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 5: Discussion

The formation of the divisome is key to cell division. At what residues division proteins interact and when they interact are central questions in this thesis. We tried to address these questions using the bacterial model system *Bacillus subtilis*. In order to address which residues interact in the divisome *in vivo*, a crosslinking method was developed to allow for residue specific crosslinking in growing cells directly in the culture (Chapter 2). This resulted in the identification of 82 uniquely crosslinked residues between proteins, although primarily between ribosomal subunits and between RNA polymerase subunits. The interaction site between the cell division protein FtsZ and its membrane anchor SepF were also investigated with residue specific crosslinking (Chapter 3). No clear interaction site could be mapped, but FtsZ did crosslink with itself and these crosslinks map to residues previously associated with lateral interactions (Monahan *et al.*, 2009). Finally, divisome assembly was imaged in single cells to further investigate the previously reported delay between Z-ring formation and late division protein recruitment (Aarsman *et al.*, 2005; Gamba *et al.*, 2009). The duration of this delay varied dramatically between cells, which was further studied in the context of cell size homeostasis (Chapter 4). The observation that Z-ring accumulation at the division site correlates with the progression through divisome assembly, linking it to cell size, is in line with a recent study that also proposes FtsZ as the defining component for cell size homeostasis (Si *et al.*, 2019). Here, we would like to discuss the cooperative assembly of the divisome in the framework of dynamic protein thresholds.

Z-ring formation

Cooperative polymerization and membrane anchoring of FtsZ ensure that a fixed concentration of division proteins is efficiently concentrated at the future division

site, leading to Z-ring formation (Chapter 4) (Levin *et al.*, 1992; Levin *et al.*, 1999; Mukherjee and Lutkenhaus, 1999; Smith *et al.*, 2002; Wu and Errington, 2004; Chen *et al.*, 2005; Pichoff and Lutkenhaus, 2005; Pichoff and Lutkenhaus, 2007; McGuffee and Elcock, 2010; Trip *et al.*, 2013). Lateral interactions between FtsZ polymers are also found to be important for Z-ring formation and these were proposed to play a role in a helix-to-ring transition of FtsZ (Monahan *et al.*, 2009). This helical localization of FtsZ is primarily seen when fused to GFP using conventional light microscopy (Thanedar and Margolin, 2004; Peters *et al.*, 2007), however not all super resolution microscopy techniques could identify this helix (Fu *et al.*, 2010; Jennings *et al.*, 2011; Holden *et al.*, 2014). Interestingly, our data shows that FtsZ laterally interacts even when not polymerizing (Chapter 3). The crosslinks that were identified map close to A240, which, when mutated to valine, leads to the inability of FtsZ to form a ring in the cell at non-permissive temperatures (Monahan *et al.*, 2009). This phenotype can be rescued by additional Ca^{2+} in the medium. In our *in vitro* crosslinking, no additional Ca^{2+} was necessary to stimulate lateral interactions. Possibly, Ca^{2+} induces lateral interactions at a different site from A240. Crosslinking an A240V FtsZ mutant in the presence or absence of Ca^{2+} could give more insight into this. Regardless of a helical-to-ring transition during Z-ring formation, additional lateral interaction sites that promote FtsZ self-interaction will promote locally concentrating FtsZ, thereby assisting Z-ring formation.

Limiting diffusion by protein interactions is not limited to FtsZ. The same is found for the FtsZ anchors FtsA and SepF, that both self-interact and interact with FtsZ as a dimer (Pichoff and Lutkenhaus, 2007; Duman *et al.*, 2013). To investigate FtsZ-SepF interactions, MBP-SepF was used since this fusion protein has been shown to interact with FtsZ (Król *et al.*, 2012), while not forming SepF

rings (Gündoğdu *et al.*, 2011), which would further complicate interpretation of any crosslinks found. The observation of MBP-SepF multimers was surprising, as only a dimer was expected (Król *et al.*, 2012). The presence of at least an MBP-SepF tetramer, the crosslink between SepF K63 and K101, the absence of any crosslinks with FtsZ K380 and the *in silico* docking of the FtsZ C-terminal peptide, together led to the proposed SepF dimer-dimer interaction site (Chapter 3). This proposed additional dimer-dimer interaction site will further increase the membrane affinity of SepF (Duman *et al.*, 2013), which will limit its diffusion, thereby locally concentrating FtsZ and promoting Z-ring formation.

This proposed interaction does not address how FtsZ polymers are positioned relative to SepF arches on the leading edge of the septum (Duman *et al.*, 2013). In SepF, there is a 36 amino acid unstructured linker between the amphipathic helix and the globular domain, while FtsZ has a 50 amino acid unstructured linker between the FtsZ globular domain and its C-terminal peptide. Both the SepF and FtsZ unstructured linkers give a large degree of freedom in positioning the two globular domains relative to each other. Resolving the position of SepF within a SepF-ring structure might give better insight into the stacking of dimers or tetramers. Doing this with crosslinking will require the ability to discriminate between peptides from the same protein and from two different proteins, which could be achieved by isotope labeling and crosslinking ^{14}N SepF with ^{15}N SepF (Ong *et al.*, 2002). Even then, any resulting crosslinks might be challenging to position within a SepF ring. A more appropriate method seems to be the use of high resolution cryo-electron microscopy (CryoEM) (Topf *et al.*, 2008), from which, in theory, the average SepF ring structure could be derived onto which the crystal structure of SepF can be fitted. Performing CryoEM in the presence or absence of either lipids or the FtsZ C-terminal peptide might give more

insight in the role of the flexible linker in the SepF ring and could show whether SepF reorients itself within the SepF-ring to accommodate membrane interaction.

It was previously shown that *B. subtilis* cells need either FtsA or SepF to form a Z-ring (Ishikawa *et al.*, 2006). However, in the absence of SepF cells do not delay the formation of the Z-ring, but rather take longer to constrict (Chapter 4). This constriction defect is maybe expected since cells lacking SepF have deformed septa (Hamoen *et al.*, 2006). This was hypothesized to be miss-positioning of the Z-ring on the leading edge of the septum (Duman *et al.*, 2013). However, with the finding that septal PG-synthesis is controlled by FtsZ-treadmilling (Bisson Filho *et al.*, 2017; Yang *et al.*, 2017), another possibility is that SepF is positively regulating FtsZ treadmilling efficiency *in vivo*. FtsZ treadmilling has been shown to be GTPase dependent, though SepF has been shown to reduce FtsZ GTPase activity *in vitro* (Singh *et al.*, 2008). However, since treadmilling will also dependent on the availability of FtsZ subunits to bind to the growing polymer, locally enrichment of FtsZ will positively affect treadmilling. It can therefore be hypothesized that the constriction defect in the absence of SepF might also be due to a reduction in FtsZ treadmilling efficiency due to lower locally enriched FtsZ as a consequence of miss-positioned FtsZ polymers on the leading edge of the septum.

Both the self-interactions between non-polymerizing FtsZ and between SepF will contribute to reaching the Z-ring formation threshold. Reaching this threshold faster by overexpression of FtsZ does not result in faster Z-ring formation, but rather increases variation in ring formation (Chapter 4). A possible explanation is that the high concentration of FtsZ most likely dilutes interaction partners away from the future division site, especially since nucleoid occlusion and the min-system might become less efficient at higher FtsZ levels. That overexpression of

FtsZ did not affect the Z-ring threshold is further emphasized by the fact that the ZapA intensity at the moment of ring-formation is unaffected in these cells (Chapter 4). This illustrates that these thresholds are probably the cumulative result of all the interactions discussed above, and are robust to the over-expression or absence of one of its components. It will be interesting to see how and if these thresholds change, e.g. at a different growth rate. The negative regulatory effect of UgtP and PDH E1α on FtsZ under rich growth conditions (Weart *et al.*, 2007; Monahan *et al.*, 2014) suggests that the effective threshold to form the Z-ring will be higher compared to poor growth conditions. Investigating the divisome assembly as done in Chapter 4 in an UgtP mutant in rich medium could confirm that indeed Z-ring formation is enhanced in such a cell, although the overexpression of FtsZ illustrates that it is maybe not that straight forward.

Late division protein interdependency

After formation of the Z-ring, the late division proteins are recruited. The recruitment of FtsL, DivIC, DivIB, Ppbp2B and FtsW is dependent on FtsZ and on each other, which results in a cooperative assembly at the division site (Katis *et al.*, 2000; Daniel *et al.*, 2000; Daniel and Errington, 2000; Gamba *et al.*, 2016). This cooperative assembly is assisted by the high number of interactions between late division proteins (Daniel *et al.*, 2006; Robichon *et al.*, 2008; Rowland *et al.*, 2010), but how these result in the observed interdependency remains unclear.

All of the late division proteins are transmembrane proteins, with 10 transmembrane helixes for FtsW, while Pbp2B, FtsL, DivIC and DivIB are all single pass transmembrane proteins. The diffusion of membrane proteins is mainly dictated by the amount of membrane helices and not molecular weight (Lucena *et al.*, 2018), thus protein interactions between late division proteins will dramatically

decrease their diffusivity. The interaction strength between late division proteins has not been studied in detail *in vitro*, however a weaker interaction would be favorable to avoid complex formation and aggregation at places other than the Z-ring. Binding to the Z-ring would focus accumulation of late-proteins at the division site, while the late division protein interactions further enhance this accumulation. Which late division protein actually binds the Z-ring is for Gram-positive bacteria so far unknown. Four observations in Chapter 4 may provide more insight into this question: i) Z-ring constriction can start before Ppb2B is localized at the division site. ii) Pbp2B recruitment to the Z-ring is not impaired or delayed in the absence of DivIB. iii) Pbp2B enriches at the Z-ring during constriction. iv) There is no enrichment of Pbp2B at the division site in a Δ*divIB* mutant. From these observations and the available literature we propose in the following discussion that several late proteins have a weak interaction for the Z-ring, and that the observed interdependency does not occur at the level of Z-ring recruitment, but is related to the retention of late proteins at the division site during constriction.

The constriction in the absence of Pbp2B (i) might be due to limitations in our method, as very low amounts of Pbp2B might be sufficient to signal constriction. Indeed, Pbp2B activity is not essential for constriction, but its presence most likely stimulates redundant activity of additional Pbps recruited to the division site (Scheffers *et al.*, 2004; Claessen *et al.*, 2008; Morales Angeles *et al.*, 2017). Alternatively, other late division proteins might have already been recruited to the division site prior to enrichment of Pbp2B, questioning the late protein interdependence in recruitment to the Z-ring. This is further illustrated by our observations that Pbp2B finds the division site without additional delay in the absence of DivIB at the permissive temperature (ii). Interestingly, the strongest Pbp2B signal is observed during constriction, where it exponentially accumulates

over time (iii). The failure to do so in the Δ*divIB* strain when grown at the permissive temperature (iv) results in a dramatic increase in constriction duration in these cells (Chapter 4). This indicates that accumulation of at least Pbp2B and possibly other division proteins is necessary for efficient constriction in *B. subtilis*. There is no effect in the initial amount of Pbp2B recruited at the permissive temperature in Δ*divIB*, only in its accumulation during constriction (Chapter 4). This could suggests that at higher (non-permissive) temperatures the constriction defect in Δ*divIB* is further exacerbated by the faster growth. This will lead to more severe cell filamentation than at lower temperature, thus dilution of late division proteins over a larger volume, disrupting the cooperative assembly at other newly formed Z-rings. Shifting Δ*divIB* cells to the non-permissive temperature in our time-lapse set up could confirm that at the non-permissive temperature there is no defect in initial Pbp2B recruitment, but that the inability of Pbp2B to localize to the division site only follows after the onset of constriction, and only for new division sites.

The interdependent localization of late division proteins was shown by depleting gene expression for several hours and then observing either the localization of other late proteins by fluorescent fusions or by immunoprecipitation (Katis *et al.*, 2000; Daniel *et al.*, 2000; Daniel and Errington, 2000; Gamba *et al.*, 2016). However, depleting *pbpB* still showed at least one division site per cell containing either DivIB, DivIC or FtsL, although cells were unable to complete constriction (Daniel *et al.*, 2000; Daniel and Errington, 2000). Residual Pbp2B localization is observed when cells are depleted of *ftsL*, although not at the same intensity as wild type (Daniel *et al.*, 2000). Some FtsW is still able to localize to the Z-ring, even after 4 hours of *ftsL* depletion, although cells are unable to divide (Gamba *et al.*, 2016). This could indicate that several late division proteins have

at least some affinity to the Z-ring and that their failure to accumulate further has a severe effect on constriction.

The accumulation of late division proteins during constriction is a surprising observation. The cell diameter at the constriction-site is getting smaller as the cells constrict further, which would limit the amount of Z-ring that can be present at the division site. Indeed, an eventual reduction in signal is observed for ZapA as it diffuses from the division site, prior to the reduction in Pbp2B signal. A similar sequential departure from the division site has been reported in *E. coli* (Söderström *et al.*, 2016). However, initially ZapA is observed to enrich at the division site, which could be explained by a constant amount of Z-ring proteins concentrating in a smaller, more in-focus, division site (Chapter 4). This increase in Z-ring concentration at the division site would stimulate the binding of late proteins and thereby favor retention and additional capture of diffusing late division proteins. The absence of Pbp2B accumulation in a Δ*divIB* mutant illustrate the necessity of additional interactions between late division proteins for this further accumulation. It would be interesting to investigate whether a similar reduction in Pbp2B accumulation is observed during the depletion of either FtsL or DivIC. This would further address if the observed interdependency of the late proteins is at the level of Z-ring recruitment or at the level of protein retention during constriction.

Interaction of late division proteins with a treadmilling polymer

The need for retaining the late proteins at the division site might point to how the Z-ring and late proteins are interacting. The previous finding that FtsZ treadmills (Bisson Filho *et al.*, 2017), and that FtsZ treadmilling drives septum synthesis in *B. subtilis* has been an important observation. However, the single molecule measurements in the paper of Bisson Filho et al. are also interesting in view of our

results: interestingly, single FtsZ and FtsA proteins are stationary in a treadmilling polymer, while single Pbp2B proteins diffuse at a speed comparable to the treadmilling speed. A direct interaction between FtsZ and Pbp2B has so far not been observed in *B. subtilis*, though the observation that FtsA is also stationary would suggest that Pbp2B does not bind FtsZ similarly to FtsA. A good control for this would be to see if SepF is also stationary within a treadmilling filament, but this would be expected given the redundant role to FtsA (Ishikawa *et al.*, 2006). Metaphorically, FtsZ and FtsA are a train track that is constantly build up and broken down, while Pbp2B is the train riding that track.

If late proteins have a weak interaction with the Z-ring, this could explain how they are not stationary like FtsA in a treadmilling filament. If the interaction is somehow strongest with the growing end of the FtsZ polymer, this would constantly favor binding further up the filament, ensuring that the train (late division proteins) does not fall off the tracks. Whether late division proteins bind as complex to the FtsZ polymer or bind individually, but are retained at or near the polymer by their pairwise interactions, are both possibilities that should be entertained. As mentioned above, the relative slowdown in Brownian diffusion in the event of multiple late division protein interactions could ensure that they do not diffuse rapidly out of the division site in case the interaction with the Z-ring is lost. Kymographs of Pbp2B localization at the division site show that it can change direction of diffusion, possibly by leaving one FtsZ treadmill and binding one going in the opposite direction. The incidence of this change in direction is a measure for how often Pbp2B would lose contact with the polymer. Investigating this in a Δ*divIB* mutant would give insight whether this leads to a higher incidence of falling of the track, which would be due to fewer interactions keeping the complex together or in contact with the Z-ring. Alternatively, the incidence of Pbp2B falling

off the track in a Δ*divIB* mutant might be similar to wild type, however retention and further accumulation at the division site would be impaired due to the a higher diffusion by lack of additional late division protein interactions. Growth at a higher temperature where diffusion is increased will further exacerbate this and result in more protein diffusing from the division site once contact is lost with the Z-ring. Identifying how these two dynamic systems, the track and train, interact will be essential to further our understanding of the divisome.

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