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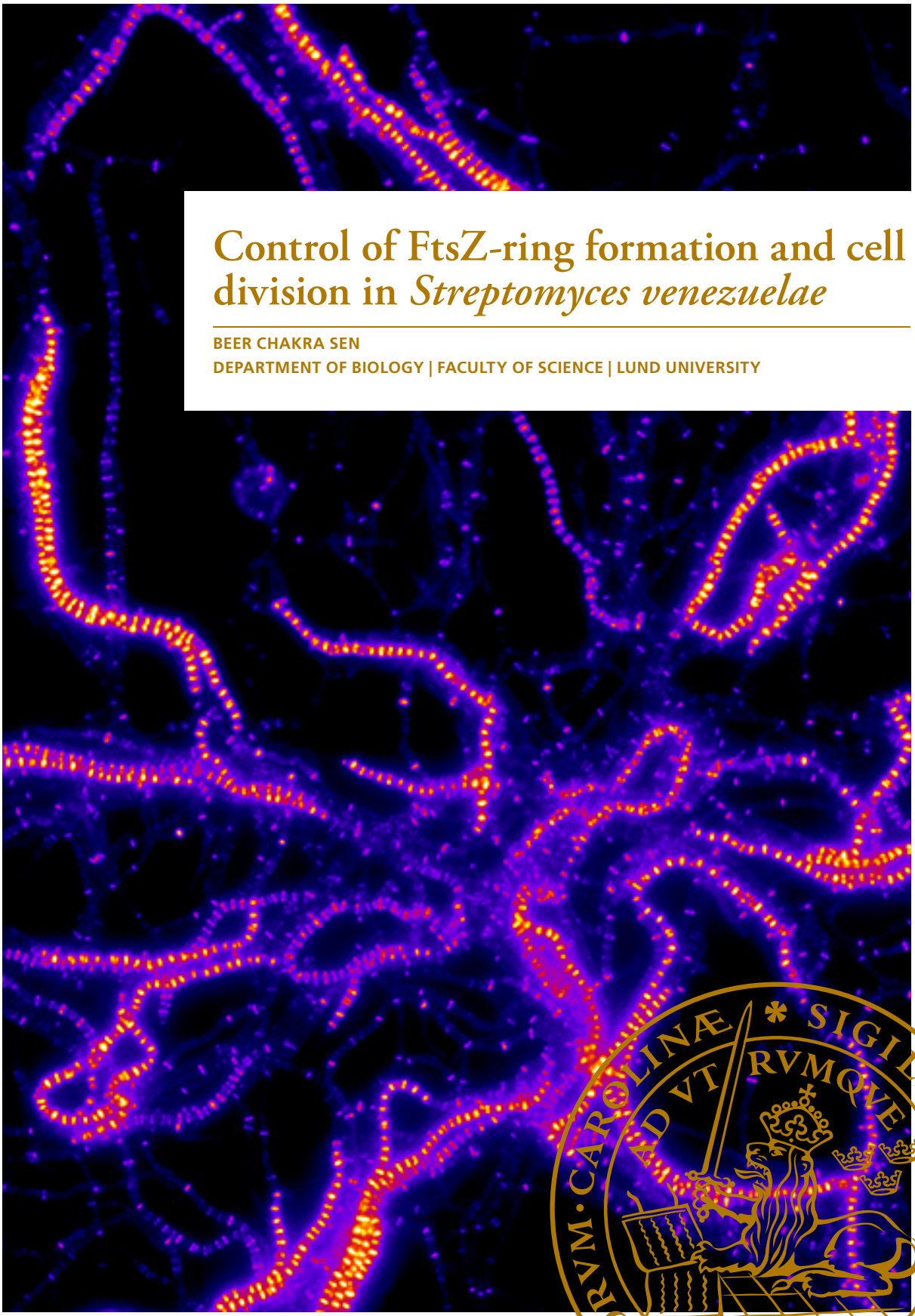

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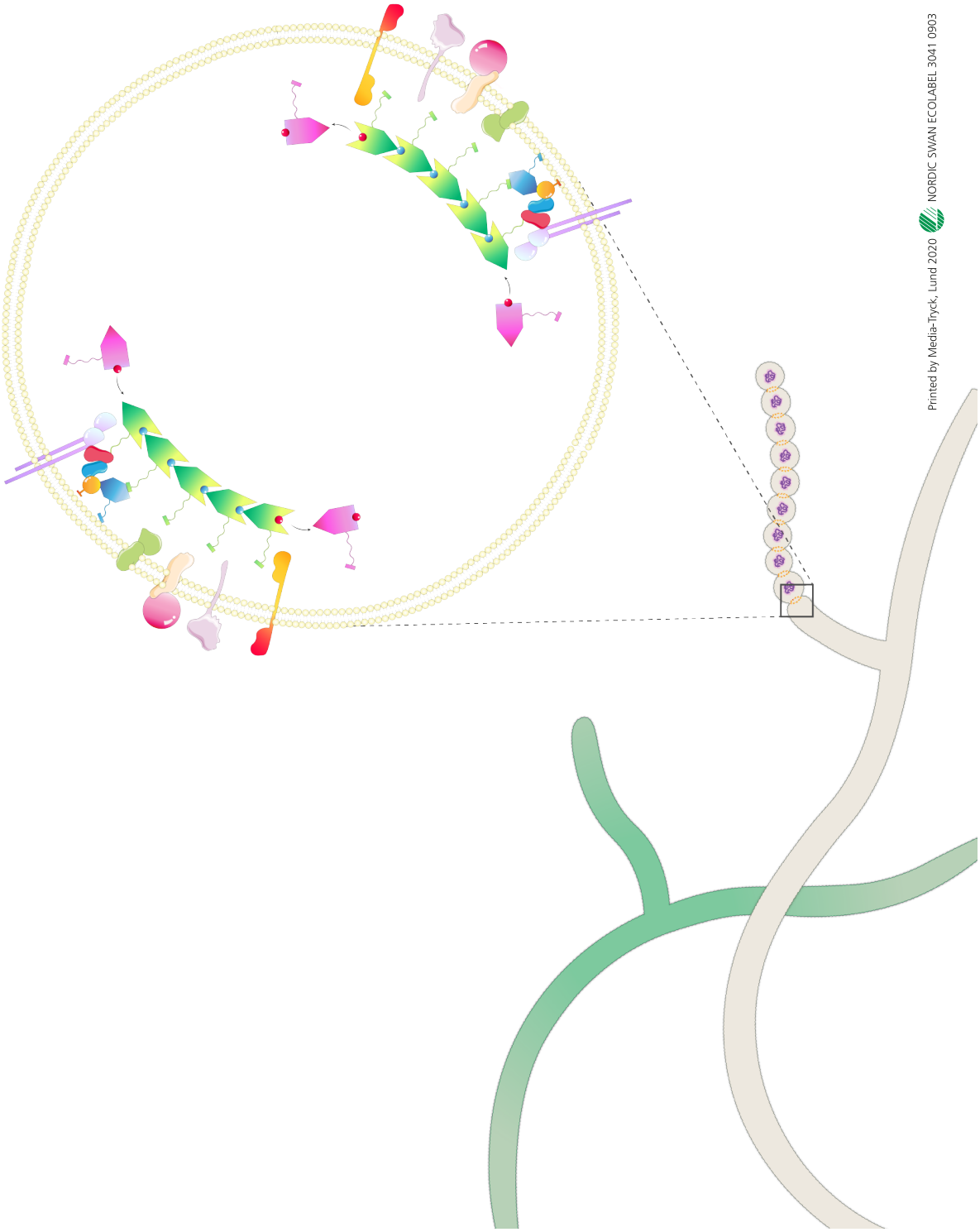

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Beer Chakra Sen

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Cell division is essential for all life forms. In bacteria, this fundamental process is precisely orchestrated by a protein, FtsZ, the ancestral homolog of eukaroytic tubulin. On the onset of division, at the division site, FtsZ assembles into a cytoskeleton structure - the Z ring - that recruits other division proteins and constricts the membrane together with cell wall synthesizing machinery, eventually splitting a cell into two. Despite billions of years of evolution, FtsZ-based division is highly conserved in most bacteria and also found in a major group of Archaea, plant chloroplasts, and mitochondria of many eukaryotes. In recent years, FtsZ has been extensively studied as an attractive antibacterial drug target.

Cell division is mainly controlled at the stage of Z-ring assembly, but the molecular mechanisms are not fully understood. For example, in Actinobacteria, the largest bacterial phylum, consisting of human pathogens such as *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis* and industrially important *Streptomyces* spp., proteins involved in the control of Z-ring assembly are not clearly known. The overall aim of this work is to shed light on the regulation of Z-ring assembly using *Streptomyces venezuelae* as a model.

First, we show that a specific region, the β strand S2, of FtsZ, is crucial for it's assembly and function. We found that a specific amino acid change in this region leads to a decrease in FtsZ's intrinsic GTPase activity and loss of critical concentration for FtsZ polymerization. This compromised assembly behaviour possibly affects cooperative assembly of treadmilling FtsZ polymers, most likely via the conformational switch. Defects in asssembly dynamics and treadmilling of FtsZ may explain the failure of the mutant to form proper Z rings, but the incorrectly shaped FtsZ strucutres that form in the cells are still able to initiate cell constrictions, leading to perturbed cell shape (Paper I). Second, we show that in *S. venezuelae*, most of the core division proteins, including FtsZ are non-essential for viability, thereby confirming that the dispensibility of division proteins for viability is a conserved cell division aspect in *Streptomyces*. Additionally, we show that the core divisome proteins are not required for Z-ring assembly (Paper II). Third, we demonstrate that SepF is a putative membrane anchor for the Z ring and is required for sporulation-specific cell division, while SepF2 and SepF3 have accessory role in *S. venezuelae* cell division (Paper III). Finally, we confirm that a septal factor SepIVA, supposedly to function in mycobacterial cell division, is not involved in *Streptomyces* cell division. Our results from SepIVA localization *in vivo* suggest that it may instead have a regulatory role in polar growth or cell wall synthesis in *S. venezuelae* (Paper IV).

Overall, this thesis work provides insights into the control of FtsZ-ring assembly and also on the functionality of several division proteins in sporulation-associated cell division in *S. venezuelae*.

Key words: *Streptomyces*, cell division, FtsZ, cooperativity, Z-ring assembly, divisome, SepF-like proteins, SepIVA

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मेरो परिवारमा समर्पित

Le rêve d'une bactérie doit devenir deux bactéries
(The dream of a bacterium is to become two bacteria)
François Jacob, 1965

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II. Stuart Cantlay, **Beer Chakra Sen**, Klas Flärdh and Joseph R. McCormick. Deletions of *ftsZ* and divisome genes in *Streptomyces venezuelae* ATCC 10712. (Manuscript)

III. **Beer Chakra Sen**, Stuart Cantlay, Joseph R. McCormick and Klas Flärdh. Elucidating the distinctive roles of three SepF homologs in *Streptomyces venezuelae* cell division. (Manuscript)

IV. **Beer Chakra Sen**, Susmita Datta and Klas Flärdh. Genetic and cell biological studies connect *sepIVA* in *Streptomyces venezuelae* to polar growth and not cell division. (Manuscript)

Contributions

I. I carried out all genetic, cell biological, and biochemical work on *S. venezuelae* FtsZ, and the biochemical experiments on *S. coelicolor* FtsZ. With input from other co-authors, I analyzed data, prepared the manuscript, and participated in the submission for publication.

II. I contributed in designing and performing all microfluidics-based time-lapse fluorescence microscopy. I also contributed in analyzing data and preparing the final draft of the manuscript.

III. The *sepF* mutants were constructed by other co-authors. I designed and carried out most of the experiments described in the manuscript. With input from other co-authors, I analyzed data, and prepared the manuscript.

IV. I designed experiments and supervised the work together with KF. With input from other co-authors, I analyzed data and prepared the manuscript.

1. Preface

Cell division (cytokinesis) is a dynamic, fundamental, and sophisticated life process. In all organisms, this process needs to be executed very precisely. In bacteria, this complex task is carried out by a macromolecular machine called the divisome, the assembly of which is directed by a protein, FtsZ. FtsZ polymerizes into a ring-shaped cytoskeletal structure the 'Z ring' that serves as a scaffold to recruit other cell division proteins, and coordinates peptidoglycan (PG) synthesis and membrane constriction, eventually resulting in cell constriction .

Cell division is mainly regulated by controlling formation of the Z ring, but despite many years of research, the molecular mechanisms are not well understood. Additionally, it remains largely unclear how cell division is regulated in the largest bacterial phylum, Actinobacteria. The phylum includes pathogenic bacteria such as *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis* and bacteria with industrial significance such as *Corynebacterium glutamicum* and *Streptomyces spp*. These bacteria lack homologs of the proteins known to control Z-ring assembly in conventional models, such as in *Escherichia coli* and *Bacillus subtilis* (discussed further in section 7). The main goal of this thesis work is to shed light into the control mechanisms of Z-ring formation and cell division in Actinobacteria. To achieve this goal, I have utilized *Streptomyces venezuelae* as a model system (discussed in section 7.1).

Here, I present first an overview of the bacterial cell division process (section 2-6). Since my work revolves around FtsZ and it is the principle driver of cell division in most bacteria, I discuss current understanding on the nature and function of FtsZ (section 3 and 4). My work also involves other cell division proteins and regulators of this process, therefore a background from the conventional models is discussed in section 5 and 6. In section 7, I discuss what is known about cell division in Actinobacteria and *Streptomyces*. In the final part, I discuss the results and analysis of my PhD research projects and put them in perspective (in sections 9 and 10).

2. Bacterial cell division: an overview

Bacterial cell division is well studied in model organisms such as *E. coli* and *B. subtilis*. Many reviews describe recent insights into bacterial cell division (Haeusser & Margolin, 2016, Ortiz *et al.*, 2016, den Blaauwen *et al.*, 2017, Du & Lutkenhaus, 2017, Errington & Wu, 2017, Mahone & Goley, 2020). Here, cell division in *E. coli* is presented as an example.

In *E. coli*, three phases can be distinguished in the cell division process, as shown in Fig. 1 .

1) First, under spatial and temporal control (discussed in section 6), FtsZ polymerizes into a ring-shaped structure - the Z ring, at the future division site (mid cell). The ring is attached to the cytoplasmic membrane with the aid of tethering proteins (discussed in section 5.1.1), forming a complex called the proto-ring.
2) Second, the proto-ring recruits other cell division proteins, such as proteins involved in cell wall synthesis and proteins involved in regulation (discussed in sections 5.1 and 5.2) to form a mature divisome complex.
3) Third, the divisome complex in coordination with septal peptidoglycan synthesis splits the cell into two.

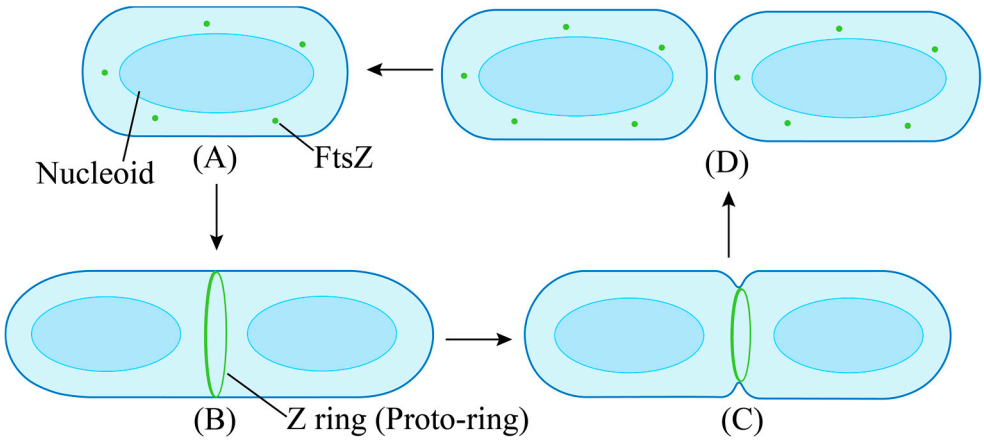

Figure 1. Schematic representation of bacterial cell division in *E. coli*. A) Typical *E. coli* cell showing cytoplasmic FtsZ and chromosomal DNA organized in a nucleoid. B) Following chromosome replication and segregation, FtsZ organizes into the Z ring at mid cell and is anchored to the membrane forming the 'proto-ring'. C) The proto-ring further recruits additional proteins forming a mature divisome that in coordination with a peptidoglycan synthesis machinery constricts the membrane and forms a septum. D) Eventually, a cell is separated into two daughter cells.

3. FtsZ: the principle cell division protein

3.1. Historical perspective

In 1960s, Van De Putte and others isolated temperature-sensitive *E. coli* cell division mutants, that later led to the identification of several genes involved in cell division (Van De Putte *et al.*, 1964, Hirota *et al.*, 1968). These conditionally lethal mutations that produced filamentous cells at non-permissive temperature were designated as *fts* (*f*ilament-forming *t*emperature *s*ensitive). One of the mutated genes was later identified and named *ftsZ* by Lutkenhaus *et al.* (Lutkenhaus *et al.*, 1980). Subsequently, Bi and Lutkenhaus demonstrated that FtsZ forms a ring structure at the cell division site and suggested that it may act as a cytoskeletal protein (Bi & Lutkenhaus, 1991).

3.2. Conservation of FtsZ

FtsZ is present in almost all bacteria, with a few exceptions like the members of the Planctomycetes, Verrucomicrobia and Chlamydiae (PVC) superphylum (Rivas-Marin *et al.*, 2016). In addition, while many of the Mycoplasmatales contain *ftsZ*, *Ureaplasma urealyticum* has lost it (Vedyaykin *et al.*, 2017). It is still unclear and poorly understood how cell division occurs in these species lacking *ftsZ.* FtsZ is also found in the Euryarchaeal group of Archaea, plant chloroplasts, and mitochondria of many eukaryotes (Margolin, 2005).

3.3. FtsZ as a drug target

In order to address the emerging global antibiotic resistance problem, development of new drugs and identifying targets with novel modes of action is of high priority. Because of its wide conservation in bacteria, FtsZ protein has been a very attractive antibacterial drug target (Casiraghi *et al.*, 2020). Several low-molecular weight

molecules have been described that target FtsZ, suggesting that it can be utilized as a drug target. One example is the benzamide derivative PC190723 that is validated to be effective against staphylococcal infection in mice model (Haydon *et al.*, 2008).

3.4. Structure

FtsZ, a tubulin homolog, is a self-activating GTPase that can bind to and hydrolyze GTP (de Boer *et al.*, 1992, RayChaudhuri & Park, 1992, Mukherjee *et al.*, 1993). It has a conserved glycine-rich G-box motif [GGGTGTG], similar to the tubulin signature motif [(A/G)GGTG(S/A)G]. Though the amino acid sequence identity between FtsZ and tubulins is relatively low (10 -18%), the first crystal structure of FtsZ at 2.8 Å resolution from *Methanococcus jannaschii* revealed high structural similarity (Löwe & Amos, 1998, van den Ent *et al.*, 2001). Strikingly, even though *E. coli* is a widely used model to study cell division, crystal structures of *E. coli* FtsZ bound to, GDP (at 1.35 Å) and to GTP (resolution of 1.4 Å) have been deduced only very recently (Schumacher *et al.*, 2020).

Based on crystal structures, the central globular part of FtsZ consists of two independent domains connected by a central core helix H7 (Löwe & Amos, 1998) (Fig. 2). The N-terminal GTPase domain has a characteristic Rossmann fold and the tubulin signature motif necessary for GTP-binding and hydrolysis. The C-terminal activation domain contains the 'synergy' or 'T7 loop' which associates with the GTPase domain in the N-terminal domain of the next subunit in a protofilament to form the active site for GTP hydrolysis (Löwe & Amos, 1998). The next ~ 50 amino acids following the activation domain are highly variable across bacterial species and can act as a flexible linker. At the C-terminal end is a highly conserved peptide (~ 17 amino acids) that has been implicated in interaction between FtsZ and several regulatory proteins, such as FtsA, ZipA, ClpX, MinC, EzrA and SepF (Erickson *et al.*, 2010).

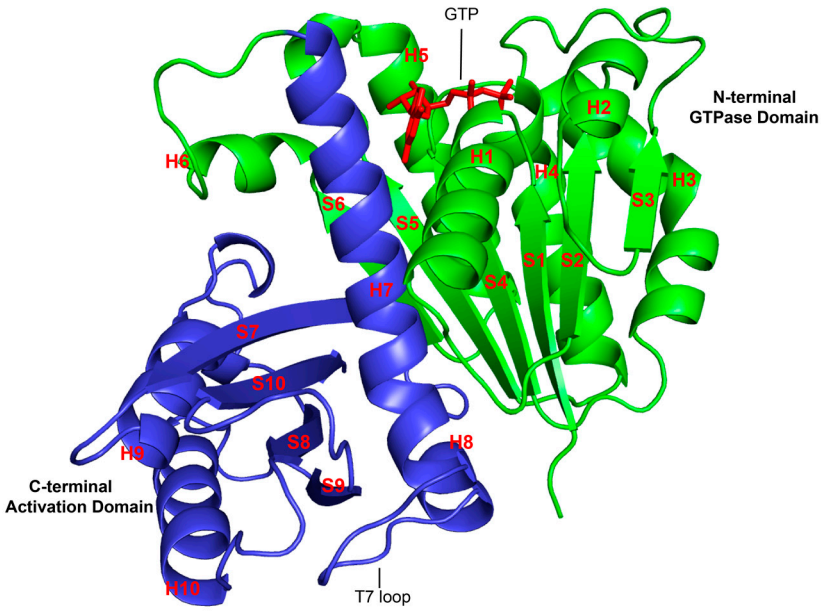

Figure 2. Ribbon cartoon of the homology model of *S. coelicolor* FtsZ. The C-terminal activation domain and H7 central helix, including the T7 loop, are shown in blue; the N-terminal GTPase domain, and the bound nucleotide are in green and red, respectively. The hypervariable flexible linker and the C-terminal conserved peptide are not shown. S and H indicate strands and helices, respectively. The model is based on the crystal structure of *M. tuberculosis* FtsZ (PDB:1RQ7) and the structure is labeled according to the common nomenclature for the tubulin/FtsZ family of GTPase by Nogales *et al* (Nogales *et al.*, 1998) (Reproduced from (Sen *et al.*, 2019), with permission).

3.5. Cooperative assembly of FtsZ

In vitro, FtsZ readily assembles into 'protofilaments' in a GTP-dependent manner (Bramhill & Thompson, 1994, Mukherjee & Lutkenhaus, 1994, Mukherjee & Lutkenhaus, 1998). During this assembly, FtsZ monomers interact in a head-to-tail manner, such that the T7 synergy loop of one FtsZ subunit associates with the GTP binding pocket of another subunit. This interaction at the longitudinal interfaces of subunits creates the active site for GTP hydrolysis (Fig. 3A).

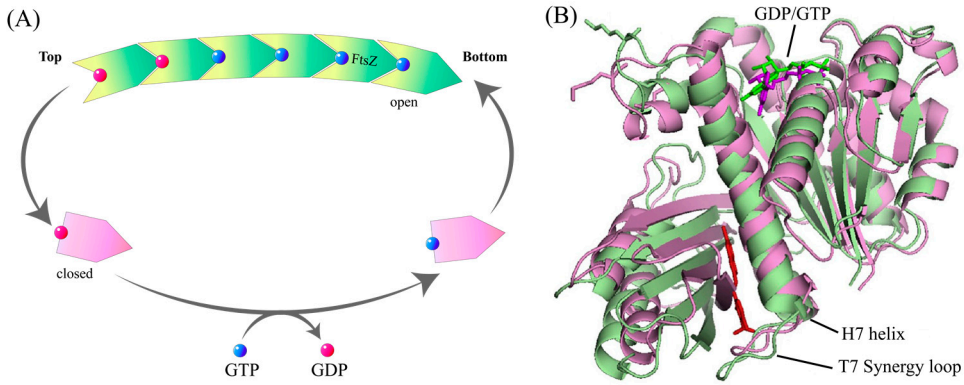

Figure 3. Polymerization–associated conformational switch in a single-stranded protofilament. A) The conformational switch allows addition of a free subunit (closed form; in pink) at the bottom end of the growing filament (kinetic plus end). The binding of the monomer switches conformation to open form in the filament (in green). Upon GTP hydrolysis, subunit dissociation occurs from the other end (kinetic minus end). The dissociated subunits change back to closed form, exchange nucleotide and bind to the growing filament. B) Two conformations of FtsZ (from *Staphylococcus aureus*), as a free monomer "closed form" (in pink; PDB:2RHL) and in association with the polymer "open form" (in green; PDB:3VOB) are superimposed. The PC190723 inhibitor (see section 3.3) is shown in red in the open form of FtsZ. (A. Image courtesy of Veronica Ekdahl, B. Reproduced from (Du & Lutkenhaus, 2019), with permission).

GTP hydrolysis occurs within the protofilament and GDP-bound FtsZ tends to depolymerize (Mukherjee & Lutkenhaus, 1998, Scheffers *et al.*, 2002). The nucleotide is then exchanged for GTP in monomeric FtsZ, which in turn leads to re-polymerization of FtsZ monomers into protofilaments (Fig. 3A). Thus the polymerization is GTP-dependent and highly dynamic (Mukherjee & Lutkenhaus, 1998). Several factors such as salt, pH, and multivalent cations such as Ca^{2+}, Mg^{2+} and DEAE-dextran affect FtsZ assembly *in vitro* (Erickson *et al.*, 2010). *In vitro*, at a high FtsZ concentration, the linear single-stranded polymers interconnect forming higher-order structures with extensive lateral interaction between the protofilaments (Chen & Erickson, 2005, Esue *et al.*, 2005). The physiological relevance of the lateral interactions between protofilaments are not well understood (Krupka & Margolin, 2018), but a recent study suggests that these interactions are critical for the assembly of a functional Z ring *in vivo* (Guan *et al.*, 2018).

Even under *in vitro* conditions when FtsZ assembles into single-stranded protofilaments, it displays cooperativity (Caplan & Erickson, 2003, Chen *et al.*, 2005). Cooperative assembly exhibits several features, such as an initial nucleation stage characterized by a kinetic lag at low protein concentration, and a growth or elongation stage that requires a critical concentration of FtsZ monomers for polymer assembly. Cooperative assembly is a common phenomenon in multi-stranded biological polymers (Oosawa & Kasai, 1962). Therefore, it is puzzling how FtsZ protofilaments that are one subunit thick can display cooperativity.

To explain cooperativity in single-stranded filaments, an allosteric model has been suggested. The model suggests that the subunits exist in two different conformations and that the conformational change approbate subunits to bind to oligomers with high affinity and to monomers with low affinity, thereby allowing cooperative assembly of single-stranded protofilaments (Michie & Löwe, 2006, Dajkovic *et al.*, 2008, Huecas *et al.*, 2008, Miraldi *et al.*, 2008). The model is confirmed by a recent study wherein FtsZ has been crystallized in two conformations: closed form (as a free monomer) and open form (in filaments) ((Wagstaff *et al.*, 2017), Fig. 3B). The study suggests that the polymerization-associated conformational switch of FtsZ between these two conformations involves opening of a hydrophobic cleft between α helix H7 and the C-terminal activation domain. The switch allows subunits to be added to one end of the protofilaments and dissociate from the other end, which is the basis for the treadmilling of FtsZ filaments ((Loose & Mitchison, 2014, Ramirez-Diaz *et al.*, 2018), (Fig. 3A)).

The polarity of treadmilling FtsZ filaments has been recently identified. Considering the N-terminal GTPase domain as top surface and the C-terminal activation domain as bottom surface, the study by Du *et al* found that subunits in closed forms are added to the bottom end of the filament and thus the kinetic plus end, and following GTP hydrolysis subunits dissociate from other end (kinetic minus end) ((Wagstaff *et al.*, 2017, Du *et al.*, 2018), Fig. 3A). In paper I, we describe *ftsZ* mutations that influence cooperative assembly.

3.6. FtsZ localization in the cell - Dynamic filaments, rings, and helices

Using immunoelectron microscopy, Bi and Lutkenhaus, showed for the first time that FtsZ assembles in a ring-shaped pattern at the future division site in *E. coli* (Bi & Lutkenhaus, 1991). Later, FtsZ rings were also visualized in *B. subtilis* utilizing immunofluorescence microscopy (Levin & Losick, 1996). Subsequently, labelling of FtsZ and other cell division proteins with fluorescent proteins, such as with green

fluorecent protein (GFP) and others, it has been possible to confirm earlier studies, and also to perform live cell imaging (Ma *et al.*, 1996, Schneider & Basler, 2016).

In recent years, data from fluorescence-based super-resolution methods such as 3D-structured illumination microscopy (3D-SIM), photo-activated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and/or stimulated emission depletion (STED) microscopy, suggest that the Z ring is rather a discontinuous structure. In this model of Z-ring organization, loosely connected FtsZ filaments are heterogeneously distributed in a bead-like pattern around the entire ring (Haeusser & Margolin, 2016, Du & Lutkenhaus, 2019, McQuillen & Xiao, 2020). However, more studies are required to conclusively determine the ultrastructure of the Z ring.

The FtsZ filaments in the discontinuous Z ring are highly dynamic as suggested by fluorescence recovery after photobleaching (FRAP) studies. The results show rapid exchange of FtsZ subunits from a cytoplasmic pool to the Z ring, with a turnover of 9 seconds in *E. coli* (Stricker *et al.*, 2002) and about 8 seconds in *B. subtilis* (Anderson *et al.*, 2004). Advanced imaging has revealed that dynamic FtsZ polymers at the Z ring undergo rapid treadmilling, leading to a circumferential movement at the division site (Bisson-Filho *et al.*, 2017, Yang *et al.*, 2017).

In addition to the dynamic rings above, FtsZ can also assemble into non-ring structures *in vivo*, such as spirals and helices. Intriguingly, though not so clearly understood, it seems that these non-ring spiral structures are utilised by cells for normal growth and development. Ben-Yehuda and Losick reported that during sporulation in *B. subtilis*, the Z ring switches from medial position to two polar Z rings via transient helical structures (Ben-Yehuda & Losick, 2002). It has also been shown that dynamic and moving FtsZ helices are part of the assembly pathway leading to the formation of regular Z rings in both *E. coli* and *B. subtilis* (Thanedar & Margolin, 2004, Peters *et al.*, 2007). Other data indicate that the FtsZ helices could be involved in remodelling of the Z ring *in vivo*. For example, the assembly of multiple Z rings during sporulation in *S. coelicolor* appeared to involve the formation and remodelling of helical FtsZ intermediates (Grantcharova *et al.*, 2005). Therefore, it is plausible to think that the formation of the dynamic helices could be an inherent property of FtsZ in these organisms. In paper I, we describe the nature of spiral-shaped FtsZ assemblies formed due to a mutation in *S. venezuelae* FtsZ.

4. What drives cell constriction?

Over the years, one of the questions about the function of Z ring has been whether it generates force for cell constriction during division. In relation to this, the "Z ring centric" model has been proposed which suggests that FtsZ ring works actively in membrane constriction and the peptidoglycan synthesis (PG) passively follows behind, mainly due to FtsZ's intrinsic GTPase activity and its dynamic polymerization property (Erickson *et al.*, 2010, Erickson & Osawa, 2017). One of the bases for this model comes from reconstitution experiments wherein fluorescently labelled FtsZ bound to a lipid bilayer, when placed inside liposomes in the presence of GTP, promotes moderate constriction (Osawa *et al.*, 2008). Further, in the presence of the membrane tether FtsA, complete liposome fission was observed (Osawa & Erickson, 2013). Similarly, FtsZ polymerized in the presence of GTP could shrink permeable vesicles containing the membrane anchor ZipA (Cabre *et al.*, 2013). These *in vitro* observations suggest that FtsA, ZipA and FtsZ could self-organize and may provide constrictive force *in vivo*. A question remains: Is this FtsZ-derived force responsible for driving cell constriction *in vivo*?

Fluorescence microscopy-based studies show that FtsZ leaves the septum before the cell wall remodeling factors, implying that FtsZ cannot constrict the membrane, at least not at the final stage of septum closure (Söderström *et al.*, 2014, Söderström *et al.*, 2016). In *E. coli*, altering FtsZ assembly dynamics, including GTPase activity (thereby treadmilling) or FtsZ concentration had no effect on constriction rate (septum closure rate), suggesting that membrane constriction is not rate-limited by FtsZ (Coltharp *et al.*, 2016, Yang *et al.*, 2017). In addition, the cell constriction rate is proportional to the cell elongation rate, which is governed by PG synthesis, indicating that PG synthesis plays a limiting role in septum closure (Coltharp *et al.*, 2016). However, in *B. subtilis*, treadmilling rate governs the rate of PG synthesis and cell division (Bisson-Filho *et al.*, 2017). Therefore, it is still puzzling what drives membrane constriction. A recent model suggesting that a combined force generated by FtsZ pulling from inside and PG assembly pushing the membrane from outside maybe sufficient to overcome turgor pressure and thus complete septation (Erickson & Osawa, 2017, Osawa & Erickson, 2018). However, further work is required to clarify what exactly drives cell constriction.

5. The Fellowship of the Z ring

As mainly studied in *E. coli* and *B. subtilis*, the Z ring at the future division site acts as a scaffold and recruits other divisome proteins in a hierarchial manner (Du & Lutkenhaus, 2017, Errington & Wu, 2017, Du & Lutkenhaus, 2019, McQuillen & Xiao, 2020). At first, the early proteins are recruited, such as FtsA, ZipA, SepF, Zap proteins, and FtsEX. After this initial assembly, several proteins arrive at the division site, namely FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI and FtsN, also referred to as late proteins, in turn forming a mature divisome complex. Finally, the mature divisome constricts the cell membrane in coordination with peptidoglycan synthesis to complete cell division. Below, I summarize various divisome proteins that participate in the hierarchial assembly of the divisome.

5.1. Early proteins

5.1.1. FtsZ anchoring proteins - FtsA, ZipA and SepF

In *E. coli,* FtsA which belongs to actin family of proteins, functions as a membrane tether for FtsZ (Pichoff & Lutkenhaus, 2005, Conti *et al.*, 2018). FtsA is a widely conserved cell division protein, but it is absent in archaea and some bacterial genera including *Mycobacterium* and *Streptomyces* (McCormick, 2009, Ortiz *et al.*, 2016).

The other membrane anchoring protein in *E. coli*, ZipA (*Z i*nteracting *p*rotein *A*), is an essential protein, but it is poorly conserved outside the Gammaproteobacteria (Ortiz *et al.*, 2016). ZipA interacts via its C terminus with FtsZ (Hale *et al.*, 2000), and this specific interaction protects FtsZ from being degraded by ClpXP (Pazos *et al.*, 2013). Recent studies suggest that ZipA acts as a linker between the cytosolic Z ring and PG synthases (Pazos *et al.*, 2018).

SepF (*Sep*tum *F*orming) on the other hand is a highly conserverved protein across Gram-positive species. It interacts with itself and with FtsZ *in vivo*, and localizes to the nascent division site in a FtsZ-dependent manner (Hamoen *et al.*, 2006, Ishikawa *et al.*, 2006). The N-terminal domain of SepF contains a membrane-binding domain, whereas the C-terminal part comprises the FtsZ-binding site (Singh *et al.*, 2008, Duman *et al.*, 2013). In *B. subtilis,* SepF is essential in cells lacking either FtsA or

EzrA (Hamoen *et al.*, 2006, Ishikawa *et al.*, 2006). The cell division defect in *ftsA* null mutants can be complemented by overexpressing SepF (Ishikawa *et al.*, 2006). SepF organizes FtsZ protofilaments into higher order structures *in vitro* (Gundogdu *et al.*, 2011). These facts together with structural analysis suggests that SepF may have an overlapping function with FtsA in the Z ring assembly (Duman *et al.*, 2013). In corynebacteria and mycobacteria, which lack FtsA, SepF is essential for cell division (Gola *et al.*, 2015, Sogues *et al.*, 2020).

5.1.2. Z-ring associated proteins

The *Z*-ring *a*ssociated *p*roteins (ZapA, ZapB, ZapC, ZapD, and ZapE), have functionally redundant roles in stabilizing Z-ring assembly (Huang *et al.*, 2013, Ortiz *et al.*, 2016)). While ZapA is highly conserved among bacteria, ZapB-E are restricted to gammaproteobacteria. ZapA is a positive regulator of FtsZ assembly that directly binds to FtsZ and promotes initial FtsZ assembly and stabilty of the Z ring (Gueiros-Filho & Losick, 2002, Low *et al.*, 2004). In addition, ZapA recruits ZapB to the divison site (Galli & Gerdes, 2010), wherein ZapB participates in releasing FtsZ from mid cell before septum closure (Pazos *et al.*, 2013). Similarly, ZapC interacts directly with the globular core of FtsZ (Schumacher *et al.*, 2016), promotes lateral interaction between FtsZ polymers and supresses GTPase activity *in vitro* (Hale *et al.*, 2011). ZapD binds to the C-terminal tail of FtsZ and bundles FtsZ at mid cell in an FtsZ-dependent manner (Durand-Heredia *et al.*, 2012, Roach *et al.*, 2016). In contrast to other Zaps, ZapE is an ATPase which in the presence of ATP destabilizes FtsZ polymers *in vitro* (Marteyn *et al.*, 2014).

5.1.3. FtsEX

FtsEX is widely conserved among bacterial species and is required for recruitment of downstream division proteins to the Z ring (for a recent review (Pichoff *et al.*, 2019)). It is an ATP-binding casette (ABC) transporter-like complex comprising of FtsE (nucleotide-binding domain) and FtsX (transmembrane domain). It antagonises FtsA for the hierarchial recruitment of divisome proteins and plays a regulatory role in cell wall hydrolysis (Yang *et al.*, 2011, Du *et al.*, 2016).

5.2. Late proteins

5.2.1. FtsK family of DNA translocases

The FtsK family of translocases have a wide distribution across bacterial species. FtsK is a multidomain protein of which the N-terminal domain serves to localize the protein to division septum and is essential for septum constriction (Wang & Lutkenhaus, 1998, Bisicchia *et al.*, 2013), whereas the C-terminal domain acts as an ATP-dependent DNA translocase (Yu *et al.*, 1998, Sivanathan *et al.*, 2006). FtsK in *E. coli* and its paralogue SpoIIIE in *B. subtilis* are involved in translocation of chromosome away from division septum and in resolving intercatenated or dimerized chromosome (Besprozvannaya & Burton, 2014, Crozat *et al.*, 2014).

5.2.2. FtsQ (DivIB), FtsL and FtsB (DivIC)

Conserved among bacteria, FtsQ, FtsL, and FtsB in *E. coli* and their homologs DivIB, FtsL and DivIC in *B. subtilis*, respectively, are bitopic membrane proteins with single cytoplasmic, transmembrane and periplasmic domains (Kureisaite-Ciziene *et al.*, 2018). In the hierarchical divisome assembly process, the FtsQLB subcomplex is recruited to the divisome by FtsK (Buddelmeijer & Beckwith, 2004). The periplasmic domain of FtsQ consists of two subdomains: α subdomain of FtsQ interacts with FtsK and β subdomain is involved in interaction with many divisome proteins including FtsL and FtsB (van den Ent *et al.*, 2008). For a long time, FtsQLB complex was believed to have scaffolding function, however a recent study suggests that the complex has a role in regulation of septal PG synthesis (den Blaauwen & Luirink, 2019). A study in *E. coli* shows that the FtsQLB sub-complex inhibits the transpeptidase domain of penicillin binding protein PBP3, suggesting that the subcomplex represses cell division until mature divisome is formed (Boes *et al.*, 2019).

5.2.3. FtsW and FtsI

FtsW is a highly conserved, transmembrane protein belonging to the SEDS (*s*hape, *e*longation, *d*ivision, and *s*porulation) family (Meeske *et al.*, 2016). *In vitro* studies suggested that FtsW is a transporter (flippase) of lipid II (the peptidoglycan precursor) (Mohammadi *et al.*, 2011), and that it's interaction with lipid II is regulated by the cognate PBP3 (encoded by *ftsI*) (Leclercq *et al.*, 2017). Recent studies revealed that FtsW is a peptidoglycan polymerase that polymerizes lipid II into PG only in the presence of its cognate PBP (Taguchi *et al.*, 2019).

5.2.4. FtsN

FtsN, found only in enteric bacteria and *Haemophilus* spp., is the last known essential protein to be recruited at the division site and is considered as a trigger for cell constriction in *E. coli* (Du & Lutkenhaus, 2017). It is a bitopic integral membrane protein with a small cytoplasmic N-terminal domain that interacts with FtsA, and a large periplasmic domain containing a C-terminal 'SPOR' domain that binds to PG strands lacking stem peptides formed by the action of amidases (Weiss, 2015). FtsN allosterically activates constriction via interactions with FtsA in the cytoplasm and with the FtsQLB complex in the periplasm (Liu *et al.*, 2015, Pichoff *et al.*, 2018).

6. Regulation of cell division

The Z ring is placed with great precision at the future division site due to spatiotemporal regulation of FtsZ assembly. In *E. coli* and *B. subtilis*, two negatively acting systems, the Min system, and the Nucleoid Occlusion (NO) system, ensure proper placement of the Z ring. Although many bacteria utilize one or both systems for Z-ring placement, in several bacteria these are either absent or redundant in function, and additional mechanisms are also involved in regulating Z-ring assembly. Below, I have briefly described the Min and NO systems and some other regulators from different bacteria.

6.1. Min system

In *E. coli*, the Min system comprises the MinC, MinD and MinE proteins, which prevent asymmetric divisions and help positioning the Z ring at mid cell by inhibiting FtsZ polymerization at other positions (reviewed in (Lutkenhaus & Du, 2017, Ramm *et al.*, 2019)). MinC, the primary inhibitor of Z-ring formation, consists of two functional domains (Cordell *et al.*, 2001), of which, the N-terminal domain interacts directly with FtsZ and inhibits assembly of FtsZ polymers, and the C-terminal domain interacts with MinD (Hu *et al.*, 1999, Dajkovic *et al.*, 2008). MinD is an ATPase that in ATP-bound form causes the MinCD complex to adhere to the cytoplasmic membrane and block FtsZ polymerization in the proximity (Hu & Lutkenhaus, 2003, Szeto *et al.*, 2003). MinE binds MinD, displaces MinC, stimulates ATPase activity of MinD, and thereby releases the MinCD complex from the membrane (Loose *et al.*, 2008, Park *et al.*, 2012). The dynamics of MinE causes MinC and MinD to oscillate from pole to pole, resulting in higher average concentration of MinCD at poles and lower at the middle of the cell, and thereby inhibiting polar FtsZ assembly.

In contrast to *E. coli*, *B. subtilis* lacks MinE and has only MinC and MinD. In *B. subtilis*, DivIVA, a protein that senses negative membrane curvature, localizes MinCD to the poles via an adapter protein MinJ (Bramkamp *et al.*, 2008, Patrick & Kearns, 2008, Lenarcic *et al.*, 2009). Thus, unlike in *E. coli*, in *Bacillus* Min proteins do not oscillate.

6.2. Nucleoid occlusion (NO) system

Another important spatial regulatory system is the *N*ucleoid *O*cclusion (NO system) (reviewed in (Schumacher, 2017)). Nucleoid occlusion is mediated by proteins SlmA (in *E. coli*) and Noc (in *B. subtilis*) that inhibit Z-ring assembly over the chromosome (the nucleoid) (Wu & Errington, 2004, Bernhardt & de Boer, 2005). Intriguingly, although SlmA and Noc show no sequence homology, they both bind to specific DNA sequences that are distributed on the chromosome except around the terminus region and occupy mid cell position during the late stages of chromosome segregation (Cho *et al.*, 2011, Tonthat *et al.*, 2013). This creates a mid cell region free of SlmA and Noc towards the final stages of chromosome partitioning, allowing there the formation of Z ring.

SlmA and Noc, however, have different modes of action. SlmA binds to a SlmA-binding sequence (SBS) in DNA and also directly attaches to the C-terminal tail of FtsZ (Schumacher, 2017). The interaction between SlmA and SBS-motifs in DNA increases the affinity of SlmA towards FtsZ. *In vitro*, SlmA depolymerizes FtsZ protofilaments independently of the GTPase activity of FtsZ (Cabre *et al.*, 2015). In contrast, Noc binds to the membrane via its N-terminus where it recruits the DNA and is suggested to create physical crowding in the space between the membrane and DNA that would in turn block cell division (Adams *et al.*, 2015).

6.3. Alternative systems in some organisms

Many bacteria lack Noc/SlmA and/or Min protein homologues. Recent studies have revealed additional negative and positive Z-ring positioning mechanisms in, for example, *Caulobacter crescentus, Myxococcus xanthus*, and *Streptococcus pneumoniae* (Hajduk *et al.*, 2016, Schumacher, 2017).

6.3.1. *Caulobacter crescentus*: MipZ

In *C. crescentus*, MipZ (*Mi*d cell *p*ositioning of Fts*Z*) establishes bipolar MipZ gradient analogous to Min system (Thanbichler & Shapiro, 2006). MipZ interacts with chromosome partitioning protein ParB at the origin (*ori*) region of the chromosome, forming dimers. The intrinsic ATPase activity of MipZ allows the dimers to dissociate and then they rebind to surrounding DNA wherein they inhibit Z-ring formation (Kiekebusch *et al.*, 2012).

6.3.2. *Myxococcus xanthus*: PomZ

In *M. xanthus*, PomZ (*Po*sitioning at *M*id cell of Fts*Z*) positively influences Z-ring positioning. PomZ localizes to mid cell, recruits FtsZ and stabilizes the Z ring (Treuner-Lange *et al.*, 2013). In another study, it has been found that PomZ forms a complex with two previously uncharacterized proteins, PomX and PomY, and that this complex directly recruits FtsZ to mid cell and stimulates Z-ring assembly (Schumacher *et al.*, 2017).

6.3.3. *Streptococcus pneumoniae*: MapZ

In *S. pneumoniae*, MapZ (*M*id cell *A*nchored *P*rotein *Z*) or LocZ (*Lo*calising at mid cell of Fts*Z*) localizes at mid cell and recruits FtsZ (Fleurie *et al.*, 2014, Holeckova *et al.*, 2014).

6.4. Other regulators

In addition to the above mentioned regulators, there are many other factors that affect FtsZ polymerization and eventual Z-ring assembly by various mechanisms. However, with the limitation of the scope of this thesis, only some regulators are briefly described below. For others, such as, response to DNA damage (including the SOS-inducible SulA), levels of FtsZ in the cell, cell size, nutrient availibilty, and stress, which generally have an effect on the assembly of Z rings, I refer to reviews (Adams & Errington, 2009, Haeusser & Margolin, 2016).

6.4.1. EzrA (*E*xtra *Z r*ings *A)*

EzrA is conserved throughout low-GC Gram-positive bacteria. In *B. subtilis*, the loss of *ezrA* leads to extra Z rings, at medial as well as polar sites and overexpression of *ezrA*, blocks Z-ring assembly, suggesting that EzrA is a negative regulator of FtsZ assembly (Levin *et al.*, 1999, Haeusser *et al.*, 2004). EzrA domain structure shows a single amino-terminal transmembrane domain followed by four coil-coiled (CC) domains (Land *et al.*, 2014). While the transmembrane domain functions in the localization of EzrA at the membrane, the first two CC domains play a role in inhibiting FtsZ assembly at poles and the last two CC domains modulate interaction of EzrA with FtsZ throughout the cell (Land *et al.*, 2014). In *S. aureus*, EzrA is also involved in coordinating cell wall synthesis with cell division in order to maintain cell size (Jorge *et al.*, 2011).

6.4.2. ClpXP

ClpXP is a proteolytic machinery found in most bacteria that degrades damaged, misfolded, or unneeded proteins. It consists of two components: ClpX - the substrate recognizer that unfolds and translocates the substrate to ClpP - the peptidase (Olivares *et al.*, 2018). ClpXP is considered to be a negative regulator of Z-ring assembly in *E. coli* and *B. subtilis* (Ortiz *et al.*, 2016). *In vitro*, ClpXP binds and degrades FtsZ monomers and also GTP-induced FtsZ polymers (Camberg *et al.*, 2009). *In vivo*, overexpression of ClpXP blocks Z-ring formation (Weart *et al.*, 2005, Camberg *et al.*, 2009).

7. Cell division in Actinobacteria

Actinobacteria is a major phylum in the domain *Bacteria*. The phylum comprises diverse groups of Gram-positive bacteria. It is phylogenetically close to another Gram-positive phylum Firmicutes, but differs from the latter in having a high GC content in DNA (Stackebrandt & Schumann, 2006, Goodfellow, 2012). Members of the Actinobacteria phylum have diverse morphology, ranging from cocci (e.g., *Micrococcus*) or rod-coccoid (e.g., *Arthrobacter*) to fragmenting hyphal forms (e.g., *Nocardia* spp.) or mycelium (e.g., *Streptomyces*, *Frankia*) (Gao & Gupta, 2012, Barka *et al.*, 2016). Bacteria of this phylum typically grow by zonal assembly of the cell wall at the cell poles (polar growth). In rod-shaped Actinobacteria, cell elongation occurs at cell poles generated by cell division, while in mycelial forms, this occurs by tip extension in combination with hyphal branching. Many, but far from all, Actinobacteria display developmental life cycles and produce spores (Gao & Gupta, 2012). The Actinobacteria are found in a wide range of ecological habitats, including terrestrial or aquatic and marine environments (e.g., *Streptomyces* spp., *Rhodococcus* spp.), plant symbionts (e.g., *Frankia* spp.), plant or animal pathogens (e.g., *Corynebacterium*, *Mycobacterium*, *Nocardia* spp), and gastrointestinal commensals (e.g., *Bifidobacterium* spp.) (Barka *et al.*, 2016).

An updated taxonomy of the Actinobacteria phylum consists of six classes, namely, Acidimicrobiia, Actinobacteria, Coriobacteriia, Nitrilruptoria, Rubrobacteria and Thermoleophilia (Gao & Gupta, 2012, Ludwig *et al.*, 2012). The class Actinobacteria represents bacteria that have industrial significance as producers of antibiotics and secondary metabolites, such as *Streptomyces* spp., and important human pathogens within the genera *Corynebacterium* and *Mycobacterium* (Gao & Gupta, 2012).

Actinobacterial species such as *Corynebacterium*, *Mycobacterium* and *Streptomyces* spp. have been extensively studied for industrial and medical purposes, but much of the molecular processes in these organisms are not clearly understood. For example, proteins involved in the regulation of cell division, and particularly in the control of Z-ring assembly, are not clear. Further, the control mechanisms of cell division differs greatly between Actinobacteria and conventional model bacteria. Though, most of the core divisome proteins from *E. coli* and/or *B. subtilis*, such as FtsZ, FtsEX, FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI and SepF are conserved in Actinobacteria, proteins known to be involved in anchoring

and assembly of the Z ring, such as FtsA, ZipA, ZapA-E, EzrA and proteins involved in Z-ring positioning, such as MinCDE, SlmA and Noc, are all absent or their homologs have not yet been identified (Flärdh & Buttner, 2009, McCormick, 2009, Jakimowicz & van Wezel, 2012, Donovan & Bramkamp, 2014, Baranowski *et al.*, 2019). A brief summary of the control and formation of Z rings during cell division in *Corynebacterium* and *Mycobacterium* is presented below. Cell division and it's regulation in *Streptomyces* is covered in sections 7.2 -7.3.

In *Corynebacterium glutamicum*, SepF is a membrane anchor of the Z ring (Sogues *et al.*, 2020). It localizes at the division site and is required for Z-ring assembly, thus forming an essential component of divisome. The study by Sogues *et al.* showed that for membrane anchoring, an interaction between SepF and FtsZ is necessary and that during this interaction two FtsZ monomers bind to the pocket formed by a symmetric SepF homodimer (Sogues *et al.*, 2020). Another protein FtsR has been shown to be a transcriptional activator of the expression of *ftsZ* (Kraxner *et al.*, 2019). It is also known that three serine/threonine kinases, PknA, PknB and PknL also regulate cell division via phosphorylation of FtsZ (Schultz *et al.*, 2009). The sites of phosphorylation represent key regions in FtsZ, such as the GTP/GDP binding/hydrolysis site, interface for oligomerization and the C-terminal end that interacts with many division proteins (Donovan & Bramkamp, 2014). In another study, WhcD (homologous to *S. coelicolor* WhiB, see section 7.3) was found to be a global regulator role of division in *C. glutamicum* (Lee *et al.*, 2017). In the study, it was shown that the transcription of division genes, *ftsI, ftsQ, ftsZ*, and *sepF* was significantly lowered (by 40-60%), suggesting that WhcD influences early stage of cell division. Another candidate suggested to have a role in Z-ring positioning is PldP. PldP localizes at the division site and its deletion results in a minicell phenotype while overexpression leads to increased cell length (Donovan *et al.*, 2010). However, further work is required to confirm role of PldP in Z-ring positioning. In the same and a later study, it was also shown the chromosome segregation genes *parA* and *parB* influence the placement of Z rings at the division site (Donovan *et al.*, 2010, Donovan *et al.*, 2013).

Similarly, in mycobacteria, the spatial-temporal regulation of Z-ring assembly is not clearly known. As in corynebacteria, SepF is essential for cell division and it's localization is dependent on FtsZ (Gola *et al.*, 2015). Another division protein FtsW has been reported to directly interact with FtsZ via their C-termini, which possibly provides a link between divisome and cell wall synthesis components (Datta *et al.*, 2002, Datta *et al.*, 2006). The serine/threonine kinases, PknA and PknB have also been implicated to have role in cell division, as shown by cell morphological defects when the levels of these kinases were altered (Kang *et al.*, 2005). Another candidate in mycobacterial cell division is FipA, a protein that interacts with FtsZ *in vivo* and is phosphorylated by PknA (Sureka *et al.*, 2010). *fipA* deletion mutants showed elongated cells in comparison to wild-type suggesting effects on cell division. The

CrgA homologue in *Mycobacterium smegmatis* interacts with FtsZ, FtsI and FtsQ, and is a part of the divisome with a role in PG synthesis and cell shape morphogenesis (Plocinski *et al.*, 2011). A distant relative of MinD, Ssd, has been implicated in mycobacterial cell division since its overexpression leads to elongated cells (England *et al.*, 2011). In another mycobacterial study, morphological landmarks (as waveform troughs) formed on the cell surface were shown to mark the sites of future cell division, however the nature and the mechanism of formation of these troughs is not yet clear (Eskandarian *et al.*, 2017). Recently, a new protein, SepIVA, was identified to be involved in mycobacterial cell division. SepIVA is essential for viability, localizes to the division septum, and its depletion results in elongated cells (Wu *et al.*, 2018). *Streptomyces* genome also contains *sepIVA* and this has been investigated in Paper IV (see section 9.4). Overall, to reiterate, we still lack a good understanding on the control and formation of Z rings in these actinobacterial species.

7.1. *Streptomyces* spp. - life cycle and study models

Streptomycetes are ubiquitous soil-dwelling bacteria that share similarities in lifestyle with filamentous fungi. These bacteria have a linear and relatively large genome, typically of size ~ 8 -10 Mb, however streptomycetes with genome size as low as 5.18 Mb and as high as 11.9 Mb have also been reported (Harrison & Studholme, 2014). These organisms are natural factories of antibiotics and secondary metabolites. Not just from an industrial perspective but also to understand various molecular processes, several *Streptomyces* species, such as *S. coelicolor*, *S. griseus* and *S. lividans* have been used as model systems (Hopwood, 2007). Over the years, many streptomycete genomes have been sequenced, further allowing to dissect key molecular pathways (Harrison & Studholme, 2014).

Streptomycetes have a complex developmental life cycle (Fig. 4). A typical life cycle begins from spores that germinate upon nutrient availability and suitable conditions and grow by tip extension into branching filamentous hyphae (vegetative mycelium). In response to nutrient starvation or other signals, the vegetative hyphae enter a developmentally controlled formation of reproductive aerial hyphae that then divide into long chains of uninucleoid compartments, which subsequently develop into spores (Flärdh & Buttner, 2009, Elliot & Flärdh, 2020).

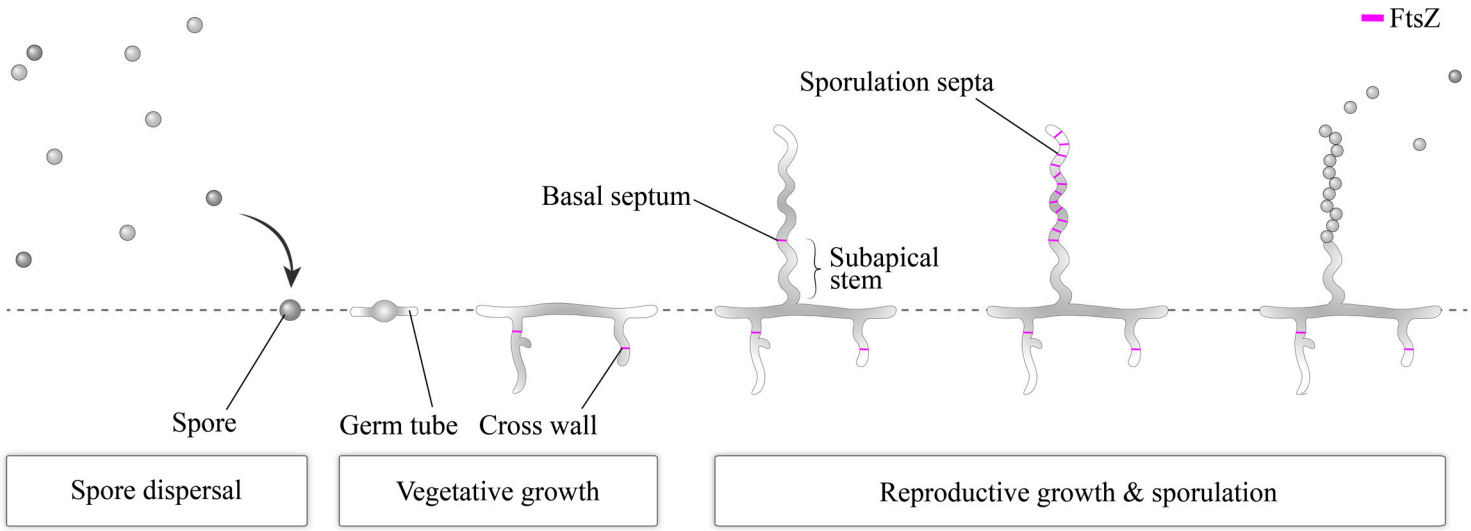

Figure 4. Illustration of developmental life cycle of *Streptomyces* spp. A spore germinates and grows out into a vegetative mycelium (vegetative growth). When exposed to unfavorable conditions, such as nutrient deprivation or other stimuli, the vegetative mycelium grows into aerial hyphae. On the onset of sporulation, a putative basal septum is first laid above the subapical stem. Further, a ladder-like FtsZ ring assembles in the sporogenic compartment. Eventually the aerial hypha is divided and differentiated into chains of equally sized spores (reproductive growth and sporulation). FtsZ (in pink) is required for the formation of infrequent vegetative cross-walls and for the sporulation septa. (Image courtesy of Veronica Ekdahl).

In contrast to most rod-shaped bacteria such as *E. coli* that grow by adding new PG at the lateral walls dependent on actin-like protein MreB (Shi *et al.*, 2018), *Streptomyces* spp. grow by hyphal tip extension (polar growth) and are independent of MreB. During polar growth in *Streptomyces*, the new PG material is added at the hyphal tips in a manner dependent on a tip-localizing protein, DivIVA (Flärdh, 2003, Hempel *et al.*, 2008). The polar growth appears to be orchestrated by a protein complex 'polarisome', constituted of three coiled-coil proteins DivIVA, FilP and Scy (Fuchino *et al.*, 2013, Holmes *et al.*, 2013). In *S. coelicolor*, two MreB homologs are found- MreB and Mbl. The MreB-like proteins are non-essential for viability and are instead involved in spore wall synthesis during sporulation in contrast to their function in rod-shaped bacteria (Heichlinger *et al.*, 2011, Kleinschnitz *et al.*, 2011).

Among streptomycetes, *S. coelicolor* is one of the most utilized models to study growth and development. Recently, *Streptomyces venezuelae* has emerged as a popular study model. *S. venezuelae* was identified in 1940s for being a natural source of the antibiotic chloramphenicol and its genome sequence has been obtained (Ehrlich *et al.*, 1948, Pullan *et al.*, 2011). In contrast to *S. coelicolor*, which differentiates only on solid medium and forms mycelial clumps in liquid cultures, *S. venezuelae* undergoes near-synchronous differentiation in liquid culture (Schlimpert *et al.*, 2016) and this property has been a boon for many studies. The use of *S. venezuelae* circumvents problems, in 'omics' approaches, where surface-grown cultures are problematic due to their developmental asynchrony and heterogeneity (Chater, 2016). Sporulation in submerged cultures has for example allowed the study of developmental regulators using microarray-based transcriptomics and chromosome immunoprecipitation sequencing (ChIP-seq) (Al-Bassam *et al.*, 2014, Bush *et al.*, 2016, Donczew *et al.*, 2016). Another advantage of using *S. venezuelae* is that key proteins in developmental life cycle can be directly and continuously observed by combining time-lapse imaging with a microfluidic cell perfusion system (Schlimpert *et al.*, 2016). Considering these advantages, I have utilized *S. venezuelae* as a model to investigate questions in relation to cell division.

7.2. *Streptomyces* cell division

The *Streptomyces* developmental life cycle involves at least two distinct types of cell division (Fig. 4). The first type is the formation of vegetative cross-walls (hyphal cross-walls), which lead to the formation of compartments in the vegetative hyphae but no physical detachment of cells from each other. The second type is the developmentally regulated sporulation septation that divides aerial hyphae into spore chains, eventually leading to physical separation of spores (Flärdh & Buttner, 2009, Jakimowicz & van Wezel, 2012). A third type of cell division has also been

suggested which occurs near the base of the aerial hypha, named the basal septum (Kwak *et al.*, 2001). The formation of the basal septum likely allows compartment-specific gene expression, either in sporogenic cell (region of aerial hyphae above basal septum) or in the subapical stem (region of aerial hyphae immediately below basal septum) (Dalton *et al.*, 2007, McCormick & Flärdh, 2012). Importantly, in all these cell divisions, FtsZ is always at the core, be it for the formation cross-walls in vegetative hyphae or for laying the basal septum, or for a developmentally controlled septation of aerial hyphae.

As for other actinobacterial species, while *Streptomyces* spp. contain core divisome proteins found in the rod-shaped models *E. coli* or *B. subtilis*, most proteins involved in the regulation of assembly and positioning of the Z ring are not clearly known yet. In contrast to conventional models, cell division in *Streptomyces* is non-essential for viability as shown by the dispensability of core cell division proteins such as FtsQ (DivIB), FtsL, FtsB (DivIC), FtsW, FtsI and FtsZ ((McCormick, 2009, Santos-Beneit *et al.*, 2017), and paper II). In paper II, we show that the core divisome proteins are not required for Z-ring assembly in *S. venezuelae*. Therefore, it remains to be shown what equivalent mechanisms or proteins are involved in the spatial and temporal control of Z-ring assembly in *Streptomyces*. Some candidates in this regulation are discussed below and in paper III.

7.2.1. SepF-like proteins

Streptomycetes lack obvious homologs of *E. coli* membrane-tethers FtsA and ZipA, and the only recognizable putative membrane-anchor for FtsZ in these organisms is SepF. Interestingly, three SepF-like proteins, SepF, SepF2 and SepF3 are found in *S. venezuelae* ((Schlimpert *et al.*, 2017) and paper III). All three SepF proteins have a conserved C-terminal domain, including residues involved in protein dimerization and interaction with FtsZ, as in the canonical SepF (Paper III). Using two-hybrid systems, it was found that three SepFs interacts with each other, but only SepF interacts with FtsZ, supporting the role of SepF in cell division (Schlimpert *et al.*, 2017). In paper III, we have investigated the different roles of these three SepF-like proteins in cell division (discussed in section 9.3).

7.2.2. SsgA-like proteins

Seven *S*sg*A*-*l*ike *p*roteins (SALPs) have been identified in *S. coelicolor*, of which SsgA and SsgB are relevant in cell division (Jakimowicz & van Wezel, 2012). Studies of *ssgA* (*s*porulation of *Streptomyces griseus A*) and *ssgB* null mutants show that they fail to sporulate, suggesting their role in sporulation-specific cell division (van Wezel *et al.*, 2000, Keijser *et al.*, 2003). A model has been proposed in which SsgA facilitates the correct placement of SsgB at the future division sites. Once

localized, SsgB directly interacts with, recruits and co-localizes with FtsZ *in vivo*. SsgB promotes FtsZ polymerization *in vitro*, suggesting a role as positive regulator of FtsZ assembly (Willemse *et al.*, 2011).

7.2.3. Dynamin-like proteins

Dynamin-like proteins are found in many bacterial species, but their role has not yet been fully understood. In eukaryotes, dynamins function in a diverse range of cellular activities such as endocytosis and cytokinesis (Bramkamp, 2012, Ferguson & De Camilli, 2012). Two dynamin-like proteins, DynA and DynB, play an important role in sporulation specific cell division in *Streptomyces* (Schlimpert *et al.*, 2017). DynA and DynB are required for regular septation, and their proper recruitment and placement depend on FtsZ. Fluorescence microscopy of *dynAB* mutants shows that once FtsZ rings are placed at regular intervals in a sporulating hypha, DynA and DynB are required for stabilization of Z rings. In addition, protein-protein interaction studies show that DynA and DynB interact both with each other and with SsgB, and DynB interacts with SepF2, thus providing indirect links via SsgB and SepFs to FtsZ (Schlimpert *et al.*, 2017).

7.2.4. SepG

SepG (formerly called YlmG) is a transmembrane protein found in Actinobacteria, Firmicutes and Cyanobacteria (Zhang *et al.*, 2016). In *Streptomyces*, SepG aids in proper localization of SsgB and thus the proper recruitment of FtsZ (Zhang *et al.*, 2016). However, more work will be needed to clarify the role of SepG in this complex cascade of cytokinesis.

7.2.5. CrgA

CrgA is a small integral membrane protein found in all actinomycetes. It has been reported to influence sporulation septation in *S. coelicolor*. Disruption of *crgA* leads to premature development of aerial hyphae and an increase in abundance of FtsZ rings, while its overexpression inhibits the FtsZ-ring formation (Del Sol *et al.*, 2006). These observations suggested that CrgA may act as a negative regulator of cell division, similar to cell division inhibitors such as MinC and EzrA, but the more recent work on mycobacterial CrgA rather indicates that it may contribute to stabilize the divisome. More work is needed to elucidate the role of CrgA in cell division.

7.2.6. ParAB proteins

The ParABS systems are involved in chromosome segregation in bacteria and archaea. ParA, an ATPase, interacts with ParB (a CTPase) bound to *parS* sites (bacterial centromeres), and thereby facilitates chromosome segregation and plasmid partitioning (Davey & Funnell, 1994, Soh *et al.*, 2019). In *Streptomyces*, ParAB proteins play a role in chromosome segregation (Jakimowicz *et al.*, 2007). Time-lapse fluorescence microscopy results suggest that the formation of Z rings in *parA* mutant is slightly delayed, while high-resolution structured illumination microscopy results show that Z-rings formed in *parB* mutant are often broken and not uniform, as compared in wild-type (Donczew *et al.*, 2016). These results indicate that ParAB proteins play an important role during Z-ring assembly.

7.3. Developmental control of cell division in *Streptomyces*

The developmental life cycle of *Streptomyces spp.* (Fig. 4) is controlled by two types of regulators, 'Bld' and 'Whi', named after the phenotypes of mutants lacking the regulators. Bld regulators are necessary for the formation of reproductive aerial hyphae from vegetative mycelium, while Whi regulators are required for the differentiation of aerial hyphae into mature spores. The *bld* mutants fail to produce aerial hyphae resulting in a 'bald' colony phenotype whereas the *whi* mutants fail to produce the characteristic dark grey spore pigment producing a 'white' phenotype (for reviews, see (Chater, 2001, Chater & Chandra, 2006, McCormick & Flärdh, 2012, Chandra & Chater, 2014, Bush *et al.*, 2015, Chater, 2016)).

Two examples of key regulators are BldD and WhiA/WhiB. The Bld regulator BldD functions as a global regulator of *Streptomyces* development. Upon forming a complex with the second messenger cyclic di-GMP (c-di-GMP), BldD controls many genes required for aerial hyphae formation and sporulation specific genes, thereby acting as a 'brake' to favor vegetative growth (Tschowri *et al.*, 2014, Bush *et al.*, 2015, Schumacher *et al.*, 2017). During the onset of sporulation, Whi regulators WhiA and WhiB work together to control a cascade of downstream genes required during sporulation, such as those for chromosome replication and segregation (e.g., *sffA* and *ftsK*), and sporulation septation (eg., *ftsW* and *ftsZ)* (Flärdh *et al.*, 1999, Bush *et al.*, 2013, Bush *et al.*, 2016).

In *S. coelicolor*, the expression of *ftsZ* is driven by three promoters, *ftsZ1p*, *ftsZ2p* and *ftsZ3p* that are located in the intergenic region between *ftsQ* and *ftsZ* (Flärdh *et al.*, 2000). During the first type of cell division (cross-wall formation during vegetative growth), *ftsZ* is expressed via promoters *ftsZ1p* and *ftsZ3p*. BldD binds

to the aerial hypha-specific developmental promoter *ftsZ2p* of *ftsZ* and represses its activity until the onset of sporulation (den Hengst *et al.*, 2010). However, during the second type of cell division, a strong up-regulation of *ftsZ* expression occurs in sporulating hyphae via the *ftsZ2p* promoter (Flärdh *et al.*, 2000). The requirement of *ftsZ2p* promoter during sporulation specific cell division in *S. venezuelae* is discussed in paper II. WhiA/WhiB-mediated activation of *ftsZ* and the release of BldD-mediated repression allows upregulation of *ftsZ* during sporulation (den Hengst *et al.*, 2010, Bush *et al.*, 2013, Schumacher *et al.*, 2017). This upregulation leads to high cellular FtsZ concentration, likely well above the required critical concentration and drives FtsZ polymerization, eventually, depositing FtsZ rings along the sporogenic hyphal compartment (Flärdh *et al.*, 2000, Kwak *et al.*, 2001). In sporogenic hyphae, initially FtsZ forms dynamic helical filaments that remodel into regularly placed dynamic Z rings (Grantcharova *et al.*, 2005).

It is not clearly understood how these helices assemble into the dynamic rings. The finding of missense mutations in *ftsZ* specifically affecting only the second type of cell division (during sporulation) but not the first type (vegetative cross-wall formation), suggest that there are different requirements for Z-ring assembly during these two types of division (Grantcharova *et al.*, 2003). Several regulators have been identified that specifically affect sporulation-specific cell division. For example, as discussed in paper III (section 9.3), depletion of *sepF* leads to the blockage of sporulation associated-cell division. During the onset of sporulation, the transcription of *sepF2* is activated by WhiA (Bush *et al.*, 2013), and *sepF3* is required for the proper distribution of Z-rings in sporulating hyphae (Paper III). Another candidate SsgB, recruited to the division site by SsgA and stabilized by SepG, is suggested to exert a positive control of Z-ring assembly (Willemse *et al.*, 2011, Zhang *et al.*, 2016). Recently, dynamin-like proteins, DynA and DynB were shown to affect sporulation-specific cell division, wherein they are required for stability of Z rings (Schlimpert *et al.*, 2017). Two-hybrid analysis showed that DynA and DynB interact with each other and with SsgB. Further, it was shown that DynB interacts with SepF2 and that all three SepF bind to themselves and to each other suggesting that a complex interaction network governs Z-ring assembly. Another candidate, CrgA has been suggested as a negative regulator of Z-ring assembly, as seen from the study wherein *crgA* overexpression leads to inhibition of FtsZ rings (Del Sol *et al.*, 2006). However, it is still unclear on how exactly the assembly of Z rings is regulated during sporulation-specific cell division.

8. Aims of this thesis work

The overall aim of my thesis work was to clarify mechanisms involved in the regulation of cell division in *Streptomyces* spp., with focus on the assembly dynamics of FtsZ. Specifically, my PhD project has addressed following questions:

(i) How does a specific mutation in *ftsZ* give rise to atypical spiral-shaped septa? (Paper I)

(ii) What effect does this mutation have on the assembly and functional dynamics of FtsZ? (Paper I)

(iii) How do the core divisome proteins affect Z-ring assembly? (Paper II)

(iv) How do the three SepF proteins affect Z-ring assembly in *S. venezuelae*? (Paper III)

(v) Does *sepIVA* have a role in *Streptomyces* cell division? (Paper IV)

9. Results and discussion

9.1. Paper I

How does a specific mutation in *ftsZ* give rise to atypical spiral-shaped septa? What effect does this mutation have on the assembly and functional dynamics of FtsZ?

The starting point of paper I was a genetic screen for *ftsZ* mutations that affect sporulation septation in *S. coelicolor* (Wasserstrom *et al.*, 2013). One isolated mutant had a very interesting sporulation phenotype as shown by spiral-shaped septa. Similar, septation had been previously reported in an *E. coli* temperature sensitive mutant (*ftsZ26*) (Addinall & Lutkenhaus, 1996), and in *B. subtilis* (Feucht & Errington, 2005), but with one difference. While a single perturbed septum per cell was observed in *E. coli* and *B. subtilis*, in *S. coelicolor* the spiral septation extended along the entire sporogenic cell. Strikingly, the mutation causing this spiral invagination phenotype reside essentially in the same position in all cases, i.e., near the α helix H1 and β strand S2 of the N-terminal GTP-binding domain of FtsZ (Addinall & Lutkenhaus, 1996, Feucht & Errington, 2005, Sen *et al.*, 2019). No mechanistic understanding was available on how these mutations alter FtsZ's function, and how do they lead to this deformed septa phenotype. Therefore, it was of interest to investigate how exactly these mutations produce the atypical septations.

More specifically, the mutation residing in the β strand S2 of *S. coelicolor* FtsZ producing spiral septation is an amino acid substitution, F37I. Coincidently, the mutations in *E. coli* (*ftsZ26*) and that in *B. subtilis* corresponds to the exact same residue F37 or the neighboring residue but in the same β strand S2 of *S. coelicolor*, respectively. This suggest that region around β strand S2 plays a crucial role in producing such a deformed phenotype. In order to investigate in more detail, we switched model system to *S. venezuelae*, which is well suited for live imaging. To clarfiy what residues in this regions are critical, we systematically mutagenized *S. venezuelae ftsZ* at and around the F37 residue on a plasmid and examined the resulting phenotypes in the *ftsZ* null background (Paper I, Table 1). We found that in addition to F37I, amino acid substitution F37R also leads to the same phenotype. However, mutagenesis of nearby residues did not show relevant phenotypes. Electron microscopy of the F37 mutants showed deep helical-invaginations in

sporulating hypha. These observations suggest that only very specific amino acid changes in F37 are responsible for spiral septation.

In order to understand how these mutations affect localization and dynamics of FtsZ *in vivo*, we utilized *S. venezuelae* due to it's benefits as described in section 7.1. The fact that the fluorescent fusions of FtsZ are not fully functional, we first introduced the mutation, *ftsZ*(F37I), into the native chromosmal locus. The mutant strain producing FtsZ(F37I)-YPet also from a plasmid was then imaged using live-cell time lapse microscopy. The microscopy showed that FtsZ assembly in the mutant during vegetative cross-wall formation was often mis-shaped (tilted) in comparision to wild-type wherein Z rings were laid perpendicular to the hyphal length axis. A much stronger effect of the mutation was seen during sporulation. While spiral-shaped FtsZ assemblies were seen in the sporogenic aerial hypha in the mutant, dynamic Z rings at regular intervals were seen in the wild-type. These results suggest that the mutation affects a key functional aspect of FtsZ *in vivo* that is involved in proper FtsZ assembly. What aspect of FtsZ could that be which is compromised due to the mutation?

To get insights on how the mutation affects FtsZ, we studied the biochemical activity and polymerization of *Streptomyces* FtsZ *in vitro*. *In vitro*, FtsZ protofilaments have been shown to interact laterally and that the lateral interaction was suggested to be important for the assembly of functional Z ring *in vivo* (Guan *et al.*, 2018, Krupka & Margolin, 2018). Interestingly, the region of mutation is located at the lateral face of FtsZ and the corresponding region in tubulins is involved in lateral interactions within microtubules (Amos, 2010). Therefore, our first hypothesis was that the mutation could affect lateral interactions formed by FtsZ protofilaments, in turn affecting FtsZ assembly *in vivo*. To test this hypothesis, we performed negative staining of polymers formed by the mutant and wild-type variants of FtsZ under different buffer conditions. The results showed that in general FtsZ polymers formed by the mutant FtsZ are shorter in length than compared to the ones formed by wild-type FtsZ, but, we did not obtain any evidence for effects on lateral interactions between FtsZ protofilaments, typically seen for example as 'doublets' of protofilaments. However, when we measured GTPase activity (using malachite-green based GTPase assay) and polymerization dynamics (using light scattering-based polymerization assay) *in vitro*, we found that the mutant FtsZs have lower GTPase activity and do not show critical concentration (Cc), a pre-requisite for cooperative assembly of FtsZ.

But how does the effect on FtsZ activity *in vitro* correlate to the observed phenotype? Our results showing that Cc and the GTPase activity is reduced suggest that the mutation may interfere with the conformational switch and thereby affects assembly cooperativity and treadmilling activity, which in turn leads to the formation of improper spiral-shaped FtsZ assemblies. As discussed in section 3.5, a

conformational switch of FtsZ is required for cooperative assembly of FtsZ polymers (Wagstaff *et al.*, 2017). The switch involves opening of a hydrophobic cleft between the N-terminal and C-terminal subdomains that also constitutes the binding pocket for FtsZ inhibitor PC190723, which upon binding force FtsZ into open form and also drastically reduces critical concentration ((section 3.5; (Elsen *et al.*, 2012, Matsui *et al.*, 2012, Wagstaff *et al.*, 2017)). The F37 amino acid substitution in our study is located in the H1-S2 loop, which is quite far away from the hydrophobic cleft but is in close proximity of helix H1 (Fig. 2 and 3B). During conformation switch, it is predicted that helix H1 and H7 move in relation to each other. Putting these together, it is plausible that even though the F37 residue is not directly inolved in the conformational switching, the mutation of this residue may have an indirect effect on this switch via helix H1 and its interaction with helix H7.

It is still puzzling: how do these mutants that possibly are affected in treadmilling dynamics of FtsZ manage to constrict, since treadmilling dynamics is crucial for proper Z-ring assembly and in driving the PG synthesis machinery (section 3.5; (Bisson-Filho *et al.*, 2017, Yang *et al.*, 2017)). It has been reported that *ftsZ* mutants with altered GTPase activity and cooperativity produce spiral-shaped constrictions (Stokes *et al.*, 2013, Adams *et al.*, 2016, Yang *et al.*, 2017). But how do such mutants, including those in our study, complete septation despite likely being defective in FtsZ treadmilling? Recently, Monteiro *et al* suggested that in *Staphylococcus aureus*, though the initial Z-ring assembly depends on FtsZ's treadmilling dynamics, the later stage of PG assembly and septum constriction is independent (Monteiro *et al.*, 2018). Thus, in our case, it is possible that the mutation affects treadmilling dynamics and therefore produce perturbed and often helical FtsZ assemblies rather than rings, but these are still capable of initiating cell constrictions.

9.2. Paper II

How do the core divisome proteins affect Z-ring assembly?

Previous studies of *S. coelicolor* mutants lacking core divisome proteins such as FtsQ(DivIB), FtsL, FtsB(DivIC), FtsW or FtsI, showed that these proteins are not required for growth and viability and are also at least partially dispensable for sporulation-specific cell division (McCormick & Losick, 1996, Bennett *et al.*, 2007, Mistry *et al.*, 2008, Bennett *et al.*, 2009). The ability to form septa in the absence of these core divisome proteins is very intriguing, since the corresponding proteins are essential or at least conditionally essential in *E. coli* and *B. subtilis* (Du & Lutkenhaus, 2017, Errington & Wu, 2017). In paper II, we confirm that the dispensability of core divisome proteins is also true in *S. venezuelae,* suggesting that

it is a conserved cell division aspect across *Streptomyces* spp. Further, the divisome mutants that were constructed and described in this study will be valuable tools in the continued investigations of the cell division process and its regulation in *Streptomyces* spp.

The *S. venezuelae* divisome mutants for FtsZ and core divisome proteins FtsQ(DivIB), FtsL, FtsB(DivIC), FtsW and FtsI constructed in our study showed condensed and well segregated DNA and regular FtsZ ladders in the sporulating hyphae, suggesting completion of cell division (Paper II, Fig. 3 and S8). How cell division is performed without the core divisome proteins, such as the FtsQ(DivIB)-FtsL-FtsB(DivIC) complex is not clearly known yet. Could there be other alternative strategies or proteins to regulate cell division in these divisome mutants. If yes, what could they be? FtsQ(DivIB), FtsL and FtsB(DivIC) are well conserved among bacterial species. The complex formed by FtsQ(DivIB)-FtsL-FtsB(DivIC) acts as a scaffold for the downstream proteins and plays essential role in divisome activation in *E. coli* (Du & Lutkenhaus, 2017). Recently, it has been shown that the complex in *E. coli* inhibits the transglycosylase penicillin binding protein PBP1b and the transpeptidase domain of PBP3 (Boes *et al.*, 2019). Therefore, in strains lacking FtsQ-FtsL-FtsB, which other protein(s) could help assemble the divisome and control PG synthesis to occur at the right time to make a septum? With respect to the dispensability of *ftsW*, it has recently been shown that FtsW is a PG synthase (with transglycosylase activity) that polymerizes lipid II into PG (Taguchi *et al.*, 2019). What could that other polymerase be that is substituting for FtsW in our *ftsW* mutants? *S. coelicolor* genome encodes four SEDS (*s*hape, *e*longation, *d*ivision, and *s*porulation)/penicillin binding protein (PBP) pairs: FtsW, RodA, SCO5302 and SCO2607 (Sfr) (Mistry *et al.*, 2008, Bennett *et al.*, 2009). Orthologs of these four SEDS proteins are also found in *S. venezuelae*. Therefore, it is likely that another pair of SEDS/PBP substitute the roles of FtsW/FtsI in cell division.

In *S. coelicolor*, during spore maturation, PG synthesis is guided by a multi-protein complex comprising of MreBCD, PBP2 and Sfr, which form an essential part of *Streptomyces* spore wall synthesizing complex (SSSC) (Heichlinger *et al.*, 2011, Kleinschnitz *et al.*, 2011, Sigle *et al.*, 2015). It has been shown that the Sfr and PBP2 proteins in the complex interact with MreB and Mbl (*M*re*B*-*l*ike) proteins. Microscopy observations suggest that the SSSC is recruited first to the septation site and then distributes along the entire spore for spore wall synthesis. Thus, could MreB and the associated cell wall synthesis proteins Sfr and PBP2 provide a possible redundant pathway in by-passing the role of core divisome proteins in the divisome mutants that can contribute to septum formation? Recently, it has been found that Chlamydial MreB directs cell division and cell wall synthesis in *E. coli* in the absence of FtsZ activity (Ranjit *et al.*, 2020). Since, sporulation and cell division are tightly connected in *Streptomyces*, it is very tempting to think that MreB

might have role in sporulation-specific cell division. Further work is required to answer these questions.

A question that is raised concerns the membrane anchor for FtsZ in *Streptomyces*, which has not been fully characterized (see paper III). In mycobacterial cell division, FtsW directly interacts with FtsZ and was suggested to be a possible anchor for FtsZ to the membrane and in linking septum formation to PG synthesis (Datta *et al.*, 2002, Datta *et al.*, 2006). However, paper II and previous studies on *ftsW* mutants of *Streptomyces* suggest that FtsW is not required for FtsZ-ring formation and cannot be a critical membrane anchor for FtsZ, at least in *Streptomyces*. So, what could that anchor be? A likely membrane anchor for FtsZ is described in paper III.

9.3. Paper III

How do the three SepF proteins affect Z-ring assembly in *S. venezuelae*?

SepF is a highly conserved protein across Gram-positives (see sections 5.1.1 and 7.2.1). So far, SepF from *B. subtilis* has been extensively studied both *in vivo* and *in vitro* (Hamoen *et al.*, 2006, Ishikawa *et al.*, 2006, Duman *et al.*, 2013). In corynebacteria and mycobacteria, SepF is shown to be essential for cell division (Gola *et al.*, 2015, Sogues *et al.*, 2020). Interestingly, three SepF-like proteins, SepF, SepF2 and SepF3 are found in *S. venezuelae* (Schlimpert *et al.*, 2017). The aim of this work was to clarify the roles of these three SepF proteins in *S. venezuelae* cell division.

The *S. venezuelae sepF*, unlike *sepF2* and *sepF3*, is in the cell *d*ivision and *c*ell *w*all (*dcw*) cluster, as is the case in *B. subtilis* and *M. tuberculosis.* In this study, we found that the *sepF* deletion mutant is blocked for sporulation-associated cell division as no spores were formed by the mutant strain (Paper III, Fig. 2). In addition, deletion of *sepF* leads to dramatic effect on DNA condensation and segregation in sporogenic hyphae, as shown by a diffuse nucleoid staining along the hyphae (Paper III, Fig. 3), very similar to the *ftsZ* null mutants described elsewhere (Santos-Beneit *et al.*, 2017). Fluorescently labeled SepF localizes as dynamic rings in a way very similar to Z-ring localization as described in Sen *et al.* (Sen *et al.*, 2019) (Paper III, Fig. 5, Movie 1 and 5) and also co-localizes with FtsZ (Paper III, Fig. 6 and S6, Movie 8). To understand what role SepF has during Z-ring assembly, we followed localization of fluorescently-labeled FtsZ-YPet in *sepF* mutant. Time-lapse fluorescence microscopy shows mainly punctuated FtsZ-YPet foci and very occasional irregularly placed Z rings during vegetative growth, but with complete abolishment of ladder-like Z-ring assembly during sporulation (Paper III, Movie 2). These observations suggest that SepF is not only required for sporulation-specific cell division; it is also important for efficient cell division during vegetative growth

(formation of hyphal of cross-walls). How exactly those occasional Z rings are laid during the vegetative stage is not clearly known. Possibly, the other two SepFs may have a role here.

In streptomycetes that lack homologs to *E. coli* membrane tethers FtsA and ZipA, SepF is a putative membrane anchor for FtsZ. SepF from *S. venezuelae* contains a partially conserved and predicted amphipathic membrane targeting helix at the N-terminus (Paper III, Fig. S1), similar to the one demonstrated in *B. subtilis* SepF (Duman *et al.*, 2013). We found that SepF(N26)-mNeonGreen fusion protein localizes in a similar way to the membrane-binding control protein SpoVM(P9A) in sporulating hyphae of *S. venezuelae* (Paper III, Fig. 8 and S8 and S9, Movie 14 and 15), suggesting that the putative amphipathic helix SepF(N26) indeed constitutes a membrane-targeting domain of SepF.

In contrast to SepF, SepF2 is devoid of the residues that constitute the amphipathic helix suggesting that SepF2 may not directly interact with the membrane. Further, *sepF2* mutants did not have any clear cell division defects, as shown by efficient formation of mature spores (Paper III, Fig. 2) and seemingly normal DNA segregation (Paper III, Fig. 3) and localization of FtsZ (Paper III, Fig. 4, Movie 3) in comparison to wild-type. Even though, we did not find any noticeable effect in division due to the deletion of *sepF2*, fluorescence microscopy shows that SepF2 co-localizes with FtsZ both at vegetative cross-walls and sporulation septa (Paper III, Fig. 6 and S6, Movie 9). Further, SepF2 is a direct target of WhiA during the onset of sporulation (Bush *et al.*, 2013), suggesting that SepF2 possibly has some role in this process. In two hybrid assay, it was shown that SepF2 interacts with SsgB and with DynB (Schlimpert *et al.*, 2017). Further, it was also shown that DynAB proteins are required for proper stabilization of Z rings. Therefore, since *sepF2* has no clear mutant phenotype, it is possible that DynAB influence Z-ring assembly via SsgB or another pathway. A clear role for SepF2 remains to be established.

Intriguingly, the *sepF3* mutant produces extremely long spore-chains. No clear effect of *sepF3* on FtsZ localization was observed during vegetative cross-wall formation, but during sporulation-specific cell division the ladder-like assembly of Z rings continues often to form through the subapical stems into the main hyphal branch (Paper III, Fig. S4, Movie 4). It is possible but unclear if the putative basal septum formation is affected in *sepF3* mutant. Another aspect of the *sepF3* mutant phenotype is that sporulating hyphae of the mutant shows irregularly segregated nucleoids and uneven spaced septa, further suggesting issues in DNA segregation and cell wall deposition (Paper III, Fig. 3). Although, SepF3-mNG showed weak fluorescence signal, it was clear that SepF3 arrives immediately but only after the localization of Z ring and dissociates before Z ring disassembles (Paper III, Fig. S6, Movie 10). These observations suggest that SepF3 is acting once the assembly of

SepF, SepF2 and FtsZ initiates where it possibly stabilizes the complex and further ensures proper DNA segregation and septal wall formation. It is tempting to speculate that SepF3 interacts with and influences protein complexes involved in DNA segregation, such as the Par proteins and division protein FtsK.

9.4. Paper IV

Does SepIVA have a role in *Streptomyces* cell division?

SepIVA is a coiled-coil protein identified in *Mycobacterium smegmatis* (Wu *et al.*, 2018). Previously, it has been found that *sepIVA* is essential in *M. tuberculosis* during growth *in vitro* (Griffin *et al.*, 2011) and also in *M. smegmatis* (Wu *et al.*, 2018). In the study by Wu *et al.*, SepIVA was found to localize at the division site and the depletion of *sepIVA* led to the formation of elongated cells that lost viability, suggesting that it may have an essential role in cell division (Wu *et al.*, 2018). Further, it was observed that SepIVA localizes at mid cell late in the cell cycle and that it dynamically moves from septum to intracellular membrane domain (IMD), a cytoplasmic membrane domain that may serve as the reservoir for cell wall precursor enzymes. The study from Jain *et al.* also showed that depletion of *sepIVA* did not have any significant effect on the localization of FtsQ, suggesting that SepIVA is recruited late in cell cycle (Jain *et al.*, 2018). These observations suggest that SepIVA is possibly involved in both cell division and cell wall synthesis. *sepIVA* is also encoded in the genomes of, *S. coelicolor* and *S. venezuelae*, suggesting that it is found across most Actinobacteria. The aim of paper IV was to find out whether SepIVA plays a role in *Streptomyces* cell division and/or cell wall synthesis or has some other effect in the model organism *S. venezuelae*.

We constructed *sepIVA* knockout mutants and analyzed macroscopic and microscopic phenotypes. Surprisingly, as compared to the mycobacterial studies mentioned above, the *S. venezuelae sepIVA* mutant does not show any obvious cell division defect(s). First, the mutant strain grows equally well as the wild-type on agar media (Paper IV, Fig. 2B) and in liquid culture (data not shown). Second, microscopic analysis of the *sepIVA* mutant shows that they sporulate normally, as no distinct effect on spores were seen upon staining cell wall and DNA (Paper IV, Fig. 3). Third, the localization of FtsZ in vegetative hyphae and during sporulation-specific cell division is not affected in the *sepIVA* mutant (Paper III, Fig. 4, Movie 1 and 2). These results suggest that *sepIVA* does not have a direct role in *Streptomyces* cell division, which is in contrast with what was suggested from the mycobacterial study.

To find clues about the role of *sepIVA* in *S. venezuelae*, we fluorescently labeled SepIVA and followed its localization through vegetative growth to sporulation

using time-lapse fluorescence microscopy. Interestingly, mNeonGreen-SepIVA localizes at the hyphal tips and no fluorescence signal was seen at the sites of vegetative cross-walls or sporulation septa (Paper IV, Fig. 5, and Movie 3). SepIVA localizes in a similar way as the tip-localizing protein DivIVA, as shown elsewhere (Flärdh, 2003, Hempel *et al.*, 2008, Fröjd & Flärdh, 2019). As also seen for DivIVA, the tip-localized SepIVA disappeared from the tips of the growing hyphae as hyphal growth ceases and reappears as soon as hyphal growth starts again (Paper IV, Fig. 6). SepIVA consists of a DivIVA domain and shares similarity with Wag31, a mycobacterial DivIVA homolog that regulates polar peptidoglycan synthesis (Kang *et al.*, 2008, Mukherjee *et al.*, 2009, Jani *et al.*, 2010). Further, in *Bacillus subtills*, *Listeria monocytogenes* and *Streptococcus pneumoniae*, GpsB protein (DivIVA homologue) regulates septation and coordinates peptidoglycan synthesis (Claessen *et al.*, 2008, Fleurie *et al.*, 2014, Cleverley *et al.*, 2019). In *Corynebacterium*, DivIVA interacts with the putative transglycosylase RodA that is involved in growth and in determining cell shape (Sieger & Bramkamp, 2014). It is also therefore possible that SepIVA in *S. venezuelae* may function as a regulator in cell wall synthesis.

Further, the tip-localization of SepIVA in a similar pattern as DivIVA suggests that SepIVA might interact with the proteins in the polarisome complex (DivIVA, FilP and Scy) (Flärdh, 2003, Hempel *et al.*, 2008, Fuchino *et al.*, 2013, Holmes *et al.*, 2013). In order to test whether SepIVA interacts with polarisome proteins at the hyphal tips, we initiated bacterial two-hybrid assays (Karimova *et al.*, 1998). The first results of the two hybrid-assays show that SepIVA interacts with itself, and that the constructs we made for *sepIVA* are functional to use in this type of assays. The ongoing work should provide clues as to whether there are interactions between SepIVA and polarisome proteins. Additionally, if SepIVA has a regulatory role in cell wall synthesis, certain difference could be expected in peptidoglycan (PG) composition of the mutant compared to wild-type. Therefore, to test this possibility, we extracted cell wall material from *sepIVA* mutant, and the PG composition of the mutant is being analyzed, as previously described (Desmarais *et al.*, 2014). From the results obtained so far, we can conclude that SepIVA is not essential and does not have a role in *Streptomyces* cell division. Instead, it appears to be associated with polar growth and if it has a regulatory role in cell wall synthesis, PG analysis results will possibly provide some clues. Also, worth to note, is that the division site in mycobacteria form the base for new pole wherein SepIVA was shown to localize late in the cell cycle in the study by Wu *et al* (Wu *et al.*, 2018). Thus, a common aspect of SepIVA in *S. venezuelae* and mycobacteria is the localization to cell poles where polar growth occurs.

10. Conclusion and outlook

Perhaps, one of the most important question that still exists in bacterial cell division is how cell division is regulated at the level of Z-ring assembly. In addition, we still lack a clear understanding of the organization of the Z ring. Certainly, more work will be needed to provide insights into these aspects of cell division, even in the well-studied model organisms *E. coli* and *B. subtilis*. Further, very little is known about the regulation in Actinobacteria, the largest bacterial phylum. In this respect, I genuinely believe that this PhD project has added some knowledge and insights into the control of FtsZ-ring formation, using *S. venezuelae* as a model system.

In paper I, we find that a previously unexplored region of FtsZ, plays a critical role on FtsZ's function and dynamics, both *in vivo* and *in vitro*. More specifically, the mutation affects a key aspect of FtsZ dynamics - the assembly cooperativity, possibly via conformational switch, a requisite for treadmilling dynamics of FtsZ. But how exactly is the treadmilling dynamics of FtsZ affected via the conformational switch? One approach that could be used is to directly observe treadmilling behavior of FtsZ using purified proteins in *in vitro* re-constitution experiments, as previously described (Loose & Mitchison, 2014, Ramirez-Diaz *et al.*, 2018). Deducing structure of the mutant protein will certainly provide an insight into how the cooperative assembly behavior FtsZ is compromised due to the mutation - is it via the conformational switch or something else?

In paper II, we show that the core cell division genes *ftsQ*, *ftsL*, *divIC*, *ftsW* and *ftsI* are not essential for Z-ring assembly during sporulation-associated cell division. Further study on the identification of additional gene(s) involved in cell division that have overlapping function with core division genes will be very interesting. A transposon-based mutagenesis could be used to identify some candidate genes. However, an efficient transposon for *S. venezuelae* is yet to be established. As discussed in section 9.2, the other three SEDS proteins and/or other proteins of *Streptomyces* spore wall synthesis complex (SSSC) should be further studied to determine whether they can contribute for the completion of division in the divisome mutants.

In paper III, we perform genetic and cell biological dissection of SepF-like proteins in *S. venezuelae*. Based on their effects on cell division, we conclude that while the putative membrane anchor SepF has a crucial role, SepF2 and SepF3 have accessory function in *Streptomyces* cell division. In corynebacteria and mycobacteria,

localization of SepF is dependent on FtsZ (Gola *et al.*, 2015, Sogues *et al.*, 2020), but it is not known whether this is also true for *Streptomyces*. Therefore, following SepF localization in *ftsZ* null mutant background would be important for answering this question. Further, the exact role of *sepF2* in sporulation-associated cell division is unclear. As discussed in 9.3, SepF2 and SepF3 may be functionally partially redundant, and studying double mutants of *sepF2* and *sepF3* for synthetic phenotype would clarify if this is the case. *In vitro* experiments of three SepF proteins, such as the established biochemical experiments from Paper I (Sen *et al.*, 2019), will provide additional information on how these proteins, each one alone or in combinations, may influence FtsZ polymerization. The role of SepF as the membrane anchor for FtsZ in *S. venezuelae* could be further supported by *in vitro* experiments as in Duman *et al.*, testing whether FtsZ is recruited to lipid membranes by SepF (Duman *et al.*, 2013). Additionally, since *sepF3* has interesting effects both on chromosome segregation and cell division, performing pull-down assays and/or two hybrid assays with SepF3 may help to identify proteins recruited to cell division sites by SepF3.

The final part of this thesis work was the investigation of the *Streptomyces* homologue of mycobacterial septation factor SepIVA. Based on the results obtained so far, we can conclude that unlike in mycobacteria, SepIVA is non-essential for viability and does not have a role in *S. venezuelae* cell division. Further investigation is required to determine whether it functions as a regulator in cell wall synthesis. The ongoing peptidoglycan (PG) analysis of the *sepIVA* mutants may clarify if the latter is true. In the long-run, bacterial two hybrid assay using *S. venezuelae* genomic library will be useful in identifying interaction partners of SepIVA in *Streptomyces*.

To summarize, an illustration is presented showing various proteins involved in *Streptomyces* cell division and more specifically during Z-ring assembly (Fig. 5). During the onset of sporulation-specific cell division, FtsZ assembles into a cytoskeletal structure - the Z ring. The assembled Z ring is anchored to the membrane by the putative membrane anchor, SepF. Notably, SepF3 has also a putative amphipathic helix and depletion of *sepF3* influences the assembly of Z rings in sporulating hyphae. Eventually, the recruitment of core divisome proteins, such as FtsEX, FtsK, FtsQ(DivIB), FtsL, FtsB(DivIC) and FtsW/I form a mature divisome. Results from Paper II suggest that there are possibly other proteins involved as the lack of core divisome proteins can still allow assembly of Z rings during sporulation. Further some known proteins in the assembly of Z ring specifically during sporulation are DynAB and SsgB. The role of proteins such as CrgA, SepG and SsgB will require further study to understand their exact role in this regulation.

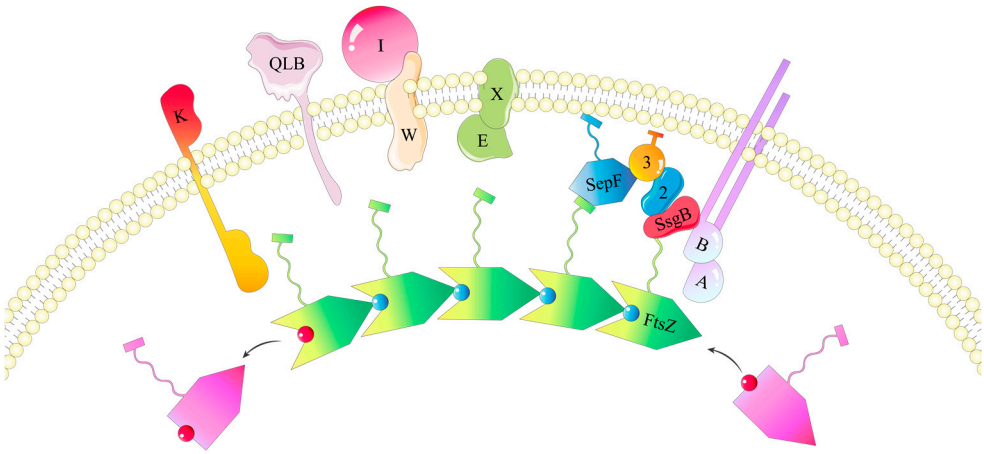

Figure 5. Illustration showing various proteins involved during Z-ring assembly in *Streptomyces*. During the onset of sporulation-specific cell division, GTP-bound FtsZ monomers (in closed form, shown in pink) assemble into a cytoskeletal structure - the Z ring. The assembly-associated conformational switch changes FtsZ monomers to open form in the filament (in green). The Z ring is then anchored to the membrane by SepF and this assembly is facilitated by a direct interaction of SsgB with FtsZ, and possibly stabilized by a complex formed by SepF, SepF2 (labeled as '2'), SsgB and DynAB, among others. The arrival of SepF3 (labeled as '3') and its interaction with SepF and SepF2 possibly further stabilizes the Z ring. Once core divisome proteins, such as FtsQLB, FtsW/I, FtsEX, and FtsK are recruited, a mature divisome complex is formed. The intrinsic GTPase activity and treadmilling dynamics of FtsZ allows removal of GDP-bound FtsZ monomers from the other end. Blue and pink circles represent GTP and GDP, respectively. (Image courtesy of Veronica Ekdahl).

Certainly, further work is required to answer the questions that have emerged during this work. This will help in better understanding of the unresolved and intriguing mechanisms that control cell division of *Streptomyces* spp. and to get insights into bacterial cell division in general. Overall, the specific contributions from my thesis work are:

1) An understanding on how a specific amino acid, F37 residing in the β strand S2 of FtsZ affects FtsZ function both *in vivo* and *in vitro*. Further, the work showed that FtsZ compromised in cooperative assembly behavior can still fulfill constriction and thereby completion of septation.

2) This work showed that in *S. venezuelae*, the Z-ring assembly does not require core divisome proteins. The cell division mutants obtained in this work will be useful in further investigation of aspects of cell division in streptomycetes.

3) This work demonstrates that three SepF-like proteins in *S. venezuelae* have distinct roles in cell division. We have shown that SepF is the putative membrane anchor for FtsZ and is crucial for cell division in *S. venezuelae*. Further, we show that SepF2 and SepF3 have accessory role in *S. venezuelae* cell division and that SepF3 influences chromosome segregation and positioning of septa during sporulation.

4) This work contributes to clarify the role of SepIVA in Actinobacteria. The results have led to a conclusion that SepIVA is not essential and is not involved in cell division but has a likely role in cell wall synthesis or in polar growth in *S. venezuelae*.

11. Popular summary

Could you for a moment imagine what those tiny cells, the building blocks of life within you, are doing right now? Perhaps, some of them are going through their cycle of life 'The Cell Cycle'. By now, we know quite a lot about what goes on during this cycle. Still, to make it simpler to understand, we can take for an example, the cell cycle of the commonly studied bacterium, *Escherichia coli*. The cell cycle begins with making a copy of the genetic material inside the cell while also growing in cell size. Eventually, when it is time to become two progeny cells from one, a ring like structure forms at the mid-point of the cell, which we call the 'Z ring'. The 'Z ring' is the central component that drives the division of a bacterium, in assistance with other cellular components and fulfils the dream of the bacterium to become two.

One might now wonder what this 'Z ring' is made up of? The Z ring is composed of protein molecule, FtsZ. FtsZ protein, the core of the Z ring, is found in most bacteria. In recent times, the FtsZ protein has been proven to be a very promising target to make new antibiotics. So far, we know about how the protein FtsZ looks like in structure and the basics of how it functions. However, the picture of the formation and control of the Z ring in the bacterial is still not so clear, despite decades of research. This thesis work attempts to contribute in the understanding of the poorly known control mechanism during the formation of Z ring. To achieve this goal, I have utilized the bacterium *Streptomyces venezuelae* as a study model.

In this thesis work, we found that a specific region of FtsZ, that had not been explored before, is crucial for its function and in the formation of the Z ring both *in vivo* and *in vitro* (**Paper I**). We show that a specific amino acid (F37) substitution in this region of FtsZ affects its dynamics and leads to irregularly shaped cell constrictions ('twisted septa'). These results are also useful in explaining similar phenotypes in other bacteria with respect to FtsZ function and Z-ring assembly. The work also showed that FtsZ with compromised assembly behaviour can still produce cell constrictions.

Further, in *S. venezuelae*, we found that the formation of Z rings can occur even without the core division proteins, which otherwise are necessary in most bacteria (**Paper II**). How the FtsZ ring is formed despite not having additional proteins is really puzzling and further work is required to shed light on this mystery. The availability of the strains lacking genes for the core division proteins will be highly

useful in understanding what other equivalent proteins/mechanisms are involved in the control for the formation of Z ring.

In **paper III**, we clarify the role of three SepF-like proteins, SepF, SepF2 and SepF3 in the formation and control of Z ring in *S. venezuelae*. We provide genetic evidence for a putative anchor role of SepF for the Z ring at the membrane. As in other Actinobacteria, this work showed that SepF is crucial for cell division. Although the exact role of SepF2 is unclear, this work showed that SepF3 has a pleiotropic effect, such as in DNA segregation, septum formation and cell separation. The mutants for *sepF3* showed a very interesting phenotype with a long spore-chain with some elongated spores devoid of DNA.

Finally, we describe the role of actinobacterial protein SepIVA in *Streptomyces* cell division (**Paper IV**). SepIVA is essential for viability and cell division in mycobacteria, including *Mycobacterium tuberculosis*, which causes tuberculosis. Our results showed that SepIVA is not essential for viability and is not involved in cell division in *Streptomyces*, and rather has a likely role in cell wall synthesis. These interpretations originate from the results that lacking *sepIVA* does not have any detectable phenotype in cell division and rather the localization of SepIVA in a cell showed tip localization wherein cell growth occurs in *Streptomyces*.

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