Investigation of protein interactions in the cell division complex of Bacillus subtilis reveals a possible mechanism for cell size homeostasis

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CHAPTER 1: General introduction

Cell division is essential to bacterial life and ensures the propagation of genetic material to the next generation. In general, a bacterial cell is born, doubles in size while replicating its circular chromosome, and divides, resulting in the birth of two daughter cells, each with a chromosomal copy. In the gram positive bacterium *Bacillus subtilis*, the divisome is the protein complex responsible for cell division. Over a dozen proteins have been identified to be part of the *B. subtilis* divisome, although not all of them essential to cell division (Den Blaauwen *et al.*, 2017). How exactly the divisome is assembled and activated are still outstanding questions, despite decades of study. New insights in the divisome of *B. subtilis* could contribute to the discovery of drug targets for gram-positive pathogens such as *B. cereus*, a causative agent of food-poisoning, *B. anthracis*, the causative agent of anthrax and *Staphylococcus aureus*, for which several multi-drug resistant strains are present in hospitals all over the world (e.g. Methicillin-resistant *S. aureus* or MRSA).

In this introduction, the current understanding of how the divisome is assembled will be discussed. Subsequently, known and putative regulators of the divisome will be addressed. The focus of this thesis will be the investigation of protein interactions in the divisome, specifically when divisome proteins interact and where the interacting residues are located.

Divisome assembly

FtsZ polymerization

A universal component of bacterial cell division is the tubulin homologue FtsZ, essential for cell division in all bacteria studied so far (Erickson, 1995). In *B. subtilis*, it is the first protein to arrive at the future division site (Gamba *et al.*, 2009). Like tubulin, it is a GTPase and its ability to bind GTP is essential for its

polymerization (Mukherjee and Lutkenhaus, 1994). For FtsZ polymerization to occur, a critical threshold concentration is necessary for its cooperative assembly (Levin *et al.*, 1999; Mukherjee and Lutkenhaus, 1999; Chen *et al.*, 2005). In the cell, FtsZ levels are tightly regulated. Deviations from the optimal FtsZ concentration lead to division defects (Beall and Lutkenhaus, 1991). A decrease in FtsZ levels will interfere with the critical concentration necessary for polymerization, while increased FtsZ levels might titrate essential interactions partners away from the division site (Ward and Lutkenhaus, 1985; Dai and Lutkenhaus, 1992). In addition, the expression of *ftsZ* corresponds to the increase in cell volume when cells grow throughout their cell cycle, resulting in a constant expression and levels of FtsZ during the cell cycle (Trip *et al.*, 2013).

In vitro, FtsZ polymers are able to self-associate through Ca^{2+}-dependent lateral interactions, and form sheets and bundles (Erickson *et al.*, 1996; Löwe and Amos, 1999). The significance of these lateral interactions is illustrated by the defect of the FtsZ(ts1) phenotype, which displays a temperature dependent division defect (Beall and Lutkenhaus, 1991). *In vitro* FtsZ(ts1) is impaired in formation of lateral interactions, and the temperature sensitive effect is rescued *in vivo* by additional Ca^{2+} (Monahan *et al.*, 2009).

FtsZ polymerization and lateral interactions are further controlled by other members of the divisome and division regulators (Fig. 1A). UgtP and MinC inhibit FtsZ polymerization interactions by binding FtsZ monomers, rendering them unable to polymerize (Weart *et al.*, 2007; Scheffers, 2008). EzrA is recruited to the divisome where it binds to FtsZ polymers, thereby disrupting lateral interactions between polymers (Levin *et al.*, 1999; Haeusser *et al.*, 2004; Cleverly *et al.*, 2014). Additionally, ZapA has been found to bind to FtsZ as a ZapA-tetramer, connecting two FtsZ polymer strands and thereby contributing to the

lateral interactions between polymers (Gueiros-Filho and Losick, 2002; Roseboom *et al.*, 2018).

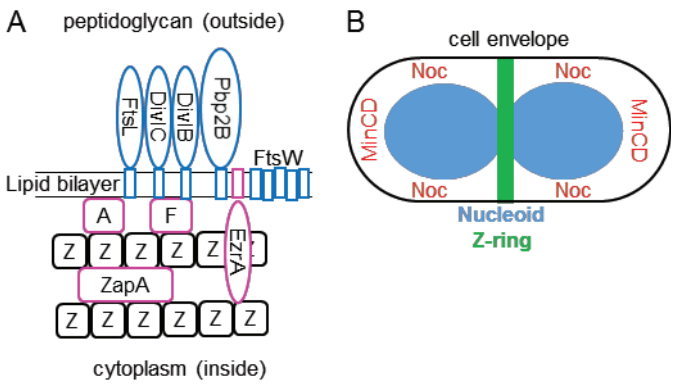

Figure 1. Overview of divisome composition and division site selection in *B. subtilis*. A) A cartoon representation of the divisome, with FtsZ (black) interacting with its direct binding partners (pink), and subsequent recruitment of the late division proteins (blue). Note that proximity in the cartoon does not necessarily mean that proteins interact, see main text for protein interactions so far characterized. B) Z-ring positioning is determined by its negative regulators (red): at the pole FtsZ polymerization is inhibited by MinCD, while Noc ensures that no Z-ring is formed over the nucleoid (blue). Therefore, the Z-ring will form in an inhibitor free zone in the middle of the cell (green).

The resulting assembly of FtsZ polymerization and lateral interactions can be observed *in vivo* as a ring at the future division site (Wang and Lutkenhaus, 1993; Levin *et al.*, 1999). This FtsZ-ring, or Z-ring, is not a continuous ring: super resolution microscopy techniques have shown gaps in this structure (Holden *et al.*, 2014). Also, the width of the ring is not uniform and has been described as beads on a string (Strauss *et al.*, 2012). These observations are in line with the lateral interactions of FtsZ polymers, which could be contributing to irregularities in ring-width.

FtsZ membrane anchoring

During cell division, the cell membrane needs to invaginate and a division septum needs to be synthesized between the daughter cells. This necessitates the confinement of FtsZ polymers close to the cell membrane (Fig. 1A). In *B. subtilis*, FtsZ is tethered to the membrane by its membrane anchors SepF and FtsA (Pichoff and Lutkenhaus, 2005; Duman *et al.*, 2013). Neither SepF nor FtsA is essential for cell division in *B. subtilis*, but cells lacking both are unable to form a Z-ring (Ishikawa *et al.*, 2006; Hamoen *et al.*, 2006). Interestingly, both FtsA and SepF self-associate and both interact with the membrane through an amphipathic helix (Pichoff and Lutkenhaus, 2005; Duman *et al.*, 2013).

FtsA is an actin homologue and its ATPase activity and self-interaction has been found to be essential for cell division in *Escherichia coli* (Pichoff and Lutkenhaus, 2005; Pichoff and Lutkenhaus, 2007). In *E. coli*, the binding of ATP stimulates self-interaction of FtsA, and an FtsA dimer subsequently interacts with the FtsZ C-terminal domain (Pichoff and Lutkenhaus, 2007). Adding purified FtsZ and FtsA on lipid surfaces resulted in dynamic spiral assemblies of approximately 1 μm in diameter, comparable to the diameter of cells (Loose and Mitchison, 2014). The importance of FtsA was also highlighted by a direct fusion between the membrane binding domain of FtsA and FtsZ. While *in vitro* this chimera is able to form constricting rings in lipid cylindrical vesicles, it cannot complement *E. coli* cells lacking FtsZ (Osawa *et al.*, 2008).

SepF forms dimers (Fig. 2C), and these dimers form larger protein rings (Gündoğdu *et al.*, 2011; Duman *et al.*, 2013). The dimerization interface of SepF can be derived from the crystal-structure (Duman *et al.*, 2013). The stacking in the crystal structure suggests that dimers interact with other dimers through their alpha-helices, which is supported by the fact that a G109K mutation is deficient in ring formation (Duman *et al.*, 2013).

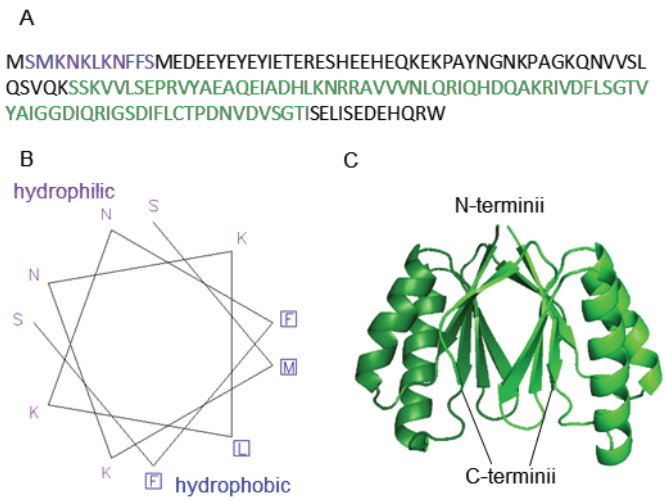

Figure 2. Schematic representation of SepF. A) amino acid sequence of SepF, with the amphipathic helix coloured in blue-purple, depending on hydrophobic (blue) or hydrophilic (purple) residues. The portion of SepF that is crystalized and makes up the more globular domain of SepF is coloured in green. An unstructured linker is present between the amphipathic helix and the globular domain. B) Helical wheel projection of the amphipathic helix of SepF (http://www.bioinformatics.nl/cgi-bin/emboss/pepwheel). Hydrophobic (blue) and hydrophilic (purple) residues are colored as in A. C) Crystal structure of a SepF dimer, with both strands colored in different shades of green (PDB: 3zih).

The membrane interaction of SepF is controlled through its N-terminal amphipathic helix (Fig. 2A-B). The amphipathic helix by itself only poorly recruits GFP to the membrane in cells. However, forced dimerization by fusing this domain with a self-interacting leucine-zipper domain greatly stimulates membrane interaction (Duman *et al.*, 2013). This might indicate that the membrane interaction is only strong when SepF is a dimer, which also seems to be the configuration in which it interacts with the FtsZ C-terminus (Król *et al.*, 2012).

The fact that SepF forms rings *in vitro* has been proposed to help position the Z-ring on the leading edge of the septum. The diameter of the SepF rings closely matches that of the septum width (Duman *et al.*, 2013). In addition, cells lacking SepF have aberrantly formed septa, which are generally much thicker than wild type septa (Hamoen *et al.*, 2006). *In vitro*, incubation of both FtsZ and SepF

leads to the formation of tubular structures with a diameter comparable to SepF rings (Gündoğdu *et al.*, 2011). In these tubules, FtsZ polymers run perpendicular to the SepF rings, although it is difficult to discriminate whether the FtsZ polymers are on the inside or outside of the SepF rings. Incubating SepF rings with lipid vesicles shows the recruitment of these vesicles to the inside of the SepF rings (Duman *et al.*, 2013). Together, these observations are in line with a model where SepF is on the edge of the leading septum, where it recruits FtsZ polymers.

Late complex formation

Once the Z-ring is formed, a second complex is recruited (Fig. 1A). This complex is responsible for synthesis of the peptidoglycan (PG) at the cell division site that will make up the division septum. It consists of the lipid-II flippase/peptidoglycan transglycosylase FtsW and its cognate transpeptidase Pbp2B, in addition to the structural proteins FtsL, DiviB and DivIC (Harry *et al.*, 1993; Katis *et al.*, 1997; Daniel *et al.*, 2000; Daniel and Errington, 2000; Mercer and Weiss, 2002; Gamba *et al.*, 2016; Meeske *et al.*, 2016). These proteins are coordinating septum synthesis, as in their absence no septation is observed, but they do not affect Z-ring formation. The exception is DivIB, which only has a temperature sensitive phenotype on late complex formation (Harry *et al.*, 1993). Interestingly, the PG transpeptidation activity of Pbp2B is not essential for a functional late complex, since an inactivated Pbp2B is still sufficient for septation (Morales Angeles *et al.*, 2017). Pbp2B and FtsW are most likely responsible for the recruitment of other PG-synthesizing proteins, such as the bifunctional class-A Pbp, Pbp1A, through interaction with EzrA (Claessen *et al.*, 2008). The late protein complex has a high number of protein-protein interactions (Daniel *et al.*, 2006). DivIC and FtsL are found to interact with each other, forming a coiled-coil heterodimer (Wadsworth

et al., 2008). In addition, FtsL dimerizes, while also interacting with DivIB and Pbp2B (Daniel *et al.*, 2006). The DivIC-FtsL heterodimer is proposed to interact with Pbp2B, and this interaction is stimulated by DivIB (Wadsworth *et al.*, 2008). In *E. coli*, the DivIB homologue FtsQ has been proposed to stabilize the FtsL-FtsB (DivIC homologue) dimer and subsequently recruit it to FtsI (Pbp2B homologue) (Wadsworth *et al.*, 2008; Glas *et al.*, 2015). However, such model it is difficult to envision for *B. subtilis* where DivIB is not essential in late protein complex formation at permissive temperatures. How FtsW binds the complex is yet unclear but studies in *E. coli* suggests that Pbp2B and FtsW directly interact (Mercer and Weiss, 2002; Pastoret *et al.*, 2004). How the late-complex is recruited to the Z-ring remains unknown, as no direct interaction between essential Z-ring proteins and late-complex proteins has been found.

Late complex interactions have been shown by yeast-two-hybrid, suggesting the complex can form in the absence of FtsZ (Daniel *et al.*, 2006). However, in cells depleted of FtsZ, no enrichment of late complex proteins is observed at a potential division site (Katis *et al.*, 2000; Daniel *et al.*, 2000; Daniel and Errington, 2000; Gamba *et al.*, 2016). Interestingly, in the absence of one of the late proteins, including DivIB at its non-permissive temperature, the other late division proteins are affected in their recruitment to the Z-ring. This is indicative of a strong protein interdependency in the late complex, and suggests cooperative protein interactions that contribute to either recruitment to or retention at the division site (Gamba *et al.*, 2016). Given this all-or-nothing phenotype, it has been challenging so far to identify the interaction site or sites between the Z-ring and the late complex in *B. subtilis* and in other gram-positive bacteria.

Late complex stability

The interdependency observed between the late divisio proteins and the all-or-nothing localization are reminiscent of the cooperative assembly of the Z-ring. This suggests that perhaps a critical concentration is necessary for late complex recruitment. Interestingly, late division proteins FtsL and DivIC are found to be highly instable, and upon depletion of FtsL, DivIC protein levels decrease rapidly (Daniel *et al.*, 1998), while FtsL levels decrease in a *divIB* null mutant (Daniel and Errington, 2000). Depleting FtsL in a *divIB* background no longer changed the protein levels of DivIC (Daniel *et al.*, 2006), suggesting that the observed FtsL-dependent instability of DivIC is mediated through DivIB. The instability of FtsL has been attributed to targeted degradation by the intramembrane protease RasP (Bramkamp *et al.*, 2006). Expression of FtsL and RasP in *E. coli* lead to degradation of FtsL, while additional expression of DivIC stabilized FtsL, suggesting that the heterodimer is more resilient to degradation by RasP (Wadenpohl and Bramkamp, 2010). Together, these observations predict a stable FtsL-DivIC-DivIB complex, while FtsL is degraded by RasP in the absence of either DivIC or DivIB.

In both *E. coli* and *B. subtilis*, a delay of approximately 25% of the cell cycle is observed between the formation of the Z-ring and the recruitment of the late complex (Aarsman *et al.*, 2005; Gamba *et al.*, 2009). The cause for this delay has so far not been addressed, but it should be viewed in context of Z-ring dependency of late complex mid-cell recruitment, the interdependency of late protein mid-cell recruitment and the instability of late proteins in *B. subtilis*. Whether this delay is a consequence of these three prerequisites or under control of a cell cycle checkpoint mechanism is so far unknown.

Constriction

Once the divisome is fully assembled, cells start to constrict. In *E. coli* this is mainly controlled by the recruitment of FtsN, which coordinates the invagination of the inner and outer membrane (Aarsman *et al.*, 2005; Goehring *et al.*, 2006; Tsang and Bernhardt, 2015; Vischer *et al.*, 2015). In *E. coli*, the rate of constriction is also directly correlated with the growth rate of the cells, suggesting that the availability of substrate could dictate constriction speed. Mutating the transpeptidase activity of FtsI (Pbp2B homologue) further reduces constriction speed in a growth rate independent way (Coltharp *et al.*, 2016). However, PG-synthesis is not the only proposed mechanism for constriction. *In vitro* experiments have shown that FtsZ can generate the force necessary to pinch in and constrict the cell membrane (Osawa *et al.*, 2008; Osawa *et al.*, 2009; Erickson *et al.*, 2010). So far no consensus has been reached on whether septal PG-synthesis pushes in the membrane during constriction, or whether FtsZ pulls in the cell membrane thereby generating sufficient free space to be filled-up by subsequent PG-synthesis.

Recent studies in both *E. coli* and *B. subtilis* have shown that the FtsZ GTPase activity drives polymer treadmilling, which is the growth of a polymer from leading end and shrinking from the lagging end (Bisson Filho *et al.*, 2017; Monteiro *et al.*, 2018; Ramirez-Diaz *et al.*, 2018). Interestingly, septal PG synthesis is found to be dependent on the rate of FtsZ treadmilling, as inhibiting treadmilling by addition of the division inhibitor PC190723 or expression of a GTPase deficient dominant negative FtsZ both lead to halted PG synthesis in *E. coli* and *B. subtilis* (Bisson Filho *et al.*, 2017; Yang *et al.*, 2017). In *S. aureus*, inhibiting FtsZ treadmilling only blocks the initial steps of constriction, while divisomes that already have recruited the lipid-II flippase/transglycosylase MurJ continue to constrict, irrespective of FtsZ treadmilling speed (Monteiro *et al.*, 2018). This is

not observed in *B. subtilis*, where all constricting divisomes are affected by inhibiting FtsZ treadmilling (Bisson Filho *et al.*, 2017). How these observations should be interpreted within the context of a possible direct interactions between the Z-ring and the late complex is so far unknown. Additionally, it remains unknown how the treadmilling of FtsZ polymers regulates septal PG-synthesis and what role additional proteins, such as MurJ, have in this regulation.

Divisome assembly regulation

Z-ring positioning

Cells need to carefully control where and when the divisome is assembled. In the rod-shaped *B. subtilis* cells divide in the middle of the cell, resulting in two equally-sized daughter cells. However, during sporulation *B. subtilis* is able to position its division septum close to the pole, generating an asymmetric division event (Beall and Lutkenhaus, 1991). Therefore, different division site selection mechanisms are in place to ensure proper Z-ring formation and positioning in this organism (Fig. 1B).

In *B. subtilis*, a negative regulator of FtsZ polymerization, MinC, is specifically recruited to the cell poles (Marston and Errington, 1999; Scheffers, 2008). Polar activity and localization of MinC is controlled by MinD (Marston and Errington, 1999). MinD in turn is localized to the pole through MinJ (Bramkamp *et al.*, 2008; Patrick and Kearns, 2008), which binds to DivIVA (Marston *et al.*, 1998). DivIVA recognizes negative curvature, and in a non-dividing cell the highest negative curvature is at the cell pole (Ramamurthi and Losick, 2009; Lenarcic *et al.*, 2009). Interestingly, upon septation the increase in negative curvature at the division site results in the accumulation of DivIVA at the base of the growing septum, and thus accumulation of MinCD (Gregory *et al.*, 2008). Why a negative

regulator of Z-ring formation is recruited to the division site during constriction is not fully understood, but the recruitment of MinCD is proposed to remove the divisome from the newly formed cell poles upon finishing division, as *B. subtilis* cells lacking a functional Min-system form minicells preferentially at the new poles (Gregory *et al.*, 2008). MinCD homologues play an important role in division site selection in *E. coli*, although no MinJ or DivIVA is present in this organism (De Boer *et al.*, 1989). Instead, polar enrichment is achieved by the oscillating MinE ring, which removes MinCD complexes when traveling from pole to pole, resulting in a relative higher MinCD concentration at the poles compared to mid-cell (Hu and Lutkenhaus, 2001).

In addition to preventing Z-ring formation at the poles, *B. subtilis* also has a mechanism to prevent Z-ring formation over the nucleoid. This so called nucleoid occlusion is facilitated by the protein Noc (Wu and Errington, 2004). Noc has been found to bind specific DNA sequences that are absent from the terminal region of the chromosome (Wu *et al.*, 2009). In *B. subtilis*, the duplicated chromosome orients itself in an origin–terminus–origin orientation along the cell axis after it has finished replicating (Wang *et al.*, 2014). The absence of Noc at the terminus region ensures that even when the chromosome has not fully segregated, the Z-ring can already start forming. In addition to binding specific sequences, Noc also binds the cell membrane and this membrane binding is necessary for it to inhibit Z-ring formation (Adams *et al.*, 2015). These large membrane attached nucleoprotein complexes are believed to interfere physically with FtsZ polymerization. A recent study has linked the segregation machinery ParAB (Spo0J and Soj in *B. subtilis*) to the activity of Noc (Hajduk *et al.*, 2019). Upon blocking early stages of DNA replication, both Noc and Spo0J are necessary to prevent Z-ring formation over unsegregated DNA, although the mechanism by which Spo0J affects Noc activity

was not addressed. In *E. coli* nucleoid occlusion is also present and is regulated by SlmA, a protein that directly binds both DNA and FtsZ, thereby interfering with Z-ring formation (Bernhardt and De Boer, 2005).

Interestingly, cells lacking both a functional Min and nucleoid occlusion system are still able to position the Z-ring between replicating nucleoids (Rodrigues and Harry, 2012). In *E. coli* a third Z-ring positioning system is observed, where FtsZ is linked to the chromosomal terminus through ZapA, which binds ZapB, and ZapB binds to the DNA terminus recognizing protein MatP (Espéli *et al.*, 2012). However, cells lacking this system in addition to a mutated minCD system and nucleoid occlusion system still efficiently position the Z-ring (Bailey *et al.*, 2014). If an additional system is responsible for Z-ring positioning in these cells, or if the nucleoid itself would generate sufficient crowding by transertion remains an unanswered question (Norris, 1995; Norris and Madsen, 1995).

Division block upon DNA damage

External and internal stresses contribute to DNA damage, which is recognized by the protein RecA (Lovett *et al.*, 1988). The resulting cellular response is the inhibition of DNA replication initiation, a halt in existing replication and a block in cell division. This SOS response is controlled by cleavage of the transcriptional repressor DinR (LexA homologue in *B. subtilis*) by the RecA-DNA complex, resulting in a broad transcriptional response (Winterling *et al.*, 1997). A RecA independent response was also observed, which included the downregulation of DnaA (Goranov *et al.*, 2005). In addition to initiating DNA replication (Fukuoka *et al.*, 1990), DnaA also functions as a transcriptional regulator that binds the promotor of *ftsL* (Goranov *et al.*, 2005). The downregulation of the *ftsL-pbpB* operon results in an rapid decrease in FtsL levels (Daniel and Errington, 2000),

unlike the more stable Pbp2B that is much more resilient to transcriptional depletion (Daniel *et al.*, 2000). This reduction in FtsL will result in an inability to recruit the late complex, thereby blocking cell division.

However, a halt in cell division upon DNA damage is not exclusively due to FtsL depletion. Deleting the *yneABC* operon was found to suppress cell filamentation in a *dinR* mutant that experiences a constant SOS response (Kawai *et al.*, 2003). Expressing specifically YneA caused cell filamentation and a stop in formation of new Z-rings (Kawai *et al.*, 2003). YneA is a transmembrane protein with an extracellular PG-binding LysM domain. The LysM domain is cleaved from the transmembrane domain (Mo and Burkholder, 2010). It is assumed that the full-length protein is inhibiting cell division while proteolytic turnover ensures that cells can leave the SOS response. This proposed recovery is promoted by the proteases YibL and CtpA, which are both found to interact with YneA (Burby *et al.*, 2018). Nevertheles, the exact mechanism by which YneA inhibits cell division is still unknown, but cells expressing YneA do not finish constricting and do not form new Z-rings (Mo and Burkholder, 2010). Interestingly, overexpression of FtsL overcomes the inhibiting effect of YneA (Kawai and Ogasawara, 2006), possibly by integrating both the RecA-dependent and -independent SOS responses to ensure efficient recovery from the cell division block.

The effect of nutrient availability on Z-ring formation

B. subtilis also controls Z-ring formation as a response to nutrient availability. The moonlighting UDP-glucose diacylglycerol glucosyltransferase UgtP is found to contribute to the communication between cell division and metabolism. Cells lacking UgtP are much shorter than wild type when grown in rich medium (Weart *et al.*, 2007). UgtP is involved in glycolipid biosynthesis and its localization is

dependent on the availability of its substrate UDP-glucose (Weart *et al.*, 2007; Chien *et al.*, 2012). When cells are grown in poor medium UgtP is localized in spots throughout the cell, while it becomes enriched at the division site in rich medium. (Weart *et al.*, 2007). The enhanced self-interaction in the absence of UDP-glucose most likely contributes to spot formation (Chien *et al.*, 2012). This ensures a weaker affinity for FtsZ, while under high UDP-glucose the affinity for FtsZ increases (Chien *et al.*, 2012). *In vitro*, purified UgtP inhibits FtsZ polymerization in a concentration dependent manner, and this inhibition is further stimulated in the presence of UDP-glucose (Weart *et al.*, 2007; Chien *et al.*, 2012). Together, these studies show that UgtP inhibits Z-ring formation in rich medium conditions.

UgtP is not the only link between Z-ring formation and metabolism. A suppressor screen for *ftsZ(ts1)* identified that deleting the pyruvate kinase Pyk resulted in growth at the non-permissive temperature (Monahan *et al.*, 2014). Deleting *pyk* also led to an increase of aberrant polar Z-rings in wild type cells, suggesting that pyruvate synthesis stimulates mid-cell Z-ring assembly. The authors suggested that this might be regulated through the pyruvate dehydrogenase subunit E (PDH E1α), which changes its localization based on pyruvate availability in the cell. In presence of intracellular pyruvate it co-localizes with the nucleoid, while in absence of intracellular pyruvate, it is excluded from the nucleus. The stimulating effect of PDH E1α on Z-ring formation was shown by depleting it in an *ezrA* background, resulting in the inability to form Z-rings (Monahan *et al.*, 2014). Unfortunately, a direct stimulation of FtsZ polymerization by PDH E1α and/or pyruvate was not shown. The presence of both negative and positive regulators for Z-ring formation that use different metabolites as input (UDP-glucose and pyruvate, respectively) allows the cell to fine-tune the necessary critical threshold concentration for Z-ring formation given the available nutrients.

The implication of cell size homeostasis on divisome assembly

An important reason to assume a link between metabolism and regulation of cell division is the observation that cells can change their average cell size as a response to nutrient availability. Over 70 years ago it was shown that *Salmonella typhimurium* increases its cell size when growing faster, but only when the growth rate was modulated by nutrient availability, not by temperature (Schaechter *et al.*, 1958). The observation that chromosomal replication in *E. coli* cannot be faster than 40 minutes, even in cells that double every 20 minutes, led to the proposal of multifork replication (Helmstetter and Cooper, 1968). Multifork replication are overlapping replication cycles occurring during the division cycle. These additional rounds of replication were proposed to contribute to the increased cellular volume for cells in order to accommodate the additional DNA in these cells. This led to the prediction of a regulator that ensures that initiation of DNA replication only occurs once per origin per division cycle, at a given cell size per origin (Donachie, 1968; Si *et al.*, 2017). How, and even if, growth rate and chromosome replication are linked to cell division is still debated (Levin and Taheri-Araghi, 2019).

In addition to having a reproducible average cell size that varies based on nutrient availability, the variation in cells size is also controlled and corrected for. Under steady state growth, cells have a reproducible cell size distribution (Collins and Richmond, 1962). Recent advances in imaging software and micro-fluidics allowed for the acquisition of large datasets on individual growing cells (Campos *et al.*, 2014; Taheri-Araghi *et al.*, 2015). These studies revealed that *E. coli* and *B. subtilis* cells grow a constant amount per division cycle, irrespective of initial birth size. This constant addition per cell cycle, an 'adder' model for cell size homeostasis, ensures that outliers return to an average size (Fig. 3). How this

constant growth per division cycle is achieved is so far unknown. Since it takes time to assemble the divisome and synthesize the septum, and since cells continue to grow during this time, size homeostasis should be connected to timing of Z-ring formation, late protein recruitment and/or constriction.

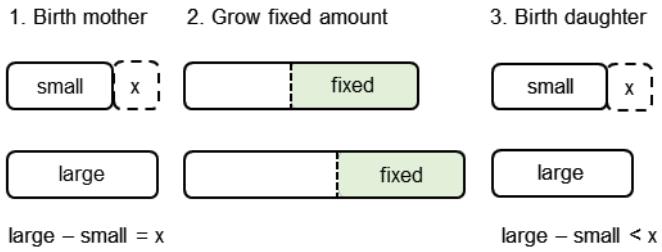

Figure 3. Schematic representation of size-homeostasis according to an adder model. 1) At birth, both mother cells (small and large) differ by size x. 2) After growing the same (fixed, green) amount during their cell cycle, they divide in equally size daughter cells. 3) The corresponding daughters still differ in size, however the difference is now smaller than size x. This ensures that each division contributes to reducing the variation between large and small cells.

Thesis overview

In *B. subtilis*, over a dozen proteins have been found to form the divisome. However, a direct interaction between the Z-ring and the late protein complex has so far not been found, although these complexes always co-localize (Harry *et al.*, 1993; Katis *et al.*, 1997; Daniel *et al.*, 2000; Daniel and Errington, 2000; Gamba *et al.*, 2016; Bisson Filho *et al.*, 2017). Here, we set out to identify these interactions, and to investigate the timing of late division protein recruitment.

In the first experimental chapter, Chapter 2, we tried to determine the molecular interactions within the divisome by the crosslinking agent bis(succinimidyl)-3-azidomethylglutarate (BAMG). BAMG specifically crosslinks lysine residues that are in close enough proximity (Kasper *et al.*, 2007). Previously, a protocol was developed to enrich for crosslinked lysines from a complex peptide

mixture and identify these by LC-MS/MS (Buncherd *et al.*, 2014a; Buncherd *et al.*, 2014b). Since the divisome is highly dynamic (Adams *et al.*, 2011), we wanted to capture these interactions *in vivo*. In order to achieve this, a modified protocol was developed to allow for residue-specific crosslinking in a growing culture without the need of washing cells. A proteome-wide crosslinking analysis was performed and this identified 82 unique crosslinks. The majority of these crosslinks are between ribosomal subunits or RNA polymerase subunits, leading to the identification of a novel putative binding site between the δ and β' subunit of RNAP. Unfortunately, a very limited number division proteins were identified, and no crosslinks between division proteins were found.

In Chapter 3, we tried to specifically enrich for division proteins in the *in vivo* crosslinking protocol. However, this was unsuccessful, and therefore we used purified FtsZ and SepF for crosslinking with BAMG, in order to investigate where the C-terminal peptide of FtsZ binds SepF. Although a putative interaction site between FtsZ and SepF was not confirmed, crosslinks between FtsZ and FtsZ in the absence of GTP were identified. These contacts did not map to the polymerization interface, but close to a residue previously reported to be involved in lateral interactions (Monahan *et al.*, 2009). We propose a possible interaction site that could facilitate lateral interactions between FtsZ in the absence of GTP.

Finally, in Chapter 4, we address the observed time delay between Z-ring formation and late protein recruitment. To do so, a timelapse method was developed to image both Z-ring formation and late protein recruitment in the same cell. It appeared that this delay was highly variable between cells. We sought to further explore this variation within the context of cell size homeostasis. Therefore, we imaged Z-ring formation and late protein recruitment in various mutant backgrounds affecting Z-ring formation and FtsL stability. From our observations

we propose a mechanistic basis of how cells can ensure size-homeostasis at the level of divisome formation. Additional observations that might give more insight into the interaction between the Z-ring and late-complex are addressed in the final discussion of this thesis, Chapter 5.

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