by GTP hydrolysis to GDP or by inorganic phosphate release (Lu et al., 2000).

The alternative hypothesis of a passive Z-ring contraction is also disputed. It is proposed that contraction takes place during the process of septum wall invagination controlled by FtsI transpeptidase bound with FtsZ and other proteins involved in septum development(Margolin, 2005). In that case FtsZ should be regarded as a marker protein determining the furrow and recruitingother division proteins rather than a key division protein in cytokinesis. However, the occurrence of FtsZ in most mycoplasmas, which lack a cell wall and many division proteins, may be in favor of the hypothesis on an active engagement of the FtsZ protein in bacterial cell division. (Vishnyakov and Borchsenius, 2007).

2.10 Hierarchy of assembly

Once FtsZ localises at the mid-cell site, it polymerizes and forms the Z-ring. The Z-ring recruits several other membrane-associated proteins like FtsA/ZipA, FtsEX, FtsQ, FtsL/FtsB, FtsW, FtsI, FtsN and AmiC in the given hierarchial and temporal order to form the division complex known as Divisome(Weiss, 2004). FtsA and ZipA are required to anchor the Z-ring to the membrane (Hale and de Boer, 1997) and are also required for all the other proteins to get recruited in the temporal order. FtsX of FtsEX complex of proteins requires FtsA and ZipA for localisation (Schmidt et al., 2004). FtsK, a transmembrane protein which coordinates the chromosomal resolution and plays an important role in segregation to cell division (Bigot *et al.*, 2004; Pease *et al.*, 2005), localises to the mid-cell site and recruits few proteins of unknown function, namely FtsQ, FtsL, and FtsB (Wang and Lutkenhaus, 1998; Chen and Beckwith, 2001). Proteins involved in peptidoglycan synthesis, namely FtsW and FtsI, are recruited to the medial ring in the same order (Wang et al., 1998; Chen and Beckwith, 2001). FtsI then recruits FtsN (Addinall *et al.*, 1997; Wang et al., 1998; Wissel and

cytoplasmic membrane to the following cell wall. An amidase (murein hydrolase), AmiC, is then localised to the ring (Bernhardt and de Boer, 2003) to cleave septal murein and separate the two daughter cells (Heidrich *et al.*, 2001). Thus, a dynamic orchestration of all the Fts proteins and other non-Fts proteins of the divisome mediate septation during binary fission in most of the bacterial systems. The precise function of many Fts proteins is yet undetermined.

The Fts proteins form the divisome complex in response to appropriate signals that are unpredicted till date. Few proteins require the presence of another specific protein that bind to the divisome apparatus previous to them. The requirement of the proteins as described by Rothfield *et al.*, 1993 is as follows:

ZipA assembly require FtsZ but not FtsA or FtsL.
FtsA assembly requires FtsZ but not ZipA or FtsL.
FtsK assembly requires FtsZ and FtsA but not FtsI or FtsQ.
FtsQ assembly requires FtsA and FtsZ but not FtsL or FtsI.
FtsL assembly requires FtsZ, FtsA and FtsQ, but not FtsI.
FtsI assembly requires FtsZ, FtsA, FtsQ and FtsL.
FtsN assembly requires FtsZ, FtsA, FtsQ and FtsA .

FtsA, ZipA and ZapA modulate the assembly state of FtsZ. A synergy of FtsA and ZipA connects the Z-ring to the cytoplasmic membrane. FtsK coordinates septation with chromosome segregation. FtsI and FtsW aid in synthesis of the peptidoglycan layer. AmiC and EnvC regulate the hydrolysis of the peptidoglycan layer to separate the two newly formed daughter cells. Functions of few other proteins are essentially unknown. (Weiss, 2004).

2.11 Inhibition of FtsZ polymerization

Substances that inhibit tubulin polymerization were added to the FtsZ to test whether or not the FtsZ polymerization is also inhibited. Common inhibitors of tubulin are classified into four categories: a) colchicine and its structural analogues, such as colcemid and podophyllotoxin, b) vinblastine and its analogues vincristine and maytansine, c) the metal ions Ca2+, Cu2+ and Hg2+ and d) aminonaphthalenes, such as bis-ANS and other inhibitors

inhibitory action of the Ca^{2+} promoted GTP-dependant polymerization of FtsZ. However, the hydrophobic probe 5,5'-bis-(8-anilino-1-naphthalenesulfonate) (bis-ANS) inhibited the FtsZ assembly despite Ca^{2+}presence. Titrations of FtsZ with bis-ANS indicated that FtsZ has one high affinity binding site and multiple low affinity binding sites. ANS (8-anilino-1-naphthalenesulfonate), a similar hydrophobic probe similar to bis-ANS had no inhibitory effect on FtsZ polymerization. Since tubulin assembly has been shown to be inhibited by bis-ANS, but not by ANS, it supports the idea that FtsZ and tubulin share similar conformational properties. Ca^{2+}, which promotes GTP-dependant FtsZ assembly, stimulated binding of bis-ANS or ANS to FtsZ, suggesting that Ca^{2+} binding induces changes in the hydrophobic conformation of the protein. Interestingly, depletion of bound Ca^{2+} with EGTA further enhanced bis-ANS fluorescence. These findings suggest that both binding and dissociation of $Ca^{2+}$are capable of inducing FtsZ conformational changes and these changes could promote the GTP-dependant assembly of FtsZ. (Yu and Margolin, 1999).

GTP binds to the minus end of an FtsZ monomer and upon hydrolysis binds another FtsZ monomer at its minus end. SulA is an inhibitor that is capable of binding to the FtsZ at its GTP binding site and thus preventing GTP to bind, thereby inhibiting FtsZ polymerization.

2.12 Mycobacterial cell division

DNA replication and chromosomal segregation in *E. coli*have been thoroughly reviewed by Bartosik and Jagura-Burdzy. The basic mechanisms appear to be similar for mycobacteria.

Replication begins at a specific stage of the cell cycle,stage C, which then progresses to cell division at stage D and then through stage G1, which encompasses the time after cell division to DNA replication. Typically, the majority of bacterial DNA is contained within a single, circular chromosome, while the remainder exists in small plasmids. However, *Streptomyces* spp. contains one large linear chromosome. The general steps of chromosomal replication occur as follows.

(i) The initiator protein, DnaA, binds to a defined site on the chromosome, known as *oriC*. Conditional depletion of DnaA in *M. smegmatis* blocks cell division, while

overexpression yields a loss of synchrony between DNA replication and division and thus multi-nucleoidal cells.

(ii) Histone-like proteins such as HU and integration host factor help unwind the DNA.

(iii) DnaC delivers DnaB, a helicase, to the unwound DNA.

(iv) DnaG, a primase, forms the initial primer and has been shown to be essential in *M. smegmatis* even though it resides in a different locus than in other bacteria.

(v) The replication machinery is sequentially loaded onto the unwound DNA, including the DNA polymerase holoenzyme, Pol III.

(vi) Replication proceeds bidirectionally from *oriC*, with two replication forks traveling in opposite directions. The replisomesare thought to be anchored near the cell center, with the DNA passing through the machinery.

(vii) The replication machinery arrives at the termination sequence, *ter*, directly opposite *oriC*, or halfway around the chromosome, and the machinery disassembles.

(viii) The replicated chromosomes are often linked and must be resolved prior to complete partitioning. In *E. coli*, this is usually accomplished by topoisomerase IV decatenating the DNA. FtsK, or SpoIIIE in *B. subtilis*, binds topoisomeraseIV and assists in decatenation. Also, dimers are resolved by the XerC and XerD site-specific recombinases .(Hett and Rubin, 2008).

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and cultures

Ampicillin (200 µg/ml),30% Acrylamide (29.2 g acrylamide, 0.8 g bis-acrylamide), 1 M Tris-HCl (pH 8.8, pH 6.8), SDS (10%), APS (10%), TEMED, 1 X SDS buffer were purchased from SIGMA, USA.

Cultures were available at Microbiology and Cell Biology Lab, Indian Institute of Science, Bangalore.

Table 3.1: List of bacterial strains used in the study

STRAIN	**RELEVANT GENOTYPE**	**REFERENCE**
E. coli C41	BL21(DE3)derivative Used for expression of toxic Proteins	J. E. Walker (Miroux and Walker, 1996)
E. coli JM109	[Δ (lac-pro) endA1 recA1 hsdR17 thirelA gyrA96 supE44/F′ lacI^{q} traD36 proAB + lacZΔM15]	Yanisch – Perron*et al.*, 1985

Table 3.2: List of plasmids used in the study

PLASMID	RELEVANT INFORMATION	REFERENCE
pET15b	Expression vector	NOVAGEN
pET15b-MsftsZ-Stop	N-terminal His-tagged *MsftsZ* gene expressed generated by cloning.	Prabuddha Gupta Doctoral thesis, 2009

3.2Competent cell (*E. coli* JM109and *E. coli* C41) preparation

Principle

The recipient cells are made competent to receive the corresponding DNA (pET15b-MsFtsZ-Stop) by calcium chloride method. $CaCl_2$dissociates into Ca^{2+} and Cl^-upon addition to the cells. The cations (Ca^{2+}) neutralize the negatively charged cell membrane (due to presence of phospholipids), thus making it neutral for entry of DNA.

Reagents

1. 0.1 M $CaCl_2$
2. LB medium

Procedure

Competent cells of *E. coli* JM109 and *E. coli* C41 were prepared using calcium chloride method. Cells were inoculated into 3 ml of LB medium from glycerol stock and incubated overnight at 37°C. One percent of the inoculum was inoculated into 100 ml of LB medium and incubated at 37°C with vigorous shaking until the O.D. at 600 nm read 0.6. The bacterial cells were transferred to polypropylene tubes and stored on ice for 10 min. The cells were harvested at 5000 rpm for 10 min at 4°C. The supernatant (medium) was decanted and

were recovered by centrifugation at 5000 rpm at 4°C for 10 min. The supernatant was decanted and 1 ml of ice-cold solution of 0.1 M $CaCl_2$ and10% glycerol was added to the pellet. The $CaCl_2$ treated cells were aliquoted and stored at -70°C for future use.

3.3 Transformation of *E. coli* JM109 and *E.coli* C41 cells with pEt15b-Msftsz-stop DNA

Principle

The pET15b-MsFtsZ-Stop DNA is incubated with the prepared competent cells. The DNA adheres to the cell surface. On giving heat shock to the mixture, the permeability of the cells increases and the adhered DNA enters into the cells. Thus, the cells are transformed with pET15b-MsFtsZ-Stop DNA.

Reagents

1. LB broth
2. LB agar containing ampicillin
3. 50 ng DNA (pET15b –MsftsZ-Stop)

Procedure

To 100 µl of competent cells ($CaCl_2$ treated), 50 ng of DNA (3 µl) was added and mixed gently by swirling and stored on ice for 20 min. The cells were subjected to heat shock at 42°C for 90 sec. LB broth (500 µl) was added to the cells and incubated at 37°C for 45 min. After incubation, the cells were plated onto LB agar containing the antibiotic ampicillin at a concentration of 20 mg/ml. The plates were then incubated at 37°C overnight. Transformed colonies that contain the DNA (pET15b-MsftsZ-Stop) and an ampicillin resistant gene grow on the medium.

3.4 Preparation of pEt15b-Msftsz-stop DNA from *E. coli*JM109 cells

Principle

The pEt15b-Msftsz-stop DNA is isolated by alkaline lysis method. Addition of NaOH increases the pH to alkalinity and SDS denatures plasmid and genomic DNA. Glacial acetic

acid is added to neutralise the pH and potassium acetate helps in renaturation of plasmid DNA. The genomic DNA is precipitated, thereby leaving the plasmid DNA intact.

Reagents

1. STE solution (0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0))
2. ALS I (50 mM glucose, 25 mM Tris-HCl (pH.8.0), 10 mM EDTA (pH 8.0))
3. ALS II (0.2 N NaOH; 1% SDS)
4. ALS III (5 M Potassium acetate; glacial aceticacid; H_2O)
5. Isopropanol
6. Chloroform and Tris-HCl saturated Phenol (pH 8).
7. Sodium acetate-3M (pH 5.2)
8. 95% Ethanol and 70% Ethanol.

Procedure

DNA was isolated from the cells by alkaline lysis method (mini prep) from overnight culture of *E. coli* JM109. The cells were harvested at 5000 rpm for 6 min. To the cell pellet, ALS-I (100 µl) was added and vortexed until the pellet was completely resuspended. ALS-II (freshly prepared) (200 µl) was then added and immediately mixed by inverting the tubes 2-3 times. ALS-III (100 µl) was then added followed by immediate mixing by inverting 2-3 times, and kept on ice for 10 min. Centrifugation was done at 10,000 rpm for 10 min at 4°C. The supernatant (450 µl) was then transferred to a fresh tube. Exactly 0.6 volume of isopropanol (270 µl) was added to the supernatant and kept at -20°C for 30 min. The sample was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was decanted. The pellet was resuspended in 50 µl of TE buffer. RNase (1 µl of 20 µg/ml solution) was added and incubated at 37°C for 3 hrs. A phenol-chloroform (1:1) mixture was added to the RNase-treated sample and spun at 10,000 rpm for 10 min at 4°C. The upper layer was carefully transferred to a fresh tube and 200 µl of chloroform was added to the new tube, followed by vortexing for a min and centrifuging for 5 min at 10,000 rpm. The upper layer was transferred to a new tube to which 3 M sodium acetate ($1/10^{th}$ volume *i.e.*, 19 µl) and 2.5 volume of 95% ethanol (475 µl) was added. Mixed well and kept at -20°C for 12 hrs. The sample was centrifuged at 10,000 rpm for 10 min. The pellet obtained after centrifugation was washed with 1ml of 70% ethanol and centrifuged at 10,000 rpm for 2 min. The supernatant was

3.5Expression of MsFtsZ-stop protein

Principle

The FtsZ protein is expressed by inducing with IPTG. IPTG can induce expression of proteins that are regulated by the lac operon. pEt15b contains lac operon and hence IPTG induction can help in expression of the FtsZ protein.

Reagents

1. LB broth containing ampicillin
2. 0.5mM IPTG

Procedure

Five colonies from the LB amp plate were inoculated into different tubes with 3 ml of LB broth containing 20 mg/ml ampicillin and incubated at 37°C for 4-5 hrs. The culture was then inoculated into tubes containing 5 ml of LB broth containing 20 mg/ml ampicillin and incubated at 37°C until an O.D. of 0.6 at 600 nm was obtained. IPTG (0.5 mM) was added to induce the expression of protein and incubated at 30°C with shaking for 4 hrs. The protein expression was analysed on SDS-PAGE and the culture showing maximum expression was inoculated into 5 ml of LB broth containing 20 mg/ml ampicillin and incubated overnight at 37°C. From the overnight culture, 2% of inoculum was inoculated into 250 ml of LB medium containing 20 mg/ml of ampicillin and incubated at 37°C until an O.D. of 0.6 at 600 nm was obtained. IPTG (0.5 mM) was added and incubated at 30°C with shaking for 4 hrs. The cells were then harvested and stored at -70°C for future use.

3.6 Purification of MsFtsz-stop protein

Principle

The protein was purified usingNi^{2+} NTA-Agarose column. $Ni^{2+}$is a transition metal and is electrophilic. It binds the His-tag protein specifically with high affinity and does not bind other proteins. When elution buffer containing imidazole at high concentration is added to the column, Ni^{2+}binds imidazole with a higher affinity, thus enabling elution of the protein FtsZ.

The eluted protein was then dialysed. The principle of dialysis is diffusion, which is the process of movement of solute from a region of higher concentration to a region of lower concentration. The proteins other than FtsZ move across the dialysis bag leaving FtsZ within.

Reagents

1.Lysis buffer (1.67 X PBS, 1 mM PMSF, 2 mg/ml Lysozyme)

PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4

2. Ni^{2+} NTA-Agarose column

3. Wash buffer (1.67 X PBS, 1 mM PMSF, 20 mM imidazole)

4. Elution buffer (1.67 X PBS, 1 mM PMSF, 500 mM imidazole)

5. Dialysis buffer (50 mM HEPES-NaOH (pH 7.2), 1 mM DTT and 10 % glycerol)

6. Dialysis bag

Procedure

The cell pellet obtained after harvesting was dissolved in 30 ml of lysis buffer and kept on ice for 1 hr. Sonication was performed at 40% duty cycles for 30 sec pulse. The procedure was repeated 4-5 times to separate the cells until a clarified solution was observed. Centrifuged at 4°C for 20 min at 10,000 rpm and the supernatant was transferred to a fresh sterile tube.

Exactly 0.5 ml of Ni^{2+}NTA Agarose slurry was equilibrated with lysis buffer. The pre-equilibrated Ni^{2+}-NTA agarose was mixed with the supernatant and kept on rotatory platform for 1 hr at 4°C. After spinning, the sample was loaded to the column and allowed to run out. Exactly 30 ml of wash buffer was added into the column and allowed to run out. Exactly 2 ml of elution buffer was added to the column and the column was kept locked for 30 min, and subsequently, the sample was eluted. Four fractions, each containing 0.5 ml, were collected. The collected fractions were kept in ice and stored.

Exactly 20 µl of each of the collected fractions (flow through, wash out, 4 eluates) was mixed with 5 µl of 5 X SDS gel loading dye and loaded on 10% PAGE. The profile revealed the fractions containing the eluted protein. All those eluates were pooled and added to the dialysis bag. The dialysis bag was placed inside the dialysis buffer and kept at 4°C on magnetic stirring apparatus. The dialysis buffer was changed once in 3 hrs, 4 times. The purified protein was loaded on SDS-PAGE. The sample was diluted into two ratios – 1:5 and

1:10 and loaded on SDS-PAGE along with different concentrations of BSA (1 μg/μl, 2 μg/μl, 5 μg/μl and 10 μg/μl).

3.7 Concentration of MsFtsZ-stop protein

Principle

The protein was concentrated using a concentrator. Removal of water molecules from the protein occurs in a concentrator by freezing the water molecules and collecting the concentrated protein.

Procedure

Since the concentration of the protein sample obtained from dialysis was low, the sample was added into the concentrator. The concentrator was placed in the cooling centrifuge and centrifuged until the volume of the sample reached 200 μl. The protein was transferred into an eppendorf tube and kept on ice. The protein was then loaded onto SDS gel along with different concentrations of BSA (2.5 μg/μl, 5 μg/μl, 7.5 μg/μl and 10 μg/μl). The gel was analysed in order to determine the concentration of the protein.

3.8 90° Light scattering assay

Principle

The protein MsFtsZ-stop was analysed in a spetrofluorimeter. Light is passed on the sample and the scattered light is analysed. Unpolymerised MsFtsZ-stop does not scatter light, whereas polymerised MsFtsZ-stop shows four-fold increase in light scattering. On analysis, it can be determined whether polymerization occurs or not.

Reagents

1.Polymerisation buffer-5X (50 mM MES NaOH (pH 6.5) buffer containing 50 mM KCl, 5 mM $MgCl_2$)

2. 10 mM GTP.

Procedure

90° LS assay (Mukherjee and Lutkenhaus, 1999) was carried out in MES-NaOH (pH 6.5) buffer. The protein concentration taken was 10 μM. The volume of the reaction mixture

respectively. Excitation and emission slit widths used were 1 nm and 2.5 nm, respectively. The reaction mixture was initially allowed to stay in the fluorimeter for 200 seconds to get a baseline. After 200 seconds, the reaction was started by addition of 1 mM GTP. Readings were recorded for every 40 seconds for an additional period of 640 seconds at 30°C.

3.9GTPase assay

Principle

Polymerization of MsFtsZ-stop occurs by binding and hydrolysis of GTP into GDP and Pi. So, the detection of inorganic phosphate that gets releases will give information about the polymerization pattern.

Reagents

1. Perchloric acid
2. HMK buffer-2X (50 mM HEPES buffer pH 7.7, 5 mM Mg(OAc) and 350 mM K(OAc))
3. Malachite green mixture (8.3 mM Sodium molybdate, 0.78 mM Malachite green, 0.05% Triton X-100, 0.7 M hydrochloric acid)
4. 10 mM GTP
5. 100 μM Potassium dihydrogen phosphate.

Procedure

GTPase assay was carried out using malachite green assay (Reddick et al., 2005) in HMK buffer. The protein concentration taken was 10 μM. The reaction mixture was fixed at 400 μl. 60 μl of reaction mixture was taken in different tubes and incubated at different time points (0 min, 1 min, 2 min, 5 min, 10 min, and 20 min). Exactly 60 μl of the reaction mixture without GTP was kept as 0^{th} min. Exactly 1 mM GTP was added to all the other tubes and kept at 37°C for the corresponding time points.

Preparation of standards for GTPase assay:

Standards were prepared from 100 μM stock solution of potassium dihydrogen phosphate. The different concentrations of the standards prepared were 5 μM, 10 μM, 15 μM

20 μM, 25 μM, 30 μM, 35 μM, and 40 μM. The standards are used for determining the phosphate concentration of evolved phosphate from the hydrolysis.

Control samples:

C_1: HMK buffer (60 μl) + water (138 μl) + perchloric acid (198 μl).

C_2: HMK buffer (54 μl) + 1 mM GTP (6 μl) + water (138 μl) + perchloric acid (198 μl).

Malachite green mixture (396 μl) was added to the GTP added tubes, standards and controls. All the tubes were kept at room temperature for half an hour. The O.D. reading was then taken at 655 nm.

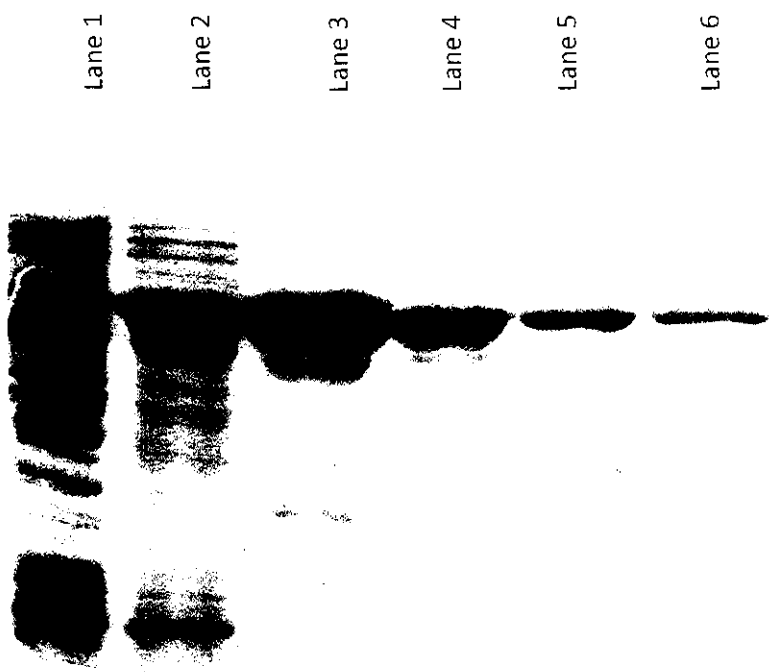
RESULTS AND DISCUSSION

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Eluted MsFtsZ-stopprotein

The protein MsFtsZ-stop was expressed by IPTG induction. It was observed that protein expression occurred and it was concluded after analysing the eluted MsFtsZ-stop protein from Ni^{2+} -NTA agarose affinity chromatography. The protein obtained from the column is relatively pure by the binding action of the His-tag attached to its C-terminal end. The profile of the eluted protein is shown in fig 4.1.

Fig. 4.1: Profile of eluted MsFtsZ-stop



Lane 1 – flow through

Lane 2 – wash out

Lane 3 – eluate fraction 1

Lane 4 – eluate fraction 2

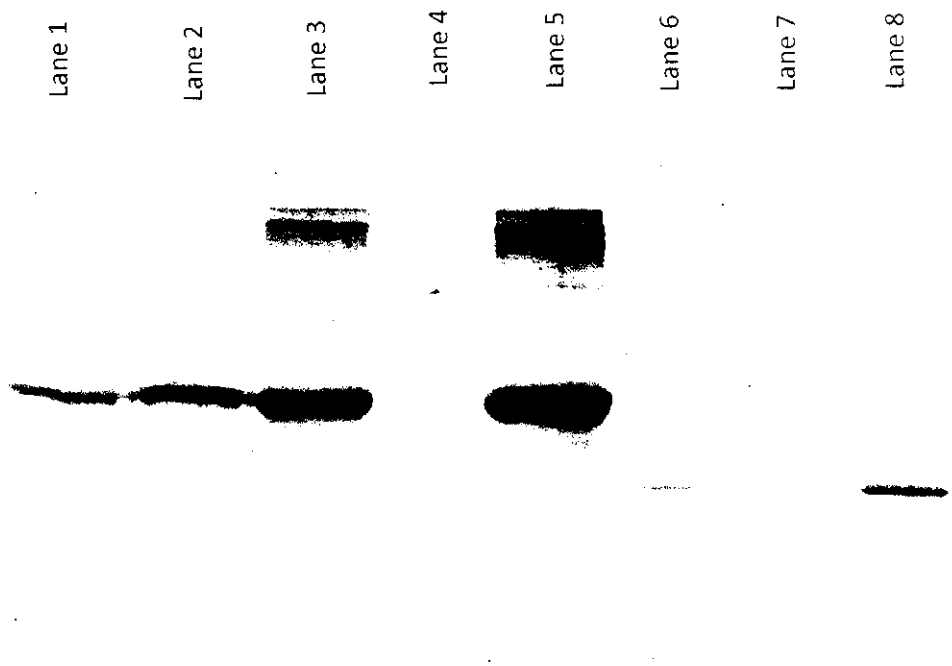
Lane 5 – eluate fraction 3

It can be seen from the profile that the eluate fractions of the MsFtsZ-stopare devoid of other protein impurities, whereas the initial flow through and wash out contain many other proteins.

4.2 Purified MsFtsZ-stop protein

The eluted protein obtainedfrom Ni^{2+} NTA agarose affinity chromatography was further purified by the phenomenon of dialysis. Dialysis purifies the protein by removing other proteins of varying molecular weight and retaining the MsFtsZ-stop. The MsFtsZ-stop protein obtained from dialysiswas analysed on SDS-PAGE at two different dilutions.The profile of the dialysed MsFtsZ-stop is shown in fig 4.2.

Fig. 4.2: Profile of purified MsFtsZ-stop



Lane 1 – BSA (1 µg/µl) Lane 2 – BSA (2 µg/µl)

Lane 3 – BSA (5 µg/µl) Lane 5 – BSA (10 µg/µl)

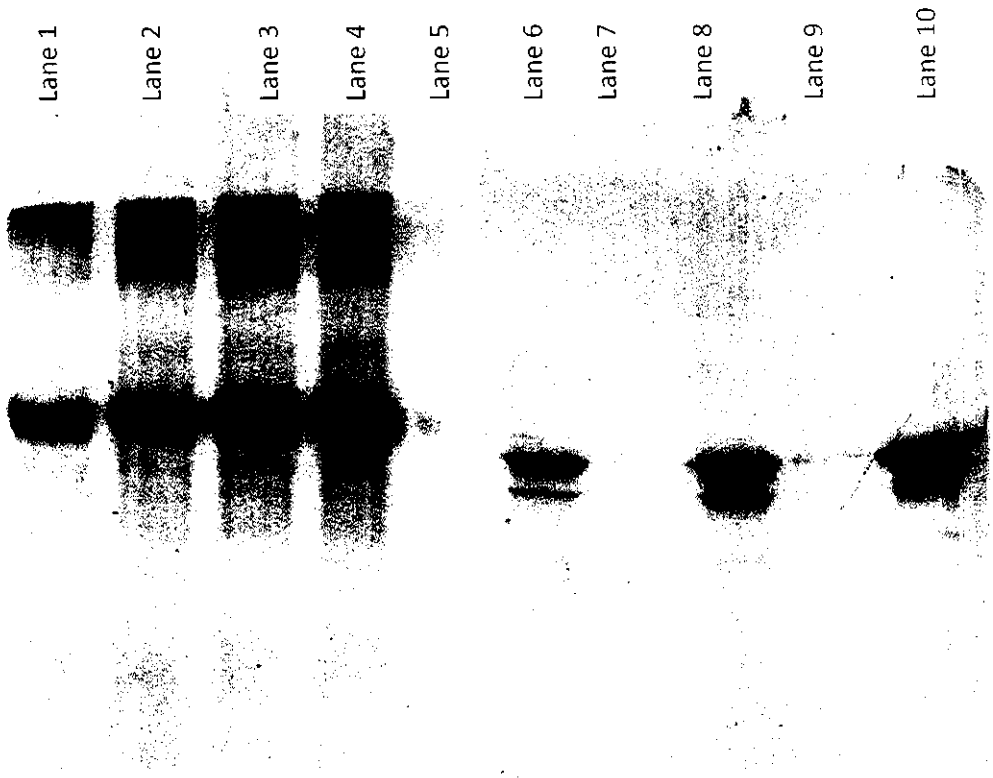
Lane 6 – MsFtsZ (1: 5 dilution) Lane 8 – MsFtsZ (1: 10 dilution)

On comparison of the concentration of the recombinant FtsZ protein with standard BSA concentration, it was found that the concentration of the protein was low.

4.3 Concentrated MsFtsZ-stop Protein

Since, the concentration of the protein was found to be low, it was concentrated. The concentrated protein was analysed on SDS-PAGE to check the concentration.

Fig 3.3: Profile of concentrated MsFtsZ-stop protein



Lane 1 – BSA (2.5 µg/µl)

Lane 2 – BSA (5 µg/µl)

Lane 3 – BSA (7.5 µg/µl)

Lane 4 – BSA (10 µg/µl)

Lane 6 – recombinant MsFtsZ (1:5 dilution)

Lane 8-recombinant MsFtsZ (1:7.5 dilution)

Lane 10 -recombinant MsFtsZ (1:10 dilution)

On comparison with BSA, the concentration of the recombinant protein *MsFtsZ* –stop was found to be 6 µg/µl.

4.4 90° Light Scattering Assay

90° LS assay was performed to observe the polymerization activity of the MsFtsZ-stop. As mentioned earlier, unpolymerised MsFtsZ-stop is unable to scatter light, since monomer units cannot scatter light. The polymerised MsFtsZ-stop, *i.e.*,after assembly of protofilaments, the protein has the ability to scatter light and the intensity of light scattering can be recorded with which conclusion can be made whether the protein has polymerised or not.

The sample was read under the spectra initially without GTP for 200 seconds. At the 200^{th} second, GTP was added to check the change in polymerization pattern. Until 200 seconds, it was observed that there was no occurrence of light scattering.In presence of 1 mM GTP, the MsFtsZ-stop (10 µM) showed polymerisation as indicated by four-fold increase of light scattering signal. The values recorded every 40 seconds are shown in Table 4.1 and the plot showing the polymerization pattern of both the samples with and without GTP is shown in Fig 4.4.

On the other hand, another samplewhen analysed without addition of GTP under the same spectra and conditions, did not show detectable polymerisation. The values recorded every 40 seconds for the reaction without GTP are shown in Table 4.2. The light scattering pattern of the sample without GTP served as the baseline for the MsFtsZ-stop polymerization.The result indicates that MsFtsZ-stop GTP is essential for its polymerization to occur. So, it can be concluded that the polymerization very much occurs in a GTP-dependent manner.

Fig 4.4: Comparison between the polymerisation of MsFtsZ-stop with and without GTP

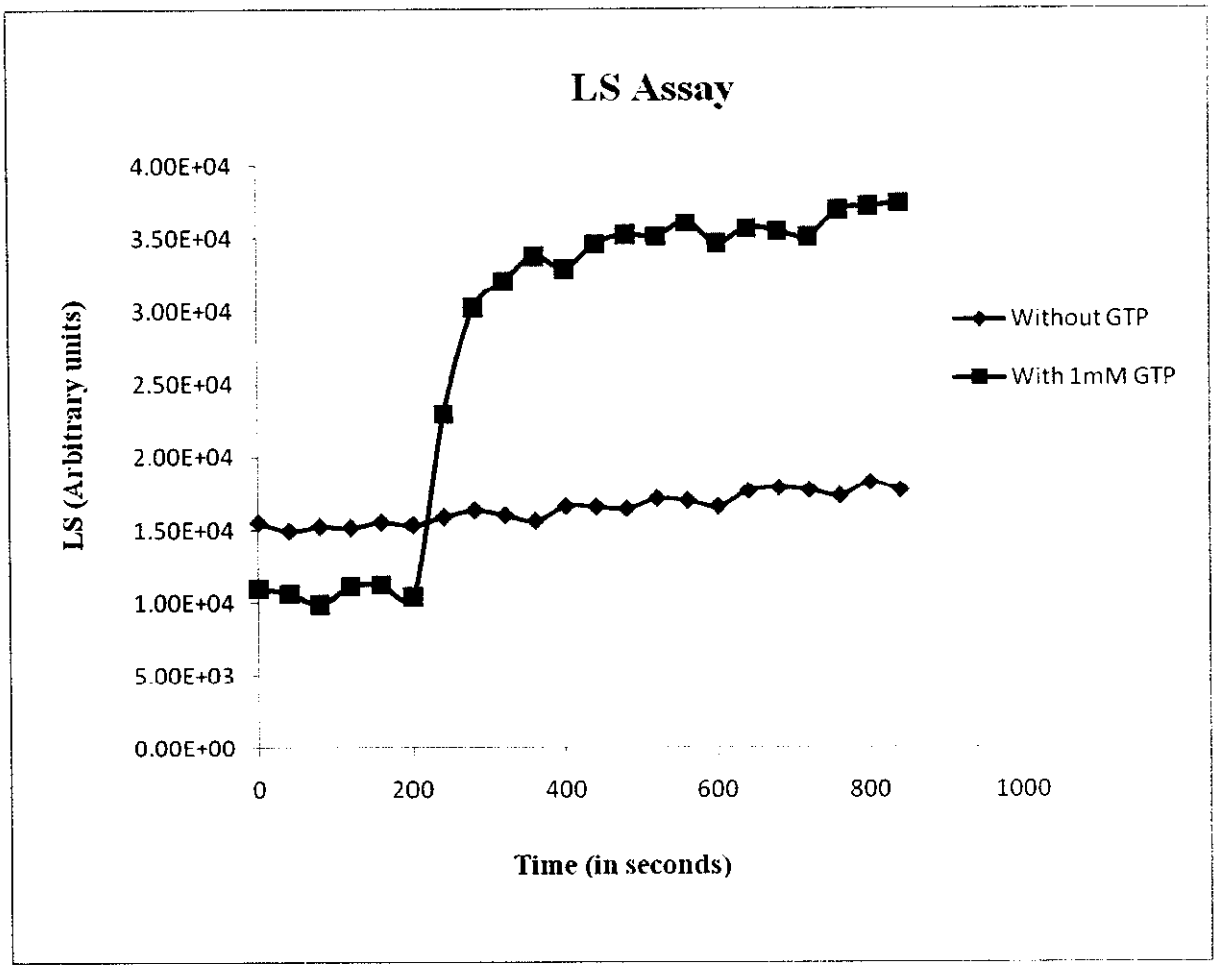


Table 4.1: The values of the LS assay obtained for the reaction with GTP

Time (in seconds)	Intensity($*10^4$CPS)
0	1.095
40	1.062
80	0.9890
120	1.111
160	1.12
200	1.041
240	2.294
280	3.026
320	3.207
360	3.377
400	3.292
440	3.459
480	3.524
520	3.51
560	3.607
600	3.463
640	3.569
680	3.547
720	3.507
760	3.694
800	3.719
840	3.743

Table 4.2: The values of LS assay obtained for the reaction without GTP

Time (in seconds)	Intensity($*10^4$CPS)
0	1.551
40	1.495
80	1.525
120	1.518
160	1.555
200	1.536
240	1.59
280	1.635
320	1.601
360	1.563
400	1.662
440	1.658
480	1.644
520	1.713
560	1.7
600	1.66
640	1.765
680	1.786
720	1.772
760	1.732
800	1.823
840	1.771

4.5 GTPase assay

GTPase assay is performed based on the principle of GTP hydrolysis for FtsZ polymerization. The concentration of phosphate evolved from hydrolysisis analysed from the assay and it correlated with the polymerization activity of the recombinant protein MsFtsZ-stop. The amount of phosphate released is directly proportional to the extent of polymerization that has occurred. The protein sample, after addition of GTP is incubated at different time points to determine the time point at which there is highest release of phosphate.

Malachite green mixture is used to assay the concentration of inorganic phosphate.Standard samples containingknown phosphate concentrations are prepared, assayed and read at 655 nm. Concentration of phosphate evolved from hydrolysis is found by comparison with the standard curve. The obtained concentrations are plotted against different time points and the plot is shown in Fig 4.5. The values of concentrations of phosphate evolved at different time points is shown in Table 4.3.

At the 0^{th} time point, no GTP was added and hence no polymerization occurred. This sample was taken as blank. GTPase activity was observed by the subsequent addition of 1 mM GTP to all other samples that are incubated at different time points.

MsFtsZ-stop showed GTPase activity such that 50.5 μM of phosphate equivalent of GTP was hydrolysed by 10 μM protein in 20 minutes.

Fig 4.5: The plot showing concentration of released phosphate at different time points.

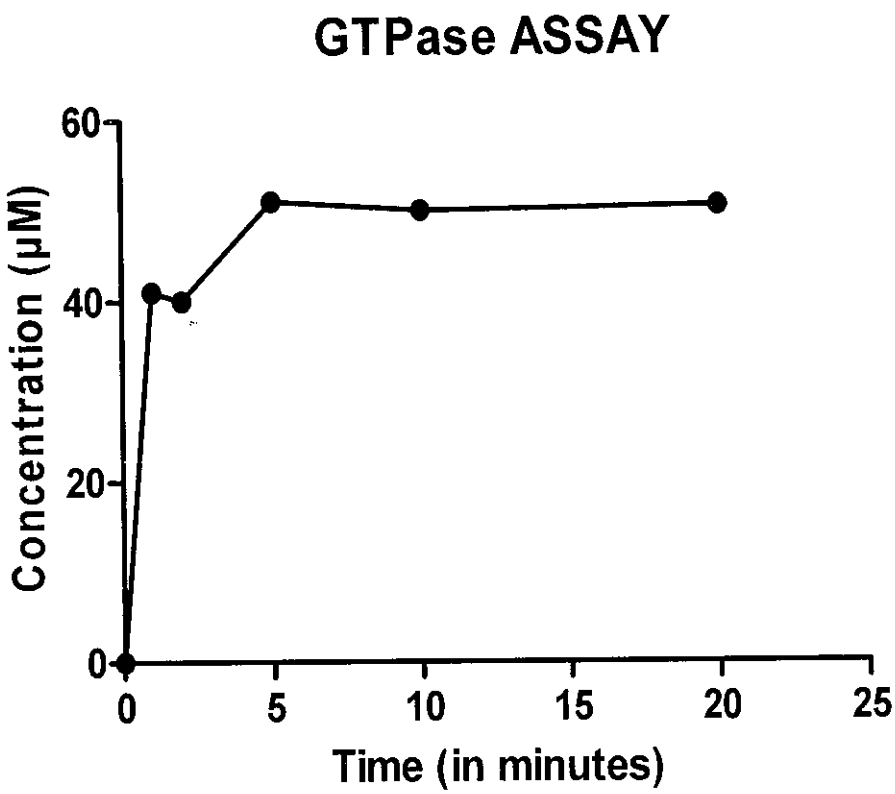


Table 4.3: The values of GTPase assay showing the concentration of phosphate released at different time points

TIME (min)	**CONCENTRATION (μM)**
0	0
1	41
2	40
5	51
10	50
20	50.5

CONCLUSION

CONCLUSION

On characterization of the cytokinetic protein FtsZ, it could be concluded that the protein is active and has polymerising activity. It is evident from the results that the FtsZ polymerization occurs in a GTP-dependant manner. Analysis from the Light Scattering assay plot revealed that without the presence of GTP, polymerization does not occur and the four-fold increase in light scattering indicates that polymerization of the protein has occurred after GTP addition. From the GTPase assay, it can be confirmed that the protein is a potent GTPase and hydrolyses GTP at a very fast rate into GDP and Pi. The GTPase plot drawn with concentration of phosphate against the different time points shows the time point at which there is release of highest amount of phosphate. The release of phosphate implies that there has been GTPase activity of the protein. Thus, the protein has been characterised of its GTP dependant polymerization activity and its GTPase activity.

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