Polymerization of the bacterial cell division protein FtsZ
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Chapter 1

Introduction

Bacterial Cell Division – get in the ring!
General introduction

Cell division is a crucial event in the life of every organism. Most bacteria and archaea divide symmetrically in a process that is subject to extensive regulation to ensure that both newly formed daughter cells contain a copy of the chromosome. Symmetric division, schematically depicted in Figure 1A, seems very simple yet is poorly understood on a molecular biological level. For the Gram-negative model organism *Escherichia coli*, numerous cell division genes were identified in *E. coli* temperature sensitive mutants cells that did not divide properly, and hence were called *fts* genes, for filamentation temperature sensitive. Currently, all identified cell division gene products have been localized at the site of cell division (18, 85, 92), but the function of most of these proteins is still elusive. By far the best-characterized cell division protein is FtsZ, which is the first to appear at the division site, where it forms a ring that localizes the other proteins. The ring formed by FtsZ is likely to consist of polymers that are similar to polymers formed by the eukaryotic homologue tubulin. The elucidation of the structures of both FtsZ and tubulin (77, 108) has greatly advanced the understanding of the polymerization of both proteins. In this introduction, the role of FtsZ in bacterial cell division and the biochemistry of FtsZ polymerization are discussed.

The role of FtsZ in cell division

In *E. coli*, *ftsZ* was identified as the first cell division gene to act during the division cycle (8, 88, 134). Now, FtsZ has been identified in most prokaryotic species studied to date, with the exception of *Chlamydiae*, a *Mycoplasma* species and crenarchaea (9, 92). FtsZ is conserved across the evolutionary divide, being essential for division of chloroplasts and mitochondria in some eukaryotes (7, 111). Groundbreaking work in the early 1990s provided important clues for FtsZ function. Immunolocalization demonstrated that in *E. coli*, FtsZ is present in a ring structure at the division site (12), which was later confirmed in other bacteria and archaea (73, 89, 112, 146). The FtsZ ring behaves dynamically during division and remains attached to the leading edge of the constricting septum (Fig. 1A) (131). The appearance of the FtsZ ring coincides with the termination of DNA replication, but a direct signal for ring assembly has not yet been identified (36). The ring structure prompted the suggestion that FtsZ is a cytoskeletal protein (12), which was augmented by the discovery that FtsZ displays a functional homology to the eukaryotic cytoskeletal protein tubulin: firstly, FtsZ was found to bind and hydrolyze GTP, sharing a nucleotide-binding motif with tubulin (34, 100, 117); secondly, FtsZ was found capable of nucleotide dependent assembly into filaments (19, 101).

FtsZ ring positioning

In *E. coli*, positioning of the ring at midcell is subject to various forms of regulation such as the *min* system (mutants give rise to minicells through division at the cell poles) and the position of the nucleoid. The *min* system consists of three proteins, MinC, MinD and MinE, which prevent cell division at the poles (reviewed in (119)). MinC and MinD act together as a negative regulator of FtsZ ring assembly, and in *E. coli* they oscillate together from cell pole to cell pole in a MinE dependent manner (62, 114, 115). MinE is present at the centre of the cell (113), where it oscillates as well (53) to repel MinCD and allow FtsZ ring assembly. MinCD prevents the correct interaction of FtsA with the FtsZ ring *in vivo* (69), and MinC has been shown to inhibit FtsZ polymerization *in vitro* (63) (discussed below). The *min* system is not strictly conserved among bacteria and archaea, suggesting the use of other topological regulators. The observation that cell division is inhibited in the vicinity of the nucleoid led to the postulation of the nucleoid occlusion model (156). This model states that the FtsZ ring is incapable of assembly in the vicinity of the nucleoid. Recent
work showing that FtsZ assembles before nucleoid separation (36), in the absence of the nucleoid and at fixed distances far away from or directly over nucleoids (28, 132), indicates that the position of the nucleoid is not the sole determinant for FtsZ spatial positioning. Most likely, negative signals from both the nucleoid and the min system co-operate, and are sufficient, to ensure proper FtsZ ring positioning (92).

**Targeting of other cell division proteins to the FtsZ ring**

Formation of the FtsZ ring is essential for correct localization of the division plane. A striking example is the ftsZ26 mutant, which contains a FtsZ spiral rather than a ring, giving rise to cells with spiral constrictions (6). After formation of the FtsZ ring all other cell division proteins localize to the division site in an ordered manner (Fig. 1B). The first two proteins that are recruited to the FtsZ ring are FtsA (5, 89) and ZipA (57, 75), followed by FtsW (143) and FtsK (144, 163), FtsQ (20, 27), FtsL (56), FtsI (143, 153, 154) and FtsN (3). All proteins are essential in *E. coli*.

Functional characterization of the cell division proteins mentioned above is far from complete (Table 1). FtsA and ZipA (for FtsZ interacting protein A) that interact directly with FtsZ will be discussed in more detail below. FtsW is a membrane protein, which is thought to stabilize the FtsZ ring in an unknown manner (16, 70). FtsK is also a membrane protein, consisting of an N-terminal membrane domain that is essential for localization (45, 163) and a large cytoplasmic C-terminal domain that is involved in the coupling of cell division to the completion of chromosome segregation, preventing chromosome scission upon septum closure (129, 164). FtsK is similar to SpoIIIE, a protein responsible for correct chromosome partitioning during *Bacillus subtilis* sporulation (127). FtsQ, FtsL, FtsN and FtsI are all bitopic proteins.

![Figure 1. A schematic depiction of cell division and the division apparatus in *Escherichia coli*. A) A model for division. The outline of the cell is indicated as a black line, the nucleoid as a grey oval, and the FtsZ ring as a grey ring. As cells grow, DNA is duplicated. Near the termination of DNA replication, the FtsZ ring assembles at midcell. Chromosome separation proceeds and all cell division proteins localize to the division site in an ordered manner (see text). After chromosome separation the division ring constricts and a septum is formed. Cell separation leads to the birth of two new cells. B) A model of a cross section of the cell at the division site. OM: Outer membrane; PG: Peptidoglycan layer; CM: Cytoplasmic membrane. The FtsZ ring is tethered to the cytoplasmic membrane via ZipA, FtsA is critical for the localization of other components. FtsI is actively involved in septal peptidoglycan synthesis.](image-url)
proteins with a short cytoplasmic domain, a transmembrane segment and a large periplasmic domain. The function of FtsN, FtsL and FtsQ is unknown. In *B. subtilis* a role for FtsL in regulation of the assembly of the division machinery has been proposed, with the FtsQ homologue DivIB acting as a chaperone for FtsL (32). FtsI (also known as PBP3) is a transpeptidase involved in synthesis of septal peptidoglycan (1). During division, the orientation of cell wall synthesis changes from parallel to perpendicular to the membrane. This requires FtsI as well as additional peptidoglycan synthetases and -hydrolyases, which can act together in multi-enzyme complex. This complex allows peptidoglycan synthesis according to the three-for-one model as proposed by Höltje (61). The other periplasmic cell division proteins have been proposed to be involved in septal peptidoglycan synthesis, since *ftsZ* is the only conserved cell division gene in organisms that lack a cell wall (92).

The metalloprotease FtsH, not a specific cell division protein in *E. coli*, was found to localize to vegetative and sporulation division septa in *B. subtilis* (151).

### Structural similarities between FtsZ and tubulin

**FtsZ polymerizes in vitro into polymers that resemble tubulin protofilaments**

FtsZ has the capacity to polymerize *in vitro* into polymers that strongly resemble tubulin protofilaments (19, 51, 101). Like tubulin, FtsZ (40.3 kDa) is a GTPase and contains the tubulin signature motif GGGTGS/TG (34). The similarity between the proteins is therefore evident, despite the low overall sequence homology (47). The polymerization behaviour prompted investigations into FtsZ self-association and polymerization to probe the similarities between FtsZ and tubulin. To describe the structural similarities between FtsZ and tubulin, the oligomeric state of FtsZ in

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CM: Cytoplasmic membrane. References in text

\( ^a \) the membrane anchor consists of a single N-terminal transmembrane segment.

\( ^b \) unconfirmed, putative function.
solution, the morphology of FtsZ polymers, and the atomic structures of FtsZ and the αβ-tubulin heterodimer will be discussed.

**Multimeric state of FtsZ in vitro and in vivo**

After purification FtsZ is present as a mixture of various multimeric association states (34), that retain 0.5 – 0.7 mol GDP per mol FtsZ ((117, 128), chapter 5). Analytical ultracentrifugation revealed that FtsZ self-association *in vitro* is concentration dependent, and shifted to the monomeric state by the addition of GDP (120, 128). The presence of Mg$^{2+}$ induces FtsZ self-association with binding of one Mg$^{2+}$ per FtsZ monomer, similar to tubulin (120).

Gel filtration of *E. coli* cytosolic extracts revealed that *in vivo*, about 75% of FtsZ is present as multimers of over 25 FtsZ subunits (69). The elution patterns of cytosolic FtsZ were concentration and temperature dependent, indicative of self-association/cold-sensitive polymerization (69). Both a λ-hybrid assay (39) and the yeast two-hybrid system (with *Bacillus subtilis* FtsZ) (145) indicated FtsZ self-association *in vivo* as well. The interaction domain is between amino acid residues 175 and 326 (100-323 for *B. subtilis* FtsZ (145)), with a role for the extreme C-terminal domain of FtsZ (39).

**Morphology of FtsZ polymers**

FtsZ polymers can form a variety of structures, depending on the polymerization conditions applied (figure 2)(101) (19, 51, 79-81, 102, 104, 137, 160)(this thesis). With GTP, FtsZ assembles into thin protofilaments that are capable of assembling further into filament sheets (figure 2)(80, 102). The dynamic assembly of polymers *in vitro* is coupled to GTP consumption: when GTP runs out.
polymers disassemble (102, 104). Assembly of filaments into polymer sheets and bundles is stimulated by millimolar amounts of Mg$^{2+}$ (104) and, more so, Ca$^{2+}$ (160) (78, 104)(chapter 2), or the poly-cation DEAE-dextran (51). The Ca$^{2+}$-induced sheets are composed of an antiparallel arrangement of double protofilaments (78). Interestingly, bundles of FtsZ filaments were also reported when FtsZ was polymerized in the presence of the FtsZ binding protein ZipA (59, 116). FtsZ GTPase activity is reduced at conditions that induce bundling, resulting in prolonged polymer presence (104) (104)(chapters 2, 4, Piet de Boer, personal communication).

FtsZ polymers formed with GTP or GDP assemble into minirings when adsorbed to a lipid monolayer (51). Polymers formed in the presence of GDP are generally more curved than polymers formed with GTP (80)(figure 2). Helical tubules of FtsZ polymers were reported after polymerization with GDP or GTP (but not nonhydrolysable GMP-CPP) in the presence of DEAE-dextran (80, 137). Similar tubes were found with FtsZ1 from M. jannaschii. Polymerization with GTP and Ca$^{2+}$ induces the formation of “closed” tubes consisting of six helically twisted, antiparallel arranged pairs of protofilaments. With GMP-CPP and Mg$^{2+}$ “open” tubes were identified, made up of three groups of four protofilament pairs, which are helically twisted around each other leaving space that appears to be filled up by additional filaments (79). A major difference between these tubes and microtubules is the fact that microtubules consist of 13 to 14 straight protofilaments, whereas these tubes consist of curved protofilaments (79). Finally, cation-induced polymerization without added nucleotide has been observed for mutants of the critical amino acid residue Asp212. Polymerization was enabled by GTP retention during purification (chapter 5).

![Figure 3](image_url)

**Figure 3.** Structures of the α,β-tubulin heterodimer (PDB entry 1TUB, 108) and FtsZ (PDB entry 1FSZ, 77). Some of the secondary structure elements discussed in the text are assigned for FtsZ according to the unifying secondary structure assignment described by Nogales et al. (106).
**FtsZ and tubulin structures**

The simultaneous elucidation of the structures of FtsZ from the thermophilic archaeon *Methanococcus jannaschii* (77) and the αβ tubulin heterodimer from bovine brain (108) provided conclusive evidence for FtsZ and tubulin homology (figure 3). FtsZ and tubulin form a distinct class of GTPases and a common nomenclature for their secondary structure elements has been developed (106). Both proteins consist of two domains formed by a β-sheet surrounded by α-helices, connected via a long “core” helix H7. The N-terminal domain (app. 200 amino acids) is involved in nucleotide binding and has a Rossmann-fold topology (122) common to many NTPases. An extra helix H0 at the start of the N-terminal domain of FtsZ protrudes from the structure and is absent in tubulin. Nucleotide binding involves many regions in the N-terminal domain. The T1-loop contacts the guanine base and the phosphates, while the T2-loop contacts the β- and γ-phosphates (only the α-tubulin structure contains GTP, both FtsZ and β-tubulin structures contain GDP) and contains conserved Asn and Asp residues (in *E. coli* FtsZ, N43 and D45, respectively). The Asp residue may be involved in the co-ordination of the Mg²⁺ ion at the nucleotide-binding site. Loop T3 contacts the γ-phosphate in α-tubulin. A recent molecular dynamics simulation has identified a conformational change of the T3-loop induced by the nucleotide γ-phosphate, which was confirmed by fluorescence spectroscopy (41). Loop T4 contains the tubulin signature motif GGGTGS/TG that is completely conserved among tubulin and FtsZ, and interacts with the α- and β-phosphates. The T5-loop shows no homology between tubulin and FtsZ except for one conserved Pro residue that contacts the ribose. In tubulin, the T5-loop also interacts with H11 (absent in FtsZ) and the following tubulin monomer in the structure. In FtsZ, the T5-loop contains a conserved stretch of positively charged residues at its end, with an Arg residue positioned similar to other GTPases (106). The guanine base is bound via hydrogen bonds to a conserved Asn in loop T6, an Asn in H7 (Asp in *M. jannaschii* FtsZ1) and a hydrophobic contact with a conserved aromatic residue (Phe182 in *E. coli* FtsZ).

The structure of the αβ-tubulin dimer showed the involvement of loop T7 and the start of helix 8 in forming nucleotide contacts with the nucleotide bound to the other monomer, which was also apparent when the tubulin dimer structure was docked onto a low resolution structure of the microtubule (MT) (107). For FtsZ a similar interaction was postulated based on a molecular modelling of its crystal structure in reconstructed electron micrographs of FtsZ protofilaments (78). This longitudinal contact is conserved between FtsZ and tubulin, and a wealth of biochemical data (discussed below) supports the interaction of the T7-loop/H8 region with the guanine nucleotide on the adjacent subunit (see chapter 4, Fig. 1). Positioning the nucleotide at the monomer interface formed during tubulin polymerization makes sense since it prevents nucleotide exchange in the microtubule, which fits the dynamic instability model for tubulin polymerization (this model is discussed below)(37, 106). In GTP-γ-S stabilized FtsZ polymers nucleotide exchange is blocked as well (chapter 3).

A comparison of the lateral interactions found in the high resolution model of the microtubule with the modelled FtsZ filaments revealed no similarities (78, 107). A possible dimer contact between two S3 sheets in two parallel protofilaments was observed for FtsZ. This contact may be calcium dependent, since it has only been observed in these calcium-induced sheets (78). The variety of possible arrangements of FtsZ protofilaments (discussed above) suggests that FtsZ may use different lateral contacts to form different types of polymers.

The C-terminal domains of FtsZ and tubulin are smaller than the N-terminal domain and consist of a four-stranded β-sheet surrounded by three helices. Following this part of the C-terminal domain there is a large difference between FtsZ and tubulin: in FtsZ the domain is
followed by two short β-hairpins (S11 and S12), whereas in tubulin it is followed by two long helices (H11 and H12). The highly acidic extreme C-termini of both tubulin and FtsZ were disordered in the crystals, so their structure has not been determined. The C-terminal tail of FtsZ is highly conserved (50) and mediates its interaction with FtsA and ZipA (discussed below). The most conserved motif in the FtsZ C-terminus is a central Pro-X-[Phe/Hydrophobic residue] motif. Co-crystallization of the C-terminal 17 residues of E. coli FtsZ with the C-terminal domain of E. coli ZipA revealed a 30 Å long peptide that binds as an extended β-strand (residues 367-373), followed by an α-helix (residues 374-383) in a shallow, hydrophobic, solvent-exposed cavity that runs along the width of the ZipA C-terminal domain (97). A major portion of this FtsZ fragment is in contact with ZipA residues mainly through hydrophobic interactions, but also via two hydrogen bonds (97). Interestingly, the highly conserved Pro residue of the C-tail of FtsZ does not contact ZipA.

**Dynamics of the FtsZ and tubulin polymers**

Microtubule dynamics: the dynamic instability model and the GTP cap.

The study of microtubule polymerization dynamics has benefited enormously from the ability to use light microscopy for real time analysis of *in vitro* and *in vivo* microtubule (MT) dynamics. The dynamic instability model for MT (de)polymerization, postulated on the basis of the length distribution in MT populations (96) was confirmed by differential interference contrast (DIC) video microscopy of individual MTs (142)(reviewed in ref. 37). The dynamic instability model postulates that single MTs never reach a steady state length but undergo phases of polymerization and depolymerization, which interconvert infrequently. MTs are polar polymers that contain a fast growing plus end and a slow growing minus end, with the β-subunit of the tubulin dimer oriented towards the plus end and the α-subunit towards the minus end. During polymerization, GTP-containing tubulin subunits add onto the polymer ends. GTP hydrolysis occurs upon polymerization or shortly afterwards. Polymerization with GMP-CPP resulted in highly stable MTs, showing that MT polymerization does not require GTP hydrolysis, but that GTP hydrolysis is essential for depolymerization and dynamic instability (65). Loss of the GTP γ-phosphate after hydrolysis would result in a less stable MT through weakening of the lateral contacts of the adjacent protofilaments (brought about by a conformational change transduced via displacement of the T3-loop) (105, 107). Combining the fact that GTP hydrolysis occurs very fast upon addition of tubulin subunits to MTs, and that MTs made up of GDP tubulin are highly unstable prompted the question how MTs are stabilized. In the original paper describing dynamic instability, the notion of a GTP-cap was coined, postulating that MTs are stabilized at their ends by tubulin subunits that contain GTP, with GTP hydrolysis lagging behind polymerization (96). Evidence that such a cap exists was obtained by severing MTs, which resulted in exposure of new, uncapped, plus ends that rapidly depolymerized and minus ends that did not depolymerize (141). Study of the minimal amount of GTP-tubulin required to function as a GTP-cap revealed that a single layer of GTP-tubulin (not GDP:P i tubulin) incorporated at the MT end (13-14 subunits) is sufficient to stabilize the MT (22, 23, 46). MT depolymerization would be the result of the loss of one of the microtubule subunits at the minimal cap.

**FtsZ dynamics: I. GTP hydrolysis**

The discovery that FtsZ is a GTPase (34, 100, 117) coupled to the observation that FtsZ forms guanine nucleotide dependent polymers prompted numerous studies on the relation between FtsZ polymerization and GTP hydrolysis activity. The GTP hydrolysis activity of FtsZ is dependent on Mg$$^{2+}$$ and stimulated by
the presence of KCl (100). Two papers described a lag in the GTPase activity that could be repressed by increasing the FtsZ concentration (34, 100). The protein concentration dependent GTP hydrolysis displayed by FtsZ from *E. coli* (81, 128), *B. subtilis* (146), *Haloflexs volanic*ii (147), *Thermotoga maritima*, *Azotobacter vinelandii* (81) and *Mycobacterium tuberculosis* (155) provided evidence for a self-activation mechanism of FtsZ GTPase. The GTPase activity was found to be stimulated at concentrations of FtsZ that allowed polymer assembly, similar to tubulin (33). Self-activation would imply GTP hydrolysis in an active site that is shared by two monomers, a model that is supported by the structural data on FtsZ (above) and mutagenesis studies. All mutations that are reported to affect FtsZ GTPase activity map at positions that were identified as part of the nucleotide binding domain based on the FtsZ crystal structure (77, 106) or in the T7-loop which contacts another FtsZ monomer in protofilaments. These mutations include changes of residues N43 and D45 (T2-loop) (145), G105 (loop T4) (117, 118), N165 (loop T6) and residues M206 (chapter 4), N207 (145) (chapter 4), D209 (chapter 4) and D212 (137) (10)(chapter 4) in loop T7. The G105S mutation, present in the temperature sensitive *ftsZ84* mutant, alters the FtsZ nucleotide specificity, converting FtsZ to an ATPase (118). Mutations in loop T7 that were found to inhibit FtsZ GTP hydrolysis activity also suppress the activity of wildtype FtsZ in mixtures of wildtype and mutant protein (chapter 4) indicating that the association of FtsZ monomers is required for GTP hydrolysis. The D212 residue is critical for cation binding at the nucleotide-binding site (chapter 5). Self-activation prompted the suggestion that the well-conserved R214 at the start of helix 8 acts as an “arginine-finger” that enhances GTPase activity, but this seems not to be the case (128). A possible role for the positive charge at this position could be the coordination of the γ-phosphate release after GTP hydrolysis (chapter 4).

Some of the mutations that severely affect GTPase activity do not inhibit the formation of a functional FtsZ ring, such as the *ftsZ84* mutation (4) and the *ftsZ2* mutation (13), suggesting that the capacity for high GTP hydrolysis rates is not essential for FtsZ function.

**FtsZ dynamics: II. Polymer formation and turnover.**

Following the discovery of FtsZ polymerization two methods have been used to study the dynamics of this process: sedimentation and light scattering (light microscopy cannot be used to visualize individual FtsZ protofilaments, chapter 2). Sedimentation, first described by Bramhill and Thompson (19) allows the quantification of amount of protein present as a polymer. A disadvantage of the method is that there is always a lag time of about 10 min due to the time it takes to spin down the samples. Light scattering, described in the same paper, but later refined by Mukherjee and Lutkenhaus (104), allows real time detection of polymers, but does not give a good indication of the amount of protein present as polymers. In the first paper that specifically addressed FtsZ polymerization dynamics, sedimentation was used to show that FtsZ polymerizes above a critical protein concentration (102) that corresponds to the concentration required for GTP hydrolysis (81). The close relation between GTP hydrolysis and polymerization was also evident from the observation that the presence of FtsZ polymers in solution is coupled to GTP hydrolysis, with polymer loss coinciding with GTP depletion (102). Mg^{2+} is not required for polymerization, but is required for the dynamic behaviour of FtsZ polymers since GTP hydrolysis is Mg^{2+}-dependent (104). Factors that influence GTP hydrolysis, such as the concentrations of Mg^{2+}, Ca^{2+} and KCl in the polymerization buffer, directly affect the polymer stability ((102, 104, 160), chapter 2).

The coupling of GTP hydrolysis to polymerization raises the question whether the FtsZ polymer contains GTP or GDP. This is
still a matter of debate. Erickson and co-workers have stated that GTP hydrolysis is coupled to a conformational change of straight to curved polymers that might drive ring constriction (80). Recently, they described isodesmic assembly of single FtsZ protofilaments of ten to one hundred subunits with GTP and GMP-CPP (121) without self-activation. This led to the suggestion of a model of polymerization with slow, lagging, GTP hydrolysis that occurs gradually throughout the FtsZ polymer (121). However, this study was performed at conditions of limited GTPase activity. Although isodesmic polymerization might account for the first steps of association of a FtsZ filament, it is very likely that lateral contacts are required for the formation of stable polymers, which would lead to a model of cooperative assembly that better reflects the self-activation described above. The self-activation of GTP hydrolysis would suggest a similar mechanism as described for tubulin, with GTP hydrolysis coupled to the addition of subunits to the polymer. The observation that FtsZ incubated with [α-32P]-GTP rapidly hydrolyzes GTP when cations are present supports the self-activation model (chapter 3). Analysis of the nucleotide content of polymerized FtsZ revealed GDP as the predominantly bound nucleotide. At limiting GTP concentrations and using conditions that slow down FtsZ dynamics, FtsZ polymers can be stabilized with GTP-γ-S, which does not exchange the polymer associated GDP (chapter 3). This finding suggests that the FtsZ polymer, like the microtubule, is stabilized by a small fraction of GTP containing subunits, probably a GTP-cap. More evidence was obtained using [γ-32P]-GTP, which confirmed the rapid hydrolysis but also showed that after GTP hydrolysis, P_i remains bound to FtsZ (D.-J.S. and A.J.M.D., unpublished), making it likely that the FtsZ polymer in fact contains GDP::P_i and that P_i release may play a critical role in polymer destabilization.

**FtsZ inhibitors**

The homology between tubulin and FtsZ prompted the search for tubulin inhibitors that also inhibit FtsZ. *In vivo*, tubulin assembly inhibitors thiabendazole and 2-methylbenzimidazole caused cell elongation in *E. coli* and cyanobacteria similar to ΔftsZ mutants, suggesting an effect on FtsZ (125). For *B. subtilis*, a similar effect was found for the ADP-ribosyltransferase inhibitor 3-methoxybenzamide (110). *In vitro*, the common tubulin inhibitors colchicine, colcemid, benomyl and vinblastine do not inhibit *E. coli* FtsZ assembly in the presence of Ca^{2+}, whereas the hydrophobic fluorescent probe 5,5’-bis-[8-anilino-1-naphtalenesulfonate] (bis-ANS) does (161). FtsZ contains one high affinity and multiple low affinity binding sites for bis-ANS suggesting a similar binding mechanism as tubulin. Binding of bis-ANS is thought to distort intermolecular FtsZ-FtsZ interactions, thereby preventing polymerization (161). The tubulin inhibitor vinblastine can induce a tubulin-FtsZ interaction when FtsZ is heterologously expressed in Chinese hamster ovary (CHO) cells, but does not induce copolymerization of FtsZ and tubulin (162). Finally, *Mycobacterium tuberculosis* FtsZ is inhibited by colchicine and ethyl 6-amino-2,3-dihydro-4-phenyl-1H-pyrido[4,3-β][1,4]diazepin-8-ylcarbamate (SRI 7614) which binds to the colchicine binding site of pig tubulin (155). Clearly more studies are required to identify universal FtsZ inhibitors.
Proteins that interact with FtsZ

SulA

Damage to cellular DNA in *E. coli* leads to the induction of the SOS-response that inhibits cell division (reviewed in (139)). Production of the SOS response protein SulA results in inhibition of cell division and filamentation. Mutations that conferred resistance to SulA were found to map in the *ftsZ* gene (67, 87), and overexpression of FtsZ resulted in SulA resistance as well (86), indicating that FtsZ is the target of SulA. Induction of SulA inhibits formation of the FtsZ ring (14), and a direct interaction of SulA with FtsZ was observed using the yeast two-hybrid system (64). *In vitro*, SulA inhibits FtsZ GTPase and polymerization activity (99, 137). An inactive SulA mutant did not inhibit FtsZ activity (99) and SulA did not inhibit the polymerization activity of two SulA resistant FtsZ mutants (99, 137). SulA overproduction in *E. coli* blocks FtsZ multimerization *in vivo*, with most FtsZ present in a possible FtsZ-SulA dimer (69). This indicates that SulA inhibits FtsZ dimerization by formation of a relatively stable complex with FtsZ. Although the interaction site of FtsZ and SulA has not been characterized yet, it is interesting to note that three out of six of the mutations that confer SulA resistance are located in the nucleotide-binding pocket of FtsZ (mutations *ftsZ1*, *ftsZ2* and *ftsZ3*) (10). The effect of SulA on FtsZ GTPase activity suggests that SulA may occupy one of the two monomer sites required for the correct FtsZ dimer interaction at the nucleotide-binding site.

MinC

MinC is part of the MinCDE system required for proper placement of the division site in *E. coli* and forms a division inhibitor together with MinD (reviewed in (119)). Overexpression of *minCD*, which blocks cell division, can be counteracted by overexpression of *ftsZ* (11, 35) and two SulA resistant FtsZ mutants are insensitive to MinCD (11). FtsZ rings did not form in *minCD* overexpressing cells (14). *In vitro*, MinC was observed to bind FtsZ and block FtsZ polymerization without inhibiting GTPase activity (63). A more recent report describes *in vivo* multimerization and FtsZ ring formation with *minCD* overexpression, presenting evidence that MinCD prevents FtsZ-FtsA interactions. Formation of FtsA rings, but not ZipA rings was blocked by *minCD* overexpression, while the cell division block induced by MinCD could be overcome by high levels of FtsA (69). These results point to a regulation of cell division by SulA and MinCD at different steps in the process.

ZipA

ZipA is the only essential cell division protein that was not identified by screening for *fts* mutants, but was obtained through affinity-blotting in a search that looked for *E. coli* proteins that interact with FtsZ (58). ZipA is a membrane protein, which consists of an N-terminal membrane anchor followed by a large cytoplasmic portion that consists of at least three domains: firstly, a Map-Tau repeat motif similar to MT binding motifs of Microtubule Associated Proteins (MAPs) and Tau, secondly, a Pro-Gln rich linker region and thirdly, a C-terminal domain (58, 116). Although ZipA is essential for division in *E. coli*, a search for ZipA homologues only revealed a ZipA in *Haemophilus influenzae* (116). ZipA localizes to the FtsZ ring in an FtsZ-dependent, FtsA-independent manner, and overexpression of zipA abolishes cell division (57, 58, 75). Increased zipA gene dosage suppresses the thermosensitivity of the *ftsZ84* mutant through FtsZ ring stabilization, and purified ZipA stabilizes both FtsZ84 and FtsZ polymers *in vitro* (116). Polymers formed in the presence of ZipA show enhanced bundling, similar to Ca$^{2+}$-stabilized polymers (59, 104, 116). The bundling is mediated by the C-terminal domain of ZipA, which contains the FtsZ interacting region as identified by affinity blotting, the yeast two hybrid system and structure determination (59, 75, 97, 98). ZipA binds the extreme C-terminus of FtsZ (59, 60). The
structure of the C-terminal domain of ZipA containing the C-terminal 17 residues of FtsZ revealed a split $\beta$-$\alpha$-$\beta$ motif with the FtsZ peptide bound in a shallow, hydrophobic, solvent-exposed cavity that runs the width of the ZipA C-terminal domain (97, 98). Various methods have been applied to characterize FtsZ residues implicated in the ZipA interaction, yielding unequivocal results. A surface plasmon spectroscopy analysis of a peptide comprising the C-terminal 17 FtsZ residues and the C-terminal ZipA domain identified D373, I374, F377 and L378 as the most critical FtsZ residues (97). In an in vivo assay, a L378A mutant interacted with FtsZ, whereas D373A, I374A and F377A mutants did not (90). Finally, in a yeast two-hybrid assay, a D373G mutant did not bind ZipA (60). The function of ZipA is still not clear, but the stabilization of FtsZ84 rings in vivo and of FtsZ and FtsZ84 polymers in vitro suggests that the interaction of FtsZ polymers with ZipA leads to the formation of a stable FtsZ ring through a reduction of FtsZ GTPase activity (P. de Boer, personal communication) which would slow down the dynamics of FtsZ polymerization.

**FtsA**

FtsA was identified in the original *fts* screen as a cell division protein acting late in the cell division cycle. *FtsA* mutants show regular constrictions (44, 136). FtsA is a member of the actin/Hsp70/sugar kinase ATPase superfamily (15), and is a cytoplasmic component of the cell division machinery. In vitro FtsA can be phosphorylated and in this state, the protein is capable of ATP binding but not of ATP hydrolysis (124). The crystal structure of FtsA (from *T. maritima* (138)) shows two domains with a common core that forms an interdomain cleft harbouring the nucleotide-binding site. This architecture is similar to proteins from the actin superfamily (15). Apart from one unique subdomain, FtsA was most homologous to actin, which reveals that two important cytoskeletal proteins in eukaryotes, tubulin and actin, have homologues in prokaryotes (recently other structural and functional actin homologues, MreB and Mbl, were identified in *B. subtilis* (68)).

The interaction between FtsA and FtsZ was demonstrated by co-localization experiments that showed that FtsA localizes in an FtsZ dependent manner, whereas FtsZ localization is FtsA independent (2, 5, 89-91). The cellular FtsA:FtsZ ratio is 1:100 and critical for correct division, since the block of septation caused by FtsA overexpression can be relieved by FtsZ overexpression (30, 38). The cytological evidence was corroborated by yeast two-hybrid interaction studies, which also identified that the extreme C-terminus of FtsZ is responsible for the interaction with FtsA (40, 90, 159). Two residues in the FtsZ C-terminus, L372 and P375, were critical for the FtsZ-FtsA interaction, interestingly these are other residues than those critical for the FtsZ-ZipA interaction (60, 90, 97).

A function for FtsA may be the recruitment of other cell division proteins to the division site, since FtsK, FtsQ, FtsI, FtsL and FtsN need FtsA for proper localization (reviewed in (92)). Dramatic bending was observed in cells that overproduce a C-terminally truncated FtsA, which is the possible result of abnormal orientation of certain cell division proteins giving rise to an aberrant septal wall synthesis (55).

**Other FtsZ interacting proteins.**

Several other proteins have been identified in *E. coli* or other bacteria that (are thought to) interact with FtsZ. MukB, a protein involved in chromosome partitioning, has been shown to bind to FtsZ in vitro (76). However, in vivo evidence for interaction of MukB with the FtsZ-ring has not been obtained, but interaction of MukB with cytosolic FtsZ could not be excluded (T. den Blaauwen, personal communication). In *B. subtilis*, EzrA was identified as a negative regulator of FtsZ polymerization (72). The critical concentration for the formation of FtsZ ring was lowered in an
ezrA null mutant, suggesting that EzrA acts to destabilize FtsZ polymers. EzrA is a cytoplasmic protein with a membrane anchor, and is conserved in several Gram-positive bacteria (72). Also in B. subtilis, a direct interaction of FtsZ and SpoIIE was observed (82). SpoIIE, a sporulation protein that is required for the formation of the asymmetric sporulation septum and the activation of the compartment specific transcription factor σF, localizes in an FtsZ dependent manner (52, 74). The formation of SpoIIE-FtsZ interactions at the site of the pre-spore septum is thought to stabilize the FtsZ ring. Two FtsZ rings are formed at both cell poles during sporulation initiation, but the pre-spore is formed at the site where SpoIIE appears first (157).

The FtsZ polymer in vivo: current models

What does the FtsZ ring look like? The variety of polymer structures that assemble in vitro raises the question which type of polymer the FtsZ ring is made up of. Electron microscopy has never revealed any sort of polymeric structure at the site of cell division, thus providing no clues for the in vivo FtsZ ring structure. This absence of in vivo data is likely due to a high cellular protein concentration, which makes it impossible to visualize cytoskeletal structures. In this respect it is interesting to note that the actin-like cytoskeleton recently identified in B. subtilis has also never been observed in electron micrographs (68). It is not necessary for FtsZ to span the membrane in a closed ring structure to generate constriction: the ftsZ26 mutant shows a spiral, nonclosed constriction and rodA mutants (a mutant that grows spherically) show constriction before FtsZ ring closure has been completed (6).

A suggestion in the literature is that a sheet of FtsZ filaments circles the cell. Since no obvious motor proteins have been found in bacteria, the force for cell constriction would be generated by the conversion of straight, GTP-bound filaments to curved, GDP-bound filaments (50, 80). There are however, numerous problems with this model. Firstly, the model implies a uniform FtsZ polymer that circles the cell as a sheet. The existence of such a polymer in vivo is questionable, given the dynamic nature of FtsZ filaments in vitro. Secondly, the curvature observed in the GDP-bound FtsZ filaments is incompatible with that of the cell surface, and it is unlikely that each subunit contact in the filament sustains the overall force that division applies to the membrane (79). Thirdly, a similar mechanism of force generation has never been described for microtubules, moreover, curved filaments at microtubule ends appear only during microtubule depolymerization (reviewed in (37)).

The observation that FtsZ ring closure is not required for constriction has led to two other
models (18) (figure 4). In the first model short filaments of FtsZ, anchored to the membrane by ZipA, slide relative to one another, aided by an as yet unidentified motor protein. In the second model, constriction is driven by depolymerization. FtsZ, anchored to the membrane (via ZipA) loses subunits through depolymerization at the anchor-site, which results in ring constriction. Several observations fit this latter model. Overproduction of FtsZ inhibits constriction of the FtsZ ring, which can be explained by the inhibition of depolymerization through the concentration increase (148). In *Xenopus* egg extracts, membrane movement could be mediated by microtubule dynamics, showing that microtubule depolymerization can lead to membrane movement (149). Microtubule depolymerization has been shown to drive the movement of latex microspheres coated with motor proteins that couple the microspheres to the microtubule (76a). Additional work on tubulin clearly demonstrated that growing microtubules exert force (42). Finally, the initiation of constriction requires only FtsZ and ZipA, since *ftsA* mutants show regular constrictions (44, 136), whereas ZipA depletion results in non-constricted filaments (75).

It is very likely that in vivo, constriction is not only dependent on FtsZ dynamics, but coupled to peptidoglycan synthesis as well. In *E. coli* K-12, septal wall synthesis starts rapidly upon formation of the FtsZ-ring (36). Ingrowth of peptidoglycan may provide (additional) force that drives constriction of the cell.

**Perspectives**

In the past decade, research in bacterial cell division has made an enormous leap forward. Structural and biochemical analyses have led to a fairly detailed picture of FtsZ polymerization and ring formation. The exact roles of GTP hydrolysis, nucleotide exchange and polymer stabilization still pose experimental challenges, given the high GTP and polymer turnover in physiological buffers. An important goal for the future will be the study of FtsZ polymerization in the presence of purified components of the cell division machinery, such as FtsA and ZipA, to identify the manner in which the polymer is stabilized and regulated in vivo. Other components of the cell division machinery, such as FtsK, FtsW, FtsL, FtsN and FtsQ still need to be characterized. The overproduction and purification of these proteins pose a major technical challenge. Reconstitution of the cell division machinery in an in vitro system using purified components would allow careful examination of the division mechanism. In the light of the poor conservation of several essential components of the *E. coli* cell division machinery in other bacteria, it will be interesting to study their role in cell division and the way that bacteria that do not contain these components complement for this absence. Finally, the search for cell division inhibitors could lead to a new generation of antibiotics.

**Outline of this thesis**

This thesis focuses on the polymerization dynamics of the *Escherichia coli* cell division protein FtsZ. In order to study FtsZ polymerization, several experimental procedures were adopted from the existing FtsZ and tubulin literature, as described in chapter 2. Sedimentation, light scattering and microscopical techniques were developed and used to study the effect of Ca$^{2+}$ on FtsZ. Chapter 2 describes the increased stability of polymers in the presence of millimolar amounts of Ca$^{2+}$, which is reflected by the inhibitory effect of Ca$^{2+}$ on FtsZ GTP hydrolysis activity. The tendency of FtsZ polymers to bundle in the presence of Ca$^{2+}$ enables their visualization using differential interference contrast microscopy, however, this method does not allow the study of polymer dynamics. To further characterize the role of Ca$^{2+}$, binding studies were performed showing that FtsZ is a low affinity Ca$^{2+}$-binding protein.

In chapter 3, the reduced FtsZ dynamics in the presence of Ca$^{2+}$ were used to study the
effect of the GTP analogue GTP-γ-S and GDP on preformed FtsZ polymers at limiting GTP concentrations. GTP-γ-S was shown to stabilize the FtsZ polymers whereas GDP induced rapid disassembly. Polymerized FtsZ was shown to contain predominantly GDP, with GTP hydrolysis immediately after GTP addition. GTP-γ-S did exchange only a small fraction of the polymer bound GDP, indicating that the FtsZ polymer is stabilized by a small GTP containing fraction of FtsZ subunits, possibly organized as a GTP cap, similar to tubulin.

The elucidation of the crystal structure of FtsZ and the organization of FtsZ monomers in protofilament sheets revealed that the T7-loop region contacts the nucleotide-binding site on another FtsZ monomer at the longitudinal monomer interface. This prompted the mutagenesis of several critical residues in the region and the characterization of the purified mutant proteins, which is described in chapter 4. Most mutants were impaired either in polymerization or GTP hydrolysis or both, showing that the loop region is indeed critical for FtsZ function. Experiments in which mutant proteins were mixed with wild type FtsZ revealed that the mutant proteins interact with wild type protein. The severe reduction of GTP hydrolysis in these mixtures indicated that the active site is indeed formed by two FtsZ monomers. Two of three mutations of a highly conserved Asp residue rendered an unusual phenotype, described in chapter 5. When the negatively charged Asp residue was replaced by a non-charged Asn or Cys, the mutant protein purified with GTP as the bound nucleotide, whereas a mutant with a replacement with the negatively charged Glu copurified with GDP, as wild type. GTP retention enabled polymerization of these mutants by the addition of divalent cations with a size exclusion limit of ~ 1.1 Å, without high GTP hydrolysis activity. These results indicate that the Asp residue plays a critical role in the coordination of cations at the nucleotide-binding site. In chapter 6, a short discussion of the results and their implications for the polymerization mechanism of FtsZ is provided.