Pneumococcal cell biology in a new light

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Chapter 1

Introduction

S. pneumoniae – history of a human pathogen

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterium that is naturally competent for DNA uptake and genetic transformation[1]. It is a colonizer of the human nasopharynx of healthy children and adults[2,3]. The nasopharynx gets densely colonized directly after birth by commensal bacteria and is the major ecological reservoir of many bacterial species[4]. The bacterial flora is in a dynamic state where bacteria are constantly acquired, eliminated and re-acquired throughout life. Although the composition differs between individuals, the general pattern of the flora remains the same[5]. Microorganisms that colonize the nasopharynx can be commensal bacteria as well as opportunistic pathogens such as *Haemophilus influenza*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Moraxella catharis*[6]. Although most carriage of the pneumococcus does not cause clinical symptoms, it can also become invasive and cause severe diseases, which lead to over one million deaths per year[7]. *S. pneumoniae* is also a leading cause of community-acquired pneumonia and other mucosal infections such as otitis media and sinusitis. In some cases, infections can become invasive and lead to sepsis and meningitis which mostly affects children of an age younger than 5 years whose immune system still has to develop, immunocompromized patients, and the elderly. A study performed in 2004 with 3200 children in the Netherlands showed that the peak incidence of pneumococcal colonization is 55% in children ≤3 years[2]. It was shown that there is a strong correlation with age and day-care attendance. During the last decades, resistance to drugs, especially antibiotics, has dramatically increased. The vaccines available today are only protective against certain sets of serotypes which led to a switch of serotypes during the past years[8]. It is important to mention that we distinguish 90 different serotypes classified on the basis of the composition of their capsule, which is an extracellular polysaccharide layer that provides protection against the immune system[9–11]. This makes it important to gain a deeper understanding of this pathogen in order to discover new drug targets.

Pneumococcal strains that do not contain a capsule are not virulent and appear transparent, whereas virulent capsular strains are opaque. Griffith[12] was the first to show opaque (smooth) to transparent (rough) phenotypic transformation of *S. pneumoniae*, although the transforming agent remained unknown at that point. Later, natural genetic transformation using exogenous DNA was described for *S. pneumoniae* by Avery et al.[1], which means that the pneumococcus can take up DNA from its environment and integrate it into the chromosome by homologous recombination. This provides a powerful strategy to

cope with selective pressures such as antibiotics and vaccines. Genetic exchange of DNA most likely takes place in the nasopharynx, the ecological reservoir of the pneumococcus[13]. These niches are inhabited by multiple clonal serotypes of *S. pneumoniae* as well as other organisms. Largely because of this genetic plasticity, multidrug resistant pneumococci are on the rise as well as non-vaccine serotypes. Thus, novel approaches to tackle this pathogen are required.

Tools to study pneumococcal cell biology

The green fluorescent protein (GFP), from jellyfish *Aequorea victoria*, was discovered in 1962 by Shimomura[14]. In 1994[15] GFP was first expressed in *Escherichia coli* and since then has become an important tool for molecular biologists. Most studies on bacterial cell biology to understand the molecular mechanisms that underlie cell division and cell growth were performed on model organisms such as *Bacillus subtilis* and *Escherichia coli*. Fluorescent proteins require post-translational oxidation for proper folding and maturation and microaerophilic conditions can have a negative effect on maturation[16]. This fact might have hindered introduction of FPs for the microaerophilic pneumococcus[17]. Therefore, most studies to visualize protein localization in *S. pneumoniae* have been performed using techniques such as immunofluorescence microscopy (IFM)[18,19]. The disadvantage of IFM is that experiments are not performed on living cells, but on fixated cells, and that it is prone to artifacts[20].

In particular, the use of fluorescent proteins, such as green fluorescent protein (GFP) and red fluorescent protein (RFP) (chapters 2 and 3), fused to a protein of investigation makes it possible to track subcellular protein localization and dynamics with fluorescence microscopy in living cells. With recent development of new molecular tools for single-cell studies, *S. pneumoniae* became more and more a model organism to study fundamental processes such as pathogenesis, DNA replication and cell division[21–23]. Time-lapse microscopy, which constitutes an important technique that allows temporal and spatial investigation of protein dynamics (Chapter 2), was also recently established for *S. pneumoniae*. These new approaches have proven to give valuable insights into the life style and cell biology of the pneumococcus[23].

Cell division and peptidoglycan synthesis

Bacteria appear in different shapes such as spherical, rod-shaped and spiral-shaped. In the past, a cell's shape was used to map its place in the phylogenetic tree,

whereas comparative analysis of ribosomal RNAs showed that similar morphologies evolved independently in different branches. Spherical bacteria, also cocci, are found in clusters of mainly rods, indicating that the ability to elongate got lost within the group of cocci[24]. Cell shape is determined by biochemical and biophysical properties of the major cell wall macromolecule, the peptidoglycan (PG). Bacterial cell wall can be compared to an exoskeleton that surrounds the cell and protects it against osmotic pressure and mechanical damage. Traditionally, bacteria were distinguished in two subdivisions, Gram-positive and Gram-negative, depending on the presence of an outer membrane[25]. The Gram-negative cell wall has an outer membrane which contains lipopolysaccharides and lipoproteins and is linked to a thin PG

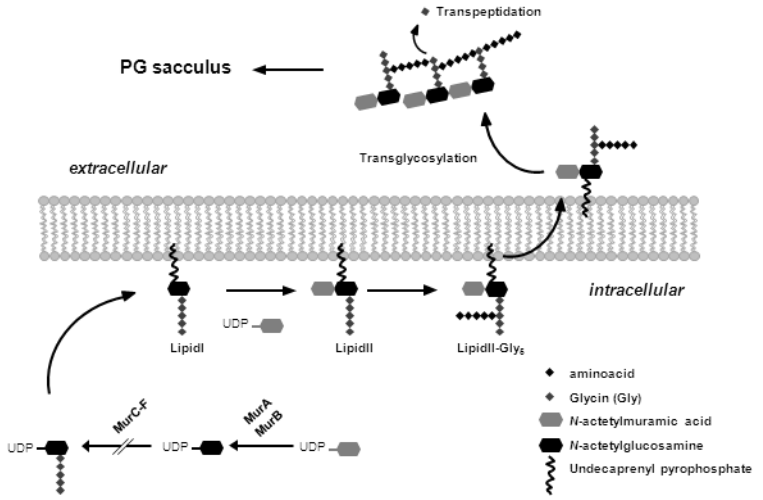

Fig. 1 PG biosynthesis. The subunits of peptidoglycan are synthesized in the cytoplasm as UDP-activated precursors. UDP-MurNAc-pentapeptide is transferred to the lipid II carrier undecaprenyl phosphate to form lipid I, which is further transglycosylated with UDP-GlcNAc to form lipid II. Lipid II is translocated across the cytoplasmic membrane by a yet unknown mechanism. The role of FtsW as flippase in this context has been discussed[26]. Two essential reactions, transglycosylation and transpeptidation, are catalyzed by the penicillin-binding proteins (PBPs) responsible for the synthesis of peptidoglycan, allowing growth of the sacculus.

layer, whereas the Gram-positive cell wall consists of a thick PG layer which makes up 30-70% of the total cell wall. Note that the *S. pneumoniae* cell wall also contains high amounts of lipoteichoic and wall teichoic acids, which might also contribute to cell shape[27].

Bacterial growth and division requires elongation of lateral cell wall and formation of a new septum, which divides an existing ancestor cell into two cells of identical size. These two processes require synthesis of new cell wall material, the so-called PG synthesis. The cell wall of Gram-positive bacteria consists of up to 30 layers of PG that form a net-like sacculus around the cell. PG is built up of glycan strands that are interlinked by short

peptide bridges[28]. Composition of PG differs per species. For *S. pneumoniae*, the sequence of the linear pentapeptide is (L-Ala)—(γ-D-Glu)—(L-Lys)—(D-Ala)—(D-Ala)[29]. The stem pentapeptide is attached to N-acetylmuramic acid and two strands are crosslinked by formation of a peptide bond between L-Lys of the donor and D-Ala of the acceptor strand (Fig. 1). Furthermore, pneumococcal PG contains peptide bridges made of branched amino acids[30]. Branched amino acids are synthesized by MurMN, by sequentially attaching L-Ala/L-Ser and L-Ala to the ε-amino group of L-Lys[31]. This provides a dynamic structure that requires constant *de novo* synthesis and remodeling by modification and hydrolysis, which allows for cell growth and division[32]. For a more detailed review see Vollmer et al[33].

PG synthesis is a complex process that takes place in three compartments of the cell. As a first step, nucleotide precursors UDP-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) are synthesized in the cytoplasm. Secondly, at the inner membrane these molecules are processed to lipid II, a disaccharide unit of N-Ac-Glc and N-Ac-MurAc with a pentapeptide chain attached and a lipid tail, catalyzed by enzymes from the *mur* family[34]. For *E. coli* it was shown that a flippase, FtsW, moves lipid II to the extracellular part of the membrane[26]. At the third stage of PG synthesis, lipid II is incorporated into a nascent PG sacculus via transglycosylation and transpeptidation reactions consecutively, which results in glycan strands that are connected via peptide bridges. These reactions are catalyzed by so-called penicillin-binding proteins (PBPs). It was reported that there is a clear correlation between cell shape and the set of PBPs[19].

So far, most research to gain a deeper understanding of cell division and morphogenesis has been carried out on rod-shaped model organisms such as *E. coli* and *B. subtilis*. The fact that a wide array of molecular and genetic tools is available for these organisms made it convenient to study their cell biology.

In rod-shaped bacteria two peptidoglycan synthesis machineries have been suggested, namely the elongasome and the divisome. Cell division, regardless of cell shape, is initiated with the assembly of the tubulin-like protein FtsZ at the new sites of cell division. FtsZ is highly conserved throughout eubacteria and archaea and can be seen as a prime organizer of cytokinesis. Polymerization of FtsZ, depending on its GTPase activity, leads to a scaffold formation, called Z-ring, at the midcell, which is anchored to the membrane via FtsA. On this structure the whole divisome assembles. The divisome is a multi-enzyme complex (MEC) that consists of conserved division proteins among which are PBPs. In rods, the MEC of the elongasome is anchored to the scaffolding protein MreB[35,36], an actin-like

protein that is assembled in patches along the peripheral cell wall[37]. Unlike rods, most coccoids lack MreB and MreB-like proteins, and staphylococci seem to have only one PG synthesis machinery located at the division sites. Nevertheless, not much is known about the morphogenesis of cocci, among which we find many pathogens.

Due to the increase in antibiotic resistances and in order to develop new potential therapeutics, the study of cell division of cocci became an important subject of interest. We distinguish cocci in truly spherical and elongated spherical bacteria. To the first we count for instance staphylococci, *Neisseria* and deinococci. Division in staphylococci occurs in 3 planes and therefore they often appear in a grape-like microcolony. Complete cell wall synthesis takes place at the division planes, which is promoted by the machinery associated with the Z-ring. Depletion of FtsZ in *S. aureus* leads to an increase of volume of the individual cells[38]. For the second group of round-shaped bacteria the term of *ovococci*, including streptococci, lactococci and enterococci as representatives, was introduced[19]. Ovococci, unlike real spherical cocci, can be distinguished in their mode of cell wall synthesis by the occurrence of peripheral growth[39]. Vancomycin is an antibiotic that specifically binds to the D-Ala-D-Ala residues of lipid II. Importantly, fluorescently labeled vancomycin (VanFL) can be used to visualize nascent PG in living cells. Staining of *S. pneumoniae* with VanFL to visualize nascent PG showed activity mostly at midcell[40,41]. Early works on ovococcal cell division were performed on *Enterococcus hirae* and *S. pneumoniae*, using mainly electron microscopy[39,42]. It was observed that first an equatorial ring is formed with little ingrowth of PG. The equatorial rings are then split in two and between them peripheral synthesis occurs. Finally, by centripetal growth a septal disc is formed and the ancestor cell eventually splits into two identical processor cells. Despite the lack of biochemical evidence, a model containing two independently acting machineries for cell wall synthesis, peripheral and septal PG synthesis, was proposed on the basis of localization studies of PBPs and other late cell division proteins[19,43–49] (Fig. 2). The division machinery guides cell pole formation by invagination and constriction of the septum, whereas the peripheral PG synthesis machinery is located more towards the edges in order to produce new hemispheres, which leads to an elongation of the cell. This two-step model of cell wall synthesis was recently confirmed by superresolution microscopy[50]. However, it remains unclear if the proteins of the peripheral growth and septal PG synthesis are organized in two distinct complexes or rather in one[51].

Unlike real cocci, the division of ovococci proceeds in one plane, perpendicular to their axis. Cells can be observed in chains or diplococci[52,53]. In order for the cells to separate, the action of murein hydrolases is required. In *S. pneumoniae* D39, 13 genes

encoding (putative) PG hydrolases have been identified[54,55]. One of those is LytB, a putative endo-beta-N-acetylglucosaminidase, that is active at the end of the cell division process[56,57]. The putative hydrolase PcsB interacts with FtsE and FtsX, both part of an ABC transporter[58]. Recently, it was proposed that the FtsEX-PcsB complex activates PG hydrolysis, which indicates a coupling of PG remodeling with PG synthesis[59]. The gene *pcsB* is part of the regulon of the two-component system WalRK (VicRK), which also controls other hydrolases[60].

Furthermore, WalRK homologues from other low GC-content Gram-positive bacteria have been shown to regulate several PG hydrolases[61,62]. Deletion of *pcsB* severely impairs growth, whereas the other hydrolases are not essential in *S. pneumoniae*[41,63].

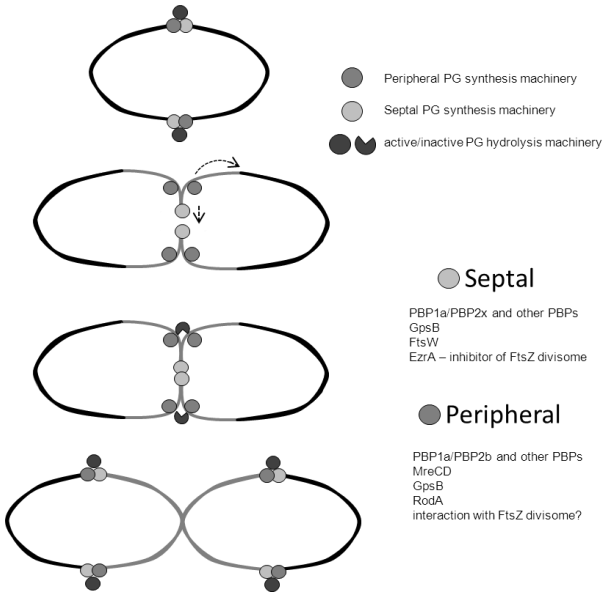

Fig. 2 Two-state model of PG synthesis of ovococci. Two machineries have been proposed to catalyze peripheral and septal PG synthesis. Initially, both machineries localize at the new sites of division. Lateral cell wall is synthesized by one machinery (*middle grey*) and causes cell elongation. Septal cell wall synthesis (*light grey*) follows later and causes cell separation, where also PG remodeling is required (*dark grey*). A spatial separation is used to illustrate the two processes although colocalization of these two complexes has been shown. The proposed protein compositions for the two machineries are mentioned based on composition in rod-shaped bacteria. Adapted from Sham et al.

Penicillin-binding proteins (PBPs)

In order for a cell to grow and divide, PG synthesis and breaking of covalent bonds in the existing PG molecule are required. Cell wall PG synthesis is highly coordinated and requires enzymatic activity of PBPs and murein hydrolases that is important for the remodeling of PG. The possibility to use fluorescent proteins to study localization of proteins that are involved in PG synthesis in growing cells brought new insights into the mechanism of cell division[64]. Also the use of fluorescently labeled vancomycin to look at the localization of newly incorporated PG in live cells helped to gain knowledge in this field.

PBPs catalyze polymerization of glycan strands (transglycosylation) and crosslinking of strands via peptide bridges (transpeptidation). Some PBPs can act as endopeptidases, on connecting stem peptides, or as D,D-carboxypeptidases, that hydrolyze the last D-Ala to regulated crosslinking. The name 'penicillin-binding proteins' derives from their ability to bind penicillin, a β-lactam antibiotic. β-Lactam antibiotics resemble the terminal D-Ala-D-Ala of the stem pentapeptide and bind to the transpeptidase domain of PBPs[65]. PBPs are classified into high molecular weight (HMW) and low molecular weight (LMW). HMW PBPs catalyze incorporation of lipid-linked PG precursors, lipid II, into nascent PG. This group of PBPs can be further distinguished into class A and class B PBPs, depending on their structure and the catalytic activity of the N-terminal domain. Class A PBPs are bifunctional transglycosylases/transpeptidases (TG/TP) and class B PBPs are monofunctional TPs. PBPs have a cytoplasmic tail, a transmembrane domain and a surface-exposed domain for PG synthesis[66–68]. The penicillin-binding domain of both classes shows TP activity. Class A HMW PBPs have an additional TG activity, while the one of class B HMW PBPs plays a role in cell morphogenesis, possibly by the interaction with other proteins[32,67,69]. LMW PBPs are monofunctional D,D-carbopeptidases.

The genome of *S. pneumoniae* encodes six different PBPs[70]: three non-essential class A PBPs, PBP1a, PBP1b and PBP2a, two class B PBPs, PBP2x and PBP2b, and one D,D-carboxypeptidase, PBP3 (*dacA*)[71] (Table 1). The function of each PBP remains, to a large extent, unknown. A global gene interaction study showed that PBP2x is an essential PBP[72]. Depletion of *pbp2b* results in small cells, indicating that PBP2b is thus essential for peripheral PG synthesis[44].

PBPs are thought to be part of MECs. The divisome, the septal PG synthesis machinery, was proposed to consist of FtsZ, EzrA, GpsB, FtsW, the heterotrimer DivIB/FtsL/DivIC and the monofunctional TP PBP2x[18,26,45,48]. FtsW is a putative flippase that transports lipid II through the membrane. EzrA is essential in *S. pneumoniae* and shown to be a negative regulator of the Z-ring. The peripheral machinery, instead, was suggested to consist of MreC, MreD, RodA, GpsB and the monofunctional TP PBP2b[19,73].

A strong role of PBP2x in cell division has been suggested earlier. Inactivation of PBP2x by methicillin, a β-lactamase, in the closely related organism *Lactococcus lactis* led to filamentation[74]. Recently, the role of pneumococcal PBP2x in cell division was confirmed[44,47,49]. Deletion of PBP2b in *L. lactis* results in spherically shaped cells, which suggests a role in peripheral PG synthesis. A similar role for pneumococcal PBP2b in cell elongation has recently been confirmed[44] and *pbp2b* mutants show lentil-like appearance

and form long chains. PBP2b-depleted cells become strongly dependent on branched muropeptides. MreCD plays a crucial role in peripheral PG synthesis. Cells depleted for *mreCD* also form spherical cells and accumulate suppressor mutations. Suppressor mutations were found in *pbp1a*, which is involved in the development of β-lactam resistance[73].

S. pneumoniae has one LMW PBP3, encoded by *dacA*[71], that does not localize at the division sites with HMW PBPs, but on the periphery. Localization of PBP3 was speculated to depend on local cardiolipin localization[75]. PBP3 cleaves off the terminal D-Ala residue of the stem peptide and in this manner regulates the amount of donor stem peptides, which are substrate for the transpeptidation reaction. PBP3 is therefore seen as a regulator for PG crosslinking. The absence of PBP3 at the cell division sites ensures availability of stem-peptides[76]. These findings of a septum-excluded localization of PBP3 were, however, not confirmed by Barendt et al[77].

In order to maintain shape, PBPs have to correctly localize to their sites of action. How PBPs localize to the sites of action is explained by two models which claim a) interaction with cytoskeleton structures as the driving force or b) substrate availability. PBPs interact with several proteins and for rod-shaped bacteria it was proposed that localization is driven through the interaction with cytoskeletal proteins MreB or FtsZ[78]. This model became less likely due to recent discoveries, where it was for instance shown that PBP2a and PbpH drive MreB dynamics in *B. subtilis*[79,80]. A second model speculates that substrate availability is the driving force for PBP localization. It has been shown for *S. aureus* that HMW PBP2 delocalizes when its binding site is blocked with oxacillin or when substrate accessibility is impaired by addition of vancomycin[38]. First, a model wherein Z-ring constriction is uncoupled from septal PG synthesis was suggested. PBP2x would herein be recruited independently of FtsZ. It was later shown that the recruitment to midcell of PBP2x depends on its extracellular PASTA domains and the interaction with StkP[49,81]. Later, PBP recruitment was suggested to be dependent on substrate availability due to action of previously recruited PBP, rather than on protein-protein interaction[76]. Later it was shown that delocalization of lipid II altered localization of PBPs in *B. subtilis* and *S. pneumoniae* (chapter 6; [82]).

Table 1 List of PBPs of *S. pneumoniae*

Protein	Locus tag	Localization	Function	Reference
HMW				
class A				
PBP2a	SPR_1823	Equatorial	Unknown, bifunctional TP*/TG**	[18,83]
PBP1a	SPR_0329	Equatorial	Unknown, bifunctional TP*/TG**	[18]
PBP1b	SPR_1909	Septal/equatorial	Unknown, bifunctional TP*/TG**; putative player in either septal or peripheral PG synthesis	[18,84]
class B				
PBP2x	SPR_0304	Septal	Essential for division, PG TP*, septal PG synthesis, part of the "divisome"	[18,19,85]
PBP2b	SPR_1517	Septal	Essential for elongation, PG TP**, peripheral PG synthesis	[18,44]
LMW				
PBP3 (*dacA*)	SPR_0776	Septal/Cell surface	D,D-carboxypeptidase; controls peptapeptide availability	[76,86]

Control and regulation of cell division and cell morphogenesis

Bacteria live in dynamic environments to which they have to respond and adapt quickly in order to survive. Adaptive potential is based on sensing and transducing external and internal signals. Signaling pathways, which are mediated by protein phosphorylation and dephosphorylation, regulate protein activity both directly and indirectly to control cellular functions. These signaling systems are most of the time built up by a sensor protein kinase and a cognate response regulator and phosphatase proteins. The best described and at the same time most abundant signaling systems in bacteria are two-component systems (TCS), which consist of a histidine kinase and a response regulator[87]. *S. pneumoniae* has 13 TCS and most of these were reported to be related to virulence. For a long time, it was assumed that this is the only signal transduction system found in bacteria.

While phosphorylation of Ser, Thr and Tyr residues in eukaryotes is widely spread, it was assumed that phosphorylation of these residues exclusively occurred in eukaryotes[88]. In 1991 *pkn1* of *Myxococcus xanthus* was the first eukaryotic-type serine/threonine protein kinase (eSTPK) to be characterized for prokaryotes[89]. The availability of prokaryotic genome sequencing led to the identification of numerous eukaryotic-type serine-/threonine (Hanks-type) kinases in many sequenced microbial genomes[90]. eSTPKs build up complex signaling networks and influence various different biological functions, such as developmental processes, secondary metabolism, cell division and cell wall synthesis.

Most importantly, eSTPKs are especially prevalent in pathogens[91]. The *Mycobacterium tuberculosis* genome includes 11 genes coding for eSTPKs. It was reported earlier for *M. tuberculosis* that PknB is essential for growth and phosphorylates proteins

that are involved in PG synthesis, cell division and stress response[92]. Overexpression of the *M. tuberculosis* PknB led to altered cell division and cell wall synthesis, which resulted in swelling of cells and incomplete formation of division septa[92]. For other bacteria, such as *Corynebacterium* and *Streptomyces,* it was reported as well that some of their eSTPKs are involved in cell wall synthesis and cell morphogenesis [review[93]]. Most substrates of eSTPKs were so far identified by phosphoproteomic approaches and *in vitro* kinase assays, whereas *in vivo* confirmation is most often lacking.

S. pneumoniae encodes a single eSTPK, called StkP, which is found in an operon with its cognate phosphatase PhpP[94,95]. StkP belongs to a subfamily of ultraconserved eSTPKs, unique to a group of firmicutes and actinobacteria that were reported to be involved in regulation of growth and cell division[96]. The eSTPKs of all Gram-positive bacteria have a common structure. They consist of a cytoplasmic catalytic kinase domain that is linked by a transmembrane segment to an extracellular domain that consists of a variable number of PASTA (penicillin-binding and serine-/threonine associated) domains (Fig. 3)[96]. PASTA domains were first found in pneumococcal PBP2x, which contains two C-terminal PASTA repeats[97]. The crystal structure of PBP2x[98] was determined with β-lactam cefuroxime bound to the first PASTA repeat, thereby forming a structural analog of unlinked peptidoglycan[99]. It was shown that PASTA domains of PknC of *B. subtilis* could bind free muropeptides and binding induced germination of spores[100]. Based on these results, proteins containing PASTA domains are thought to bind PG fragments, which act as signaling molecules. In line with these findings, it was later shown for *S. pneumoniae* that PASTA domains of StkP can bind specifically to crude pneumococcal PG fragments and β-lactam antibiotics that mimic the structure of lipid II[101]. So far, substrates of StkP have been identified by phosphoproteomic approaches and *in vivo* kinase assays[102,103]. The identification of substrates still bears some difficulties, for instance when substrate could be only detected in either the *in vitro* or the *in vivo* assay. Furthermore, it is poorly understood how phosphorylation affects the activity of the substrate proteins. Nevertheless, several potential substrates were identified that play a role in cell wall metabolism and cell division. Cell division protein FtsZ has been identified in an *in vitro* kinase assay[102]. FtsA was also identified as a potential substrate in an *in vitro* assay[43]. GlmM, which catalyzes the conversion of N-acetyl-glucosamine-6-P into glucose-N-1-P, a precursor for cell wall synthesis, was detected by 2D electrophoresis and in an *in vitro* kinase assay[95]. Another important player involved in cell division, DivIVA, was found to be a substrate of StkP by mass spectrometry, *in vivo* 2D electrophoresis and an *in vitro* kinase assay[104]. Furthermore,

the orphan response regulator RitR[105], involved in iron transport, and MurC, involved in synthesis of cell wall precursors, are both phosphorylated by StkP *in vitro*[106]. In a global assay of the pneumococcal phosphoproteome, the murein hydrolases LytA and LytB were identified[103]. Interestingly, transcriptome analysis also showed an upregulation of *lytB* and the hydrolase *pcsB* in an *stkP* null mutant[107].

Activation mechanisms of Ser/Thr protein kinases still remain unclear. Ligand-promoted dimerization was for example shown for the human TGF-β and dimerization is important for its activity (Fig. 3)[108]. But it was reported that eSTPKs that lack a ligand-binding domain are still activated as shown for PknB from *M. tuberculosis*[109].

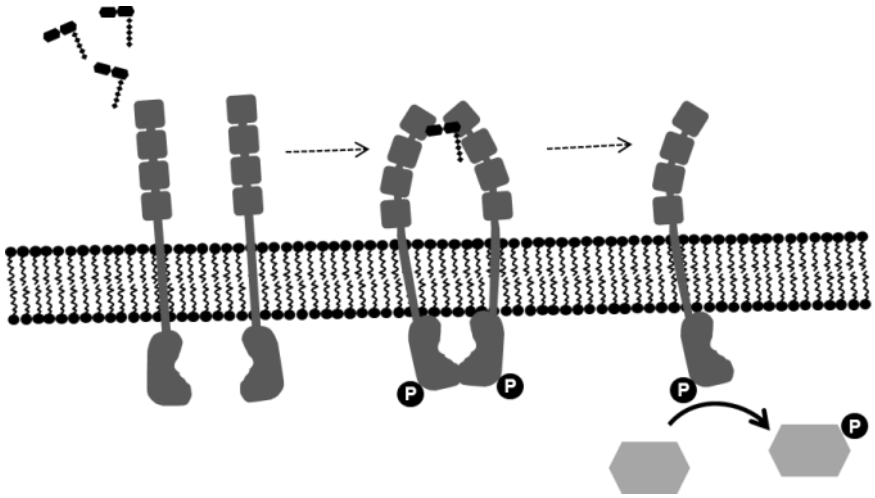

Fig. 3 Model of eSTPK activation. In the presence of a ligand, here a PG fragment (black), two eSTPK monomers (dark grey) dimerize upon ligand binding to their extracellular PASTA domains. The intracellular catalytic domains subsequently get in closer contact and the kinases are activated by autophosphorylation and substrates are subsequently phosphorylated (light grey). The figure was adapted from Pereira et al.[110]

StkP forms dimers *in vivo*, which was suggested to promote autophosphorylation activity and substrate targeting[111]. The cognate mammalian-type PP2C-type phosphatase PhpP is dephosphorylating StkP[95], and PhpP and StkP were shown to form a functional signaling couple *in vivo*[112]. Although *stkP* is not essential for *S. pneumoniae*, mutants are more susceptible to stress, such as acid, osmotic, heat and oxidative stress[107]. In absence of *stkP*, the phenotype shows elongation of cells, which indicates a defect in cell division[111]. Interestingly, colocalization of FtsZ and StkP to new division sites was shown by co-immunofluorescence microscopy[102].

At an early stage of cell division StkP gets recruited to new division sites via recognition of PG precursors by its PASTA domains[43]. Ligand binding induces dimerization of

StkP, which triggers autophosphorylation. For the model bacteria *B. subtilis* and *E. coli* it was reported that division proteins assemble in discrete steps[113,114]. For *S. pneumoniae* we were able to show a three-step assembly wherein StkP localizes to the midcell after the early cell division protein FtsA, but before DivIVA[43]. Cells depleted of *stkP* are still able to divide, but nevertheless their size is heterogeneous and cells often appear elongated. Elongated cells show multiple unconstricted septa and septal closure can only be completed after peripheral PG synthesis[43]. Together with another study on StkP[115], it was concluded that StkP plays a crucial role in cell division by controlling septum closure and progression (Chapter 4)[43].

Cell wall stress response - CiaRH

Two-component systems are found in archaea, plants and lower eukaryotes. In bacteria, TCSs are often involved in regulation of virulence factors[116]. CiaRH (*c*ompetence *i*nduction and *a*ltered cefotaxime susceptibility) was the first out of 13 TCSs that was described for *S. pneumoniae*. It was identified in a screen for mutants that developed a spontaneous resistance to the β-lactam antibiotic cefotaxime[117]. The *cia* operon consists of *ciaH*, encoding the histidine kinase, and *ciaR*, encoding the cognate response regulator. Mutations in *ciaH,* like in *pbp2x,* are causing resistance to cefotaxime. If *ciaR* is disrupted, no expression of *ciaH* is detected and *ciaH*-dependent resistance to β-lactams is lost[118]. Furthermore, a loss of spontaneous competence development was observed for the *ciaH* mutants, which indicated a negative regulation of competence by CiaRH[117]. Under lysis-inducing conditions, *ciaRH* is crucial for maintenance of cell integrity[119,120]. Furthermore, *ciaRH* is involved in bacteriocin production, virulence, and host colonization[121–123]. The core promoter sequence that is regulated by CiaR has a characteristic direct hexamer repeat (TTTAAG-N5-TTTAAG) 10 bps upstream of the -10-sequence[124]. 15 promoters that are controlled by CiaR were identified with one of those being negatively regulated[124]. A core regulon, including the gene *spr0931*, the *htrA-parB* operon, and small RNAs *ccnA-E*, is highly dependent on CiaR, showing up to 40-fold upregulation.

The specific trigger for CiaRH induction is unknown[118]. It has been shown that serine protease HtrA, which is part of the CiaRH regulon, is upregulated upon vancomycin-induced cell wall stress in *S. pneumoniae*, *S. aureus*, and *B. subtilis*[125–127]. Another antibiotic that has an effect on PG synthesis, penicillin, was shown to induce CiaRH[128]. Expression of *ciaRH* and thus *htrA* is induced during competence and their role in repression of the

competence state has been discussed[129,130]. The repression of the competent state can be explained by the degradation of the competence stimulating peptide (CSP)[131] by HtrA[129,132].

HtrA is an outer surface located serine protease of the DegP family of proteases and highly conserved in *S. pneumoniae*. Although *htrA* is not essential in lab conditions for *S. pneumoniae*, it is important for nasopharynx colonization[123]. HtrA also plays a role in the repression of pneumocin production[121], probably by the same mechanism as was described for competence suppression.

Interestingly, we found an upregulation of CiaRH under StkP hyperphosphorylation conditions (Chapter 5). At the same time StkP turnover was increased which led us to hypothesize that a protease actively degrades StkP~P. Furthermore, HtrA was found to localize at midcell[133], comparable to StkP (Chapter 4). Whether StkP is a *bona fide* substrate of HtrA needs to be verified, but it is tempting to speculate that the CiaRH system, via HtrA, controls cell division under cell wall stress, regulating the levels of StkP~P (Chapter 5).

Thesis outline

Single-cell methologies to study for instance subcellular protein localization in bacteria brought many insights on how bacterial cells are organized. Nevertheless, most molecular tools to study bacterial cell biology were mainly developed for model organisms such as *Bacillus subtilis, Caulobacter crescentus* and *Escherischia coli*. For a long time the lack of molecular tools for *S. pneumoniae* limited the possibilities to study its cell biology and most single-cell techniques to study protein localization required fixation of cells. Maturation of fluorescent proteins (FPs) requires post-translational oxidation. This fact might have hindered the employment of FPs for this microaerophilic organism[17]. To study protein localization on living cells, fluorescent proteins and time-lapse microscopy are useful and essential tools. In **chapters 2** and **3** we describe new tools and techniques for single-cell studies on living cells for *S. pneumoniae*.

The first study on protein localization in live pneumococcal cells was only published in 2009[21]. However, the tools that are available for single-cell studies in this organism remained limited. Different GFP and RFP variants were benchmarked and signal to noise ratios were determined to know which of the available variants are the most suitable for the pneumococcus (**chapters 2 and 3**). (Fluorescence) time-lapse microscopy gives the possibility to study protein localization in a temporal context as well as gene expression. We

were able to set up a protocol to perform time-lapse microscopy on *S. pneumoniae* (**chapter 2**).

Bacterial cell division is a highly regulated process that is governed by multienzyme complexes in a hierarchical manner. This complex interactive network depends on temporal as well as on spatial regulation. **Chapters 4** and **6** address key questions on the regulation of cell division in *Streptococcus pneumoniae* and *B. subtilis* using single-cell techniques.

The regulation of cell growth and cell division in bacteria is often controlled by reversible protein phosphorylation that is catalyzed by protein kinases and protein phosphatases. In many Gram-positive bacteria eukaryotic-type serine/threonine protein kinases were shown to play an important role in cell division and cell cycle control. Pneumococcal StkP and its cognate phosphatase PhpP target important cell division proteins such as GlmM, DivIVA, FtsA and FtsZ. Phenotypic studies of *stkP* null mutants revealed its role in cell division[107]. The extracellular PASTA domains are essential for targeting StkP to the midcell (**chapter 4**). It had been hypothesized and shown that PASTA domains of *B. subtilis* PrkC can bind to PG stem peptides[96,100,134]. For *S. pneumoniae*, it was reported that PASTA domains can bind PG precursors, which are most likely the substrate of StkP[101]. By using single-cell methodologies for *S. pneumoniae* we were able to show that StkP localizes to new cell division sites depending on substrate availability (**chapter 4**). Using β-lactam antibiotics to block substrate binding sites showed that binding of unlinked PG precursors is important for septal localization. By overexpressing StkP or PhpP, cell shape was changed, which demonstrated a direct link of this signaling couple in controlling cell shape. Furthermore, we identified a hierarchical mode of divisome assembly.

The bacterial cell wall surrounds the cell and maintains its shape. Peptidoglycan (PG) is a macromolecule and main component of the cell wall that forms a net-like sacculus. The synthesis of PG requires Penicillin-binding proteins (PBPs) that incorporate PG precursor lipid II. PG synthesis in rod-shaped and round (or oval) bacteria takes place at distinctive locations. For *B. subtilis*, a model organism for rod-shaped bacteria, PG synthesis can be divided into two parts: elongation and division. It was shown that cell elongation takes place at lateral cell wall attached to MreB, whereas the division machinery is located at midcell, along the Z-ring. In ovoid bacteria, on the other hand, septal and peripheral PG synthesis machineries are located at midcell, recruited to the Z-ring.

In **chapter 6** we addressed the question of how PBP localization is guided to the sites of PG synthesis. Two models for PBP localization have been proposed wherein the

localization depends on a) cytoskeleton proteins MreB and FtsZ or b) substrate availability. We showed that by delocalizing the substrate, lipid II, using nisin, the localization of PBPs in both the rod-shaped *B. subtilis* and the ovoid pneumococcus is altered. These data support the hypothesis wherein the localization of PBPs is substrate-driven.

Finally, we observed that in cells where StkP~P is highly active, which we call a hyperphosphorylated state, the turnover of StkP~P is increased. This hyperphosphorylated state is obtained upon induction of StkP and in absence of the phosphatase *phpP*. Cells appear significantly shorter, which indicates induced cell division by StkP-dependent phosphorylation. DNA-microarray experiments revealed that the *ciaRH*, encoding the TCS CiaRH, is upregulated upon accumulation of StkP~P. The operon encoding the serine protease HtrA and the partitioning protein ParB is part of the *cia* regulon. We hypothezised that the activity of StkP in dividing cells is controlled by the protease HtrA. To confirm this hypothesis we performed several experiments (chapter 5). By co-immunofluorescence microscopy we could show that both StkP and HtrA localize to cell septa. Nevertheless, our data do not support our hypothesis wherein HtrA degrades StkP. Therefore, it might be that another yet unknown protease controls cell-cycle progression in the pneumococcus or perhaps we have not found the right conditions for HtrA-dependent degradation of StkP~P yet.