# Interactions of cell division protein FtsZ with large and small molecules

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# Summary

The first known step in bacterial cell division is the assembly of the FtsZ protein into a ring-like structure, the so-called Z-ring, in the middle of the cell. Assembly of FtsZ is a complex process that involves the polymerization of FtsZ into long protofilaments, which are tethered to the inner membrane by additional proteins like FtsA, ZipA or some others. Next, single FtsZ filaments are cross-linked by other proteins or interact with each other via lateral bonds to form thicker structure. Upon formation of this complete Z-ring structure, other cell division proteins are recruited, which results in a big complex called the divisome. The divisome machinery is responsible for membrane constriction and cell wall ingrowth, which results in the splitting of the original 'mother' cell into two equally sized 'daughter' cells. FtsZ is a key player in this process. It is a very conserved essential protein, whose assembly and disassembly is highly regulated by other proteins in order to ensure proper cell separation. In the presence of FtsZ inhibitors or when FtsZ is depleted, cells are not able to divide, elongate into long filaments, and eventually die [1].

## THE FTSZ PROTEIN



FtsZ consists of 5 domains, all of which are crucial for the proper functioning of FtsZ and the division machinery. FtsZ assembles into long filaments in the presence of GTP, through longitudinal interactions between the globular domains of FtsZ that assemble on top of each other. These interactions activate FtsZ GTP hydrolysis, which occurs when the T7 loop (placed on the C-terminal globular core) from the top monomer enters the GTP binding site (present in the N-terminal domain) on the bottom monomer [1]. Lateral interactions between protofilaments of FtsZ represent another kind of interaction. Here, the extreme C-terminal part of FtsZ is involved, in particular, the last 4-6 residues of the FtsZ protein (CTV) [2]. The CTV is preceded by a very short α-helix (CTT), built up of 9 residues which was shown to be involved in many interactions with FtsZ binding partners (see [1] and **chapter 3**). The CTT and CTV are connected to the globular domain via the intrinsically disordered C-terminal linker which is important for the formation of protofilaments and the Z-ring architecture *in vivo* [3].

The most characteristic feature of FtsZ is the formation of long filaments, which makes it interesting protein for *in vitro* studies. Several techniques are

commonly used to study assembly and activity of FtsZ. The most common are: sedimentation of polymers, which allows the quantitation of the fraction of polymerized protein; light scattering, which measures assembly and disassembly of FtsZ in real time; negative stain electron microscopy (EM), which allows visualization of polymers in the sample; and free phosphate release assays, in which the total phosphate released from GTP hydrolysis can be measured and correlated to GTPase activity of FtsZ. In **chapter 2**, protocols to study the biochemistry of FtsZ in various buffers using above methods are discussed. The assembly of FtsZ from two model organisms, *E. coli* ($FtsZ_{Ec}$) and *B. subtilis* ($FtsZ_{Bs}$), at various pH (6.5, 6.8 and 7.5) and various KCl concentrations (50 mM, 300 mM) is compared. Even if $FtsZ_{Ec}$ forms longer polymers than $FtsZ_{Bs}$, as visualized using EM, the light scattering signal of $FtsZ_{Ec}$ is around 40-fold lower than that of $FtsZ_{Bs}$ at 50 mM KCl and pH=6.5. This is an effect of the bundling of FtsZ protofilaments into larger structures driven by electrostatic forces. The CTV of $FtsZ_{Bs}$ is positively charged at pH=6.5 while the charge of the CTV from $FtsZ_{Ec}$ is neutral. In the model proposed by Buske and Levin, the polymer core formed by the assembled globular domains of FtsZ carries a net negative charge at physiological pH [2] . A positively charged CTV interacts with the core of the adjacent polymer, causing bundling [2]. Bundling of $FtsZ_{Bs}$ polymers reduces the GTPase activity compared to non-bundled polymers of $FtsZ_{Ec}$. At an increased salt concentration (300 mM), $FtsZ_{Bs}$ displays reduced polymer bundling and the light scattering signal of both proteins was comparable. EM analysis showed that protofilaments of $FtsZ_{Bs}$ are shorter than those of $FtsZ_{Ec}$. Shorter filaments of $FtsZ_{Bs}$ gave slightly a lower light scattering signal at 300 mM KCl and the polymers disassembled more quickly comparing to the ones formed by $FtsZ_{Ec}$. The GTPase activity of both proteins was similar at high KCl concentrations, indicating that bundling of filaments and the concomitant decrease in subunit turnover of FtsZ monomers caused the decrease in GTPase activity of $FtsZ_{Bs}$. All methods described in **chapter 2**, except the sedimentation assay, give clear results at all chosen conditions. In the sedimentation assay, preassembled FtsZ polymers are spun down and separated from the monomers and shorter polymers that stay in solution. Polymers from both $FtsZ_{Bs}$ and $FtsZ_{Ec}$ could not be recovered in this assay at high KCl concentrations. This may be because of the reduction in the formation of lateral bonds between polymers

and the formation of shorter polymers or a higher turnover of subunits. Some of the methods described in **chapter 2** were used in the following three chapters. Knowledge about the behaviour of FtsZ under various conditions allowed a quick selection of the best conditions for the interaction studies with other proteins (**chapter 3** and **chapter 4**) and small molecule inhibitors (**chapter 5**). These protocols were also successfully adapted in our laboratory to study FtsZ from some other organisms (not shown).

## FTSZ INTERACTING PROTEINS

At least nine proteins directly regulate assembly of FtsZ in *B. subtilis* [1]. FtsA, EzrA and SepF are membrane anchors for FtsZ in cell during vegetative growth [1]. SpoIIE is an additional membrane anchor for FtsZ that functions in the relocation of the Z-ring from the mid-cell to the cell poles during sporulation [4,5]. Most of these proteins (excluding EzrA) "positively" regulate assembly of FtsZ, which means that they support bundling or polymerization of FtsZ. Another positive regulator is the cytoplasmic protein ZapA, that stabilizes FtsZ structures by cross-linking of protofilaments [1]. The role of the negative regulators is to block FtsZ polymerization in places where, or at moments when FtsZ should not assemble. MinC, for example, blocks FtsZ polymerization at cell poles ensuring proper placement of the Z-ring at mid-cell during vegetative growth [1]. Another example is UgtP, which blocks assembly of the Z-ring in fast-growing cells "to give time" to the cell to reach its appropriate size before cell division occurs [6]. Many FtsZ interacting proteins bind to the the C-terminus (CTT +/- CTV) of FtsZ. This short peptide is considered to be a "landing pad" for the interacting partners of FtsZ [1]. It is impossible that all these proteins simultaneously bind to such a small region. Thus, it is likely that FtsZ is regulated in time by competition of the interacting proteins for the C-terminus. In fact, FtsZ cannot function in cells without its extreme C-terminus, because proteins like ZipA or FtsA that serve as membrane anchors also bind to this region [7]. In **chapter 3**, a pull-down strategy to find binding partners for the extreme C-terminus of FtsZ is described. The last 69 amino acids of FtsZ, comprising the C-terminal non-conserved linker and the CTT+CTV (16 aa) sequences were fused to a Halo-

-tag. The HaloTag consists of a modified dehalogenase that covalently binds to sepharose beads via a chloroalkane ligand [8]. A control fusion protein contained only the linker sequence without the final 16 C-terminal amino acids. Lysates of *E. coli* cultures in which the FtsZ interacting partners EzrA, MinC and SepF were overexpressed were passed over sepharose beads with the bound bait proteins. Although both EzrA and MinC were previously shown to interact with the C-terminus of FtsZ [9,10] , in the pull-down assay only SepF was recovered. It is likely that MinC and EzrA do not strongly bind the C-terminus and thus were lost during washing steps. The recovery of SepF was surprising, especially because a previous study by Singh *et al*. indicated that SepF protein binds to the globular domain of FtsZ [10]. To evaluate which amino acids in the C-terminus are involved in the interaction with SepF, every single amino acid in the C-terminus was mutated to Alanine and the same pull-down assay was performed using this single mutant collection. Mutation of the two highly conserved residues P372 and F374 fully abolished binding of SepF to the C-terminus. Surprisingly, mutation of most of the other residues (11 out of 16) also affected the binding to SepF to some extent. This suggests that the secondary and tertiary structure of the C-terminus are important for the interactions with SepF. Recently, Duman *et al*. screened FtsZ binding sites on SepF using a yeast two hybrid screen [11]. Many residues on SepF were found to be important for FtsZ binding, suggesting a similar requirement for folding of the SepF domains in the interactions with FtsZ [11].



A reverse pull-down assay was used to prove that the interaction between SepF and the C-terminus of FtsZ is specific and this is the only place where SepF is able to bind FtsZ. MBP-SepF immobilized on amylose resin was incubated with FtsZ, FtsZP372A (full-length FtsZ with mutated conserved Pro372 into Ala) and FtsZΔ16 (FtsZ without the CTT and CTV). After several washing steps, full-length FtsZ remained bound to the resin while the other two mutants were not retained by MBP-SepF. This proves that the globular domain of FtsZ is not sufficient to (strongly) bind SepF.

Another binding partner of FtsZ that has not been studied in much detail is the sporulation protein SpoIIE. SpoIIE is a membrane protein and is thought to consist of three domains: the membrane domain I, the FtsZ-interacting and oligomerization domain II and the phosphatase domain III. It has been known

for a long time that FtsZ and SpoIIE co-localize *in vivo* and that SpoIIE is involved in relocation of FtsZ from mid-cell to the cell poles during sporulation in *B. subtilis* [12]. After formation of asymmetric septum, SpoIIE performs its second function. It activates $\sigma^F$ by dephosphorylation of the anti-sigma factor antagonist SpoIIAA. Direct interaction between SpoIIE and FtsZ *in vitro* was shown by Lucet *et al*. using a pull-down strategy and gel filtration analysis [13]. However, another group which studied the cytoplasmic domain of SpoIIE ($SpoIIE_{cyt}$) *in vitro* could not reproduce these findings [14]. Therefore, whether or not, and how, these two proteins interact is still not fully clear. One reason for this may be that $SpoIIE_{cyt}$ is highly unstable and forms inclusion bodies when overexpressed in *E.coli* [13,14]. Levdikov *et al*. showed that domain III on its own can function as phosphatase and that this function depends on its cofactor $Mn^{2+}$ [15]. In **chapter 4** the interactions between FtsZ and SpoIIE are studied. The cytoplasmic part of SpoIIE (domains II and III) was purified using a fusion to MBP to improve solubility and a strep-tag for purification (Ms-$SpoIIE_{cyt}$). The purified protein strongly binds metal ions, which were incorporated into the protein during folding in *E. coli*, indicating that the $SpoIIE_{cyt}$ domain is properly folded and possibly active. Metal binding enhances oligomerization of strep-$SpoIIE_{cyt}$, which is an indication that domain II, involved in oligomerization of SpoIIE, and the manganese-dependent phosphatase domain III are not completely independent but might influence each other. The phosphatase domain of SpoIIE is involved in the activation of $\sigma^F$, which activates most of the sporulation specific-genes, that drive the sporulation process in the prespore. Activation of $\sigma^F$ is achieved by dephosphorylation of the SpoIIAA protein, a process dependent on $Mn^{2+}$ [16]. In the absence of $Mn^{2+}$ sporulation is blocked and can be supported only by high concentrations of iron in the sporulation medium [17]. However, at which stage of sporulation is the process blocked is still not fully understood. Here, it is shown that in the absence of $Mn^{2+}$ formation of the asymmetric septum is delayed and that less asymmetric Z-rings are formed compared to when $Mn^{2+}$ is included in the medium. This phenotype resembles the SpoIIE knock-out phenotype. The result suggests that metal binding by SpoIIE is not just required for its phosphatase activity, but also critical for SpoIIE functioning in asymmetric division, either by influencing SpoIIE folding, oligomerization and/or interaction with FtsZ. However, which of these processes is affected is not clear.

The interactions between FtsZ and strep-SpoIIE$_{cyt}$ were analysed in more detail *in vitro*. Preliminary data on strep-SpoIIE$_{cyt}$ strongly suggested a direct interaction between FtsZ and strep-SpoIIE$_{cyt}$. However, strep-SpoIIE$_{cyt}$ alone, after cleavage of the MBP tag was unstable and required high concentrations of KCl and the presence of Triton X-100 in order to remain soluble, components that influence FtsZ activity. High concentrations of the wild type protein could not be obtained. As Ms-SpoIIE$_{cyt}$ could be purified in large amounts and was stable in variety of buffers, this protein was used to study the interactions with FtsZ using assays described in **chapter 2**. FtsZ interacted with Ms-SpoIIE$_{cyt}$ only in the presence of GDP, as interactions between protofilaments of FtsZ and Ms-SpoIIE$_{cyt}$ in the presence of GTP could not be detected in any of the standard assays. The large MBP tag, which is ~40 kDa, possibly (partially) covered the domain II of SpoIIE, which contains the FtsZ binding site. This shielding might make the binding site not accessible for FtsZ polymers but only for FtsZ monomers and small oligomers. However, these findings are preliminary and should be further analysed. Most of the standard assays and conditions used in **chapter 2** could not be used to study interactions with SpoIIE or should be adapted for these studies. All assays contain $Mg^{2+}$ because GTP hydrolysis of FtsZ is dependent on the presence of this metal. This complicates sedimentation assays as Ms-SpoIIE$_{cyt}$ is always oligomeric in the presence of metal and is thus recovered in the pellet fraction in all sedimentation assays. Second, because both proteins oligomerize and scatter light, the analysis of the results of light scattering experiments is difficult as the measured light scattering signal might be either a sum of the individual signals or an effect of the interactions between these two proteins. Finally, in the presence of Ms-SpoIIE$_{cyt}$, the free phosphate level in GTPase assay was always higher than the background signal. It is unlikely that SpoIIE is GTPase. The free phosphate levels may be higher because Ms-SpoIIE$_{cyt}$, as phosphatase, may be co-purified with free phosphate bound to the protein. The free phosphate can be released from the protein in the assay, although there is no phosphate released from Ms-SpoIIE$_{cyt}$ in the presence of EDTA. However, a direct interaction between SpoIIE$_{cyt}$ and FtsZ is evident and the findings described in **chapter 4** may inform other studies on FtsZ and SpoIIE interaction.

## SMALL MOLECULE INHIBITORS OF FTSZ

FtsZ has been studied as an attractive target to develop novel antibiotics. Most attention is focused on human pathogens, especially from the group of multidrug resistance pathogens like *S. aureus* or *M. tuberculosis*. In **chapter 5** the possible activity of semi-synthetic compounds, alkyl gallates, against FtsZ is studied. Alkyl gallates are derivatives of gallic acid, which is an intermediate of the tannin biosynthesis pathway in plants. Alkyl gallates are easily hydrolysed to gallic acid and corresponding alcohols and, though of limited use in medicine, could provide good environment-friendly alternatives for pesticides used in agriculture. Alkyl gallates have been shown in various studies to have a broad spectrum activity against Gram-positive and Gram-negative pathogens [18,19]. Recently, Silva *et al*. noted that alkyl gallates with a side chain length between 5 and 8 carbons, may target cell division proteins in the Gram-negative plant pathogen *Xanthomonas citri* [20]. In **chapter 5** the activity of pentyl, hexyl, heptyl and octyl gallates (called drug 8, 9, 10 and 11 to keep in line with the previous report from Silva *et al*.) is further investigated using the model organism *B. subtilis*. To visualize cell division in live cells, a FtsZ-eYFP fusion was used. In *B. subtilis*, cell division is affected when bacteria are treated with alkyl gallates, with treated cells not only significantly longer than the cells treated with the control (1% DMSO), and FtsZ-eYFP localization to the Z-ring was disrupted with FtsZ-eYFP present in the cytoplasm in randomly distributed patches. The standard assays described in **chapter 2** were used to study the effect of alkyl gallates on FtsZ *in vitro*. EM analysis showed that alkyl gallates cluster FtsZ monomers and bundle preformed FtsZ polymers. Additionally, the compounds effectively inhibit GTPase activity of FtsZ. There was a correlation between the activity of alkyl gallates *in vivo* and *in vitro* as heptyl gallate had the lowest MIC, the lowest binding constant and the strongest activity against FtsZ *in vitro*. Combined, these findings indicate that FtsZ is a direct target for alkyl gallates. In a previous study by Takai *et al*. it was shown that alkyl gallates may target cell membranes [21], which was confirmed here using a membrane permeability assay. However, as the delocalization of FtsZ-eYFP from mid-cell by alkyl gallates is not a secondary effect of disruption of the cell membrane integrity, it can be concluded that alkyl gallates kill bacteria through a combi-

nation of mechanisms: disruption of the cell membrane integrity and a block in cell division. The length of the side chain of alkyl gallates is important for their activity both against FtsZ and cell membrane. Pentyl gallate, the drug with the shortest side chain had the highest activity against the membrane, whereas heptyl gallate was most effective against FtsZ. Drugs with shorter and longer side chains had lower activity against FtsZ. Therefore, heptyl gallate is an attractive compound for further development and studies against cell division in bacterial pathogens. Heptyl gallate and other gallates used in **chapter 5** are attractive compounds for the use in agriculture against plant pathogens. First, some time after introduction into the environment they will be hydrolysed to gallic acid and corresponding alkohols which makes them safe for use. Second, their multi-target mechanism makes it difficult for bacteria to develop resistance. And finally, they are effective against broad spectrum of bacteria and fungi, which makes them good compounds to be used against a broad range of plant pathogens.



## CONCLUSION AND FUTURE PERSPECTIVES

The results described in this thesis bring new information into the FtsZ research field in three different FtsZ areas: the interactions between FtsZ and partner proteins, the use of FtsZ as antibacterial target, and the standardization of FtsZ assay conditions.

Based on the results described in this thesis and on the literature it can be concluded that FtsZ is regulated by most of the interacting partners via the small region placed at its C-terminus (see Table 1 and fig. 2 from Introduction). Most of the regulation via this region occurs through membrane-associated proteins (eg. FtsA, ZipA, SepF, EzrA, MinC, etc. (see Table 1 and fig. 2 from Introduction). However, some cytoplasmic proteins, like ClpX or SlmA, also bind to the C-terminus of FtsZ, whereas other proteins bind to the globular domain of FtsZ (ZapA and MciZ). An interesting example is MinC, which is a cytoplasmic protein but that regulates FtsZ from the membrane through formation of a complex with MinD. MinC binds to two regions on FtsZ, placed on the globular domain and the C-terminus (see Table 1 and fig. 2 from Introduction). The

extreme C-terminus of FtsZ is highly conserved. In **chapter 3** we showed that SepF, which is present only in Gram-positive organisms can still bind to the C-terminus of *E. coli* FtsZ. Whether this cross-interactivity is also a feature of the other C-terminus binders is not known. Most of the binding sites for FtsZ partners were discovered. The proteins of which the binding sites on FtsZ remain unknown are UgtP, SpoIIE and ZapA.

Most of the FtsZ interacting partners have an additional function apart from the regulation of FtsZ. It is possible that FtsZ also regulates these proteins, or at least that interaction with FtsZ is necessary for the localization of the specific protein to the division septum in order to perform its function. One of such an example is SepF which is responsible for proper septum formation in *B. subtilis* [22]. SepF is not absolutely required for FtsZ function as cell division occurs also without this protein. The exact function of the FtsZ-SepF interaction is still to be discovered. The current focus in the field is on effects of FtsZ regulators on FtsZ because assays to detect FtsZ polymerization, bundling and GTPase activity are well established. Thu, it is easy to study changes in these features caused by other factors. The features of the FtsZ binders are not so well defined and standard assays are lacking, which makes it difficult to study of the changes in their behaviour caused by FtsZ. Therefore, this area remains open for future work.



The study of the interactions of FtsZ with regulatory proteins is interesting from a mechanistic point of view. It brings additional information to understanding of the cell division process. Protein-protein interactions were also studied in order to find new inhibitors that could specifically target FtsZ-essential protein interactions. So far, only FtsZ-ZipA interaction sites were targeted with specific drugs [23]. The reason for this is that this approach requires more detailed analysis of the target, good knowledge of the interaction and rational drug design process. Nowadays, more focus is put on the screening of the drugs that target FtsZ alone. In **chapter 5,** a series of *in vivo* and *in vitro* assays was performed to find the target for alkyl gallates, semi-natural compounds that were identified by screening of a library of drugs against the plant pathogen *X. citri*. FtsZ was identified as a direct target for alkyl gallates but these compounds also targeted bacterial cell membrane. A series of controls was performed to prove that targeting FtsZ by alkyl gallates is not a false positive

event. It is important to study FtsZ inhibitors using both *in vitro* and *in vivo* methods, as some of the drugs might give false positive results. For example, some screens for FtsZ inhibitors are based on cell elongation or FtsZ delocalization, which might be a secondary effect of pore formation in the membrane or DNA damage. On the other hand, studying FtsZ inhibitors only *in vitro*, using purified FtsZ does not give significant and sufficient information as well.

There is still a long way to go for FtsZ researchers before FtsZ will get enough attention from pharmaceutical companies. In a recent review by Walsh and Wencewicz, FtsZ was mentioned as a target which did not give promising results [24]. Therefore, the area needs some improvements in order to bring FtsZ into a level of significance in antibacterial area. First, the amount of false positive FtsZ targeting drugs must decrease. Anderson *et al*. proposed a simple improvement in the *in vitro* assays by introducing Triton X-100 into the buffers [25]. Also, both *in vitro* and *in vivo* controls should be performed, and a more systematic approach is required. One solution is unification of the conditions in the FtsZ assays in order to simplify comparison of the effectiveness of drugs between laboratories. A good start for that are method papers and protocols which could be used by various laboratories. One such an example is presented in **chapter 2.** This protocol was used in **chapter 5** in combination with *in vivo* studies to find new FtsZ inhibitors.



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