Cell division in walled Bacillus subtilis and cell wall-lacking Acholeplasma laidlawii

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CHAPTER 1

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INTRODUCTION

Cell division of most walled bacteria is initiated by polymerization of the tubulin homologue FtsZ into a ring-like structure at midcell, which functions as a scaffold for the recruitment of membrane proteins that synthesise the septal cell wall \(^1,2\). But there are some exceptions, such as \textit{Streptomyces}, which possess an FtsZ-independent division machinery and proliferate via branch formation \(^3\).

There also exist cell wall-deficient bacteria, such as the \textit{mycoplasmas} and the so called bacterial L-forms. These bacteria lack a peptidoglycan synthesis machinery and require osmotically supportive medium for propagation. \textit{Mycoplasmas} have an FtsZ homologue, but L-forms can proliferate without FtsZ and instead use membrane blebbing to divide. Here, we review bacterial cell division mechanisms based on the presence or absence of a cell wall.

1. Cell division in walled bacteria

Cell division is best studied in the Gram-positive \textit{Bacillus subtilis} and Gram-negative \textit{Escherichia coli} model systems. Both are rod-shaped and divide symmetrically along their short axis into two daughter cells of equal size. \textit{B. subtilis} can also divide asymmetrically in order to form endospores. The process of bacterial cell division can be dissected into three sequential steps: (i) FtsZ, the bacterial homolog of the eukaryotic protein tubulin, polymerizes into a ring-like structure at midcell \(^1\). (ii) Once this Z-ring is assembled, the so called late cell division proteins are recruited \(^4\). (iii) This is then followed by septum synthesis.

1.1 FtsZ and the Z-ring

\textit{ftsZ} was first described as a cell division associated gene in 1980 \(^5\). The breakthrough came when, using electron microscopy (EM) and immunogold staining, it was shown that FtsZ forms a ring structure at nascent division sites in \textit{E. coli} \(^6\). The protein is universally
conserved among almost all bacteria (rod-, round-, or more complex-shape species), and is also found in mitochondria and chloroplasts of several eukaryotes. FtsZ has two domains, one of which is a GTPase domain located at its N-terminus. FtsZ monomers bind GTP through their GTP-binding domain and then polymerize in a head-to-tail conformation to create a tubulin-like loop that functions as GTP hydrolase. This GTP hydrolysis is not essential for polymerization, and an ftsZ mutant that is impaired in GTP hydrolysis, but not in GTP binding, is still capable of self-polymerization. However, GTP hydrolysis plays a regulatory role. The Z-ring is not a static structure and undergoes constant remodelling with rapid turnover dynamics, which depends on the GTPase activity of FtsZ. In addition, nucleotide hydrolysis by FtsZ polymers was also proposed to contribute to the force generating during cell division. EM studies showed that upon GTP hydrolysis, FtsZ polymers transform from straight protofilaments to curved filaments, and it was proposed that this conformational change can transmit mechanical force to the membrane, providing energy for the constriction of the Z-ring. This assumption was confirmed by Osawa et al., who constructed a membrane binding FtsZ variant, by fusing a membrane targeting amphipathic helix to its C terminus, and mixed this FtsZ mutant with lipid vesicles, which resulted in visible constrictions of liposomes, a reaction that required GTP hydrolysis.

The C-terminus is the binding domain for many FtsZ-interacting proteins, such as FtsA and ZipA in E. coli, and FtsA, SepF and EzrA in B. subtilis. A small region in the C-terminus, called the C-terminal variable (CTV) region, was shown to affect polymerization of FtsZ and differs between E. coli and B. subtilis. The CTV in E. coli FtsZ is neutral whereas this domain is positively charged in B. subtilis. Interchanging the CTV domains completely abolished the self-interaction of B. subtilis FtsZ, suggesting that electrostatic forces are important for FtsZ polymer assembly in this organism.

In vitro, FtsZ protofilaments tend to form lateral interactions, and under the electron microscopy, FtsZ polymers can form very different conformations in different buffers, including filaments, sheets with a lattice similar to that microtubules, tubular structures...
with longitudinal arrays of protofilaments \(^{24}\), and ribbons \(^{25}\). Addition of special metal ions, like Ca\(^{2+}\) and Rb\(^{+}\) can also stimulate the GTP-dependent polymerization of FtsZ \(^{26,27}\).

To examine Z-ring formation \textit{in vivo}, fluorescence microscopy was used using cells expression GFP tagged FtsZ \(^{6,28,29}\). After assembly into a ring-like structure at midcell, the diameter of the ring becomes smaller as cell division progresses, and finally only a fluorescent dot is visible that disappears after the daughter cells separate \(^{30}\). Recent super-resolution microscopy revealed that the structure of FtsZ rings in both \textit{E. coli} and \textit{B. subtilis} is discontinuous, displaying large nodes of high concentration separated by thinner regions of low concentration \(^{31}\). This was also found in other bacteria, including \textit{Caulobacter crescentus} \(^{32}\) and \textit{Streptococcus pneumonia}, in which FtsZ forms patchy foci \(^{33}\). A curious FtsZ assembly pattern occurs in the longitudinally dividing symbiotic bacteria like \textit{Candidatus Thiosymbion oneisti} and \textit{Thiosymbion hypermnestrae}, which grow on the surface of marine nematodes \(^{34,35}\). In these bacteria, FtsZ polymerises into a discontinuous arc-like structure along the long axis of the cell (Fig. 1).

### 1.2. FtsZ regulators

Polymerization of FtsZ into a Z-ring establishes the future division site. Due to the fact that the expression levels of FtsZ in both \textit{B. subtilis} and \textit{E. coli} is constant throughout the whole cell cycle \(^{11,36}\), Weart and Levin proposed that the timing of FtsZ assembly is governed primarily through cell cycle-dependent changes in polymerization kinetics of FtsZ regulated by specific regulatory proteins \(^{36}\).

#### 1.2.1 Membrane anchors of the Z-ring

To form a Z-ring and to transmit the constrictive force to the membrane, it is essential for FtsZ polymers to be anchored to the cell membrane \(^{18,37-39}\). The main membrane anchor is FtsA, which is widely conserved throughout the bacterial kingdom \(^{40,41}\). SepF is a membrane anchor present in Gram-positives and cyanobacteria \(^{42}\). \textit{B. subtilis} has both SepF and FtsA, \textit{E. coli} has only FtsA, but has a transmembrane protein, ZipA, that also binds to FtsZ \(^{43,45}\).
Fig. 1. Schematic representation of perpendicular and longitudinal cell division

(A) Rod-shaped bacteria such as *E. coli* and *B. subtilis*, grow in length prior to FtsZ assembly into a ring-like structure (red circle), and Z-ring mediates the septum synthesis at midcell, producing two identical daughter cells.

(B) The marine ectosymbiont, *Candidatus Thiosymbion oneisti* attaches at one pole to the surface of the nematode *Laxus oneistus*. This bacterium grows wider along its short axis prior to FtsZ assembly (red), which occurs at midcell parallel to the long axis of the cell, presumably to permit both daughter cells to remain adhered to the host.

1.2.1.1 FtsA

The gene coding for FtsA is often found upstream of *ftsZ*, in an operon in the division cell wall (dcw) cluster. Its localization at midcell depends on the presence of FtsZ. Unlike FtsZ, FtsA is not an essential protein in all bacteria, e.g. deletion of *ftsA* in *B. subtilis* results in elongated non-sporulating cells, but the mutant is still able to survive. The reason for this is that *B. subtilis* also contains SepF, the alternative FtsZ membrane anchor.

On the basis of its fold, FtsA was originally classified as a member of the actin/HSP70 protein family. The crystal structure of FtsA from *Thermotoga maritima* revealed that the protein is very similar to that of eukaryotic actin. Indeed, FtsA is able to form actin-like protofilaments. FtsA comprises two main domains, each of which can further be divided...
into two subdomains: 1A, 2A, 2B and 1C \cite{52}. The two main domains are connected by a cleft region that was found to contain both ATP and an Mg\textsuperscript{2+} ion, strongly suggesting that this is the ATP-binding site \cite{51,53,54}. The 2B subdomain was reported to be responsible for binding to the 16 amino acid C-terminal tail of FtsZ \cite{50,55}. The 1C subdomain of \textit{E. coli} FtsA is required for recruitment of late division proteins such as FtsN and FtsI \cite{56,57}. However, so far there is no evidence that \textit{B. subtilis} FtsA also interacts with late proteins.

FtsA forms extremely stable polymers in the presence of ATP, but less stable when mixed with ADP \cite{54}. However, a clear ATPase activity has only been reported for \textit{B. subtilis} FtsA \cite{51}, and for example \textit{Streptococcus pneumoniae} FtsA does not exhibit ATPase-dependent polymerization \cite{58}. However, mutations in the ATP-binding domain of FtsA abolishes self-assembly and the interaction with FtsZ \cite{55}.

FtsA contains a membrane-targeting amphipathic alpha helix at its extreme C-terminus, and this motif is separated from the core protein by a flexible linker region \cite{50}. Binding of this amphipathic helix to the cell membrane is sensitive for the membrane potential, and addition of the proton motive force neutralizing ionophore CCCP completely abolished its septum-localization \cite{59}. Deletion of this domain renders the protein inactive \cite{40}, however, the amphipathic helix can be replaced by various unrelated membrane-targeting amphipathic helices without affecting its activity \cite{40,53}.

The key function of FtsA is to anchor FtsZ protofilaments to the cell membrane. To determine whether this interaction can result in membrane invagination, Osawa \textit{et al.} fused a functional FtsZ-YFP reporter protein with a C-terminal amphipathic helix and showed that this fusion protein is able to constrict tubular liposomes \cite{60}. In later experiments, the same group reconstituted FtsA and FtsZ-YFP without the amphipathic helix, and again they were able to show division like events with liposome \cite{61}. These experiments strongly suggested that the increased curvature of GTP hydrolysing FtsZ polymers is able to constrict cells when these polymers are attached to the cell membrane by FtsA.

\textbf{1.2.1.1 SepF}
sepF is located upstream of the cell division regulator gene divIVA in the dcw cluster. The gene is highly conserved in Gram-positive bacteria and cyanobacteria, and its role in cell division was first discovered in *Streptococcus pneumoniae* and *Synechococcus elongates*\(^{62,63}\). In *B. subtilis*, disruption of sepF is synthetic lethal when combined with an ezrA mutant, resulting in filamentous cells\(^6^4\). In addition, Ishikawa *et al.* showed that a sepF ftsA double deletion is also synthetic lethal and abolishes Z-ring assembly, and that overexpression of SepF compensates the filamentous cell phenotype caused by deleting ftsA\(^6^5\), indicating an overlapping function for FtsA and SepF. In *B. subtilis*, SepF is regarded as the second membrane anchor for FtsZ. However, not all bacteria contain FtsA, and these bacteria generally contain a SepF homolog, such as Mycobacteria and Streptomycetes species, in which SepF is an essential protein\(^6^6\).

Similar to FtsA, SepF contains a membrane-targeting amphipathic helix, but at its N-terminus\(^4^2\). With its conserved C-terminal domain the protein directly binds to the last 16 residues of the C-terminus of FtsZ\(^2^0,4^2\). EM images revealed that purified SepF can polymerize into large regular ring structures with an average inner diameter of 40 nm, which is close to the average thickness of septa (33 nm)\(^4^2,6^7\).

Interestingly, when SepF rings were mixed together with lipid membranes, the liposomes were strongly deformed, forming amorphous elongated tubular structure, and some small liposomes were found inside SepF rings\(^4^2\), suggesting that the N-terminal amphipathic helix domain is located inside the protein rings. Furthermore, SepF appeared to increase the bundling of FtsZ polymers as well\(^6^8\). SepF rings were able to bundle FtsZ protofilaments into strikingly long and regular tubular structure with an average diameter of 48 nm, close to that of SepF protein rings. Based on these observations, it was proposed that SepF does not form rings, but assembles into arcs (semi-circles) on top of the leading edge of nascent septa, thereby attaching polymerized FtsZ to the cell membrane, and helping them to align parallel to the plane of division\(^4^2\). When SepF is absent cells form very irregular septa\(^6^4\).
1.2.1.1 ZipA

ZipA is a transmembrane FtsZ-interacting protein and conserved in the Gram-negative gammaproteobacteria \(^{43}\). It is composed of a single N-terminal transmembrane domain and a large globular cytoplasmic C-terminal domain that is connected by a linker domain mainly consisting of proline and glutamine residues \(^{43,69,70}\). ZipA is dispersed throughout the cell membrane until cell division is initiated, when it is captured by Z-rings by direct interaction with the C-terminal tail of FtsZ \(^{43-45,69-72}\). This recruitment does not depend on the presence of FtsA, suggesting that ZipA can also function as membrane anchor for FtsZ polymers \(^{43-45}\). Indeed, a mixture of purified ZipA and FtsZ inside giant unilamellar vesicles (GUVs) caused invagination, resembling the constriction of the cytoplasmic membrane \(^{73,74}\).

The crystal structure of ZipA revealed a hydrophobic cleft contained in its C-terminus with a similarity to the fold found in many RNA-binding proteins \(^{69,70}\), and this domain appeared to function as the binding pocket for FtsZ. The C-terminal ZipA domain has been shown to promote the assembly of FtsZ protofilaments into thick bundles and sheets \(^{71,75}\), and overexpression of ZipA rescues the division defect of a thermosensitive \(ftsZ84\) mutant \(^{75}\). ZipA is also required for the recruitment of the late division proteins FtsK and FtsQ to the Z-ring \(^{76,77}\).

1.2.2 Other regulators of Z-ring assembly

1.2.2.1 ZapA

Z-ring assembly is regulated both negatively and positively to assure the proper timing and correct localization at midcell. One of the positive regulators is ZapA, which is composed of only 85 amino acids, and was discovered in \(B.\ subtilis\) when screening for proteins that, when overexpressed, would restore cell division caused by overproduction of the negative FtsZ regulator MinD \(^{78}\). ZapA is widely conserved among Eubacteria \(^{78}\), and is not essential, but its deletion results in conditionally lethality in various sensitized backgrounds, e.g. a \(zapA\) null mutant becomes lethal when expression of FtsZ is reduced \(^{78}\), and is unable to form
normal Z-rings when combined with other cell division mutants, including \textit{ezrA} and \textit{whiA} knock outs (see below) \cite{78,79}.

The crystal structure of ZapA from \textit{Pseudomonas aeruginosa} revealed that it consists of an N-terminal globular domain and C-terminal coiled-coil protrusion, and that it forms dimers by contacts between the small, globular N-terminal domains \cite{80}. A pair of ZapA dimers can form an anti-parallel tetramer by association through the C-terminal coiled-coil domain \cite{80}. ZapA tetramers are thought to link FtsZ polymers \cite{78,80}, and purified ZapA stimulates FtsZ polymerization and polymer stability \cite{78,81,82}.

\subsection*{1.2.2.2 ZapB, ZapC and ZapD}

In \textit{E. coli} several other cell division proteins have been identified and named Zap: ZapB, ZapC and ZapD. Like ZapA, they play a positive role in Z-ring polymerization, and they are conserved among gammaproteobacteria \cite{83-86}.

ZapB is also a small protein and consists of only 81 amino acids. \textit{zapB} null mutants appears slightly elongated, resulting in less and abnormal Z-ring formation, such as spirals and short helices \cite{83,87}. ZapB can form large protein bundles \textit{in vitro} and can directly interact with ZapA through its N-terminus \cite{83}. It has been proposed that ZapB increases the stability of the Z-ring by crosslinking ZapA molecules \cite{88,89}. In fact, recruitment of ZapB to the Z-ring depends on the presence of ZapA, and not on other early division proteins like FtsA and ZipA \cite{83,88}. Interestingly, detailed microscopic studies have indicated that ZapB forms a ring inside Z-rings \cite{88}. Overproduction of ZapB results in condense nucleoids, indicating a potential effect on the regulation of chromosome replication and/or segregation. In fact, it was found that ZapB directly interacts with MatP, a DNA-binding protein that binds the chromosomal terminus region and condenses this Ter region to ensure proper segregation of chromosomes \cite{90}. Recently, Buss \textit{et al.} found that ZapA and ZapB form a diffuse accumulation at nascent division sites independent of the presence of a Z-ring \cite{91}. A ZapA N60Y mutant, defective in the interaction with FtsZ, also accumulated at mid-cell, preceding Z-rings, and this localization required the presence of ZapB and MatP, providing further
support for the notion that the ZapA-ZapB-MatP complex coordinates chromosome segregation and cell division \textsuperscript{91}.

ZapC is larger protein containing 180 amino acids \textsuperscript{92}, and identified in \textit{E. coli} as being another protein that is recruited to Z-rings via direct interaction with FtsZ \textsuperscript{84,85}. Overproduction of ZapC resulted in elongated cells and abnormal Z-ring structures \textsuperscript{84,85}. Purified ZapC bundles FtsZ protofilaments and reduces the GTPase activity of FtsZ \textsuperscript{84}. ZapD is the last identified member of Zap proteins \textsuperscript{86}, and its co-localization with Z-rings is achieved through the interaction with the conserved C-terminal tail of FtsZ \textsuperscript{86}. Similar to other Zap proteins, its overexpression represses cell division and its deletion, combined with the heat-sensitive allele \textit{ftsZ84}, causes growth and division defects \textsuperscript{86}. Purified ZapD forms dimers in solution and increases bundling of FtsZ protofilaments by lowering the GTPase activity. Of note, these three Zap proteins (B, C and D) do not exist in Gram-positive bacteria.

\textbf{1.2.2.3 EzrA}

EzrA functions as a negative regulator of Z-ring assembly in \textit{B. subtilis} and was discovered in a study that looked for suppressor mutations of a temperature sensitive \textit{ftsZ} mutant \textsuperscript{93}. Overexpression of EzrA results in filamentous cells due to blockage of Z-ring assembly \textsuperscript{94}, and purified EzrA suppresses FtsZ polymerization \textsuperscript{95,96}. Detailed analysis revealed that EzrA reduces the affinity of FtsZ for GTP and accelerates the rate of GTP hydrolysis, which promotes disassembly of FtsZ polymers \textsuperscript{95}.

Although these reports suggest that Z-rings are more stable in the absence of EzrA, a deletion of \textit{ezrA} results in elongated cells, and such mutation is synthetic lethal when combined with mutations in other cell division genes, including \textit{sepF}, \textit{zapA} and \textit{whiA} \textsuperscript{64,79}.

EzrA contains an N-terminal transmembrane helix, and is recruited to the division site in an FtsZ-dependent manner \textsuperscript{93}. Its large cytoplasmic C-terminal domain consists of 4 coiled-coil regions \textsuperscript{93,94}, and is conserved throughout the low-GC-content Gram-positive bacteria \textsuperscript{94}. Cleverley \textit{et al.} have solved the crystal structure of the cytoplasmic domain of \textit{B. subtilis} EzrA and showed that the structure resembles eukaryotic spectrin, which connect the
actin cytoskeleton to the membrane-bound integrin proteins, stimulating membrane fission. EzrA interacts with FtsZ through a conserved 7 amino acids residue region at its C terminus, the so called QNR patch. Deletion of this QNR patch abolishes the recruitment of EzrA to the Z-ring and results in longer cells, typical for ezrA null mutants. Finally, EzrA has also been shown to play a role in the recruitment of penicillin binding protein PBP1 from the lateral wall to the division site, by interacting with the transmembrane domain of PBP1. This process requires the protein GpsB that bind to both EzrA and PBP1.

1.2.2.4 FtsEX

The ATP binding cassette (ABC) transporter complex FtsEX was first identified as cell division associated proteins in E. coli. An ftsEX deletion blocks cell division, and the FtsEX complex is recruited to Z-rings after the recruitment of FtsA and ZipA. The complex was found to act on FtsA and an ATPase mutant of FtsE appears to lock FtsA in an inactive form. Overexpression of a variety of divisome proteins, including FtsZ and FtsA, but also the late cell division proteins FtsQ and FtsN (see below), can restore this division defect in low-osmolality medium. The ATPase activity of FtsEX is also required for the interaction between the periplasmic loop of FtsX and the N-terminus of EnvC, an activator of the cell wall amidases AmiA and AmiB which are required for cell separation. Similarly, FtsX also acts as a regulator of PG hydrolysis in Streptococcus pneumonia, in which FtsX interacts with the N-terminal coiled-coil domain of PcsB, a putative CHAP (Cys, His, Asp) peptidase assumed to hydrolyse PG cross-links. In Mycobacterium tuberculosis, the extracellular domain of FtsX binds to the N terminal domain of the PG peptidase RipC, thereby activating this enzyme.

In B. subtilis, FtsEX was shown to play a role in sporulation initiation, and its absence results in a delay in sporulation. This sporulation defect can be bypassed by activating the key sporulation regulator Spo0A. B. subtilis FtsEX can also activate the endopeptidase CwlO, which is an ortholog of S. pneumoniae PcsB. The null mutant of
either *ftsE* or *ftsX* results in shorter and fatter cells, which can be rescued by addition of 
$\text{Mg}^{2+}$.

### 1.2.2.5 FtsK, SftA and SpoIIIE

Chromosome segregation occurs before cell division, but some DNA can remain in the vicinity of the closing septum and be trapped. DNA translocases are required to rescue such septum-trapped chromosomes. FtsK is the integral membrane ATP-dependent translocase in *E. coli*, and its absence results in filamentous cells 113, because of the blockage in the later stages of cell division 114-116. FtsK contains 4 transmembrane domains at its N-terminus, and a C-terminal nucleotide-binding domain involved in DNA translocation 117. FtsK is recruited to the division site via its N-terminal domain, which is essential for cell division 118,119. The N- and C-terminal domains are connected by a long 600 amino acid long linker domain that interacts with several different cell division proteins, including FtsI and the FtsB-FtsQ-FtsL complex 120,121.

In *B. subtilis*, two proteins share homology with FtsK, SpoIIIE involved in chromosome segregation during sporulation, and SftA 117,122-124. SpoIIIE is only essential for sporulation as it pumps the chromosome through the sporulation septum into the forespore 125-127. SftA functions during vegetative growth. It is not essential for cell division in *B. subtilis*, although *sftA* mutants are longer. The absence of SftA leads to a relative small increase in trapped chromosomes and the evidence for a role as DNA pump are rather limited 122. SftA does not contain transmembrane domains and it is assumed that the protein associates with the Z-ring by direct protein interactions 128. Recently, it was found that FtsA plays a role in this recruitment, based on the evidence that an *ftsA* deletion leads to a significant reduction in septal SftA recruitment 129.

### 1.3. Regulation of Z-ring placement

The correct placement of the Z-ring at mid-cell is primarily orchestrated by two systems, the Min system that prevents Z-ring formation near the polar region of the cell, and nucleoid
occlusion (NO) that avoids division occurring over the nucleoid (Fig. 2). It is likely that other systems are in place, since cell division in *B. subtilis* still occurs at mid-cell in the absence of both the Min and NO systems.\(^{130}\)

### 1.3.1. Min system

The Min system consists of the Z-ring assembly inhibitor MinC,\(^ {131}\), and a membrane-associated ATPase, MinD, which recruits MinC to the plasma membrane. In *E. coli*, the protein MinE is involved in the polar accumulation of the Min system, and in *B. subtilis* this is organized by the proteins DivIVA and MinJ. The absence the Min system results in division close to cell poles, generating anucleate minicells.\(^ {132,133}\)

MinC interacts directly with FtsZ by its N-terminal domain,\(^ {134-137}\), and its inhibitory effect on FtsZ polymerization has been revealed both *in vivo* as well as *in vitro*.\(^ {131,138,139}\) Its C-terminus is required for MinD interaction.\(^ {136}\) MinD is an ATPase belonging to the ParA/MinD family, and binds to the cell membrane via a C-terminal amphipathic alpha helix.\(^ {140}\) This membrane binding requires the membrane potential.\(^ {59}\)

In *E. coli*, the peripheral membrane protein MinE stimulates the ATPase activity of MinD, resulting in the disassociation of MinD from the membrane.\(^ {141,142}\) Subsequently, cytosolic MinD binds to ATP, forming a dimer that stimulates membrane association. This dynamic MinE-MinD interaction results in an oscillation of Min proteins from one pole to the other, causing a low time-averaged concentration of MinCD complex at mid-cell where the Z-ring assembles.\(^ {2}\) This dynamic self-organization of the Min system has also been shown *in vitro* using only MinD, MinE, ATP and a phospholipid membrane support.\(^ {143}\) The *B. subtilis* MinCD complex uses MinJ and DivIVA as topological markers, instead of MinE.\(^ {144-147}\) DivIVA is a membrane bound protein, and shows its preference for negatively curved membrane regions found at nascent division sites and cell poles.\(^ {148,149}\) MinJ is a trans-membrane protein that forms a molecular bridge between DivIVA and MinD.\(^ {146,147}\) The polar localization of DivIVA is static and the *B. subtilis* MinCD complex does not oscillate between cell poles.\(^ {150,151}\) Bacterial two-hybrid experiments suggested that MinJ can also interact with other...
division proteins like FtsA, FtsL, EzrA and Pbp2B\textsuperscript{147}, and it was later proposed that MinJ stimulate the disassembly of divisome complex after cell division\textsuperscript{152}.

1.3.2. Nucleoid occlusion

Another system that regulates proper division position is the nucleoid occlusion (NO) system. The NO proteins Noc in \textit{B. subtilis}\textsuperscript{153}, and SlmA in \textit{E. coli}\textsuperscript{154} were discovered by searching for functional overlapping protein of Spo0J, which involves in chromosome and plasmid partitioning\textsuperscript{155}. Absence of NO proteins increases the chance of division to occur over the nucleoid\textsuperscript{153,154}. Both Noc and SlmA bind to DNA. In \textit{B. subtilis}, about 70 Noc binding sites, with a 14 bp palindromic consensus sequence, have been identified via chromatin immune precipitation combined with microarray analysis (Chip-on-chip analysis)\textsuperscript{156}. Likewise, there are around 24-52 palindromic SlmA-binding sites in \textit{E. coli}\textsuperscript{157,158}. Strikingly, the Noc and SlmA binding sites cover the entire chromosome but are noticeably absent from the DNA replication termination (Ter) sites, which are located midcell between partially segregated daughter chromosomes. Therefore, Noc/SlmA has also been regarded as a timing regulator that couples the closing stages of DNA replication to the initiation of cell division\textsuperscript{156-158}. Despite their similar roles during cell division, Noc and SlmA show no homologies and they belong to the ParB and TerR DNA-binding protein families, respectively\textsuperscript{153,154}. SlmA interacts directly with FtsZ, and it was shown that the FtsZ binding site is locate close to the helix-turn-helix DNA-binding domain. Based on the crystal structure and EM experiments, it was suggested that SlmA forms dimers that link FtsZ protofilaments in an anti-parallel configuration, as such blocking the formation of functional FtsZ filaments\textsuperscript{157}, although another study showed that purified SlmA actively disassembles FtsZ filaments\textsuperscript{158}.

There is no indication that Noc directly binds to FtsZ in \textit{B. subtilis}, or to any other divisome protein. Noc contains an N-terminal amphipathic helix that anchors Noc to the cell membrane, so Noc recruits DNA loops to the cell membrane, and there is evidence that these large membrane-associated nucleoprotein complexes physically block the assembly of the Z-ring\textsuperscript{159}.
1.4. Late division proteins

Some time after the formation of a Z-ring, a period that can span at least 20% of the whole cell cycle, the late division proteins are recruited to the Z-ring (Fig. 2). These membrane proteins are involved in synthesis of the septum. Recent studies have shown that the septal peptidoglycan (PG) synthesising proteins move directionally along the division plane in a manner dependent on FtsZ treadmilling, which influences the rate of PG synthesis. So a more inclusive model was proposed to reconcile the previous FtsZ-centric model and this PG-centric force: In the initial FtsZ treadmilling-dependent stage, FtsZ could generate a small constrictive force for membrane deformation, but attachment of the membrane to the rigid PG would keep this deformation invisible. Invagination of the cell envelope is subsequently driven by active PG synthesis.

1.4.1. DivIB-DivIC-FtsL complex

A crucial set of late division proteins in *B. subtilis*, is formed by the bitopic transmembrane proteins DivIB, DivIC and FtsL. DivIC and FtsL tend to form a heterodimer, which is stabilized by interacting with DivIB. Their recruitment to the Z-ring depends on one another. FtsL is unstable and its cytoplasmic N-terminal domain can be recognized and then cleaved by the regulatory protease RasP. FtsL requires the presence of its partner proteins DivIB and DivIC to prevent proteolysis. DivIB is only required for temperatures above approximately 37 °C. In turn, the presence of FtsL is also necessary for the stability of DivIC. It is believed that this ternary complex acts as an important checkpoint for cell division in *B. subtilis*. Recently it was shown that the DivIB-DivIC-FtsL complex attracts the lipid II flippase MurJ (see below) to the septum in *Staphylococcus aureus*.

The homologues of DivIB-DivIC-FtsL complex also exist in Gram-negative bacteria like *E. coli*. Here they are called the FtsQLB complex. FtsQ, the DivIB homolog, is essential for cell division in *E. coli* and the localization of FtsQLB depends on the presence of FtsK.
via a direct interaction between FtsK and FtsQ \textsuperscript{174}. In addition, the FtsQLB complex is also required for the recruitment of the late proteins FtsW and FtsI \textsuperscript{175-177}.

Fig. 2. Summary of the division process in \textit{B. subtilis}.

FtsZ assembles into Z-rings anchored by FtsA and SepF. ZapA crosslinks FtsZ polymers. EzrA functions as negative regulator, and is also required together with GpsB, for the mid-cell recruitment of penicillin binding protein PBP1. The DNA translocase SfIA also localizes at the septum via direct interactions with FtsA. The Min system inhibits Z-ring formation at both poles and Noc-mediated nucleoid occlusion assures that the Z-ring fails to form over the chromosome. After Z-ring assembly, the late division DivIB-DivIC-FtsL complex, FtsW, and Pbp2B are recruited to the Z-ring in an interdependent manner. The flippase MurJ/Amj also plays an important role in septum synthesis.

1.4.2. Lipid II flippase MurJ and Amj, and the peptidoglycan polymerase FtsW

Cell wall synthesis begins on the inner face of the cytoplasmic membrane \textsuperscript{178} where a set of highly conserved enzymes catalyse the synthesis of the lipid-linked peptidoglycan precursor lipid II, which is composed of undecaprenyl-pyrophosphate linked to N-acetylglucosamine-N-acetylmuramic acid pentapeptide. The lipid II is then translocated to the outer surface of the membrane, and incorporated into the existing peptidoglycan by the cell wall synthetic machineries composed of penicillin-binding proteins (see below) and additional factors \textsuperscript{179}. This lipid II translocation process requires the assistance of lipid II flippases.

The protein MurJ functions as the flippase in \textit{E. coli} and contains 14 transmembrane domains \textsuperscript{180}. It localizes both in the lateral membrane and at midcell, and its septal recruitment requires FtsW (see below) and lipid II synthetic machinery \textsuperscript{181}. Cells depleted for
MurJ or having an inactivated murJ variant, showed a severe defect in lipid II translocation and incorporation into the peptidoglycan \(^{182,183}\). The essentiality of MurJ has also been observed in *S. aureus*, in which the septal recruitment of MurJ by the DivIB-DivIC-FtsL complex drives peptidoglycan incorporation at midcell \(^{163}\). So the recruitment of MurJ has been regarded as a checkpoint in *S. aureus* cytokinesis.

However, in *B. subtilis*, cells lacking MurJ are still viable and have no detectable defect in cell wall synthesis \(^{184,185}\). This is because of the presence of Amj, which was identified as a novel type of lipid II translocase, and its absence is synthetic lethal when *murJ* is deleted \(^{186}\). Importantly, the expression of either *B. subtilis* MurJ or Amj in *E. coli* supports lipid II flipping and viability in the absence of *E. coli* MurJ \(^{186}\), indicating that two flippases, MurJ and Amj, can translocate lipid II in *B. subtilis*.

For a long time FtsW was also regarded as the candidate flippase in *E. coli*, based on the fact that it also showed lipid II flippase activity \(^{187}\). But a recent study of *E. coli* FtsW suggested that FtsW functions as a peptidoglycan polymerase that works together with class B penicillin-binding proteins (PBPs, see below) to synthesize septal peptidoglycan \(^{188}\).

### 1.4.3 Penicillin binding proteins (PBPs)

After lipid II is translocated by flippases to the extracellular membrane surface, it is incorporated into peptidoglycan \(^{189}\). This peptidoglycan synthesis process requires penicillin-binding proteins (PBPs), which were initially identified as targets of β-lactam antibiotics such as penicillin. In *E. coli*, The PBPs comprise several type of enzymes, of which the most important ones are class A PBPs (aPBPs) and class B PBPs (bPBPs) \(^{190}\). The aPBPs consist of a peptidoglycan glycosyltransferase domain for lipid II polymerization and a transpeptidase domain that crosslinks the resulting glycan strands \(^{191}\). The bPBPs contain a transpeptidase domain and a domain of unknown function.

Of all the PBPs in *B. subtilis*, only PBP2B and PBP1 are the ones known to be involved in septal peptidoglycan synthesis. PBP2B is an essential protein with a transpeptidase domain \(^{192}\), and in *B. subtilis* its depletion results in filamentous cells that will
eventually lyse \(^{171}\). It is recruited to the division site by interacting with other component of the division machinery, including DivIB-DivIC-FtsL \(^{164}\). Recently, it was shown that a mutant of Pbp2B, with inactivated transpeptidase activity, is still viable, but now needs the assistance of the non-essential PBP3, to synthesise a septum \(^{193}\). Another cell division associated PBP in \textit{B. subtilis} is PBP1, encoded by the gene \textit{ponA}, which contains both transglycosylase and transpeptidase activities \(^{194}\). Unlike PBP2B, PBP1 depletion showed no significant effect on cell survival, but instead resulted in slower growth \(^{194}\). The recruitment of PBP1 to the divisome depends on the presence of other late proteins including Pbp2B, DivIC and DivIB \(^{195}\), as well as the early proteins EzrA and GpsB \(^{99}\).

2. Cell division in cell wall-deficient bacteria

Cell wall-deficient bacteria are pleomorphic and occur naturally, like the \textit{Mycoplasmas}, or they can be generated by cell wall-inhibiting antibiotics resulting in so called L-forms. Some of these cell wall-deficient bacteria can propagate without an FtsZ-based division machinery. E.g. the bacterial L-form cells proliferate by random membrane extrusions and blebbing, and some mycoplasmas, such as \textit{Mycoplasma pneumonia} \(^{196-199}\), \textit{Mycoplasma mobile} \(^{200}\), \textit{Mycoplasma genitalium} \(^{201}\) and \textit{Mycoplasma penetrans} \(^{202}\), use a structure, known as the attachment organelle to glide along surfaces, and which is able to pull cells apart and as such separates the two daughter cells.

2.1. Proliferation in L-forms

L-forms were identified from patients containing β-lactam antibiotic-resistant infections. In the beginning, people were confusing L-forms with \textit{mycoplasmas}, which are naturally existing cell wall-deficient bacteria that have undergone millions of years of evolution to adapt to a life without a cell wall. However, L-forms are generated artificially and are generally, also able to switch back to the walled state.

The general principle to generate L-forms is to inhibit cell wall synthesis by e.g. blocking the synthesis of the peptidoglycan precursor lipid II by depletion of the \textit{murE} operon
or by taking advantage of antibiotics. A range of organisms, including *Corynebacterium glutamicum*, *S. aureus* and *E. coli*, are able to switch to the L-form state by addition of antibiotics such as phosphomycin or D-cycloserine, which repress the synthesis of lipid II. Another method to obtain the L-form is to increase the accumulation of membrane lipid, leading to the increase in the ratio of cell surface area to the volume. To some degree, the above two ways for L-form generation is through the same mechanism, since repression of PG precursor synthesis was discovered to result in increased membrane synthesis indirectly, although the detailed mechanism is unclear yet. *B. subtilis* L-form generation also requires the inactivation of *ispA* encoding an intracellular protease. Kawai *et al.* discovered that a blockage of cell wall synthesis results in the increased level of reactive oxygen species (ROS) originating from the electron transport pathways, and this can be mitigated by inactivating expression of the electron transport chain. *IspA* is a component enzyme in the polyprenoid synthetic pathway, and this pathway leads to the formation of heptaprenyl diphosphate, a substrate for synthesis of menaquinone which is involved in the electron transport chain (ETC) system. Deletion of *ispA* is able to reduce the ROS originating from ETC pathway.

L-forms are able to proliferate without FtsZ. When the cellular localization of FtsZ was monitored using a FtsZ-GFP reporter fusion, no regular ring-like structures were observed at constrictive site, instead, FtsZ filaments were found randomly distributed over the cells. Time-lapse experiments showed that *B. subtilis* L-forms form membrane protrusion that expand into long tubular shapes, which then resolve into a chain of small cells. In some cases, the bulges or dimples erupt at multiple points along the cell surface, followed by the formation of multiple progenies (Fig. 3).

To determine what is necessary for this alternative division process, Mercier *et al.* screened mutants that were unable to survive as an L-form but could grow normally in the walled state, and found that the gene *bkd*, required for branched-chain fatty acid (BCFC) synthesis, was inactivated, affecting the ratio of anteiso- versus iso-branched chain fatty acids. This ratio plays an important role in controlling membrane fluidity in Gram-positive
bacteria, and an increase in anteiso-branched chain fatty acids increases the fluidity of the cell membrane, thus facilitating membrane extrusions in L-forms.

2.2 Cell division in mycoplasmas

Mycoplasmas are naturally existing bacteria without cell wall, and only have a cell membrane protected by a layer of polysaccharides and carbohydrates. They are classified as mollicutes and are assumed to that have undergone a reductive evolution from the firmicutes. Interestingly, in the well-studied mycoplasmas, such as Mycoplasma genitalium, FtsZ is not essential for growth. This species is motile and glides over surfaces using a membrane protrusion formed at one cell pole, also called attachment organelle (AO), and it is assumed that this machinery forces cell division in the absence of FtsZ, as schematically outlined in Fig. 4. ΔftsZ mutants exhibited reduced gliding motion and are fixed at the same location for extended periods of time. In addition, a knockout of ftsZ is synthetic lethal in cells that have an impaired attachment organelle.
Fig. 4. Proposed model for *M. pneumoniae* cell division

In the predivisional cell (A), attachment organelle (AO) locates at one pole with chromosome (Red) attached. (B) The AO replicates into a second AO with newly replicated DNA attached. (C) The movement of the cell pulls the newly generated AO to the other cell pole. (D) The AOs continue to stretch and pull the cell body into two nascent daughter cells, meanwhile the Z-ring (blue) is forms at mid-cell. (E) The Z-ring constricts, eventually resulting in two nascent daughter cells (F).

Based on this, it has been proposed that FtsZ and the attachment organelle work together to effectuate cell division. As shown in Fig. 4, cell division is then initiated with the formation of AOs at the cell poles that pulls the cells in opposite directions. Finally, FtsZ provides the constriction force to separate the two daughter cells.

Many mycoplasmas are non-motile and it is assumed that they use only an FtsZ-depending mechanism for division. However, the non-motile *Mycoplasma mycoides* has been shown to proliferate when *ftsZ* was deleted. How this mutant divides is unknown, but it might use the membrane blebbing mechanism found in L-forms.
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