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Pneumococcal cell biology in a new light

Beilharz, Katrin

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Chapter 7

Discussion and Summary

Chapter 7

Streptococcus pneumoniae is a major human pathogen that can cause severe infections such as pneumonia, meningitis, otitis media and sepsis. Especially in developing countries, septicaemia caused by S. pneumoniae is responsible for 25% of all preventable death in children less than 5 years[3]. The emergence of multiple antibiotic-resistant strains during the last decades stress the importance of designing new efficient strategies to fight pneumococcal infections[241]. Therefore, it is essential to understand the mechanisms with which these bacteria survive and cause disease within the host.

Molecular tool development

Molecular mechanisms underlying cell division, chromosome segregation, cell growth and pathogenesis in S. pneumoniae are still poorly understood. One reason for this is that molecular biological tools to address these questions are still limited. The discovery of the green fluorescent protein (GFP) and later other fluorescent proteins changed the field and brought new possibilities to study live single cells. However, for S. pneumoniae, as a microaerophilic organism, the use of GFP was introduced only recently[21]. What might have hindered the introduction of GFP in the field is that for proper GFP maturation oxidation of its fluorophore is required. Earlier studies where protein localization was investigated involved mainly immunofluorescence microscopy. The limitation of this technique is that prior to microscopy, cells have to be fixed and lysed in order for the antibodies to find their target inside the cells. Fluorescent microscopy using FP-tagged proteins shows clear advantages in the aspect that it can be performed on living cells.

In chapter 2 and 3 we describe the properties of different GFP and RFP variants for use in S. pneumoniae. These fluorescent proteins allow not only for studying protein localization but also gene expression in live single cells. In addition, in order to allow investigation of single-cell protein dynamics during cell growth, we provide a protocol for time-lapse microscopy with S. pneumoniae (chapter 2). Thus, the tools presented in this thesis make it possible to study spatial as well as temporal protein localization throughout the S. pneumoniae cell cycle. Here we focused on the use of GFP and RFP variants, whereas for multi-label experiments other wavelength FPs might be worth considering. For instance, blue and yellow variants recently have successfully been used to visualize protein localization in S. pneumoniae[22].

Although (fluorescence) light microscopy is a powerful tool to study single-cell biology, it is limited by its resolution. The resolution of conventional light microscopes is determined by the diffraction limit of light and for most fluorophores this is ~200 nm (half

of their emission wavelength). This limitation in spatial resolution is particularly important for small bacteria like *S. pneumoniae*, whose length is only about 1 μm. The more recent development of superresolution microscopy approaches through single molecule localization, such as photo activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), can help to us to study cell division or chromosome segregation at an even more detailed level since these techniques provide spatial resolutions of around 10-50 nm. PALM and STORM are based on photocontrollable FPs which can be switched on and off in a controlled way using different wavelengths[242]. A set of photoswitchable fluorescent proteins, including monomeric forms, has recently been developed and this type of FPs is suitable for techniques to study single-molecule localization[243]. PALM has been suggested an important and useful technique to study the cell division machinery and a superresolution image for the structure of the Z-ring has been shown in *E. coli*[244], but the technique has yet to be implemented in *S. pneumoniae*. Another superresolution fluorescence microscopy technique is structured illumination (SIM), which improves the resolution limit with a factor of two compared to conventional light microscopy and has already successfully been used for protein localization studies in *S. pneumoniae*[245]. However, superresolution techniques so far are applied mainly to fixed cells. Development of PALM/STORM to allow also live-cell imaging would therefore greatly facilitate future investigation of pneumococcal as well as bacterial cell biology in general.

Bacterial cell division

One of the most fundamental processes of the bacterial life cycle is the division of the cell. This involves a well-coordinated and regulated interplay of complex protein machineries. Many aspects of this process have been extensively studied, mainly in rod-shaped model organisms such as the Gram-negative *Escherichia coli* and *Caulobacter crescentus* as well the Gram-positive *Bacillus subtilis*, and many key players of cell division and PG synthesis have been identified and characterized[222]. Much of our current knowledge on cell division derives from these studies and has been beneficial to the understanding of cell division in general but also to transferring the gained insights to other organisms. Nevertheless, the available knowledge remains limited, especially when we want to understand how cell shape is maintained, for instance when we are looking at cell division of ovococci such as *Streptococcus pneumoniae*. Although a set of conserved cell division proteins has been investigated in ovococci, mechanisms that govern cell division and peptidoglycan synthesis are poorly understood. One big question that we partly addressed

in this thesis is how peripheral and septal cell wall synthesis are coordinated in such ellipsoid bacteria.

For ovococci, two modes of PG synthesis, namely septal and peripheral, have been suggested[52,74]. The current model suggests that the cell elongates due to action of peripheral PG synthesis. Interestingly, no homologues for MreB have been identified in *S. pneumoniae* or other (ovo)cocci, whereas cell elongation of rod-shaped bacteria depends on MreB. MreB forms patches along the lateral cell wall where the elongation machinery attaches. For ovococci it was therefore assumed that PG synthesis is coordinated by FtsZ and that the machineries will assemble along the Z ring. A two-state model for the PG synthesis has been proposed wherein we find two machineries that are responsible for either peripheral or septal cell wall synthesis. In analogy with the composition of the elongation and division complexes in *E. coli* and *B. subtilis*, two complexes that are required for septal and peripheral PG synthesis have been suggested. Accordingly, the septal machinery consists of FtsZ, EzrA, the DivIVA paralog GpsB, the lipid II flippase FtsW, the complex of DivIB/FtsL/DivIC and the transpeptidase PBP2x[18,45,48,246]. The proposed peripheral synthesis machinery includes MreC/MreD, the lipid II flippase RodA, GpsB and the monofunctional transpeptidase PBP2b[19,73,247].

However, the regulation and coordination of these two machineries remains unclear. Land and Winkler showed that PBP2x and PBP1a both localize in a similar localization pattern, but they show different patterns during septum closure. Similar observations were made by Peters et al., where PBP2x and PBP1a colocalized in most cells. On basis of these data the two-state model has been strengthened, with PBP1a being shuttled between peripheral and septal PG synthesis.

Impact of Ser/Thr phosphorylation by StkP on cell division

Protein phosphorylation by protein kinases and phosphatases is a widely used strategy to transmit cell cycle signals in order to respond to the environment. Two-component systems (TCS) were the first-described signalling systems for prokaryotes and are also the most abundant ones[87]. More recently, eukaryotic-type serine threonine protein kinases have been discovered[110]. *S. pneumoniae* encodes only a single eSTPK, StkP, and its cognate phosphatase PhpP.

The first bacterial phosphoproteome describing serine, threonine and tyrosine phosphorylations was published for *B. subtilis* in 2007[248]. Since then, this approach has been applied to a couple of other organisms; among them *S. pneumoniae*[205]. Using different *in*

vivo and/or *in vitro* assays, targets of StkP phosphorylation involved in cell division and cell wall synthesis, such as DivIVA, FtsZ, FtsA and GlmM, have been identified[43,95,102,104,205]. The identified targets, that represent important cell division proteins, and the fact that StkP-depleted cells appear elongated[104] indicate a strong link of StkP with cell division regulation.

As mentioned above, *S. pneumoniae* has a characteristic ovoid shape which is likely obtained by a controlled interplay of peripheral and septal peptidoglycan synthesis, but how these two processes are controlled and coordinated remains unclear. In chapter 4 it is described that StkP plays an important role in coordinating cell wall synthesis during cell growth and division.

StkP is part of the family of ultraconserved Ser/Thr kinases in Gram-positive bacteria, which all consist of intracellular kinase domains and extracellular PASTA domains that are linked by a transmembrane domain. It was shown that PrkC, an eSTPK of *B. subtilis*, gets activated upon binding of free muropeptides[100], which leads to germination. Interestingly, the PASTA domain of StkP can bind PG subunits and β-lactam antibiotics. In chapter 4 we describe that StkP localizes to cell division sites and its activation is dependent on the cell cycle and substrate (unlinked PG) availability. Cells that lack *stkP* show a severe cell division defect and cells have an elongated phenotype.

Finally, we showed that *stkP* is essential for correct septum progression and closure. Therefore, we assume that StkP acts as a molecular switch between peripheral and septal PG synthesis that controls cell division (chapter 4). This can be explained as a cumulative effect of the lack of well-timed phosphorylation of cell division proteins. For the closely related oval-shaped organism *L. lactis*, it was shown that an unbalanced PG synthesis activity results in elongated, thus rod shaped cells[74]. For correct cell cycle progression, well-coordinated assembly and disassembly of the division machinery is of relevance and might be controlled by phosphorylation and dephosphorylation by StkP and its cognate phosphatase PhpP. The exact mechanism by which StkP regulates cell division and PG synthesis remains unclear. In a more recent publication the group of C. Grangeasse (Lyon) proposes a mechanism wherein StkP is directly linked with the control of septal and peripheral PG synthesis via DivIVA and the shuttling protein GpsB. Phosphorylation of DivIVA and GpsB has previously been described for other organisms, such as *C. coelicolor*, *B. subtilis* and *M. tuberculosis*[248–251]. Nevertheless, phosphorylation of GpsB by StkP has not been shown for *S. pneumoniae*. The identification of phosphorylated residues remains tricky and, moreover, phosphorylation sites are not conserved among different species. Interestingly, deletion of GpsB and DivIVA both lead to drastic morphological changes,

where GpsB was shown to be a negative regulator of DivIVA and prevents the cells from elongation. In *B. subtilis*, GpsB is, together with EzrA, involved in shuttling PBP1[252]. Furthermore, PBP2x, FtsW or PBP2b, RodA cannot be depleted in mutants of *divIVA, gpsB* or *divIVA/gpsB* which strongly suggests that the two modes of PG synthesis cannot be separated. Thus, on basis of their findings, the two-state model of PG synthesis has been challenged and a single PG machinery has been proposed and it has been suggested that GpsB/DivIVA/StkP act in a triad as a way to fine-tune septal and peripheral PG synthesis and thereby precisely control cell shape[166].

MreC and MreD are essential in encapsulated strains D39 and TIGR4, but not in unencapsulated R6. It was shown that the differences in essentiality are not dependent on the capsule but rather on the accumulation of suppressor mutations[73]. Suppressor mutations were found in the bifunctional class A PBP1a and it is assumed that its proper positioning and activity is dependent on the presence of MreC and MreD. For *B. subtilis* it was shown that PBP1 localization depends on MreB and that most likely MreC, which interacts with both MreB and PBP1, is involved[73]. It is interesting to mention that in coccoid bacteria that lack peripheral PG synthesis, such as *S. aureus*, MreC/D are present but not essential[253]. It was proposed that MreC and MreD, together with PBP1a and other proteins, are involved in peripheral PG synthesis. 2D and 3D SIM immunofluorescent microscopy, comparing PBP2x and PBP1a localization, showed that at mature septa, PBP2x and PBP1a reveal different localization patterns, which rather supports their function in two different PG machineries for PG synthesis[245].

Overexpression of StkP~P results in the opposite phenotype compared to the StkP depletion and cells appeared significantly shorter (Chapter 5) and a higher turnover of StkP has been detected, which hints to the action of a protease degrading StkP~P. Under StkP~P overexpression conditions we found that the two-component system *ciaRH* was upregulated. Interestingly, the regulatory system of CiaRH is involved in response to several stresses, including cell wall stress, and autolysis and the sensitivity to β-lactam antibiotics[120]. HtrA is part of the CiaRH regulon. HtrA is member of a family of serine proteases that play an important role for protein quality control[254]. It is therefore possible that proteolysis of StkP~P by HtrA is used to set up a finely controlled feedback system to control cell-cycle progression in *S. pneumoniae.* However, although the TCS *ciaRH* and as a results also *htrA* was upregulated, we could not confirm StkP~P as a bona fide target of HtrA. Nevertheless, this might still be true since it is possible that we could not observe any degradation of StkP~P due to some technical issues that we were not able to tackle.

Interestingly, HtrA localizes at the division sites; the sites where PG synthesis takes place[133], and colocalizes with StkP (chapter 5). Furthermore, immunofluorescence microscopy revealed that the subunits of the Sec machinery, SecA and SecY, and HtrA coincide in growing cells[133]. Thus, it has been suggested that HtrA is important for protein quality control of Sec translocated proteins[133]. Peters et al. showed that GFP-PBP2x derivatives are degraded by HtrA, but nevertheless no direct degradation of PBP2x or GFP-PBP2x has been detected[49]. The protein turnover at division sites is assumed to be high and HtrA plays an important role in regulating cell cycle indirectly by recognizing misfolded proteins and degrading them. Since competence stimulating peptide CSP is directly degraded by HtrA, another role is to control competence development[123,132]. Also in intra-species competition HtrA has its input by controlling expression of bacteriocins[121].

Positioning of Penicillin-Binding Proteins

PG synthesis is catalyzed by high molecular weight PBPs, although the specific role of most individual PBPs remains elusive. During PG synthesis, the lipid II PG precursor is incorporated into the growing PG mesh by transpeptidation and transglycosylation reactions. These reactions are promoted by PBPs. For rod-shaped organisms, such as *B. subtilis*, it was shown that different sets of PBPs are involved in PG elongation and division. For *B. subtilis*, PBP2b is involved and essential for division[213], whereas PbpH and PBP2a are required for PG synthesis during elongation[214]. In *S. pneumoniae* and other ovococci, PG synthesis occurs mainly at cell division sites and the two new hemispheres are synthezised between the two splitting old hemispheres[113,222]. However, two key questions remain open: How is their activity regulated and how are they directed to the sites of action?

Two different models have been proposed to answer these questions, wherein PBP localization is either driven by cytoskeletal structures or the substrate itself. PBPs interact with cytoskeletal structures and form dynamic structures that were assumed to drive localization of PBPs[78]. Other studies showed that for the dynamics of MreB active synthesis of PG, by the action of PbpH and PBP2a, is required[79,80,215]. The proposed model wherein PBP positioning depends on substrate availability has been proposed earlier[216]. In order to test this hypothesis (chapter 6) we made use of the fact that nisin, a post-translationally modified antimicrobial peptide, binds and delocalizes lipid II[220], the substrate of PBPs. By following localization of *B. subtilis* PBPs that are known to drive MreB dynamics we could show that placement of these PBPs rather depends on substrate than on MreB. Also several *S. pneumoniae* PBPs were delocalized by nisin (Chapter 6). Together with

previous work on *S. aureus*[38] and *S. pneumoniae*[76], the data of chapter 6 strongly suggest that lipid II is driving the localization of key PBPs. Substrate-dependent localization was also reported for StkP probably via its extracellular PASTA domains, that have first been discovered in PBP2x[43,47]. Morlot et al. showed by immunofluorescence microscopy that PBP2x delocalized upon deletion of the D,D-carboxypeptidase PBP3. It was thus proposed for PBP2x that its localization depends on recognition and binding of uncrosslinked PG by its PASTA domains (Maestro et al 2004)[101]. It still remains an open question what drives the specific localization of lipid II at the new division sites.
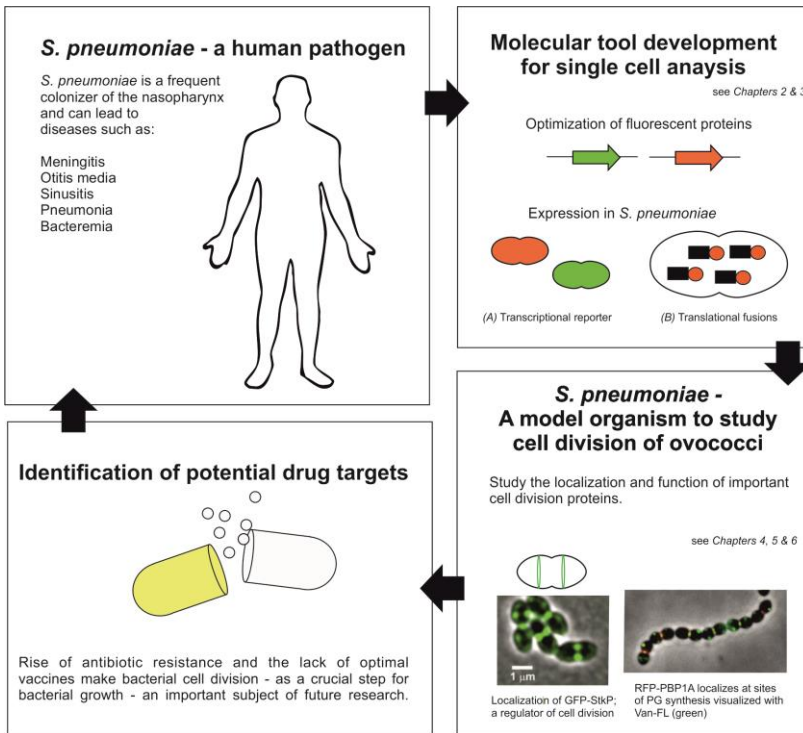

Fig.1 Thesis overview

Concluding remarks

Bacteria appear in various shapes – rod, oval and round – and each shape requires different mechanisms in order to divide. Cell division, which is intertwined with DNA replication, chromosome segregation and cell wall synthesis is crucial for cell growth. With the development of new powerful molecular tools, the human pathogen *S. pneumoniae* has been developed into an important model organism for cell biology of oval-shaped bacteria (Fig. 1).

 Work presented here in this thesis and other recent publications gave important insights into cell division of ovococci in particular as well as bacteria in general (Fig. 1). Importantly, we have addressed how peripheral and septal cell wall synthesis are coordinated. *S. pneumoniae* lacks MreB, which is a cytoskeletal protein organizing longitudinal growth in rod shaped bacteria. For ovococci, however, it is proposed that cell division proteins can be divided into groups that act in peripheral and septal synthesis, respectively. This requires a finetuned coordination, and we showed that StkP plays an important role in this coordination, most likely by phosphorylation of key proteins in cell division. Furthermore, it has been shown that, in analogy to what was already known for rod-shaped organism *B. subtilis*, PBP2x and PBP2b act in septal and peripheral synthesis, respectively. Still it remains unclear how most of the division proteins are positioned and one model suggested that cytoskeletal proteins MreB is the driver for localization of PBPs. For *B. subtilis*, however, our data suggested that the substrate (lipid II) of PBPs drives the localization, and this model is further strengthened by the fact that in *S. pneumoniae* we show that localization of StkP and PBP2x depends on availability of lipid II.

Cell division is a complex process that involves numerous players and requires a well-controlled coordination of their actions. Future work to address these questions is still needed and the use of new superresolution microscopy techniques as well as novel genetic screens can be important tools to further progress on our understanding of this process. Knowing that antibiotic resistance among pneumococci as well as other bacteria are rising, research into mechanisms controlling and driving the cell cycle is important to identify novel antimicrobial targets as well as new approaches to target these pathogens (Fig. 1).