Interactions of cell division protein FtsZ with large and small molecules

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CHAPTER

Introduction

1. GENERAL INTRODUCTION TO CELL DIVISION

Cell division is a fundamental process in the life of all bacteria. In rod-shaped bacteria it is often simplified to a three steps event: cell elongation, septum formation and division into two equal daughter cells [1]. In fact, to complete each of these steps, bacteria have to actuate complicated biomolecular machineries, which orchestrate different series of complex events. At the heart of these processes lies a 40 kDa cytoplasmic GTPase called FtsZ (Filamentous Temperature Sensitive protein Z) [1,2]. At the onset of cell division, FtsZ localizes to the mid-cell and polymerizes into a ring-like structure, the so-called Z-ring. FtsZ is tethered to the membrane via other membrane proteins and establishes the future division site. During the process of division, the Z-ring constricts and disassembles to move to the new division site in the daughter cell (see Fig. 1A, B) [1].

FtsZ is almost universally conserved in Bacteria. Homologues of FtsZ have also been found in a group of Archaea, in chloroplasts, and in mitochondria of some eukaryotes. FtsZ is an essential protein for cell division in most bacteria. It has a dual function during cell division, as a scaffold for the recruitment of other cell division components, known as divisome, and as a force generator for membrane invagination [1-3]. FtsZ is also tightly regulated by other proteins, which make sure that the Z-ring assembles in the correct place (usually in the middle of the cell) and time (after chromosome segregation is complete) and which control the dynamics of the Z-ring (see below and Table 1) [1-5].

Understanding the role and mechanism of Z-ring assembly is not just an interesting topic. It is also crucial for answering further questions about other processes during cell division, including remodeling of the cell envelope. Recently, the study of FtsZ also gained special attention in antibacterial research, since FtsZ and other essential cell division proteins may be promising targets for the development of new antibiotics [6-12]. Here, I describe the nature of FtsZ and its behaviors *in vitro*, and in the cell, as well as advances in the research of antibacterial compounds which target FtsZ. I focus mainly on the model rod-shaped bacteria, *Bacillus subtilis* and *Escherichia coli*. Any data or conclusions described here are derived from work on these organisms unless otherwise noted.

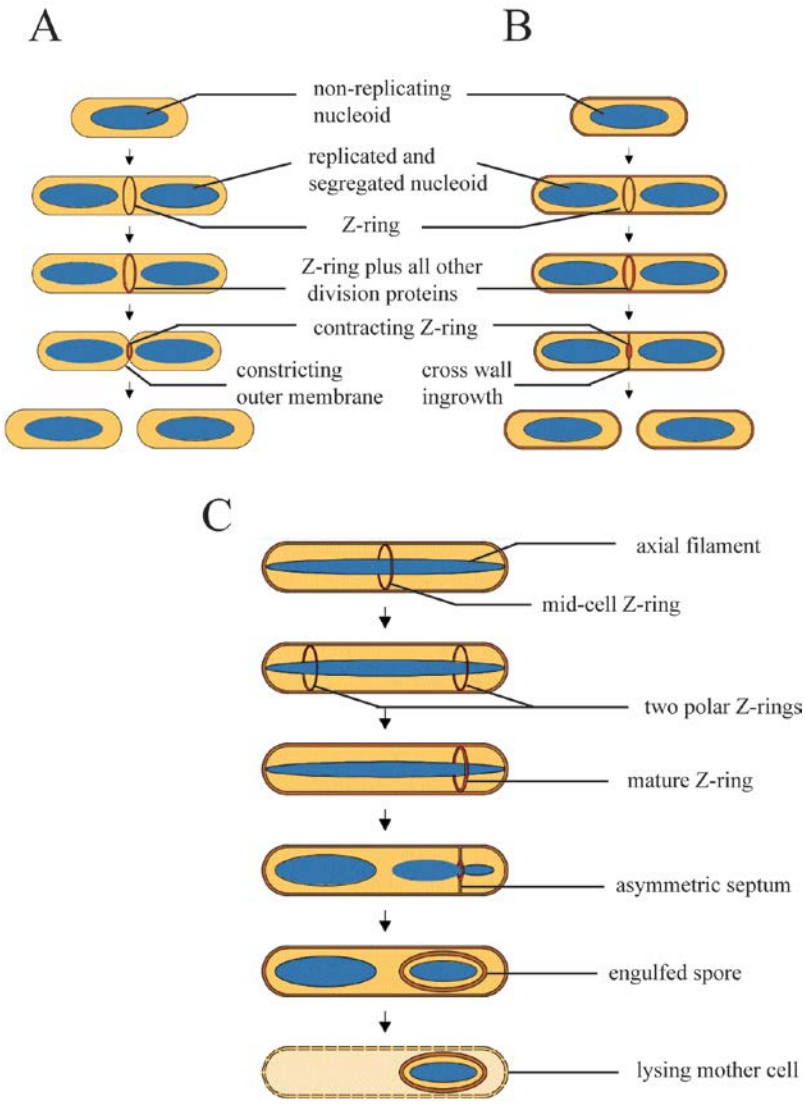

Figure 1. Schematic representation of different modes of cell division. Representation of cell division during vegetative growth in *E. coli* (A) and *B. subtilis* (B), and during sporulation in *B. subtilis* (C). Vegetative cell division starts with cell elongation and chromosome replication and segregation, followed by assembly of the Z-ring in the middle of the cell. After that, other cell division proteins are recruited to the Z-ring to form divisome. The divisome drives cell separation by cell wall ingrowth in *B. subtilis* (B) or cell envelope invagination in *E. coli* (A). The process is finalized by the formation of two equal daughter cells. (C) Sporulation begins with the formation of an axial DNA filament. After that FtsZ is relocated to the poles of the cell. One of the Z-ring matures and drives asymmetric septum formation. The process is completed when the forespore is engulfed in the mother cell and the mother cell has lysed to release the matured spore to the environment.

2. FTSZ

2.1 CRYSTAL STRUCTURE

The first crystal structure of the FtsZ molecule was obtained of FtsZ from the archaeon *Methanocaldococcus jannaschii* [13]. The three-dimensional structure of FtsZ appeared to be similar to the structure of eukaryotic α- and β-tubulin, which were presented at the same time [14], although the sequence homology of these proteins is weak. Based on the crystal structure, presented in Fig. 2, two domains can be distinguished: the N-terminal domain – a nucleotide-binding domain (blue) and the C-terminal globular core (cyan). Both domains are separated by a central α-helix. The GTP/GDP nucleotide binding site is built up of four T-loops (phosphate-binding loops), a sugar recognition sequence (placed in the N-terminal domain), and a guanine recognition sequence (placed in the α-helix that connects both domains) [13].

GTP BINDING

The FtsZ monomer alone can bind GTP/GDP (Fig. 2, orange), however, for GTP hydrolysis at least two monomers are necessary. Activation of GTP hydrolysis occurs when the T7 loop (Fig. 2, red) from one monomer (placed on the C-terminal domain, following the central α-helix) enters the GTP binding site of another monomer [2,4,13]. Nucleotide binding does not cause any conformational change within the FtsZ monomer [15].

2.2 THE EXTREME C-TERMINAL PART

The extreme C-terminal part of FtsZ was not resolved in the crystal structure due to its high flexibility [13]. However, this part of FtsZ plays a very important role in FtsZ assembly, its interaction with many cell division proteins, and is crucial for cell division [2,16-22]. The extreme C-terminus may be divided into 3 domains as proposed by Buske and Levin: an unstructured C-terminal linker region of variable length, a highly conserved C-terminal tail (CTT) and a short variable region at the extreme C-terminus (CTV) (Fig. 2) [16]. Although some of these domains consist of only a few residues (in *E. coli*, the CTT is formed

of 9 residues and the CTV of only 4), distribution of the extreme C-terminal part into 3 independent domains is important as each of the domains has its own function in cell division/FtsZ assembly and interaction with other proteins [2,16-18].

C-TERMINAL LINKER

The structure of the C-terminal linker cannot be resolved in crystal structures (Fig. 2, purple) and its sequence conservation is weak, and so this part of FtsZ has received relatively little attention. The length of the linker may range from 2-330 residues [23], but most FtsZs have linkers with a length of 50 to 100 residues (for *E. coli* and *B. subtilis* the linker sequence is about 50 residues). Recently the function of the C-terminal linker was determined [17,18,24]. It was shown that the C-terminal linker plays a role in the formation of FtsZ protofilaments and the architecture of the FtsZ ring *in vivo*. Apparently, the C-terminal linker is crucial for FtsZ assembly into a ring structure and cell division. It was proposed that the C-terminal linker is intrinsically disordered [17,18]. Flexibility and disorder of the linker is critical for proper FtsZ functioning as replacing the linker with helical repeats leads to the filamentation of *B. subtilis* cells and a significantly decreased GTP hydrolysis rate by chimeric FtsZ [17]. The length of the linker is important for lateral interactions of protofilaments and cell division [17]. Introducing extra amino acids into the flexible linker increases the distance between the globular N-terminal domain and the extreme C-terminus, that binds to the membrane anchors of FtsZ, like FtsA or ZipA [25,26]. Szwedziak *et al*. found that increasing the length of the C-terminal linker changes the distance of FtsZ protofilaments from the cell membrane from 16 nm to a variable distance between 16-21 nm [25]. Recently, it was shown that the C-terminal linker is required for the coordination of peptidoglycan synthesis in *Caulobacter crescentus* [24].

C-TERMINAL CONSERVED TAIL

Following the C-terminal linker, there is a highly conserved set of residues called the C-terminal conserved region (CTT) [16]. It is defined as 9-amino acid motif, with a completely conserved proline at position 6 and highly conserved residues at positions 4, 5, 8 and 9 [23]. This part of *E. coli* FtsZ has been crystalized in a complex with cell division protein ZipA revealing an helical structure of

this region (Fig. 2) [27]. However, the structure of the CTT alone, or in complex with other proteins, was never determined.

C-TERMINAL VARIABLE REGION

At the very end of the C-terminus, a small region called the C-terminal variable region (CTV) is present. It may contain 1 to 13 poorly conserved residues (4 in *E. coli* and 6 in *B.subtilis*) [16,23]. Buske *et al*. showed that the charge of the CTV is crucial for the formation of lateral interactions between FtsZ protofilaments [16]. It was shown that a positively charged CTV is involved in promoting lateral interactions (*B. subtilis* FtsZ) while a negatively charged (*E. coli* FtsZ) or neutral CTV does not promote lateral interactions between single FtsZ filaments [16].

The extreme C-terminus of FtsZ (CTT and CTV) serves as a link between FtsZ and the other cell division components since most of the FtsZ interacting proteins bind to this region of FtsZ (FtsA, ZipA, SepF, EzrA, ClpX(P), SlmA, etc.) (see below) [19,20,22,27,28].

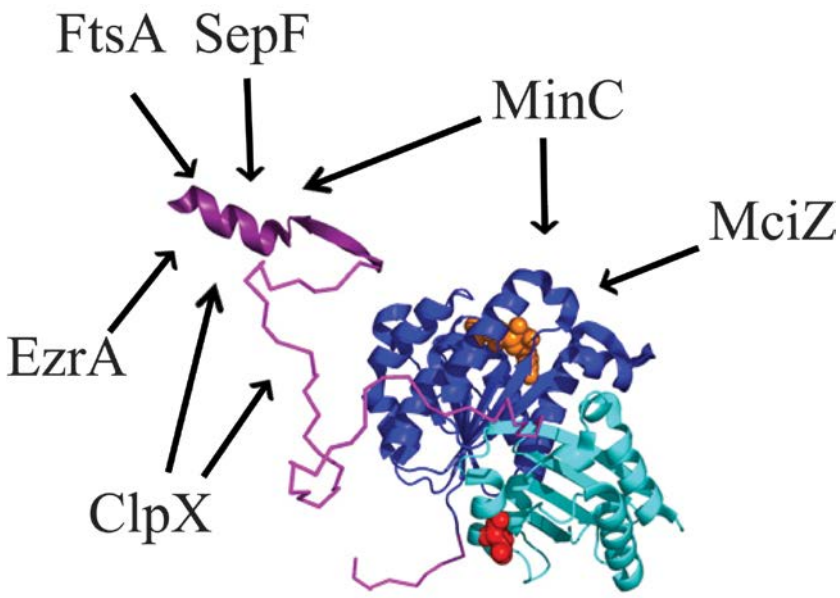

Figure 2. Cartoon format of the 3D structure of FtsZ molecule from *Pseudomonas aeruginosa*. The N-terminal domain is marked in blue, the C-terminal domain in cyan. A bound GDP molecule is marked in orange and the T7 synergy loop in red. Binding regions for interacting proteins are indicated with arrows. The structure of FtsZ was reprinted with permission from [2].

3. ASSEMBLY OF THE Z-RING AND REGULATORY SYSTEMS

3.1 SPATIAL REGULATION

During vegetative growth, Z-ring formation occurs precisely in the middle of a bacterial cell (Fig. 1A, B). How FtsZ is directed with such a high precision to the mid-cell has been studied for decades in two rod shaped model organisms, *E. coli* and *B. subtilis* [29]. For a long time it has been thought that the spatial regulation of Z-ring positioning is a combination of two negative molecular systems, the Min system, which prevents formation of the Z-ring at the cell poles, and nucleoid occlusion (NO), which prevents constriction of the Z-ring over nucleoids [4,29]. Positive regulatory systems were found only in *Actinomycetes* (SggAB system) and *Myxococcus xantus* (PomZ) [29]. Recently, an additional system, Ter linkage, was shown to positively regulate Z-ring placement in *E. coli* [30].

MIN SYSTEM

The Min system prevents Z-ring formation at the cell poles by directly inhibiting the polymerization of FtsZ via the MinC protein. MinC interacts with a membrane associated ATPase MinD. The third component of the Min system is a topological factor that localizes the MinCD complex to the poles. In *B. subtilis*, the DivIVA protein senses negative curvature of the polar membrane and localizes MinD to the cell poles via a bridging protein called MinJ. DivIVA does not exist in *E. coli* and instead, the MinE protein plays the role of topological determinant. MinE oscillates from pole-to-pole and displaces MinD from the membrane. MinD assembles at the membrane distant from MinE, creating a dynamic oscillating system. The result is a concentration gradient of MinCD with a minimum around the mid-cell and maximum at the cell-poles. There are many reviews that comprehensively describe the Min systems from *E. coli* and *B. subtilis* [29,31-34]. Here, the direct inhibition of FtsZ by MinC is shortly described.

MinC. MinC consists of two domains of approximately equal size [35,36]. Both domains are necessary for the inhibition of Z-ring formation [36-38]. The N-terminal domain of MinC ($MinC^N$) blocks FtsZ assembly into a Z-ring *in vivo* and blocks polymerization *in vitro* (Fig. 3) without influencing the GTPase activity of

FtsZ (Table 1) [36]. The C-terminal domain of MinC ($MinC^C$) affects FtsZ assembly only in the presence of MinD [38]. The $MinC^C$/ MinD complex binds to the extreme C-terminus of FtsZ, whereas the binding site for $MinC^N$ is placed on the C-terminal globular domain of FtsZ (Fig. 2) [39,40]. Binding of the $MinC^C$/ MinD complex blocks lateral interactions resulting in less bundled single protofilaments [37,38] (Fig. 3). MinC interacts preferentially with GDP-bound FtsZ and shortens FtsZ filaments possibly by partially destabilizing them [41].

NUCLEOID OCCLUSION

The second regulatory system prevents formation of the cell division septum over the nucleoid and, in consequence, guillotining the chromosome. Nucleoid occlusion is mainly driven by the DNA binding proteins Noc (*B. subtilis*) and SlmA (*E. coli*). Despite their similar roles, Noc and SlmA belong to different groups of DNA binding proteins and inhibit cell division by different mechanisms [42-45].

E. coli **SlmA.** SlmA specifically binds DNA in the regions distributed all over the chromosome except for the replication terminus region. DNA binding activates a SlmA dimer to inhibit FtsZ [45]. The inhibition of FtsZ occurs in two steps. In the first step, DNA-bound SlmA binds to the C-terminus of FtsZ, competing with its membrane anchors and several other proteins. In the second step, SlmA breaks/disassembles FtsZ protofilaments without affecting its GTPase activity [44,46]. In this way, FtsZ formation is inhibited everywhere over the chromosome, except for the replication terminus region, where cell division can occur. Althoug a lot of work has been done on SlmA in *E. coli*, the exact mechanism of protofilament breakage as well as *in vivo* mechanism of SlmA action are still not fully clear.

B. subtilis **Noc** also controls Z-ring formation in regard to chromosome segregation. However, the mechanism of control is different from SlmA. There is no evidence of direct interaction between FtsZ and Noc. Recently, Adams *et al*. proposed a model, in which they explain how Noc inhibits cell division [47]. Noc is a peripheral membrane protein that is able to recruit DNA to the cell membrane. As a result, large nucleoprotein complexes are formed over the nucleoid, which cause physical crowding. The physical crowding is thought to act as short-range inhibitor of cell division [4,42,47].

THE TER LINKAGE

Neither Min nor NO systems are essential in *E. coli* and *B. subtilis*. Moreover, in the absence of both systems, the Z-ring is still positioned with high precision at mid-cell between segregated nucleoids in slow growing *E. coli* cells [30]. This suggested the presence of another regulatory mechanism. Recently, Espeli *et al*. found that the MatP protein serves as a linkage between the Z-ring and the chromosome in *E. coli* [48]. MatP binds DNA in the replication terminus region and is connected to the Z-ring via ZapB and ZapA [48]. Interestingly, MatP and SlmA binding sites on the chromosome are complementary, making them positive and negative sites for regulation of Z-ring formation [48].

The Ter linkage is also not essential in *E. coli*. Moreover, deletion of *slmA*, *minCDE* and Ter linkage genes (*matP*, *zapB* and *zapA*) does not affect viability of *E. coli* in slow growth conditions [43]. Taken together, these facts indicate the presence of additional mechanism or that there are no indispensable mechanisms that place the divisome in the correct position in cells [43].

3.2 OTHER REGULATORY SYSTEMS

CELL SIZE CONTROL, NUTRIENT AVAILABILITY

Bacterial cell size is coupled to nutrient availability and, in consequence, growth rate. Cells grown on a nutrient-rich medium are approximately 2x as voluminous compared to cells grown on a nutrient-poor medium, and cell division is inhibited until the cell reaches its appropriate size. This means that cells contain a mechanism which transmits the information about their metabolic status and growth rate to the division machinery to delay cell division until the cell reaches the correct size. Part of the mechanism is Uridine-5'-phosphoglucose (UDP-glucose), a small molecule that is produced in nutrient-rich conditions and that signals the cells metabolic status. In *B. subtilis* and *E. coli*, Ugtp and OpgH respectively bind UDP-glucose and are recruited to the nascent septal site to inhibit Z-ring formation in fast-growing cells [49-51].

UgtP is a membrane-associated glucosyltransferase, a part of the conserved glucolipid biosynthesis pathway. In nutrient-rich conditions, UgtP localizes to the division site and inhibits cell division by directly interacting with FtsZ until the cell reaches its appropriate size. In nutrient-poor conditions UgtP

is sequestered away from mid-cell in randomly distributed foci. UDP-glucose influences the FtsZ-UgtP interaction by reducing the affinity of UgtP for itself and making it more available for interaction with FtsZ. However, the interaction is not dependent on UDP-glucose. *In vitro*, UgtP inhibits FtsZ polymerization but does not have significant influence on its GTPase activity [49,51].

OpgH is the functional homologue of UgtP in *E. coli*. It is an inner-membrane protein and interacts directly with FtsZ via its N-terminal domain in a UDP-glucose dependent manner. Similar to UgtP, it localizes to mid-cell in fast-growing cells and is sequestered away from the Z-ring in slow-growing conditions. Unlike UgtP, OpgH localization is not dependent on UDP-glucose. *In vitro*, it inhibits assembly and GTPase activity of FtsZ [50].

Another link between nutrient availability and cell division in *B. subtilis* was recently discovered by Monahan *et al.* It was shown that the E1α subunit of pyruvate dehydrogenase influences cell division in a pyruvate-dependent manner. Whether the influence is direct or indirect is still an open question [52].

SOS RESPONSE

In response to DNA damage, cells activate an SOS mechanism, the goal of which is to repair the DNA damage and to inhibit cell division until the repair is complete. An SOS component that directly targets cell division is **SulA**. SulA is a small protein induced in response to DNA damage and removed by Lon protease when DNA is repaired [53]. It inhibits cell division by directly interacting with FtsZ [54,55], at the site bound by another FtsZ monomer during polymerization, thus increasing the critical concentration for FtsZ assembly [54]. In the presence of SulA, the GTPase activity of FtsZ is about 50% reduced [55].

4. THE Z-RING *IN VIVO*

4.1 DYNAMICS OF THE Z-RING

Pioneering studies on FtsZ *in vivo*, using immunofluorescence and Green Fluorescent Protein (GFP) fused to FtsZ, revealed localization of FtsZ to mid-cell during most of the cell cycle [76-78]. Only 30% of the total FtsZ available in the

Table 1. Summary of FtsZ interacting proteins present in *B. subtilis* and their mode of action on FtsZ.

Protein	Size [kDa]	Function *in vivo*	Knock-out phenotype	Mode of action on FtsZ		Binding site on FtsZ	Structure (PDB entry)
				GTPase activity	Polymerization		
Positive regulators							
FtsA [56-58]	48	• Tethering FtsZ to the membrane	• Elongation in *B. subtilis*, • Elongation and cell death in *E. coli*	No	• Bundling	CTT	1E4G (*T. maritima*)
ZapA [59-61]	10	• Z-ring stabilization, • A role in septum formation in *E. coli*	• No phenotype • Lethal with *ΔezrA*	• Strong inhibition at 10 mM $MgCl_2$ • Slight inhibition at 5 mM $MgCl_2$	• Cross-linking polymers, • Bundling at 10 mM $MgCl_2$ • No effect at 5 mM $MgCl_2$	Not known	1T3U
SepF [19,62,63]	17	• Tethering FtsZ to the membrane, • Formation of division septa	• Deformed division septa, • lethal with *ΔftsA* or ΔezrA	No	• Large tubular structures	CTT	C-terminal part (aa 61-140): 3ZIH
SpoIIE [64,65]	92	• Relocalization of FtsZ to the cell pole, • tethering to the membrane	• Delay in the formation of asymmetric septum	No	• Bundling	Not known	Phosphatase domain: 3T91
Negative regulators							
EzrA [22,66-68]	65	• Inhibition of the formation of extra Z-rings in cell	• Extra Z-rings formed along cell	• Increase	• Disassembly of preformed polymers • Inhibition of polymerization	CTT	Cytoplasmic domain: 4UY3
MinC [20,35,41]	25	• Spatial inhibition of Z-ring formation	• Minicells	No	• Depolymerization of protofilaments, • Decrease in bundling	K243, D287 – Helix 10, R376 - CTT	1HFZ
UgtP [49,51]	44	• Inhibition of Z-ring according to nutrient availability	• Shorter cells in nutrient-rich medium	No	• Not determined	Not known	-
ClpX [21,49,51,70]	46	• Maintaining dynamics and disassembly of the Z-ring	• Strong filamentation in *ΔminC* strain	No	• Disassembly of FtsZ polymers • Inhibition of polymerization	CTT and C-terminal linker	3HTE
MciZ [72-74]	4	• Inhibition of FtsZ assembly in mother cell during sporulation	• Polar Z-ring formation after engulfment of a spore by mother cell	• Increase at low and decrease at high concentrations of MciZ	• Shortening of FtsZ polymers	C-terminal globular core, Asp280	2MRW

cell is incorporated into the Z-ring [2,79]. The rest of FtsZ is present in the cytoplasm as a pool of monomers and short polymers [80] which are continuously exchanging with the Z-ring subunits with half-time between 8-9 sec [79]. Not only FtsZ monomers are exchanged but also the overall architecture of the Z-ring constantly changes with time and the Z-ring remains dynamic during constriction [81]. It is not fully clear whether the Z-ring disassembles during constriction or only after constriction is complete. Strauss *et al*. have noted that the total intensity of FtsZ-GFP within the ring remains constant during constriction, suggesting that the total amount of FtsZ within the ring does not change [81]. However, the data was limited only to large rings (800-900 nm in diameter) [81] and previous studies have noted disassembly during constriction [82,83]. After constriction, FtsZ immediately reassembles at mid-cell in daughter cells. What drives the dynamics and disassembly of the Z-ring? It was suggested that the FtsZ interacting proteins ClpX(P) and *B. subtilis* EzrA may be involved in this process [21,69].

ClpX(P) is a part of the ClpXP protease complex, in which it serves as a substrate recognition domain. ClpX may also function in a ClpP-independent manner. The mode of action of ClpX on FtsZ in *E. coli* and *B. subtilis* seems to be completely different. In *B. subtilis*, ClpX blocks FtsZ polymerization independently of ATP and ClpP, whereas in *E. coli* both ClpP and ATP are necessary for FtsZ inhibition. However, both modes of action suggest a role of ClpX(P) in the modulation of the Z-ring disassembly with a possible role in maintaining dynamics and subunit turnover between the Z-ring and cytoplasmic FtsZ (Fig. 3) [21,69]. ClpX(P) interacts with extreme C-terminal part of both monomeric and polymeric forms of FtsZ. Therefore, it has dual role in inhibition of FtsZ polymerization; one is degradation of FtsZ monomers and prevention of FtsZ polymerization and the second one is breakage of the previously assembled polymers (Table 1) [21].

EzrA (Extra Z-rings A) is a membrane protein present in Gram-positive organisms and one of the first proteins to localize to the Z-ring in *B. subtilis*. In *ezrA* null mutant cells, extra Z-rings are formed at cell poles and the medial Z-ring becomes more stable (Table 1). Thus, EzrA is considered an inhibitor of FtsZ in the cell (Fig. 3) [84]. *In vitro*, EzrA inhibits the assembly and increases the GTPase activity of FtsZ. Similarly to ClpX(P), EzrA can also break previously assembled polymers by interacting with the C-terminus of FtsZ (Fig. 2 and Table 1) [22].

Recent studies suggest a second role of EzrA in coordination of divisome assembly with lateral cell wall synthesis [85,86].

4.2 ARCHITECTURE OF THE Z-RING

FtsZ assembly has been extensively studied *in vitro*. However, assembly of the Z-ring and its architecture *in vivo* is still not clear. Together with the development of super-resolution techniques (like PALM – photoactivated localization microscopy, 3D-SIM – three-dimensional-structured illumination microscopy, cryotomography, the understanding of FtsZ-ring formation *in vivo* and *in vitro* is increasing. It has been known that FtsZ polymers are tethered to the cytoplasmic membrane by membrane (binding) proteins like FtsA or ZipA (*E.coli*), and FtsA, SepF, and EzrA (*B. subtilis*) [26,58,63,84]. The Z-ring filaments are placed at a distance of ~15-16 nm from the inner membrane (observed for *E. coli* and *C. crescentus*) [25,87]. Up to now, it was thought that FtsZ forms a continuous filament at mid-cell. Recently, research based on super-resolution microscopy revealed that FtsZ localizes into patches instead of a continuous ring [80,81,88,89]. These patches are most likely composed of short overlapping FtsZ polymers [25,87]. It was proposed that gaps existing between FtsZ beads may be necessary for FtsZ ring constriction [3,80]. FtsA and ZipA in *E.coli* [80] as well as EzrA and PBP2 in *S. aureus* [81] form similar patches. The FtsA and ZipA patches overlap with FtsZ and with each other in *E. coli* [80].

In contrast to previous studies, the recent cryotomography work revealed that the structure of the Z-ring is continuous and encircles the whole division site [25]. Both techniques, however, show that the Z-ring is formed of shorter overlapping filaments [25,80,81,87,88]. Cryotomography work revealed that the Z-ring is formed of single layered bands that are 5-10 filaments wide [25]. It is possible that both models are correct and that either structure may exist during different stages of cell division [88].

5. ASSEMBLY OF THE Z-RING DURING SPORULATION

A completely different type of cell division is observed in *B. subtilis* during sporulation (Fig. 1C). Sporulation is an adaptive process undertaken by *Bacillus* and

its relatives under starvation conditions [31,90]. It begins with the switch from medial to polar Z-ring formation through a spiral-like intermediate, a process which is indirectly controlled by the protein Spo0A [77,90]. In *spo0A* null mutants, the Z-ring is not directed to the polar sites and cell division is completed at mid-cell. The positional switch is mediated by the sporulation specific protein SpoIIE, which is expressed under Spo0A control [77,91]. Asymmetric septation leads to the formation of two unequal-sized daughter cells, a larger mother cell and a smaller forespore. The forespore is then engulfed by a mother cell in a process resembling phagocytosis. Subsequently, the mother cell lyses and the spore is released to the environment, where it can survive indefinitely in a state of dormancy (Fig. 1C) [31,92]. During sporulation, chromosomes do not segregate in a manner observed for vegetative growth. Instead, *oriC* regions migrate to the opposite poles of the cell and chromosomes form an elongated structure known as the axial filament. The axial filament is bisected by the asymmetric septum and one-third of the chromosome ends up in the forespore (Fig. 1C). The remaining two-third is later transferred to the forespore by a conjugation-like mechanism directed by SpoIIIE [93,94]. It was suggested that the asymmetric septation uses the same cell division machinery as the vegetative one, except for an additional component, SpoIIE, which localizes to the asymmetric division site in a ring-like structure called the E-ring. However, the asymmetric septum is a much thinner structure than the vegetative septum and most of the peptidoglycan formed to separate the two lipid bilayers is removed soon after septation completes [31]. Also, the recently discovered septum-forming protein SepF was not yet studied during sporulation, indicating that more differences between vegetative cell division and division during sporulation exist.

SpoIIE is a 92 kDa membrane protein, which consists of three domains: the N-terminal membrane domain with 10 membrane-spanning segments, the central domain involved in SpoIIE oligomerization and interaction with FtsZ and a PP2C-type phosphatase domain at the C-terminus [65]. SpoIIE is expressed at the onset of sporulation and has two functions. One is redeployment of FtsZ from a medial to two polar Z-rings, one of which eventually constricts. The second role is activation of the transcription factor σ^F in the forespore [64,91,95]. Initially, FtsZ assembles into a ring-like structure at mid-cell. SpoIIE co-localizes

with FtsZ via a direct interaction. Instead of constricting at mid-cell, FtsZ and SpoIIE redeploy near both cell poles in the form of E-rings. One of the E-rings dissolves and the other matures into the sporulation septum (Fig 1C) [91].

MciZ (Mother cell inhibitor of Z). MciZ is a 40-aa peptide expressed during sporulation to block Z-ring formation in the mother cell. It binds to the C-terminal globular core of FtsZ (Fig. 2) and functions as a filament capping protein. It was shown that MciZ shortens FtsZ polymers without competing with GTP for binding to FtsZ (Fig. 3). Interestingly, low MciZ concentrations promote the GTPase activity of FtsZ, while high concentrations of MciZ inhibit FtsZ GTPase (Table 1) [73].

6. FOLLOW-UP PROCESSES

Once FtsZ is present at mid-cell, it becomes a scaffold for the recruitment of other "divisome" components (Fig. 1A, B) [1]. In *B. subtilis*, the assembly of cell division proteins occurs in two steps. First, the proteins FtsZ, FtsA, ZapA and EzrA (early cell division proteins) are recruited to mid-cell and after that, a second set of proteins arrives to the division site, including GpsB, FtsL, DivIB, FtsW, Pbp2B and DivIVA (late cell division proteins) [96]. In *E. coli* the assembly was first thought to be more hierarchical: [FtsZ, FtsA/ZipA] > [FtsK > FtsQ > FtsL/B > FtsW > FtsI] > FtsN. However, Goehring *et al.* found that proteins FtsK, Q, L, B, W and I may assemble together independently on FtsA and be recruited together, as a complex, to the established Z-ring (the complexes are marked in square brackets) [97].

6.1 EARLY CELL DIVISION PROTEINS

MEMBRANE TETHERS

Several proteins function as membrane tethers for FtsZ, **FtsA** and **ZipA** in *E. coli*, and FtsA, **EzrA** and, as recently discovered, **SepF** in *B. sutbilis*, reviewed in [1]. Mutation of *ftsA* in *E. coli* prevents cell division. The Z-rings are still formed via ZipA but are not able to complete cell division. In the absence of both proteins, cells are unable to form the Z-ring [58].

FtsA is an ATPase that is structurally related to actin. ATP binding is crucial for the FtsZ-FtsA interaction. FtsA binds to the membrane via an amphipathic helix at its C-terminus [26]. Recently, it was shown that FtsA recruits FtsZ filaments, but not monomers to a lipid bilayer *in vitro*, and that FtsA and FtsZ together form highly dynamic spirals on the lipid bilayer [28]. In contrast, the transmembrane protein **ZipA** is able to recruit FtsZ monomers to the membrane and to bundle FtsZ filaments. Thus, the interaction between ZipA and FtsZ is stronger than between FtsA and FtsZ [28]. FtsA and ZipA recruit downstream division proteins to the divisome [58].

Even though FtsA is essential in *E. coli*, it is not in *B. subtilis*. What is more, an *ftsA* and *ezrA* double mutant is viable in *B. subtilis*. However, either *ftsA* and *sepF* or *ezrA* and *sepF* double knockouts are lethal. These findings, and the fact that overexpression of SepF may restore deletion of *ftsA*, strongly suggest that SepF complements the function of FtsA in *B. subtilis*. SepF mutant cells are viable but have deformed division septa [63].

SepF is a 17 kDa protein which assembles into large (>2 MDa) rings at physiological conditions *in vitro* [63]. The protein is composed of two domains. The N-terminal domain consists of a highly conserved region which forms a membrane binding amphipathic helix [62]. The C-terminal domain is involved in SepF ring formation and interactions with FtsZ. The crystal structure of the C-terminal part of the SepF monomer reveals two α-helices and a five-stranded β-sheet arranged into a compact α/β-sandwich. SepF is organized into a tight dimer with the interface formed by β-sheets and the α-helices placed at the outside of the dimer. The SepF rings are formed by interactions between conserved glycine residues (G109) in the external helices. The C-terminal domain of SepF is also involved in the interactions with the C-terminus of FtsZ (Fig. 2) [19]. Several SepF residues involved in the interaction with FtsZ were identified in a yeast two-hybrid screen, out of which half (V64, F126, I118) are placed on the internal β-sheet and half (D105, F106, G116) are placed on the external α-helices. Together, SepF and FtsZ self-organize into long, ~50 nm-wide tubules reminiscent to eukaryotic microtubules [63]. Several facts indicate that SepF is an unique membrane anchor for FtsZ with a clear structural role. First, the organization of SepF into rings as well as the polymerization of FtsZ (in the presence of Mg^{2+} and GTP) are required for tubule formation *in vitro*. Second, the

Figure 3. Schematic representation of the process of FtsZ assembly into a ring. FtsZ monomers (black spheres) assemble into short polymers, which are tethered to the membrane via membrane proteins (red oval), which is and followed by lateral association of the filaments to form a mature Z-ring. Positive (+) and negative (-) regulators are indicated at each stage of FtsZ assembly.

organization of SepF into a ring structure is also important for normal SepF function *in vivo*. Mutation of a conserved glycine to asparagine (G135N) at the C-terminus of SepF does not abolish FtsZ binding but this mutant is defective in ring formation *in vitro* and cannot support cell division in a Δ*ftsA* mutant. In addition, Duman *et al*. found an intriguing correlation between the size of SepF rings (~40 nm diameter) and the width of septa which is in the range of 43 nm and proposed a model in which arcs of SepF polymers would fit on top of the leading edge of developing septum [62,63].

Interestingly, all membrane tethers for FtsZ bind to the same region, the extreme C-terminal part of FtsZ (Fig. 2) [19,58]. It is not possible that all proteins bind to one region at the same time, it is likely that Z-ring formation is regulated from the membrane via this part of the protein by activators and inhibitors of FtsZ.

Z-RING ASSOCIATED PROTEINS (ZAP)

ZapA is a non-essential, highly conserved, protein recruited early to the division site via direct interaction with FtsZ. A knock-out of *zapA* does not exhibit a phenotype unless it is combined with an *ezrA* mutant which is lethal [98]. ZapA forms tetramers *in vitro* and bundles FtsZ protofilaments into thick branched higher order structures [99]. It is thought that the role of ZapA is to stabilize the Z-ring *in vivo* (Fig. 3) [99].

Recently, three other Zap proteins were discovered in *E. coli*, two of which directly interact with FtsZ (**ZapC and D**) [100,101] and one (**ZapB**) that is associated

to the Z-ring via ZapA [102,103]. ZapB forms long cables *in vitro,* which are bundled by ZapA. Together with FtsZ both proteins form an interactome with highly ordered long cables and bundles [103]. ZapC and D bind to FtsZ independently of ZapA and their role is stabilization of FtsZ protofilaments [100,101]. Why *E. coli* needs so many different proteins with similar functions is not fully resolved. However, it is known that FtsZ lateral interactions play a critical role for Z-ring stability *in vivo* [83]. It was shown that the CTV is important for lateral interactions of FtsZ protofilaments *in vitro*. Lateral interactions between *B. subtilis* FtsZ protofilaments are stronger compared those of *E. coli* [16]. Durand-Heredia *et al.* suggested that Zap proteins compensate for weak lateral interactions of *E. coli* FtsZ *in vivo* [100].

6.2 LATE CELL DIVISION PROTEINS

Z-ring formation at mid-cell promotes the recruitment of other components of the cell division machinery. This includes late cell division proteins which together with FtsZ and its interacting partners form a complex called divisome [104]. The divisome contains over 30 different proteins out of which almost half are essential in *E. coli* [105-107]. FtsK is thought to be recruited first and FtsN last of the downstream essential cell division proteins [104,105,108]. However, recent findings reveal interactions between FtsN and early cell division proteins and the hierarchy of assembly seems to be more complex than previously thought [109]. After FtsN is recruited to the septal ring, the constriction of the cell membrane and remodeling of the cell wall begins [105,110-112]. This stage is likely to be closely regulated so that the development of potential error is minimal and can be corrected quickly [104,105,112].

FtsK is a bifunctional protein involved in cell division and chromosome segregation [107]. It is thought that membrane/periplasmic domain of FtsK is involved in stabilizing late cell division proteins and the recruitment of the FtsQLB complex to the division site [113,114]. The cytoplasmic domain forms hexamers involved in DNA transport [115] associated with chromosome segregation during division.

FtsQLB. Highly conserved among bacteria, the FtsQLB subcomplex (in *B. subtilis* DivIB, DivIC, FtsL) is formed independently of its localization to

mid-cell. FtsL is involved in many protein-protein interactions within the divisome [116]. Therefore, for a long time it was thought that the role of FtsQLB complex is scaffolding the divisome components together. However, recent findings by Tsang and Bernhardt suggest that the complex plays an important role in activating the divisome to begin constriction [117].

FtsW belongs to the SEDS (Shape Elongation Division Sporulation) family of membrane proteins [107,118]. Mohammadi *et al.* identified FtsW as a transporter (flippase) of cell wall building blocks across the cytoplasmic membrane [119].

FtsN was thought to be recruited to the division site as the last component of the divisome [97]. However, recent findings indicate that small amounts of FtsN are recruited to the division site earlier via interaction with a FtsA monomer [120]. It has been suggested that FtsN allosterically activates constriction via two interactions, one with FtsA in the cytoplasm and another with the FtsQLB complex in the periplasm [105,109,111].

In contrast to cell membrane constriction and cell wall ingrowth in *E. coli*, in *B. subtilis* septal cross-wall synthesis is completed before daughter cell separation. Gram-positive bacteria contain most of the essential late cell division proteins except for FtsN [107]. It was shown that the FtsZ-interacting protein EzrA, together with GpsB, plays a role in the switch from lateral to septal cell wall synthesis by recruiting the major peptidoglycan synthase PBP1 [85]. The misshaped septa formed in a *sepF* mutant [63] suggest that SepF also plays a role in septum closure. However, the exact mechanism of septum synthesis and closure is still to be discovered [62,63].

The next stages of cell separation involve peptidoglycan synthesis and septum cleavage to complete cell separation. This stage was extensively described in a review by Egan and Vollmer [107] and are beyond the scope of this thesis.

7. FTSZ AS A FORCE FOR MEMBRANE CONSTRICTION

Even though proper cell envelope constriction and cell separation requires a number of accessory proteins, Erickson has proposed that FtsZ may generate the constriction force for the membrane by itself [2,121]. The "Z-centric hypothesis" was supported by works of Osawa *et al.*, Hsin *et al.* and Szwedziak *et al.*,

that showed that a nucleotide-dependent bending of FtsZ protofilaments is enough to provide a mechanical force for membrane constriction [25,122-124]. Dynamic simulations provided indications that GDP-bound FtsZ filaments form more curved filaments than GTP-FtsZ [122], in line with *in vitro* data obtained earlier [125]. Moreover, overexpression of FtsZ and FtsA is enough to generate extra septa in *E. coli* cells [25]. However, the force produced by FtsZ is not enough to completely close the septa and cell wall ingrowth might be crucial to push the septum toward closure [2]. Another question is that, if FtsZ is enough to begin membrane invagination, then why does constriction begin only after divisome assembly is completed [105]? Thus, we come back to the possible role of FtsN in activation of FtsA – a membrane anchor for FtsZ. It is also possible that FtsZ bending is blocked by another divisome component like ZapA, until the divisome is fully assembled. The initiation of membrane constriction *in vivo* is still not fully understood.

8. FTSZ ASSEMBLY *IN VITRO*

The crystal structure of the FtsZ monomer is similar to the structure of eukaryotic tubulin [13]. FtsZ and tubulin also share some other properties. Both proteins assemble into long straight protofilaments in the presence of GTP [126]. The formation of a longitudinal bond between monomers is necessary for GTP hydrolysis *in vitro*. After GTP hydrolysis, GDP-bound FtsZ and tubulin protofilaments adopt a curved conformation [125]. In contrast to the conserved longitudinal bonds, the lateral associations between tubulin or FtsZ filaments seem to be completely different. Tubulin assembles into microtubules of regular cylindrical shape while FtsZ filaments may form a variety of more or less defined structures: single straight or curved filaments, sheets, tubes, minirings and small helices [125,127].

8.1 ASSAYS TO STUDY UNMODIFIED FTSZ

FtsZ structures and activity have been extensively studied *in vitro* using several standard assays and methods. The most common assays for studying native

FtsZ include sedimentation of polymers, 90° angle light scattering, visualization of FtsZ polymers using Electron Microscopy (EM) and a phosphate release assay that measures the GTPase activity of FtsZ [2,9,16,19,22,63,101,128]. Each of the methods, when used alone, gives only partial information about FtsZ activity in solution. However, the combination of all of them gives sufficient information about the behavior of FtsZ under chosen experimental conditions. For example, the phosphate assay may indicate a decrease in the GTPase activity of FtsZ under specific experimental conditions (low pH or the presence of inhibitors). To understand whether the changes in activity are due to bundling of FtsZ protofilaments, aggregation or the blockage of FtsZ oligomerization may be confirmed using EM. On the other hand, visualization of polymers by EM will not reveal anything about the dynamics of polymer formation and polymer disassembly in time, which can be studied using light scattering [128].

Other, less generally employed methods were used by several groups to study unmodified FtsZ in more detail. These methods include linear dichroism (LD) [129], dynamic light scattering [130] and analytical ultracentrifugation [131]. These methods have some advantages over standard methods. For example, LD distinguishes between FtsZ polymers and less-well defined aggregates while light scattering shows only the difference between FtsZ monomers and higher order structures [129]. However, they require access to more specialized equipment and further data analysis while standard assays are easy to perform and commonly used. All methods currently used to study unmodified FtsZ *in vitro* are summarized in Table 2.

8.2 FACTORS THAT INFLUENCE FTSZ IN VITRO ACTIVITY

Standard assays for FtsZ *in vitro* studies require the presence of special low molecular weight components, among which the most important are GTP or its analogues. The right choice of divalent and monovalent cations is crucial, ex. replacement of potassium with sodium may completely abolish FtsZ GTPase activity. Anions do not have significant influence on FtsZ assembly of GTPase activity. The most important factors for FtsZ assays are described below.

Table 2. Assays to study the biochemistry of unmodified FtsZ in vitro with examples of references in which these methods were used.

Method	Purpose	Advantages	Disadvantages
Sedimentation	Quantification of FtsZ present in polymers	• Easy • Possible to study several conditions at the same time • Requires simple equipment	• Cannot distinguish polymers from aggregates • Cannot distinguish short bundled polymers from long single protofilaments • No information about activity
90° light scattering	Quantification of polymer (dis) assembly in time	• Easy • Detection of polymer formation and disassembly in real time	• Difficult to distinguish polymers from aggregates • Cannot distinguish short bundled polymers from long single protofilaments • No information about activity • Limited amount of samples and conditions
Phosphate assay (GTPase assay)	Measurement of FtsZ activity	• Quantifiable • The only assay to study activity rather than structure of FtsZ	• No information about polymer formation
Electron microscopy	Visualization of polymers and structures adsorbed on surface	• High resolution • Observation of real structures • Small amount of sample necessary	• Specialized equipment needed • Surface contact may increase bundling and polymerization • Not possible to study polymerization in real time • No information about activity • Dependent on preparation of the sample
Analytical ultracentrifugation [131]	Quantification of polymer assembly	• Quantitative • Characterization of the size distribution and shape of individual polymers in solution	• Complicated analysis • Specialized equipment needed • Not possible to study polymerization in real time • No information about activity • GTP can be depleted in the experiment
Dynamic light scattering [131]	Quantification of polymer assembly and length in time	• Detection of polymer formation • Measurement of length distribution of polymers in sample	• Difficult analysis • Requires specialized equipment
Linear dichroism [129]	Quantification of polymer assembly and length in time	• Detection of polymer formation in real time • Distinguishes between FtsZ polymers and less well-defined aggregates	• Difficult analysis • Requires specialized equipment

GUANINE NUCLEOTIDES

FtsZ is its own GTPase-activating protein (GAP). In the presence of GTP, FtsZ forms long straight protofilaments with a broad length distribution. Oligomerization into filaments is necessary for GTP hydrolysis. Single protofilaments are visible on an EM grid when low concentrations of FtsZ are used. At higher concentrations protofilaments start to form lateral bonds and bundle into higher order structures. Bundling reduces subunit turnover in protofilaments and is accompanied by a decrease in GTPase activity [16,128]. Several nonhydrolyzable GTP analogues were used to induce oligomerization of FtsZ, including GMPCPP (guanylyl--(alpha, beta)-methylene-diphosphonate), GMPPNP (5'-Guanylyl imidodiphosphate) and GTPγS (guanosine 5'-O-[gamma-thio]triphosphate) and GDP-AlF (GDP in complex with AlF) [132-134]. FtsZ polymerizes well in the presence of GMPCPP and this GTP analogue is hydrolyzed 50 times slower than GTP. GMPPNP and GTPγS alone cannot support assembly of FtsZ. GMPPNP binds to FtsZ too weakly and GTPγS requires the presence of GTP to induce polymerization [133]. GDP bound to aluminium fluoride (GDP-AlF) resembles a nonhydrolysable form of GTP and supports polymerization well but with slower kinetics [132]. It was also shown that FtsZ does not hydrolyze GTP in the absence of Mg^{2+}, while assembly of FtsZ still occurs. Therefore this is also a way to form stable FtsZ protofilaments [135]. FtsZ also assembles in the presence of GDP, but GDP binding is weaker than GTP. In the presence of GDP, FtsZ polymers are much shorter and form minirings [2,126].

CATIONS

The major cytoplasmic cation in *E. coli* and *B. subtilis* is potassium. The concentration of K^+ varies between 0,4 and 0,76 M depending on external osmolarity [136]. It is also one of the monovalent cations that favor FtsZ polymerization. Another monovalent cation which was shown to support polymerization of FtsZ is Rb^+. However, GTP hydrolysis in the presence of Rb^+ is at least 5 times lower comparing to K^+. Potassium is the only cation which supports both polymerization and GTP hydrolysis of FtsZ [137]. In the presence of Na^+, GTP hydrolysis is completely blocked at low FtsZ concentrations [137] and the critical concentration for assembly is raised from 1 to 20 μM [2]. The concentration of K^+ is also important because it influences activity, assembly, bundling and subunit exchange in protofilaments [2,128]. The GTP hydrolysis activity is connected to the

K^+ concentration, with hydrolysis occuring faster at increasing K^+ in a range of 0 to 0,5 M K^+ [2,128].

Magnesium is another important factor influencing the assembly of FtsZ. Mg^{2+} is not necessary for FtsZ assembly but it is required for GTP hydrolysis [135]. It was shown that Mg^{2+} facilitates depolymerization of GDP bound FtsZ [138]. The Mg^{2+} concentration in various *in vitro* studies varies from 2,5 mM to 10 mM and influences FtsZ assembly. At lower Mg^{2+} concentrations *E. coli* FtsZ forms single filaments while higher concentrations cause bundling of protofilaments [2].

PH

The first *in vitro* assays of *E. coli* FtsZ were performed at pH=6.5. At this condition the assembly of FtsZ is more robust because the negatively charged FtsZ protofilaments form more lateral bonds, which reduce subunit turnover and GTPase activity [128]. Currently, pH close to neutral is used because it is more similar to the internal cytoplasmic pH of *E. coli* and *B. subtilis* [2]. At higher pH, FtsZ forms shorter polymers and bundles less compared to lower pH buffers. Another important fact is that some of the FtsZ interacting proteins, such as MinC and SepF, are pH sensitive [63,139]. Therefore, for interaction studies, stability of the interaction partner must be also taken into consideration.

Many factors influence FtsZ polymerization, bundling properties and GTPase activity. In all previous studies on FtsZ *in vitro*, various K^+ (from 0 to 500 mM), Mg^{2+} (from 2 to 10 mM) and GTP (from µM to mM range) concentrations and pH (from 5.8 till 7.5) were used [2]. It is difficult to compare assembly dynamics and activity between studies from various groups because a single variation in buffer composition may affect FtsZ assembly and activity. For example, at pH=7.5 FtsZ bundles less than at pH=6.5, but when simultaneously a Mg^{2+} concentration close to 10 mM is used, bundling will be predominant. Therefore, FtsZ may form similar structures at pH=7.5 and 10 mM Mg^{2+} as at pH=6.5 and 2 mM Mg^{2+}, whereas pH=7.5 and 2 mM Mg^{2+} will give a clear difference in polymer structure. On the other hand, using the same pH but different KCl concentrations will also influence activity, assembly and bundling properties of FtsZ [128].

9. CELL DIVISION AS TARGET FOR ANTIBIOTICS.

9.1 THE NEED FOR DEVELOPMENT OF NEW ANTIBIOTICS

Antibiotics represent a special class of drugs, whose incorrect use affect the broader community, not just an individual person [140]. The misuse of antimicrobial drugs, both in humans and in livestock, is one of the main causes of the rise in antibiotic resistance among pathogenic bacteria which in turn is developing into one of the biggest problems in public health. Previous studies showed that around 50% of antibiotic prescriptions by general practitioners may be improper [141]. Multidrug resistance among pathogenic bacteria is the cause of around 25,000 deaths each year in European hospitals [142]. Next to the importance of promoting the correct use of antibiotics [142], there is a clear need to develop new effective classes of antimicrobials with possible new targets [140,143]. Unfortunately, many pharmaceutical and biotechnology companies have left the area of antibiotic develoment as it is a time consuming process (approx. 10-15 years) involving huge costs (up to 1,3 billion dollars) [140], with relatively low expected gains [12,143]. Another cost-related problem is the focus on development of broad-spectrum rather than narrow-spectrum antibacterials. The narrow-spectrum drugs would be more specific and would probably avoid resistance development for a longer period of time, but will not provide a return on investment [12]. The current drugs target several conserved synthesis pathways: protein, DNA or RNA, cell wall, and folate synthesis pathways, and additionally, disruption of bacterial membrane integrity [140]. However, most of the new antibiotics which target these pathways are variants of older scaffolds (*e.g*. cephalosporins, β-lactams that target cell wall synthesis, have already reached the 5th generation of modification). This raises additional problems of quicker resistance development to these drugs, and modifications of core scaffolds cannot last forever. Therefore, new core scaffolds are needed [140]. Previously, the approach of drug development was not focused on specific targets, with first finding a compound with antibacterial activity followed by the discovery of its mode of action. Nowadays, facilitated by the sequencing of the genomes of most of the common bacterial pathogens, a target-centered approach is leading with an identification of novel targets first, and the development of target-specific drugs after [9,12,140,143,144].

9.2 CELL DIVISION AS ANTIBACTERIAL TARGET

Early cell division proteins are potential targets that have not yet been targeted by clinically approved drugs [12,140]. Most of the cell division proteins are essential for viability, highly conserved among bacteria and absent in eukaryotic cells and many proteins are accessible from the outside of the cell which relieves the need for the compound to enter the cytoplasm. All these features make cell division proteins good candidates as antibacterial targets [12].

FtsZ is the cell division protein against which most of drugs which target cell division were discovered. Even though FtsZ is a cytoplasmic protein, it has several features which make it a very promising target among all essential cell division components. First, it initiates assembly of the cell division machinery and interacts directly with many other essential proteins. Interactions between FtsZ and other components have been well characterized. Second, FtsZ biochemistry and interactions have been studied for years and there is a large body of knowledge about its structure and mode of action. And third, FtsZ purification and assays to study FtsZ are well established and thus suitable for high-throughput screening of potential drugs. *In vivo*, cells have a clear elongation phenotype when FtsZ is affected [12]. Nevertheless, there are also some problems when studying FtsZ as an antibacterial target. First, FtsZ is a close structural homologue of tubulin. However, the sequence homology between these two proteins is low and many drugs which target FtsZ were shown not to affect tubulin at all [12]. Second, FtsZ is a cytoplasmic protein and drugs targeting FtsZ must be enough small to go through the cytoplasmic membrane and reach the cytoplasm. And the third problem is that some of the *in vivo* and *in vitro* FtsZ screening assays generate many false positive hits. In *in vivo* assays, this is because cell elongation may be a secondary effect of another mechanism, for example the SOS response to DNA damage. *In vitro*, high-throughput assays using GTPase inhibition as readout resulted in various false positive hits because drug aggregation caused non-specific inhibition of FtsZ activity. This problem was identified by Anderson *et al.*, who improved these assays by addition of 0,01% Triton X-100 which breaks small molecule aggregates but does not significantly inhibit FtsZ GTPase activity [6].

10. DISCOVERY OF FTSZ INHIBITORS

Many approaches have been used to find molecules that target FtsZ. Rational drug design resulted in a number of GTP analogues with substitution of the C8 group of guanine. Other methods are based on high throughput screening (HTS) and include whole-cell antibacterial assays, bioinformatics tools (docking of known molecules into the crystal structure) and biochemical assays (ex. GTPase assay). All these approaches led to the identification of a huge number of compounds with various properties [6,9,12]. Anderson *et al*. divided them into seven different structural groups: cationic dyes (1), nucleoside and nucleotide analogues (2), drug-like heterocycles (3), phenolic natural products (4), miscellaneous high-throughput screening hits (5), anionic dyes (6) and quaternary alkaloid natural products (7) [6]. Among these FtsZ inhibitors are natural (such as chrysophaentins), synthetic (like PC190723) and semi-synthetic compounds (such as derivatives of sanguinarine). There are many extensive reviews on the topic, which include an explanation of the screening methods and some of the most promising hits [6,9,12,144]. Therefore, specific FtsZ-targeting drugs will not be described here in further detail.

Despite extensive studies and an enormous amount of publications on novel drugs that target FtsZ, none of the discovered compounds was chosen for further preclinical development [140]. There are several reasons for that. The first reason comes from the FtsZ studies. A big influence of buffer composition on mode of action of FtsZ (mentioned in section 7) makes comparison of all inhibitors difficult. Another reason is a huge number of false positive hits [6,9] due to compound aggregation as explained above. A third reason is that some of the positive hits specifically inhibit FtsZ but with a high IC_{50} value (*e.g.* Zantrin Z3 with an IC_{50} of 20 μM) and need to be developed further in order to obtain better inhibitory effects [6]. The last reason is that antibiotic development groups and companies prefer to work on known targets (or explore further the known targets) and scaffolds to reduce risk [140]. However, in the longer term FtsZ inhibitors may be also of interest for companies as multidrug resistance becomes a big problem and novel targets are needed.

Although FtsZ does not yet seem to be considered a good target for one-drug therapy, it would be a very good target for combination therapies with oth-

er antibiotics. The mutation rate of 10^{-6} per bacterial cell division cause problems in current antibiotic therapy [140]. Even partially blocking cell division would reduce the risk of mutations and developing resistance to antibiotics targeting other essential pathways. Recently, a quinuclidine-based FtsZ inhibitor was shown to synergistically act with β-lactam antibiotics against antibiotic-resistant strains [145] and alkyl gallates were shown to restore the β-lactam sensitivity of MRSA [146]. Alkyl gallates and possibly other natural compounds targeting FtsZ are also good candidates to fight plant diseases against plant-pathogenic bacteria [147].

SCOPE OF THIS THESIS

The aim of this thesis is to gain more insight in the biochemistry of FtsZ, the interaction of FtsZ with its partners, and to study molecules that could be potential antibacterial drugs targeting FtsZ.

Chapter 2 provides a detailed description of the standard assays used to study FtsZ: sedimentation, light scattering, electron microscopy and GTPase assays. Various assay conditions are tested on FtsZs from two model organisms: *E. coli* and *B. subtilis*. These assays were used in chapters 3, 4 and 5 to study *B. subtilis* FtsZ ($FtsZ_{Bs}$) and may be used to study the biochemistry of isolated FtsZ, or of FtsZ in the presence of interacting partners and/or small molecule inhibitors.

In **chapter 3** a novel pull-down strategy to find interacting partners for FtsZ C-terminus is described. A strong interaction between SepF and the FtsZ C-terminus was found and the amino acids responsible for this interaction were mapped by alanine scanning of the FtsZ C-terminus. The amino acids identified, P372 and F374, are highly conserved among various FtsZ proteins from different species.

In **chapter 4** the purification of the cytoplasmic domain of sporulation protein SpoIIE ($SpoIIE_{cyt}$) and the characterization of its interaction with FtsZ are described. Oligomerization of $SpoIIE_{cyt}$ is dependent on the presence of its cofactor Mn^{2+} or other divalent cations. It is hypothesized that the metal-dependent oligomerization of SpoIIEcyt may influence the interaction with FtsZ, as the SpoIIE-dependent relocalization of FtsZ to the cell pole is delayed when sporulation is induced in the absence of Mn^{2+}.

Chapter 5 focuses on a special class of antimicrobial compounds – alkyl gallates, and their inhibitory effect on *B. subtilis* cells. Alkyl gallates were identified as potential cell division inhibitors [148] and in this chapter the mode of action is determined in more detail. Alkyl gallates affect multiple targets in *B. subtilis*, one of which is FtsZ. We show that heptyl gallate is the most potent inhibitor of *B. subtilis* FtsZ and the most promising scaffold for development of more effective compounds.

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