Cell division in *Escherichia coli*

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Cover: Homemade bread. Dividing.
Baked by the author.

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I Estimating Z-ring radius and contraction in dividing *Escherichia coli*. Strömqvist J, Skoog K, Daley DO, Widengren J, von Heijne G. *Mol Microbiol*. 2010 76(1):151-8

II Sequential closure of the cytoplasm then periplasm during cell division in *Escherichia coli*.
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III Penicillin-binding protein 5 can form a homo-oligomeric complex in the inner membrane of *Escherichia coli*.
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IV The *Escherichia coli* cell division protein ZipA forms homo-dimers prior to association with FtsZ.
Skoog K, Daley DO. 2011. (*submitted to Biochemistry*)

* These authors contributed equally to the work

Abstract

The Gram-negative bacterium *Escherichia coli* is a model system to describe the biochemistry and cell biology of cell division in bacteria. This process can be divided into three major steps. The first step involves the replication of the DNA, followed by an elongation step in which the cells become twice as long. In the last step the elongated cell constricts in the middle and the two daughter cells are separated. The cell division process in *E. coli* has been extensively studied for at least 50 years and a lot is known, however many details are still vague. New proteins involved in the process continue to be identified and the number of these proteins as well as the interactions among them are not yet fully known. It is therefore not completely understood how the contraction proceeds to form two daughter cells. In this thesis, I have carried out experiments that contribute to our understanding of cell division in *E. coli*. Using fluorescence microscopy I show that the contraction of the inner membrane in dividing *E. coli* proceeds in a linear fashion and that the periplasm closes after the cytoplasm. I have also analyzed the oligomeric state of two proteins involved in the cell division and I show that the early cell division protein ZipA can dimerize. This could explain how this protein can bundle FtsZ protofilaments, as it could bridge two protofilaments. Penicillin-binding protein 5 (PBP5) has been found to localize to the septum and it has been suggested to be connected to cell division. I have found that PBP5 forms a homo-oligomeric complex, most likely a dimer. The dimer can be modeled in a back-to-back conformation with the catalytic domains being flexible. This allows PBP5 to reach for pentapeptides of the peptidoglycan at different distances from the membrane. An understanding of the mechanisms used by the cell division proteins and their protein: protein interactions can be a first step towards determining new antibiotic targets.

Contents

Abbreviations

Å	Ångström
BN	blue native
CL	cardiolipin
DOPC	dioleoylphosphatidylcholine
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
FZB	FtsZ binding domain
GFP	green fluorescent protein
Glc*N*Ac	*N*-acetylglucosamine
LPS	lipopolysaccharides
m-A_2pm	meso-diaminopimelic acid
Mur*N*Ac	*N*-acetylmuramic acid
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin binding protein
PE	phosphatidyletanolamine
POTRA	polypeptide transport-associated
SDS	sodium dodecyl sulphate
SEDS	shape, elongation, division and sporulation
WACA	Walker A cytoskeletal ATPase

Escherichia coli

Escherichia coli is a bacterium that normally grows in the lower intestine of warm-blooded organisms and it is named after its finder, the German scientist, Theodor Escherich. *E. coli* is essential for producing vitamin K in its hosts, as infection of germ-free rats deficient in vitamin K with *E. coli* could restore the vitamin K-levels [2]. It has been suggested that *E. coli* compete with more harmful bacteria in the gut and it has been shown that the presence of *E. coli* in the intestine can increase the survival rate during *Salmonella* infections in mice [3].

E. coli is one of the best-studied organisms. Due to its robustness, fast growth and relatively simple genetics it has been used as one of the key model systems to study and describe life. As a result several important discoveries made in *E. coli* have been awarded the Nobel Prize. Furthermore the discovery of cloning in *E. coli* was one of the foundations in establishing biotechnology, which has grown to an important industry field during the last 35 years [4].

The *E. coli* chromosome (the nucleoid) is a single circular DNA molecule that is located in the cytoplasm. The genome was one of the first to be completely sequenced and it is composed of 4.6 million bases that form 4288 protein-coding genes [5]. In addition to the chromosome, *E. coli* cells also have extrachromosomal plasmids, which are circular double-stranded DNA molecules [6]. Many plasmids do not give an obvious advantage to the cell, although some are beneficial as they encode antibiotic resistance.

An *E. coli* cell is rod-shaped and roughly 2 μm long and somewhat less than 1 μm wide (Figure 1). It is a Gram-negative bacteria and the cytoplasm is surrounded by the cell envelope composed of an inner membrane facing the cytoplasm and an outer membrane facing the extracellular *milieu*. The compartment in between the two membranes, called the periplasm, contains the peptidoglycan layer (Figure 1). The cell envelope both protects the cell and facilitates fundamental contact with the surroundings.

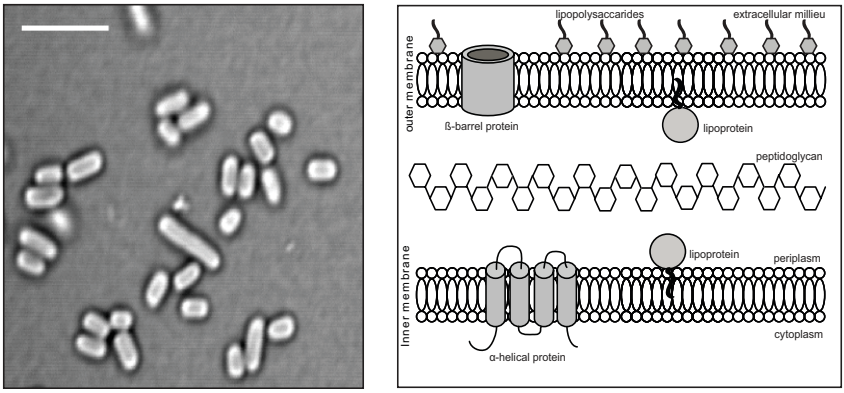

Figure 1. The *E. coli* cell and its cell envelope. The left panel shows *E. coli* cells photographed using a light microscope. The scale bar represents 5 μm. The right panel contains a cartoon of the *E. coli* cell envelope. It consists of an inner membrane an outer membrane and the peptidoglycan layer located in the periplasm. The different protein content between the membranes can also be observed in this illustration adapted from [7].

The membranes

Membranes act as barriers for the passage of polar molecules and ions. These are formed by lipids, which hinder the passage of polar and other compounds such as ions, water and nutrients. The membranes are semi-permeable to uncharged, non-polar molecules such as CO_2 and O_2, which diffuse freely through the membrane. The transport of polar compounds is carried out by membrane proteins acting as transporters, pumps and channels.

Membrane lipids are characterized by a polar (hydrophilic) entity and a non-polar (hydrophobic) entity (Figure 2A). The most common lipids in membranes, glycerophospholipids, are composed of two acyl chains (fatty acids) connected by ester bonds to the first and second carbon of glycerol. Furthermore, there is a polar or even charged group connected by a phosphodiester bond to the third carbon of glycerol [8]. The properties of the lipids can be changed by varying the acyl chains, in length and the degree of unsaturation, and by varying the polar group.

As a result of the hydrophobic effect membrane lipids will form a bilayer in aqueous solutions. The bilayer is formed by two monolayers (leaflets) of lipids arranged in such a way that the hydrophobic acyl chains are in the middle of the bilayer and the polar groups are facing the aqueous surroundings. The thickness of the hydrophobic core has been estimated in an artificial membrane composed of the lipid dioleoylphosphatidylcholine (DOPC) to be 30 Å whereas each hydrophilic interface has been estimated to be 15 Å thick on each side (Figure 2B) [9]. Membranes have for a long time been described by the

fluid mosaic model [10]. This model describes the membranes as a 'sea' of lipids with a low concentration of monomeric membrane proteins that are dispersed throughout the lipid sea. With the increased amount of experimental data the fluid mosaic model has been refined and nowadays membranes are thought to be rather crowded with membrane proteins [11].

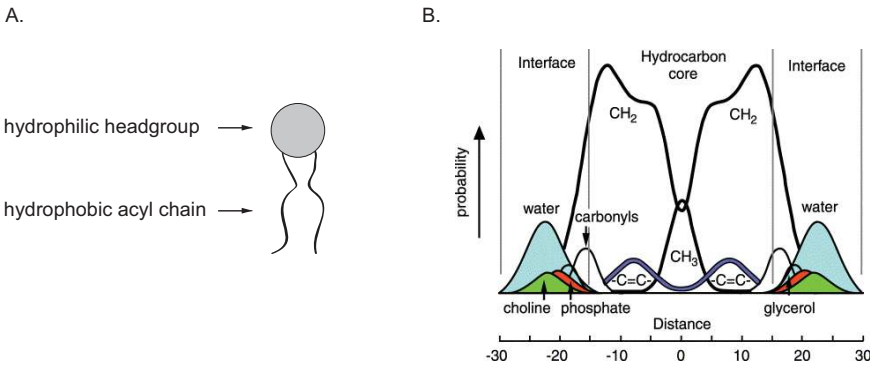

Figure 2. The biological membrane. The membrane is built up from lipids that consist of a hydrophilic headgroup and hydrophobic acyl chains (A.). The structure of the DOPC membrane (B.) [1]. (B.) is reprinted with permission from Elsevier.

The lipid composition of the *E. coli* inner membrane varies from 70 to 80% phosphatidylethanolamine (PE), 20 to 25% phosphatidylglycerol and 5 to 10% cardiolipin (CL) [12]. The outer membrane, on the other hand, has a higher content of PE than the inner membrane and it is enriched in saturated acyl chains [13]. Another feature of the outer membrane is that lipopolysaccharides (LPS) are present in the outer leaflet. Therefore, the outer membrane is asymmetric with an inner leaflet mainly containing glycerophospholipis and an outer leaflet mainly containing LPS [14]. LPS are oligosaccharides attached to Lipid A (glucosamine disaccharide acylated with two fatty acids). There are strong lateral interactions between LPS molecules giving the layer a compact structure. The rigidity explains the low permeability through the outer membrane for small hydrophobic compounds such as antibiotics (reviewed in [15]).

The protein content also differs between the two membranes of *E. coli*. Inner membrane proteins are of two types, integral membrane proteins with membrane spanning domains composed of α-helices (Figure 3) or lipoproteins that are anchored to the outer leaflet of the inner membrane [7]. The functions of many inner membrane proteins are similar to proteins located in the organelles of eukaryotic cells, including the electron transport chain, oxidative phosphorylation and some protein translocation systems. Furthermore, many inner membrane proteins are

involved in the transport of small molecules in and out of the cell [7]. The integral outer membrane proteins are different. Their membrane spanning domains are composed of antiparallel β-strands forming a barrel and as a result many of the outer membrane proteins function as porins (Figure 3) [7, 16, 17]. Furthermore the outer membrane also contains the majority of the lipoproteins of the cell, which are anchored to the inner leaflet of the membrane [7, 17].

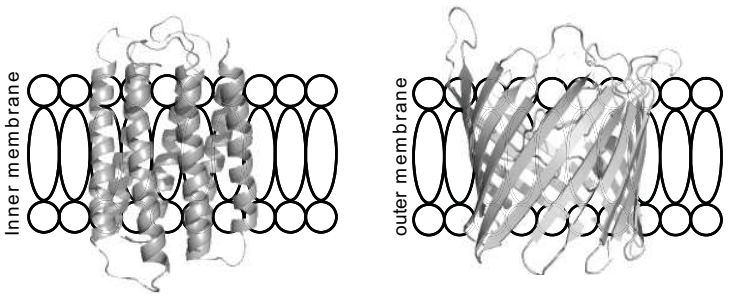

Figure 3 There are two main classes of integral membrane protens. The α-helical membrane proteins (PDB: 2BRD) reside in the inner membrane (to the left) and the β-barrel proteins (PDB: 1RPN) reside in the outer membrane (to the right).

The peptidoglycan layer

In the periplasm of Gram-negative bacteria there is a bag-shaped macromolecule called the peptidoglycan layer (or the murein sacculus). The main function of the peptidoglycan is to encase the cytoplasmic membrane and to protect the cell from rupture by its internal pressure (turgor pressure). As a result, inhibition of peptidoglycan biosynthesis (by antibiotics or protein mutations), or specific degradation (by lysozyme), will cause cell lysis [18-21]. Other features of the peptidoglycan layer are to contribute to the maintenance of the cell shape [20, 22] and to anchor components of the cell envelope (reviewed in [23]).

As inferred by its name, the peptidoglycan layer consists of a peptide moiety and a glycan moiety. The entire layer is formed by cross-linking linear glycan strands by short peptides [24]. The glycan strands are formed by oligomerization of monomeric disaccharide peptide units. The disaccharide units are composed of *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) residues linked by β- 1→ 4 bonds [24]. The chain length of the glycan strands varies with strains and growth conditions and newly synthesized peptidoglycan has an average chain length of 50 to 60 disaccharide units in *E. coli* [25]. In cells grown into the stationary phase the average chain length decreases to 18 disaccharide units [26]. In another study of the glycan strand chain length, 70% of the glycan strands consist of 1-30 disaccharide units with an average length

of 9 disaccharide units, whereas 30% of the glycan strands are longer than 30 units with an average chain length of 45 disaccharide units [27].

There are currently two models describing the architecture of the glycan strands in the petidoglycan layer. The layered model suggests that the glycan strands and the peptide cross-links run parallel to the inner membrane (reviewed in [28]). More recently, the scaffold model was proposed [29-33], in which the glycan strands are suggested to run perpendicular to the membrane whereas the peptide cross-links run parallel to the membrane. There is no consensus in the field on which model to favor. However, since more than 50% of the glycan strands are longer than the width of the periplasm the layered model is more likely (Figure 4) [21].

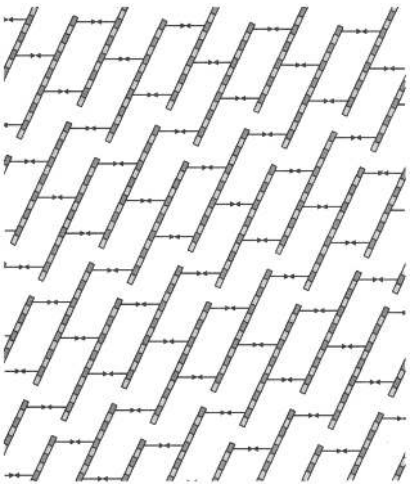

Figure 4. The peptidoglycan is most likely described by the layered model in which the glycan strands run parallel to the membrane. Mur*N*Ac and Glc*N*Ac are illustrated by dark grey bars and light gray bars, respectively. The cross-linking peptides are illustrated by arrows [28]. Reprinted with permission from American Society for Microbiology.

The peptide moiety is attached to the disaccharide units by amide linkage to Mur*N*Ac. The peptides in the peptidoglycan are unusual as they contain rare D-amino acids. Initially, the sequence of the peptide is L-Ala – D-Glu – m-A_2pm – D-Ala – D-Ala (m-A_2pm stands for the dibasic meso-diaminopimelic acid) [19, 20]. However the fraction of pentapeptides in *E. coli* is low in the peptidoglycan, as they are rapidly converted into tetrapeptides (L-Ala – D-Glu – m-A_2pm – D-Ala), tripeptides (L-Ala – D-Glu – m-A_2pm) and dipeptides (L-Ala – D-Glu) [19, 20].

The amount of disaccharide peptide units in the peptidoglycan layer of an average *E. coli* cell has been estimated to be 3.5 million units [34] and 20-30% of these form a cross-link with another peptide [26]. The majority

of all cross-links are formed by covalent bonds between the ω-amino group of the diamino acid (m-A_2pm) in position 3 of the pentapeptide (acyl acceptor) and the carboxyl group of D-Ala in position 4 of the pentapeptide (acyl donor) [24]. Furthermore, cross-links between m-A_2pm in two different peptides also occur, but more rarely [26]. The glycan strands are twisted and a recent study performed on synthetic peptidoglycan fragments show that each disaccharide peptide unit is rotated with an estimated rotation angle of 120° [33]. The twisted glycan strands also causes the peptide chains to rotate and it is therefore unlikely that adjacent peptidoglycan strands are cross-linked by consecutive disaccharide peptide units.

Peptidoglycan biogenesis

The biogenesis of the peptidoglycan layer can be divided into two steps. The first step, synthesis of the peptidoglycan precursor, occurs in the cytoplasm, whereas the second step, polymerization of peptidoglycan precursors to form the peptidoglycan layer, occurs in the periplasm [35].

The peptidoglycan precursor is called lipid II. It is a disaccharide pentapeptide linked to an undecaprenylphosphate lipid and it is formed by a number of events (Figure 5). First UDP-*N*-Glc*N*Ac is formed from fructose-6-phosphate [36-39]. In the second event UDP-Mur*N*Ac is formed from UDP-*N*-GlcNAc in two steps, catalyzed by the enzymes MurA and MurB [40-43]. The peptide is then attached to UDP-Mur*N*Ac by stepwise addition of L-Ala, D-Glu, the diamino acid m_2Apm, and a dipeptide D-Ala – D-Ala. Each addition is catalyzed by a specific synthetase (MurC, MurD, MurE and MurF respectively) using ATP. All four proteins utilize a similar chemical mechanism to form an amide or peptide bond via an acylphosphate intermediate [44-50]. After the formation of the UDP-Mur*N*Ac-pentapeptide, the phospho-Mur*N*Ac-pentapeptide is transferred to an undecaprenylphosphate molecule and UMP is released. The product of this reaction is called lipid I and the reaction is catalyzed by MraY [51]. Thereafter, the Glc*N*ac moiety is attached to lipid I by MurG to form the complete peptidoglycan precursor lipid II containing the disaccharide-pentapeptide moiety attached to the lipid [52].

The mechanism by which lipid II is flipped across the inner membrane has been unknown for a long time, but recently MurJ (also called MviN) was proposed be the putative lipid II flippase [53, 54]. However there is a controversy in the field, as MurJ homologues in *Bacillus subtilis* are not essential for growth, indicating that they are not involved in the flipping of lipid II in all organisms [55]. Furthermore, the cell division protein FtsW has been proposed to be involved in the translocation of lipid II [56]. Recently, a study using Förster resonance

energy transfer (FRET) could also show that FtsW can translocate lipid II across the membranes in lipid vesicles [57].

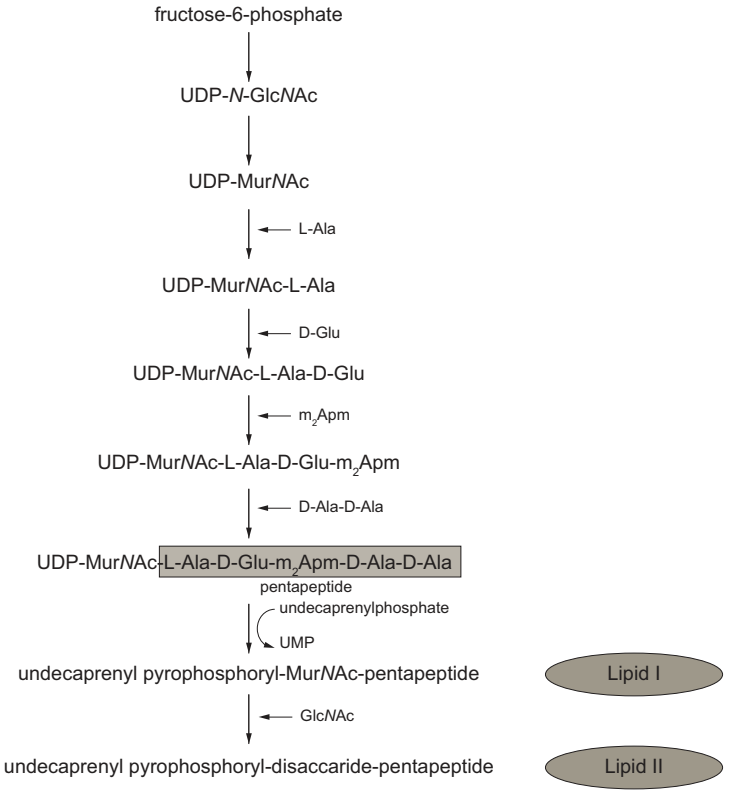

Figure 5. The biogenesis of the peptidoglycan precursor Lipid II. Lipid II is synthesized in the cytoplasm in a series of steps (see the text for details). The figure is adapted from [35].

Penicillin-binding proteins

After being translocated across the inner membrane into the periplasm, lipid II has to be polymerized. This process is performed by a group of proteins called murein synthases. These proteins are monofunctional transglycosylases, bifunctional transglycosylases/transpeptidases or monofunctional transpeptidases. The copy number of the murein synthases has been estimated to 120-220 per cell [19, 58]. The murein synthases that function on the peptide moiety belong to the group of penicillin-binding proteins (PBPs). Their ability to bind penicillin and other β-lactam antibiotics is due to the structural similarity of β-lactam antibiotics to the terminal D-Ala – D-Ala of the pentapeptide side chain [19]. The *E. coli* genome encodes at least 12 PBPs including endopeptidases

and $_{D,D}$-carboxypeptidases [59, 60]. PBPs can be divided into two subclasses based on their relative mobilities in SDS-PAGE [61, 62]: The high molecular weight PBPs PBP1A, PBP1B and PBP1C, all contain transglycosylase and transpeptidase activities, whilst PBP2 and PBP3 contain transpeptidase activities. The low molecular weight PBPs are PBP4, PBP5, PBP6, PBP6B, PBP7 and PBP8. These are endopeptidases and $_{D,D}$-carboxypeptidases processing the peptide chains holding the glycan strands together or cleaving off the final residue from the pentapeptide [63]. Therefore the high molecular weight PBPs are involved in the peptidoglycan synthesis, whereas the low molecular weight PBPs have been suggested to regulate the peptidoglycan cross-linking [64].

High molecular weight PBPs

The high molecular weight PBPs can be further divided into class A and class B PBPs [19]. The proteins in the two classes both have a short N-terminal cytoplasmic region followed by a single transmembrane helix, but the periplasmic domains are different. The properties of the non-penicillin binding motif of the periplasmic domains determine which class the protein belongs to [19, 65]. The class A PBPs contain a transglycosylase domain, whereas the class B PBPs are monofunctional transpeptidases [19].

It is considered that PBP1A (encoded by *mrcA*) and PBP1B (encoded by *mrcB*) are the enzymes with the highest murein synthesis activity. Although neither enzyme is not required for cell growth, it is essential to have at least one of them expressed [66, 67]. PBP1A seems to have a higher affinity for β-lactams than PBP1B, as deletion of PBP1B leads to higher sensitivity to β-lactams than a deletion of PBP1A [67, 68]. Both proteins are built up of the N-terminal transmembrane domain, followed by the transglycosylase domain and the C-terminal transpeptidase domain (both located in the periplasm) [69]. Despite this, there is a major structural difference between the proteins, as PBP1B contains a 100 amino acid long linker between the transmembrane domain and the transglycosylase domain (the structure of PBP1B can be seen in Figure 6) [70]. PBP1C (encoded by *pbpC*) is a non-essential protein, which cannot support cell growth in the absence of PBP1A and PBP1B [71]. Recently LpoA and LpoB, two outer membrane lipoproteins, were found to be essential for the transpeptidase activities of PBP1A and PBP1B, respectively [72, 73]. These two proteins are the first outer membrane proteins known to regulate the peptidoglycan synthesis.

The monofunctional transpeptidases PBP2 and PBP3 (encoded by *mrdA* and *fts1* respectively) are involved in the cell cycle of *E. coli*. PBP2 is involved in the synthesis of peptidoglycan in the cylindrical part of the cell and is essential for the cell elongation that occurs prior to cell

division [74]. When this protein is mutated or inactivated by the antibiotic mecillinam, rod shaped cells become spherical [74, 75]. PBP3 is a cell division specific transpeptidase and it is a component of the protein complex (the divisome) that handles cell division in *E. coli* (a hybrid cartoon of PBP 3 can be seen in Figure 6) [76-78]. When the *ftsI* gene is mutated, cells form filaments indicating that PBP3 is involved in cell division [79].

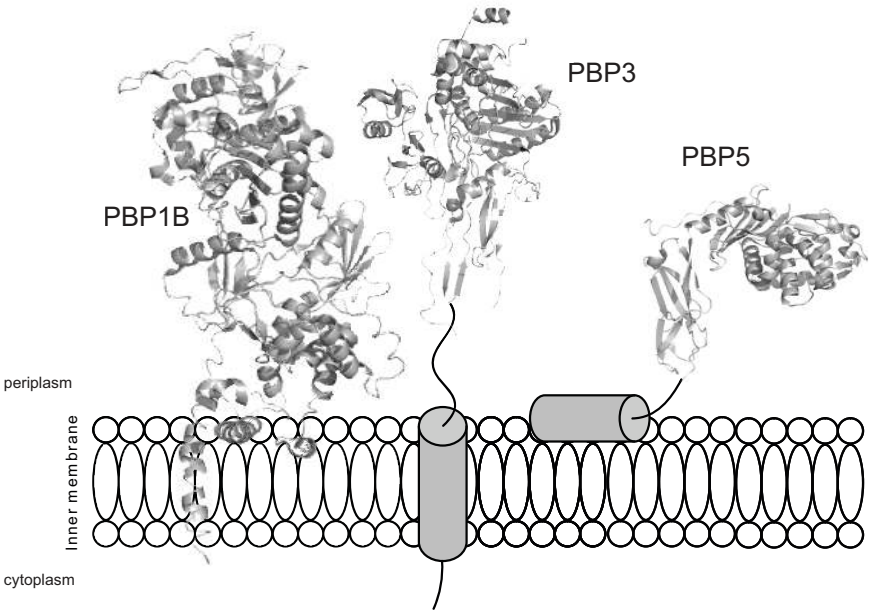

Figure 6. Structural information of PBPs from different classes shown as hybrid cartoons. The crystal structure of PBP1B lacking the N-terminal 57 amino acids (the N-terminal is located in the cytoplasm) (PDB: 3FWL). The crystal structure of the periplasmic domains of the PBP3 homologue in *Streptococcus pneumoniae*, PBP2x (PDB: 1K25) is fused to a cartoon of the remaining protein. The crystal structure of the periplasmic domains of PBP5 (PDB: 1NZO) is fused to a cartoon representing the membrane anchor.

Low molecular weight PBPs

In contrast to the high molecular weight PBPs, the different roles of the low molecular weight PBPs are less clear.

PBP4 (endcoded by the *dacB* gene) has been assigned with both an endopeptidase and a $_{D,D}$-carboxypeptidase activity *in vitro*, but the carboxypeptidase activity has been questioned [62, 80]. Deletion of the *dacB* gene itself does not cause any morphological changes. Although, when *dacB* is deleted together with PBP5 and especially if PBP7 is also deleted, there are severe morphological defects [81, 82].

PBP5 is the best studied low molecular weight PBP and the most abundant penicillin-binding protein within the cell [83]. PBP5 has a $_{D,D}$-carboxypeptidase activity that removes the terminal amino acid (D-Ala) of the peptide chain in the disaccharide-pentapeptide unit [62]. It has been shown to have an important role in maintaining the morphology of the cell [82, 84, 85] by preventing excessive or inappropriate transpeptidation and thereby regulating the number of pentapeptides available for cross-linking [82, 86]. Recently, PBP5 was suggested to be involved in maintaining resistance towards β-lactam based antibiotics, as *E. coli* cells deleted for PBP5 are more susceptible to these antibiotics [87].

The structure of PBP5 has been thoroughly studied and there are 11 different crystal structures deposited in the protein data bank (as of August 2011). The structures indicate that PBP5 is composed of two periplasmic domains orientated in right angles to one another (the structure of PBP5 can be seen in Figure 6) [88, 89]. The $_{D,D}$-carboxypeptidase activity is mediated by the N-terminal domain whereas the C-terminal domain has not been assigned any function. However, it has been suggested that the C-terminal domain can be involved in interactions with other cell wall synthesizing proteins, or acting as a linker allowing the active site in the N-terminal domain to be closer to the peptidoglycan [88]. PBP5 is attached to the outer leaflet of the inner membrane by an amphipatic anchor located at the C-terminus of the protein. The membrane anchor seems to be crucial for the function of PBP5 *in vivo*, as the truncated protein cannot rescue morphological phenotypes in PBP-depletion strains [82]. The structure of the membrane anchor was recently determined by NMR. It forms a helix-bend-helix-turn-helix motif and the structure reveals that the anchor enters the membrane as an amphiphilic structure within the interface of the hydrophobic and hydrophilic regions close to the lipid head groups [90]**.** In this thesis I have studied the oligomeric state of PBP5. We suggest that it forms homo-oligomers and most likely a homo-dimer. By modeling our cross-linking data, we have been able to propose a back-to-back conformation in which the C-terminal domains interact and the catalytic domain has the freedom to move from the position seen in the crystal structure (Paper III).

PBP6 (encoded by *dacC*) and PBP6B (encoded by *dacD*) share high amino acid identity with PBP5 [62, 91, 92]. Initial studies suggested that the functions of PBP6 an PBP6B were similar to PBP5 but both proteins exhibit weaker $_{D,D}$-carboxypeptidase activity than PBP5 [91, 93]. The idea that PBP6 and PBP6B function with lower efficiency has been named the substitution hypothesis [82]. However many reports would suggest that this is not the case [59, 82, 94, 95]. It has further been suggested that each of the $_{D,D}$-carboxypeptidases acts on specific subsets of peptidoglycan

pentapeptides, but this has to be confirmed experimentally [62]. PBP6 and PBP6B have been found to play different roles than PBP5 in maintaining resistance towards β-lactam based antibiotics in *E. coli* cells. Cells in which PBP5 has been deleted are more susceptible to antibiotics and this can be rescued by heterologous expression of PBP5 and partially rescued by PBP6B, but not by PBP6 [96].

PBP7 (encoded by *pbpG*) is a periplasmic protein that is loosely attached to membranes [97]. Isolation of membranes in the presence of 1M NaCl releases the protein from the membrane fraction [98]. PBP7 is further shown to exhibit endopeptidase activity [98]. PBP8 is a short form of PBP7 that is processed by the protease OmpT [99]. Little is today known about the physiological roles of these two proteins.

Cell division

Cell division, or cytokinesis, is an essential event in the cell cycle of prokaryotes. In this event a mother cell is divided into two daughter cells by following a tailored pathway to ensure that the progenies are similar to the mother cell. For this to happen division has to be tightly regulated in time and space so that the cells divide at the correct position when the chromosome has been replicated.

Cell division in *Escherichia coli* is conducted by a large protein complex called the divisome [100], which is a dynamic hyperstructure [101]. The divisome has been investigated during the past decades and initially this was mainly done by genetic approaches. Most of the genes involved were originally identified as temperature sensitive mutations. At non-permissive temperatures cells continued to grow filamentous, as cell division was blocked. Hence, most genes involved in cell division are named *fts*, meaning filamentous temperature sensitive [102, 103]. With the development of green fluorescent protein (GFP), fluorescence techniques could be dramatically improved. Today, the use of fluorescence techniques based on GFP has intensified studies of the components and the dynamics of the divisome. So far, at least 10 essential proteins are known to be incorporated into the divisome in *E. coli* [100, 104]. In addition, there are approximately 15 non-essential proteins that localize to the midcell and play different roles in the division process [105-115].

FtsZ

The essential protein FtsZ is at the heart of the division process and is the first protein to localize to the site where the division is to take place [116-118]. FtsZ assembles to what is referred to as the Z-ring. The Z-ring functions as a scaffold for the recruitment of downstream proteins in the divisome and it persists until the division is completed, guiding the synthesis, location and shape of the division septum [119]. FtsZ is highly conserved throughout most of the major groups of bacteria as in the Euryarchaeal branch of Archaea but is absent in the Crenarchaea and a few bacterial groups [120]. As a result of their endosymbiotic origin, chloroplasts in most photosynthetic eukaryotes use a nuclear-encoded FtsZ for chloroplast division (reviewed in [121]). FtsZ has also been found to be involved in mitochondrial division in several primitive eukaryotes [122-124]. However mitochondrial FtsZ appears to be lost in higher eukaryotes including fungi, animals and plants [121]. In the search for new antibiotics one has attempted to inhibit cell division by developing compounds that target FtsZ, as FtsZ is widespread among prokaryotes, (reviewed in [125]).

FtsZ is a soluble protein with a structure similar to tubulin (Figure 7) [126]. The crystal structure of FtsZ revealed three domains: (i) a variable N-terminal domain, (ii) the tubulin-like core domain and finally (iii) a C-terminal peptide that is essential for interactions with other cell division proteins (i.e. FtsA and ZipA) [127]. As for tubulin, the core domain of FtsZ binds and hydrolyzes GTP [126, 128-130] and binding of GTP induces self-assembly of FtsZ into protofilaments similar to the tubulin protofilaments [131-134]. The protofilaments bundle and cross-link, which is promoted by ZapA *in vitro* [110, 135]. Furthermore ZapB interacts with ZapA and it has been suggested that ZapB stimulates the bundling of FtsZ protofilaments by bridging ZapA molecules [108, 136]. Recently ZapC was also identified to interact with FtsZ, to promote lateral interactions of FtsZ protofilaments and suppress the FtsZ GTPase activity [113, 114].

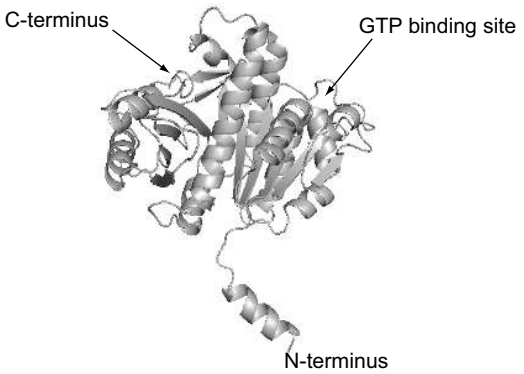

Figure 7. The crystal structure of FtsZ (PDB: 1FSZ) from *Methanocaldococcus jannaschii*. The GTP binding site and the C-terminus are indicated. The C-terminal peptide that interacts with FtsA and ZipA is disordered in the crystal and not shown.

It has been estimated that *E. coli* contains 3000-20,000 copies of FtsZ per cell [137-139]. For comparison 10,000 copies of FtsZ would generate a single protofilament of 40 μm, which is enough to encircle a bacterium of 0.6 μm in diameter 20 times and even if 2-4 FtsZ protofilaments bundle into sheets, there will still be enough to encircle the cell [131]. So far, the architechture and assembly of the Z-ring is not fully known. However cryo-electron microscopy studies of *Caulobacter crescentus* indicate that the Z-ring consists of a large number of short, overlapping protofilaments rather than a single continuous protofilament [140].

A fundamental aspect of the Z-ring assembly is the association of FtsZ to the cell membrane. FtsZ alone does not seem to have any affinity for the membrane, but all models of the formation of the Z-ring require attachment to the membrane to maintain its structural integrity during the

septation and most likely also to transmit a constrictive force on the cell membrane [102, 140-142]. In *E. coli,* FtsZ is anchored to the inner membrane by two proteins, FtsA and ZipA [143]. Both of these proteins are essential for cell division [142, 143]. In this thesis I have studied the progression of the Z-ring contraction in dividing *E. coli* using a new approach based on fluorescence recovery after photobleaching (FRAP). This work suggests that the contraction is a linear process on a population average, however it is more complex when studying on the single-cell level (Paper I).

ZipA

ZipA (FtsZ interacting protein A) is a bitopic membrane protein composed of four domains: an N-terminal transmembrane anchor, a charged domain, a domain enriched in proline and glutamine and a C-terminal globular domain (Figure 8) [142]. The membrane topology of ZipA is rare in bacteria with the amino terminus located in the periplasm and the carboxy terminus located in the cytoplasm [142].

ZipA is dispersed throughout the inner membrane but upon cell division initiation, it is recruited to the division site by FtsZ to form the Z-ring [142, 144]. Furthermore, the recruitment of ZipA to the emerging Z-ring is independent of the other FtsZ-interacting protein FtsA (see below) [144, 145]. The C-terminal peptide of FtsZ interacts with the C-terminal globular domain of ZipA, named the FtsZ binding (FZB) domain [146]. As the interaction between ZipA and FtsZ is only conducted via the FZB domain, this domain is both necessary and sufficient for the recruitment of ZipA to the Z-ring [146]. In addition to the role of anchoring FtsZ to the inner membrane, *in vitro* studies of purified FtsZ and ZipA have showed that ZipA induces bundling of FtsZ protofilaments [146, 147]. Furthermore, the ZipA-induced bundles of the FtsZ protofilaments have been estimated to contain 10-20 protofilaments per bundle [147] and the bundling of the protofilaments have been suggested to increase the stability of the Z-ring [143].

ZipA is not widely conserved outside γ-proteobacteria [142], suggesting that it has either been replaced by other proteins or has become redundant. One interesting feature of FtsA, which is widely conserved throughout bacteria [148], is that a single amino acid substitution (R286W) is sufficient to bypass the requirement for ZipA in *E. coli* [149]. This suggests that bacteria outside of the γ-proteobacteria family may have a version of FtsA with properties similar to the FtsA R286W, thereby bypassing the need for ZipA [149]. In this thesis I have studied the oligomeric state of ZipA *in vivo*. Our data suggest that ZipA exists both as monomers and dimers. Furthermore the dimerization could be a

possible way for ZipA to bundle FtsZ protofilaments, as the dimer could bridge two FtsZ protofilaments (Paper IV).

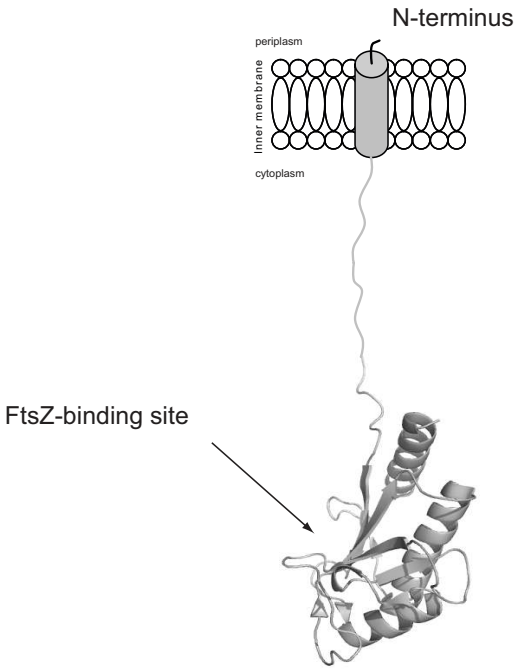

Figure 8. The known structural information of ZipA summarized in a hybrid cartoon. ZipA is a single-spanning transmembrane protein with an N-terminal transmembrane anchor and a C-terminal FtsZ-binding domain. These are linked by a long unstructured region containing the charged domain and a PQ-rich domain. Notably, the crystal structure has only been solved for the FtsZ-binding domain (PDB: 1F47).

FtsA

The crystal structure of FtsA from *Thermotoga maritima* has been solved, indicating a structural similarity to actin [150]. The protein consists of two domains with a common core forming an interdomain nucleotide-binding site (Figure 9). Furthermore, each domain can be further divided into two subdomains [150]. One of the solved structures contained ATP in the nucleotide-binding site, supporting previous biochemical data and indicates that FtsA has the ability to bind ATP [151-153].

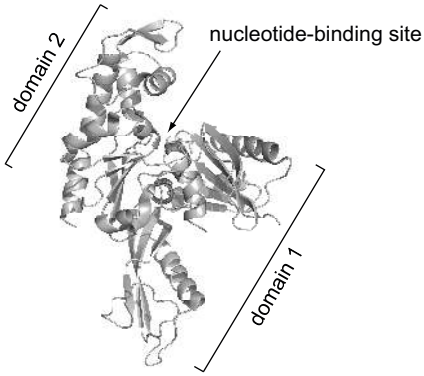

Figure 9. The crystal structure of FtsA from *T. maritima* (PDB: 1E4G). The two domains as well as the nucleotide-binding site are indicated.

When the crystal structure of FtsA was solved the C-terminus was found to be disordered [150]. Hovever, a later study showed that the extreme C-terminus contains 15 amino acids that are conserved and present in all FtsA sequences and which form an amphipatic helix [154]. This is the membrane targeting sequence of FtsA and it is important for cell division, since FtsA lacking the amphipatic helix causes the protein to form deleterious cytoplasmic rods rather than interacting with the membrane [154]. A region on FtsA containing several charged residues is proposed to be involved in the interaction with FtsZ [155].

The levels of FtsA in the cell have been estimated to be approximately 700 copies giving a 5:1 FtsZ:FtsA ratio [139], whereas ZipA has been estimated to be present at 1000-1500 copies [139, 145]. This indicates that a limited number of the FtsZ molecules will be interacting with either ZipA or FtsA. Furthermore, the balance of these proteins (i.e. the ratios) is crucial since overexpression of either of FtsA or FtsZ has been shown to be toxic. This toxicity can be counteracted by overproducing the other protein to restore the FtsZ:FtsA ratio [137].

Various studies have shown that FtsA self-interacts to form dimers or higher oligomeric states. In *Bacillus subtilis*, FtsA has been shown to be a dimer [151]. Furthermore, several studies using bacterial two-hybrid assays have shown self-interaction of FtsA [152, 155-161]. The *E. coli* FtsA dimer has been modeled suggesting residues important for protein: protein interactions within the dimer [302]. Furthermore, dimerization of FtsA has been suggested to be important for Z-ring integrity, as substitutions destabilizing the FtsA dimer also destabilize the Z-ring [159]. In *Streptococcus pneumoniae*, FtsA has been seen to polymerize into large complex helices with corkscrew-like structures formed by pairs of paired filaments [152]. These polymers form in a nucleotide-dependent manner and

are more stable when formed in the presence of ATP than ADP [152]. To further support the importance of nucleotide-binding in the self-interactions of FtsA, mutations that prevent FtsA to bind ATP eliminates dimerization [155].

Once the Z-ring is assembled and tethered to the membrane at the midcell, the remaining essential division proteins are recruited. These proteins are either single-pass or multi-spanning inner membrane proteins [162]. These proteins (FtsK, FtsQ, FtsL, FtsB, FtsW, PBP3 (FtsI) and FtsN) are briefly described below.

FtsK

The segregation of the bacterial chromosomes into the daughter cells has been described as one of the most mysterious events during the bacterial cell cycle [163]. A recent theory is that chromosome segregation is mainly driven by entropy and proteins found to be segregation factors function to create the right physical conditions for the entropy-driven chromosome segregation to occur [164]. FtsK is a septum-located DNA translocase that is composed of three domains: an N-terminal integral membrane domain with four transmembrane helices, a proline- and glutamine-rich linker and a C-terminal domain, a DNA translocase involved in chromosome segregation (Figure 10) [165-167].

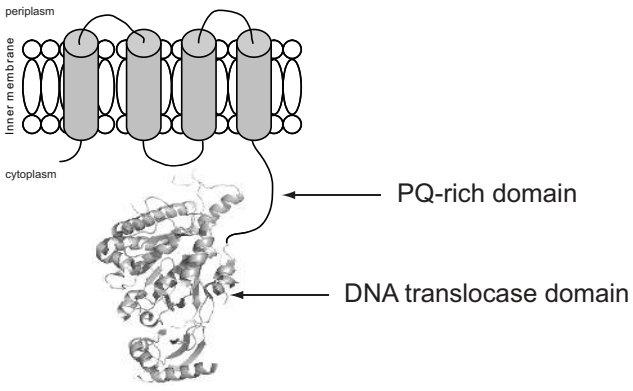

Figure 10. Structural information on FtsK summarized in a hybrid cartoon. The protein consists of four N-terminal transmembrane helices followed by a PQ-rich domain and a DNA translocase domain located in the cytoplasm (PDB: 2IUS).

The N-terminal domain of FtsK is essential for cell viability in wild-type *E. coli* whereas deletion of the other parts of FtsK is not lethal [168]. Studies perfomed on the C-terminal DNA translocase domain have shown that it can form hexameric ring-structures and that DNA duplexes can pass through the ring [169, 170]. As FtsK assembles at the midcell during a late

stage of the cell division, it seems likely that normal DNA replication, followed by decatenation and the segregation of the sister chromosomes will have been completed prior to its arrival [171, 172]. On the other hand, if the final steps of replication or decatenation are delayed or if chromosome dimers have formed, DNA will remain in the septal region where FtsK becomes available to speed up the segregation of the two chromosomes [172].

FtsQ, FtsL and FtsB

The three bitopic membrane proteins FtsQ, FtsB and FtsL form a subcomplex within the divisome (described below), which appears to bridge the cytoplasmic cell division proteins and the periplasmic cell division proteins [173]. A bioinformatic analysis of nearly 400 bacterial genomes showed conservation of homologues of FtsQ, FtsB and FtsL in the majority of the bacteria examined [174]. It is interesting to note that these homologues were also present in bacteria that divide in a different manner from the binary fission in *E. coli* [174].

FtsQ has a central role in the formation of the divisome and it is present in approximately 20-25 copies per cell [175]. FtsQ is a bitopic membrane protein consisting of a short cytoplasmic tail, a transmembrane region and a periplasmic domain (Figure 11) [175, 176]. The crystal structure of the periplasmic domain of FtsQ from *E. coli* has been solved and it reveals two domains. One of them is strikingly similar to a POTRA (polypeptide transport-associated) domain [177]. These domains are usually involved in chaperone-like functions but whether this is the case for FtsQ is however still to be proven [178].

Although it has become evident that FtsQ plays a key role in the interaction web between all proteins within the divisome, the exact role remains unknown. FtsQ has been shown by bacterial two-hybrid analyses to interact with FtsA, FtsK, FtsX, FtsL, FtsB, FtsW, PBP3, FtsN and YmgF [156, 157, 179]. Some of these proposed interactions were further confirmed by co-immunoprecipitation, such as those between FtsQ, FtsB and FtsL [173] and between FtsQ and FtsW [179].

FtsL is composed of a small N-terminal cytoplasmic domain of 37 amino acids followed by a transmembrane helix and a periplasmic C-terminal domain of 64 amino acids (Figure 11) [180]. The domains of FtsL have been studied to understand the interactions with other cell division proteins. The far C-terminal part of the periplasmic domain seems to be important for interactions with FtsQ but not with other cell division proteins. The N-terminal cytoplasmic domain is important for recruitment of the later cell division protein FtsW but not for the interaction with FtsQ and FtsB. The interactions between FtsL and FtsB

are suggested to take place mainly through the transmembrane helix and a part of the periplasmic domain containing coiled coils [174].

As for FtsQ and FtsL, FtsB has a short N-terminal cytoplasmic domain and a large periplasmic domain (Figure 11). The C-terminal domain of FtsB is important for its interactions with FtsQ. The amino terminus, the transmembrane domain and the membrane proximal portion of the periplasmic domain interact with FtsL, PBP3 and presumably FtsW [181]. Beyond their interactions with the divisome, the functions of FtsB and FtsL are not clear [162]. It has been suggested that they have no other function than being parts of a scaffold for the recruitment of other proteins to the divisome [181]. Although, a recent study indicates that heterologous expression of FtsL confers a Zn^{2+} sensitive phenotype suggesting a possible role in Zn^{2+} transfer across the membrane [182].

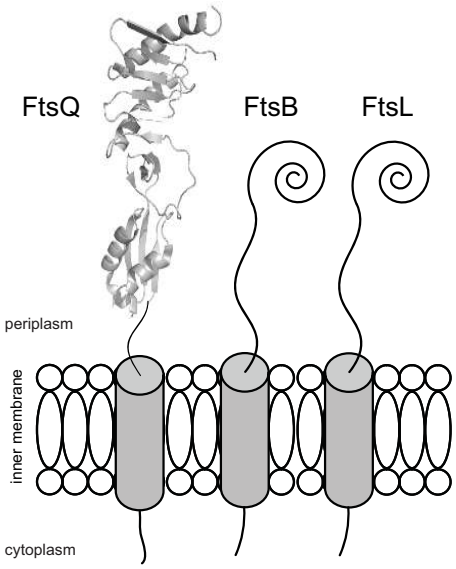

Figure 11. Structural information of FtsQ, FtsB and FtsL. All three proteins consist of a short N-terminal cytoplasmic sequence, a transmembrane helix and a larger periplasmic domain. The crystal structure has only been solved for the periplasmic domain of FtsQ (PDB: 2VH1).

FtsW

When the divisome constricts to form a septum, new peptidoglycan has to be synthesized for the new cell poles. FtsW is a transmembrane protein with ten transmembrane segments (Figure 12) [183]. It is involved in the recruitment of PBP3 to the midcell, which is the penicillin-binding protein that catalyzes the peptidoglycan cross-linking in the septal peptidoglycan synthesis [184, 185].

FtsW is present in virtually all bacteria that have a peptidoglycan and is an essential cell division protein [186]. FtsW has been assigned to a protein family called SEDS (for shape, elongation, division and sporulation) [187]. Proteins within the SEDS family appear to work in concert with a PBP catalyzing the peptidoglycan synthesis during the cell cycle, however their mechanisms of action are not known [188]. As no function has been assigned to FtsW, it has been speculated that it can integrate signals between the cytoplasmic and periplasmic cell division proteins [189]. Furthermore, it has been proposed that FtsW can be involved in the translocation of lipid-linked peptidoglycan precursors (lipid II) through the cytoplasmic membrane [56]. This proposal has been further strengthened by a recent study using FRET to show the involvement of FtsW in the lipid II flipping [57]. Although, MurJ (also called MviN) has also been proposed as a putative flippase for translocation of lipid II (see above).

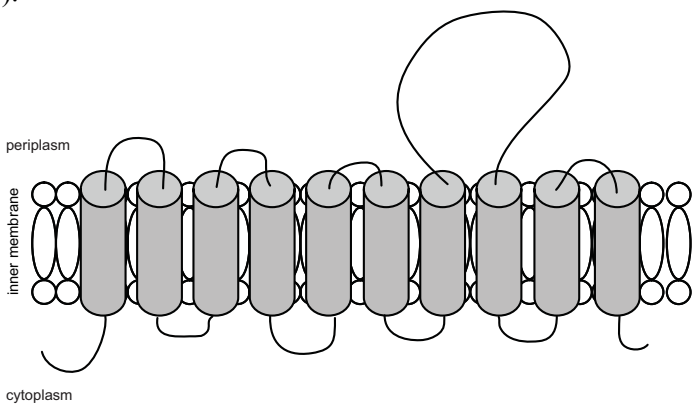

Figure 12. The topology map of FtsW according to [183]. The protein has 10 transmembrane helices and a large loop between helix 7 and 8.

Septal penicillin-binding proteins

Early studies of penicillin-binding proteins identified two proteins important for cell shape maintenance and cell division. When the gene *mrdA* (encoding penicillin-binding protein 2, PBP2) was mutated a round-shaped phenotype was observed. This indicates that the protein is involved in the synthesis of the peptidoglycan within the cylindrical part of the cell [74]. When the gene *ftsI* (encoding penicillin-binding protein 3, PBP3) was mutated, peptidoglycan synthesis during division ceased and filamentous cells were observed [79]. This indicates that PBP3 is involved in the synthesis of the septal peptidoglycan. PBP2 and PBP3 belong to the same class of penicillin-binding proteins (class B) sharing peptidoglycan transpeptidase activities, i.e. that cross-link the peptide moieties within the peptidoglycan layer [190].

PBP3 has been estimated to be present in the order of 100 copies per cell in fast-growing cells [58, 191], which is 30- to 200-fold less than the copy number of FtsZ (see above).

PBP3 and its homologues have a short intracellular N-terminus and a single-spanning transmembrane domain followed by a periplasmic domain of approximately 200 amino acids. This domain has a poorly understood function and is called the non-penicillin-binding domain. The C-terminal domain of approximately 300 amino acids contains the catalytic residues and is called the penicillin-binding domain (Figure 6) [190, 192]. The first 56 residues (including the transmembrane domain) are required to target PBP3 to the division site [193]. Furthermore, when the first 41 amino acids are replaced by another single-spanning transmembrane domain, the interactions between PBP3 and FtsW are lost [194]. Additionally, point mutations in or near the transmembrane helix impair localization to the division site [195], showing the importance of the transmembrane helix in the localization of PBP3. Two of these substitutions (R23C and L39P) have been showed to decrease and impair interactions between PBP3 and FtsW [196].

The transpeptidase activity of PBP3 is limited to the division site and its catalytic activity depends on the division status of the cell [76-78]. Notably, the transpeptidase activity of PBP3 is different compared to PBP2. When the active site of PBP3 is replaced by the active site of PBP2, the function of PBP3 is disrupted [162, 192]. It has been suggested that these two proteins differ in their substrate specificity with PBP3 exhibiting a preference for peptidoglycan precursors with a tripeptide side chain and PBP2 for pentapeptide side chains [76, 197].

The interactions of PBP3 with other cell division proteins have been studied using bacterial two-hybrid [156, 157], genetic and biochemical assays

[137, 185, 198, 199]. These studies show that PBP3 interacts with FtsA, FtsN, FtsQ and FtsW. A recent publication shows that FtsW and PBP3 form a subcomplex within the divisome [196]. Further, using FRET they showed that PBP3 forms homo-dimers, which have been suggested previously [156, 157].

PBP3 is not the only penicillin-binding protein that localizes to the division site during septation. PBP1B (encoded by *mrcB*) is a class A penicillin-binding protein with the ability to both polymerize the glycan strands of the peptidoglycan by transglycosylation as well as cross-link the peptide moieties by transpeptidation (for protein structure see Figure 6) [190]. PBP3 interacts directly with PBP1B and it has been suggested that these two penicillin-binding proteins act in concert to enlarge the peptidoglycan during cell division. [107]. Furthermore, PBP1B interacts with FtsN, which in turn interacts with PBP3 [200].

The $_{D,D}$-carboxypeptidase PBP5 that has a role in maintaining the cell morphology by regulating the number of available pentapeptides for transpeptidation (see above) was also recently found to localize to the septum. [201]. Furthermore, a connection between PBP5 and the cell division machinery has previously been suggested. When the function of FtsZ is partially lost in cells deleted for PBP5, this causes a morphological phenotype including branching and abnormalities [202].

FtsN

FtsN is the last of the known essential cell division proteins to be recruited to the septum [203, 204] and is only found among the γ-proteobacteria [189]. FtsN is a membrane protein with a single-spanning transmembrane helix. The N-terminus is located in the cytoplasm whereas the C-terminus is located in the periplasm. In the periplasm, the protein consists of a long proline and glutamine-rich linker followed by a domain with peptidoglycan-binding activity, known as the SPOR domain (Figure 13) [205-207].

FtsN was originally found as a multi-copy suppressor for a thermosensitive mutation in *ftsA* [208]. Several copies of FtsN were also found to compensate for loss of function in FtsQ and PBP3 [208], and complete loss of FtsK [168].

It has been suggested that FtsN has a 'triggering role' in the constriction of the Z-ring and that the SPOR domain has an ability to specifically bind septal peptidoglycan, which is transiently available during the constriction process. The SPOR domain has been found in three additional proteins in *E. coli* (DamX, DedD, and RlpA) which also localize to the septal ring [209, 210]. Furthermore, FtsN has been assigned a

role in maintaining the stability of the divisome, as depletion of FtsN causes disassembly of the divisome in an ordered way [211]

FtsN interacts directly with the FtsA, FtsQ and PBP3 [107, 156, 157, 179] and it has been found that FtsN fails to be recruited to the divisome if FtsA or FtsQ is missing [100, 212].

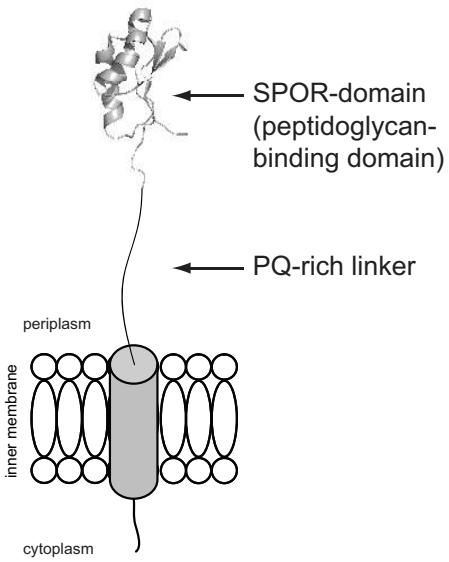

Figure 13. The known structural information of FtsN illustrated in a hybrid cartoon. The protein has its N-terminal in the cytoplasm, followed by a transmembrane helix. In the periplasm, the transmembrane helix is connected to the SPOR-domain through a PQ-rich linker. The structure has only been solved for the SPOR-domain, using NMR (PDB: 1UTA).

The assembly of the divisome

Early fluorescence microscopy studies on the assembly of the divisome indicated a specific pathway in which proteins are recruited in a linear fashion to form the complete divisome [213].

As previously described, FtsZ is thought to be the first protein to localize at the midcell upon initiation of cell division [116-118]. Following FtsZ, FtsA and ZipA assemble into the ring and bind directly to FtsZ. These assemblies are solely dependent on the localization of FtsZ and not on any of the downstream proteins [145, 214]. The three proteins are important for the recruitment of other proteins to form the complete divisome.

The recruitment of FtsK to the Z-ring depends on the prior localization of FtsZ, FtsA and ZipA but not on the downstream proteins PBP3 and FtsQ [143, 204, 215]. Then follows FtsQ, which requires FtsK, but not FtsL, PBP3 and FtsN [204, 216]. As previously described, FtsQ is thought to act as an interaction hub within the divisome. FtsQ is needed for the recruitment of FtsL and FtsB. Furthermore FtsL and FtsB require each other for their localization [217]. After FtsL and FtsB follows FtsW, which requires both the preceding proteins to localize [185, 217] and PBP3 needs FtsW to localize [185]. Finally, the last essential protein to be recruited to the divisome is FtsN and this is dependent on PBP3 [203].

During the last years, the specific pathway has been further developed into a model in which the cell division proteins are thought to be recruited to the divisome in the form of sub-complexes. The model is based on the findings that FtsQ, FtsB and FtsL form a sub-complex in the absence of any other cell division protein [173, 212]. This subcomplex has been modeled and it was suggested that the subcomplex is either present as a ternary complex (FtsQ:FtsB:FtsL: 1:1:1) or as a hexameric complex (FtsQ:FtsB:FtsL: 2:2:2) [218]. Furthermore, FtsW and PBP3 form another sub-complex and can reciprocally recruit each other [212]. From these studies it could not be detected if FtsN and FtsK are constituents of a sub-complex forming the divisome. The assembly order and the subcomplexes are illustrated in Figure 14.

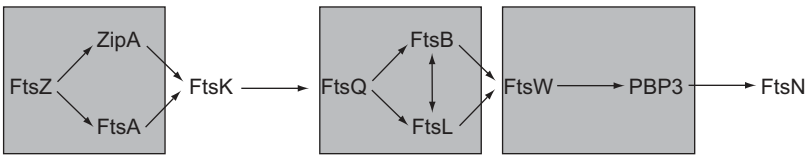

Figure 14. The assembly order of the cell division proteins in *E. coli*. The specific pathway for the recruitment of the proteins is illustrated with arrows and the subcomplexes are illustrated with grey boxes.

The network of interactions between proteins forming the divisome has been studied mainly via bacterial two-hybrid assays [156, 157]. The many interactions formed generate a complex network, which further support the model of sub-complexes. If the proteins were recruited in a linear fashion (one by one), mechanisms would be needed to prevent simultaneous assembly, which has not been found [219].

Invagination of the outer membrane

In Gram-negative bacteria there is a need to invaginate the outer membrane and the inner membrane as well as the peptidoglycan layer during division. None of the known components of the divisome are outer membrane proteins. Therefore it has been suspected for a long time that the invagination of the outer membrane occurs passively via lipoprotein linkages between the peptidoglycan layer and the outer membrane [104, 220]. Braun's lipoprotein (Lpp) is one of the most abundant proteins in *E. coli* and plays an important role in maintaining connections between the outer membrane and the peptidoglycan layer, also at constriction sites [221-224]. Lpp is covalently attached to the m-A_2pm residue of a peptidoglycan peptide [225]. Although Lpp contributes to proper outer membrane invagination, it is dispersed along the cell envelope [226, 227] and is therefore not considered as a component of the cell division machinery.

The Tol-Pal system is widely conserved among Gram-negative bacteria [228] and is required for maintaining the integrity of the outer membrane [229, 230]. It consists of at least five proteins: TolA, TolQ and TolR are inner membrane proteins that form a complex via interactions between their transmembrane helices [231-234]. TolB, a periplasmic protein, and Pal (peptidoglycan-associated lipoprotein), an abundant outer membrane lipoprotein, form another complex associated with the outer membrane [235-237]. The two complexes are connected via interactions mediated by the extended periplasmic C-terminus of TolA and Pal [238, 239] as well as via interactions between TolA and TolB [240, 241]. Furthermore, Pal interacts with the peptide moiety of the peptidoglycan layer via strong non-covalent interactions [235, 242-245].

Recently, the proteins in the Tol-Pal system were found to localize at the division site. This localization required FtsN to be present at the division site [109]. As the Tol-Pal system connects the inner membrane with the outer membrane, it was suggested that the Tol-Pal system could pull the outer membrane onto the invaginating peptidoglycan layer and inner membrane during septum formation.

Since the deletion of components of the Tol-Pal system is not lethal, it was speculated that there must be other lipoproteins involved in tethering the outer membrane to the peptidoglycan during constriction [109]. The lipoprotein LpoB has been reported to localize at the septum, to interact with PBP1B and with the peptidoglycan layer [72, 73]. Furthermore, a double-knock out of LpoB and Pal cause cell lysis and therefore it was suggested that LpoB-PBP1B could be another system promoting the outer membrane constriction [72]. This ability could also explain why the Tol-Pal system is not essential.

In this thesis I have studied the timing of the closure of the periplasm at the late stages of cell division in *E. coli*. Based on dual-colour FRAP experiments, we suggest that the cytoplasm closes before the periplasm (Paper II).

Regulation of cell division

To ensure that the progenies are identical copies of the mother cell, cell division has to be tightly regulated. It is especially important for rod-shaped bacteria to ensure that the division occurs in the midcell in between the segregated chromosomes. This phenomenon was observed early in the studies of bacterial cell division [246, 247]. Along with the increased knowledge of the division in rod-shaped bacteria, two systems involved in the spatial regulation of cell division were revealed. The Min system prevents cell division at the cell poles [248, 249] while nucleoid occlusion prevents cell division from occurring across the nucleoids [250, 251].

Min system

The Min system in *E. coli* is known to consist of three proteins encoded by the *min* operon: MinC, MinD and MinE [249]. MinC and MinD form a division inhibitory complex whereas MinE restricts the action of the MinCD complex to the cell poles.

MinC is the actual inhibitor in the Min system [252, 253]. *In vitro* studies of MinC and FtsZ show that MinC antagonizes the polymerization of FtsZ without affecting the GTPase activity of FtsZ [254]. The protein consists of two domains: an N-terminal domain and a C-terminal domain, both of which antagonize the assembly of the Z-ring [255-257]. The N-terminal domain is responsible for breaking FtsZ polymers whereas the C-terminal domain interacts with the extreme C-terminal tail of FtsZ to displace ZipA and FtsA [258, 259]. A mutation analysis of the C-terminal domain showed loss of interaction with MinD, indicating this domain to be responsible for the interaction with MinD [260]. Furthermore, both genetic and biochemical analyses of MinC indicate that the protein most likely forms homo-dimers [256].

The inhibitory effect of MinC is actually relatively weak in the absence of MinD. MinC has to be present at levels 25 to 55 times higher than normal to cause inhibition in the absence of MinD [253]. This implies that MinD has a role in triggering the activity of MinC. MinD is a membrane protein (see below) and the triggering effect can (at least partly) be induced by attaching MinC to the membrane. In addition, the triggering effect can also be obtained by fusing MinC to a transmembrane helix [261, 262]. Two recent reports indicate that MinC

inhibits cell division by two mechanisms: (i) by displacing FtsA and probably also ZipA from the Z-ring [258] and (ii) by preventing interactions between FtsZ polymers [263].

MinD is an ATPase belonging to protein family called WACA (Walker A cytoskeletal ATPase) as it contains a Walker A motif and can polymerize into large coiled-coil structures *in vivo* [264-267]. A recent crystal structure of MinD shows that it forms a dimer in the presence of ATP [268]. This finding is supported by previous FRET experiments [269] and other proteins in the same family have also been found to dimerize when ATP is present [270, 271]. Although, dimerization can be species-dependent, as two crystal structures of MinD from Archea only show monomers [272-274]

MinD binds phospholipid vesicles in an ATP-dependent manner and upon binding it assembles into polymers [275]. Furthermore, it has been shown that the C-terminal end of MinD is required for binding of MinD to the membrane [276, 277]. This C-terminal is predicted to form an amphipatic helix and is therefore the membrane targeting sequence of MinD. A model for the binding of MinD to the membrane has been proposed in which it has to dimerize, in an ATP dependent manner, in order to bind the membrane [262, 276]. This model is supported by the observation that a single membrane targeting sequence cannot bind the protein to the cytoplasmic membrane but a tandem repeat of the membrane targeting sequence associates MinD to the membrane [262]. The crystal structure of MinD reveals the binding sites of MinC and MinE. The binding sites overlap and are located at the dimer interface so that the complete binding sites are only formed upon dimerization [268]. This is supported by previous yeast two-hybrid experiments in which single amino acid substitutions in MinD hindered interactions with both MinC and MinE [278].

MinE is a protein of 88 amino acids that forms a dimer [279]. The protein has two separable functional domains: the N-terminal 32 residues counteract the MinCD activity. The C-terminal part (residue 32-88) ensures that MinCD is only counteracted at the midcell [280, 281]. The N-terminal domain has also been found to form electrostatic interactions with the membrane and these interactions are necessary for the localization and oscillation of MinCD (further described below) [282]. MinE stimulates the ATPase activity of MinD in the presence of phopholipids [283], which causes MinD (and hence MinC) to be released from the membrane into the cytoplasm [283, 284]. Furthermore, MinE can release MinC from the membrane-bound MinD in an ATP-independent manner [285, 286]. A recent NMR structure of the full-length MinE from *Neisseria gonorrhoeae* shows that the protein consists of a three-strand β-sheet packed against an α-helix, which is almost in a parallel orientation to the

β-sheet [287]. This is further supported by an X-ray crystal structure of a truncated form of MinE from *Helicobacter pylori* [288]. Most interestingly, both structures indicate that the N-terminal residues involved in counteracting MinCD activities are buried, indicating that a conformal change is needed in order to increase their accessibility [287].

So, how does the Min-system actually inhibit division at the cell poles and at the same time allow it in the midcell? In the early reports of the Min-system the models were static with MinCD localized at the cell poles whereas MinE was acting in the midcell [249, 281, 289]. Over time, the model has been refined and the finding of the rapid oscillation of MinD between the cell poles was a break-through. During the oscillation, MinD forms a polar zone on the membrane that is extending towards the midcell. This polar zone will start to shrink towards the cell pole to establish a new polar zone at the other cell pole and the cycle is then to be repeated [284]. It has also been shown that MinC oscillates in the same pattern as MinD, indicating it to be a cargo to MinD during oscillation [254, 290]. Later studies revealed that MinE also oscillates in a ring-shape in front of MinD [291-293]. This suggests that MinE works by a stop-growth mechanism that prevents the MinCD polar zone to extend beyond the division site at the midcell. By using more refined fluorescence microscopy, the oscillation of the components of the Min-system has been shown to be organized into membrane-bound coiled structures rather than being randomly distributed throughout the polar zones [267]. Although it has been shown that expression of MinCD (from a plasmid) in cells in which the *min* operon is deleted, the Δ*min* phenotype can be rescued [294]. This indicates that MinCD can have different sensitivity for polar and non-polar Z-rings. The Min-system is described in Figure 15. Recently, the Min-system was studied *in vitro* and by simultaneously monitoring the Min-proteins with fluorescent probes using confocal and single-molecule microscopy [295]. Their results indicate that the Min proteins propagate as waves over the membrane *in vitro* and that MinE is in close contact with MinD at the rear of the wave causing a displacement of MinC.

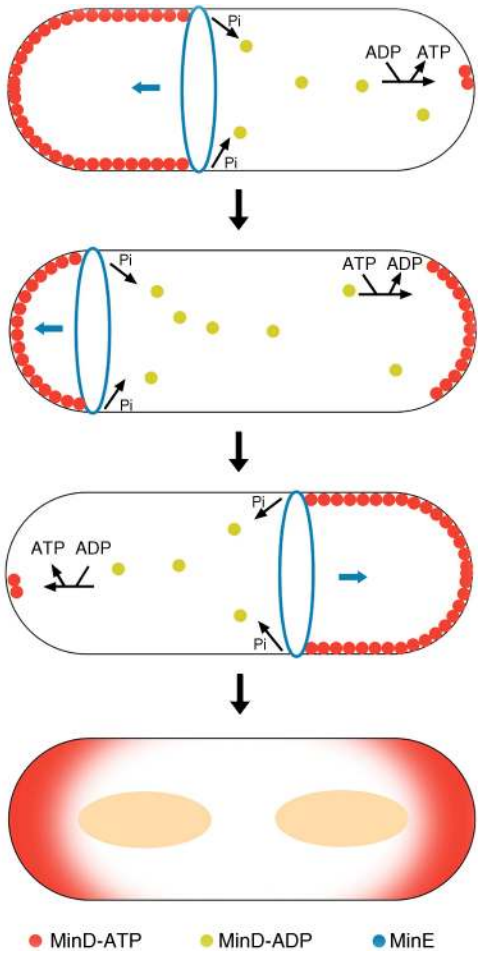

Figure 15. The Min-system inhibits cell division at the cell poles by an oscillating mechanism of MinCDE between the cell poles. MinE, illustrated by the blue elipse, binds MinD-ATP. This triggers ATP hydrolysis followed by a release of MinD-ADP and its passenger MinC from the membrane. The simple oscillation will generate a gradient of MinCD over time, which is lowest at the midcell [296]. Reprinted with permission from Elsevier.

Nucleoid occlusion

The model of nucleoid occlusion was proposed 20 years ago [251], but it was not until 2005 that the actual protein (SlmA) involved was identified [250].

The protein SlmA was identified when searching for mutations synthetically lethal with a defective Min system (*slm* mutants) [250]. It was found that cells lacking both the Min system and SlmA had a dramatic increase in FtsZ structures, compared to cells only lacking the Min system. The majority of these structures were located across the nucleoids. Furthermore, this study showed that a significant number of cells lacking SlmA formed a midcell septum across the nucleoid, indicating that SlmA is involved in an anti-guillotine mechanism [250].

Recently, the crystal structure was solved for SlmA and it shows that the protein is divided into two domains: a small N-terminal domain (residues 1-53 and a C-terminal domain (residues 54-198) [297]. Within the N-terminal domain there is a canonical helix-turn-helix motif that has been shown to bind DNA [250, 297]. A hydrophobic region of the C-terminal domain mediates dimerization of SlmA [297]. Furthermore, the crystal structure shows that SlmA dimerizes [297].

SlmA binds specifically to a palindromic DNA sequence that is found at 25-50 positions in the *E. coli* chromosome [297, 298]. The SlmA-DNA-binding sequences cluster within and around the origin of replication (ori), but are absent within and around the termination of replication (Ter) of the chromosome [297]. Furthermore, it has been shown that SlmA can bind DNA and FtsZ simultaneously [297] and that DNA binding activates the anti-FtsZ activity of SlmA [298]. Furthermore, DNA binding of SlmA increases the GTPase activity of FtsZ and once significant amounts of GTP is hydrolyzed, the FtsZ polymers disassemble [298, 299]. Interestingly, SlmA does not interact with the C-terminal tail of FtsZ [297], in contrast to FtsA and ZipA [127]. Electron microscopy studies indicate that SlmA causes FtsZ to form long helical-like structures with an anti-parallel arrangement [297]. These helical-like structures are significantly different from the normal bundling of FtsZ polymers. Thus, it seems that SlmA does not prevent FtsZ from polymerizing, rather it affects the higher order assembly of the FtsZ polymers [297]. Although, a recent publication proposes another mechanism in which SlmA is monomeric and inactive in the cytoplasm. Upon binding of SlmA to its DNA binding sequence, it dimerizes and this disrupts the interactions between FtsZ molecules and GTP is hydrolyzed [298].

Concluding remarks and future perspectives

The aim of this thesis is to better describe and understand the cell division event in Gram-negative bacteria. Although this process has been extensively studied for at least 50 years, several fundamental questions have not been fully addressed. These include how the contraction process occurs in dividing bacteria and how the cell division proteins interact to form a functional division complex.

In paper I and II, I have focused on describing the contraction process. First, the diffusion of GFP through the septum of dividing cells was studied using FRAP. This approach enabled us to determine the septal radii in each cell and a population average for the Z-ring contraction. The population average indicated a linear contraction process, however this process was more complex in individual cells (Paper I). The timing of the contraction of the outer membrane in dividing *E. coli* has been discussed for a long time and studies have indicated that the inner membrane and outer membrane either constrict together or sequentially [117, 300, 301]. Both these models suggested that at the very late stage of cell division the cytoplasm closes before the periplasm, although this has never been addressed experimentally. We again used our FRAP approach on a large number of dividing cells and could conclude that the cytoplasm closes before the periplasm (Paper II). Both these findings have given new perspectives on the contraction process and opens up new questions. For instance, does the entire divisome assemble before the septum starts to contract? It would be interesting to determine if the proteins suggested to perform the contraction of the outer membrane assemble at a later stage, after the contraction of the inner membrane is initiated. Furthermore, it is not known how the cell division proteins assemble at the septum. Do they form discrete protein clusters or a large continuous biomass? Both these questions could be answered by modifying the FRAP assay used in this thesis. For instance, the localization of cell division proteins can be monitored simultaneously as the contraction process by combining our FRAP assays with fusions of these proteins with fluorescent proteins. Furthermore the diffusion in the inner membrane, which can be studied by fusing a membrane protein to GFP or by using a lipid dye, may give insights to if the cell division proteins form clusters or a continuous biomass.

In paper III and IV, I have focused on describing protein: protein interactions among the cell division proteins. These interactions have previously been studied using genetic approaches of modified proteins (see for instance [156, 157]). In our lab we have developed methods to study protein oligomerization of membrane proteins, based on a combination of BN-PAGE and cysteine cross-linking. In these two studies I have

mapped the oligomeric state of PBP5 (Paper III) and ZipA (Paper IV). We concluded that PBP5 is a homo-oligomer, most likely a dimer. The cross-linking data can be used to map the dimer into a back-to-back conformation of the stem domains, while the catalytic domains have the freedom to move at the same time. ZipA was also found to homo-dimerize. Its dimerization was independent of FtsZ and the dimerization of ZipA could be a possible way for ZipA to bundle the FtsZ protofilaments, as the dimer could bridge two FtsZ polymers. The BN-PAGE and cross-linking approach that we used to determine that the homo-oligomeric state for both PBP5 and ZipA can also be used to further map the protein: protein interactions and oligomerization among other cell division proteins. Several of these important protein: protein interactions have not been fully identified and the responsible protein motifs are not known. Furthermore a future aim should be to isolate the entire division complex for instance by cross-linking, to determine all proteins involved in the division process.

All four studies presented in this thesis have contributed to the field of cell division. This research field has become more important as the problems with antibiotic resistance have become more severe. The components of the bacterial cell division machinery are new antibiotic targets as cell division is an essential cellular event and the proteins involved are unique to bacteria [125]. To understand the mechanisms used by the cell division proteins and the protein: protein interactions among them can be a first step in determining new antibiotic targets. These new antibiotic compounds could be small molecules that stop the contraction process in dividing bacteria or inhibit protein: protein interactions at the division site.

Populärvetenskaplig sammanfattning på svenska

Biokemi är läran om kemin i levande organismer. Den handlar om att förstå och beskriva det stora antal olika molekyler som en cell är uppbyggd av: hur de reagerar med varandra, hur de bildas och bryts ned.

Bakterien *Escherichia coli* återfinns normalt i tarmarna hos varmblodiga djur och är oftast ofarlig för värddjuret. *E. coli* är ett utmärkt modellsystem inom biokemin. *E. colis* DNA kodar för omkring 4600 proteiner medan människans DNA kodar för 22 000 proteiner, trots detta har många proteiner i *E. coli* har likheter med proteiner i människor. Därmed kan man genom att studera enskilda proteiner och större mekanismer i *E. coli* öka förståelsen för biokemin i människor. *E. coli* används även som modellsystem för att förstå hur bakterier fungerar och vilka specifika mekanismer som sker i dessa celler.

En mekanism som studerats särskilt noggrant är hur bakterier förökar sig, vilket sker genom celldelning. Celldelningen är en för bakterien livsviktig process och om den stoppas kommer bakterien att dö. Processen har studerats i *E. coli* sedan mitten på 1900-talet, trots detta återstår det mycket forskning för att få full förståelse hur celldelningen sker. De proteiner som styr och utför celldelningen har på senare tid föreslagits som målproteiner för ny antibiotika. Problemet med antibiotikaresistens blir allt vanligare vilket gör det extra angeläget att hitta dessa nya målproteiner.

Jag har i min forskning undersökt de mekanismer som leder till att bakterien *Escherichia coli* delar sig och hur de proteiner som är involverade i celldelningen samarbetar. Förhoppningen är att den ökade förståelse för celldelningen som mina studier gett kan bli ett steg mot utvecklingen av ny antibiotika.

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