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Diverse Paths to Midcell: Assembly of the Bacterial Cell Division Machinery

Review

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At the heart of bacterial cell division is a dynamic ring-like structure of polymers of the tubulin homologue FtsZ. This ring forms a scaffold for assembly of at least ten additional proteins at midcell, the majority of which are likely to be involved in remodeling the peptidoglycan cell wall at the division site. Together with FtsZ, these proteins are thought to form a cell division complex, or divisome. In *Escherichia coli*, the components of the divisome are recruited to midcell according to a strikingly linear hierarchy that predicts a step-wise assembly pathway. However, recent studies have revealed unexpected complexity in the assembly steps, indicating that the apparent linearity does not necessarily reflect a temporal order. The signals used to recruit cell division proteins to midcell are diverse and include regulated self-assembly, protein–protein interactions, and the recognition of specific septal peptidoglycan substrates. There is also evidence for a complex web of interactions among these proteins and at least one distinct subcomplex of cell division proteins has been defined, which is conserved among *E. coli*, *Bacillus subtilis* and *Streptococcus pneumoniae*.

Introduction

In the 1960s, researchers set out to characterize many of the cellular processes of the bacterium *Escherichia coli* by obtaining a large collection of thermosensitive mutants affecting the growth and replication of the organism. Among these mutants was a class of strains specifically defective in cell division which produced long filamentous cells at high temperature [1,2]. The mutations allowed identification of a set of genes (filamentation thermosensitive, *fts*), the products of which are essential for cell division. Mutants defective in these genes replicate and segregate their chromosomes normally, but are unable to divide and thus exhibit a characteristic filamentous phenotype (Figure 1A,B).

In a seminal experiment performed more than a decade ago, the first and most abundant of these cell division proteins, FtsZ, was shown to localize to the bacterial midcell (Figure 1C) [3]. In the ensuing years, work by various laboratories, using classical genetic approaches as well as novel tools including fluorescence microscopy and bioinformatics, has led to the identification of at least 15 proteins that play a role in proper division of the cell [4,5]. Because these proteins co-localize at midcell throughout the division process, it is generally assumed that they assemble into a large cell division complex or divisome.

Much remains to be discovered about the nature of the divisome, including the precise molecular functions of many of its components (Figure 2) and the mechanisms by which these proteins are assembled at the cell center. Progress has, to some degree, been slowed by the very nature of the problem — the complex consists of cytoplasmic, inner membrane-embedded and periplasmic components and the majority of these components are essential for viability. Moreover, the existence of this complex may well be fundamentally linked to the spatial organization of the membrane and peptidoglycan at the nascent division site.

New approaches have been critical to furthering our understanding of bacterial cell division. These include the use of computational biology, small molecules both to inhibit protein function and as imaging reagents, advanced *in vivo* imaging, which has allowed an appreciation of the dynamics of division, and biochemical assays to physically assess both the functions of and interactions between these proteins. At the same time, the application of genetic techniques, including mutant analysis, synthetic lethal and two-hybrid screens, as well as gene fusions, has continued to be invaluable for identifying novel proteins involved in dividing the cell and for understanding the web of interactions among the divisome components. Finally, many of these proteins are widely conserved, while others appear largely confined to one or another bacterial subgroup [6]. Examination of this process in a variety of diverse organisms has been particularly useful in defining both the core division pathway and how it has been adapted to fit the shape and cell envelope structure of each species (Box 1).

Establishing the Division Site

FtsZ

At the center of the cell division process is the GTPase FtsZ. It was the first cell division component found to be localized specifically to the midcell [3], where it forms a ring-like structure known as the Z-ring. The ~15,000 copies of FtsZ per cell are more than sufficient to span the circumference of the cell at least several times, which is consistent with the Z-ring being composed of multiple strands of FtsZ polymers [7]. It remains unclear, however, whether the Z-ring is composed of a single linear filament or an assembly of short protofilaments, as has been proposed recently [8]. Close examination of FtsZ outside the Z-ring or of cells overproducing FtsZ shows that FtsZ can form a helical polymer, suggesting that the basic form of the FtsZ ring is actually a tight spiral rather than a closed ring [9–11]. The Z-ring is subsequently used as a scaffold for assembly of the remaining cell division components.

FtsZ is conserved in all bacteria, with a few notable exceptions, such as *Chlamydia* and some archaeal

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species [6]. There are also homologues in higher eukaryotes where its role in cell division has been maintained for dividing plastid organelles and mitochondria [12]. In organisms without a cell wall, FtsZ is the only conserved protein of the cell division machinery, suggesting that it may provide the major force in constricting the cytoplasmic membrane. This force has been postulated to derive either from a shift in polymer curvature due to GTP hydrolysis or via a purse string model driven by depolymerization of the FtsZ ring [13].

Early work in the field noted some similarities between FtsZ and tubulin, but it was not until the crystal structure was solved that the similarity between these two proteins was fully appreciated [14]. Like tubulin, FtsZ polymerizes in a GTP dependent fashion and both binds and hydrolyzes GTP [15,16]. Perhaps not surprisingly, given these similarities to tubulin, the Z-ring is a highly dynamic structure. Only about 30% of FtsZ monomers are associated with the Z-ring at a given time and the FtsZ within the ring undergoes rapid exchange with a cytoplasmic pool of FtsZ with a half-life of approximately eight seconds [8,13,17]. Experiments using a temperature-sensitive allele of *ftsZ* indicated that FtsZ is capable of rapid assembly and disassembly [18]. Also, time-lapse microscopy of FtsZ–GFP in *E. coli* revealed the presence of helical FtsZ polymers that periodically extend out from and retract back to the central Z-ring [9]. Hence, FtsZ is undergoing constant assembly and disassembly, presumably allowing the cell to rapidly regulate Z-ring formation by modifying assembly and disassembly rates (Figure 3).

Proteins Affecting FtsZ Polymerization

The assembly and disassembly rates of tubulin in eukaryotes are modified by a large number of tubulin and microtubule binding proteins. Likewise, FtsZ is influenced by a group of proteins that are likely to shift the equilibrium of FtsZ between an unassembled cytoplasmic pool and the assembled ring (Figure 3). These include stabilizing factors, such as ZapA, ZipA, FtsA and SpoIIE, as well as destabilizing factors, such as SulA, EzrA and MinCD. Overexpression of stabilizing factors can result in aberrant FtsZ structures that extend outside the midcell, while overexpression of destabilizing factors can abrogate Z-ring formation (reviewed in [13]).

Several of these FtsZ-interacting proteins play conserved roles in the division of *E. coli* and are discussed below. SulA, a key component of the SOS response, binds FtsZ and prevents its polymerization [19,20], ensuring that cells with damaged chromosomes do not divide. This is particularly important when DNA fails to segregate properly, because division under these conditions would act as a guillotine, with the constriction cutting the DNA remaining at the division site — a fatal event. ZapA is a widely conserved FtsZ binding protein that stabilizes FtsZ rings *in vivo* and promotes the bundling of FtsZ protofilaments *in vitro* [21]. Although it is not essential in the two organisms examined, deletion of *zapA* in *B. subtilis* renders the cells sensitive to reduced levels of

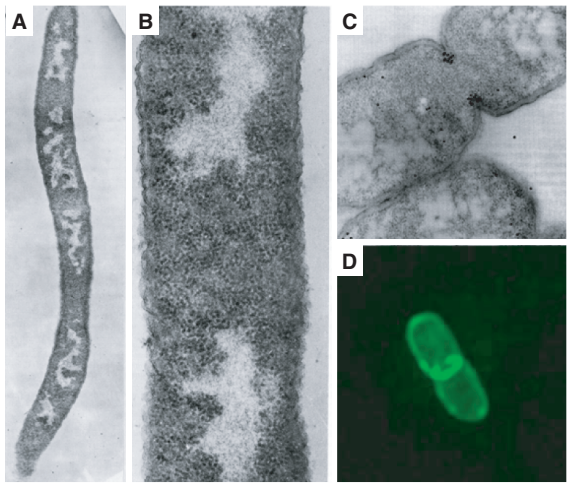

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Figure 1. Mutations affecting bacterial cell division.

(A,B) Mutation of *fts* genes leads to growth without division, giving rise to filamentous cells in which DNA (light regions) is well separated, but between which no cell wall ingrowth is observed (reproduced with permission from [1]). (C) Of the *fts* gene products, FtsZ was the first to be localized at the division site to the leading edge of the invaginating membrane by immunogold electron microscopy (reproduced with permission from [3]). (D) A cell expressing a GFP fusion to FtsL shows the ring-like localization pattern exhibited by cell division proteins (reproduced with permission from [130]).

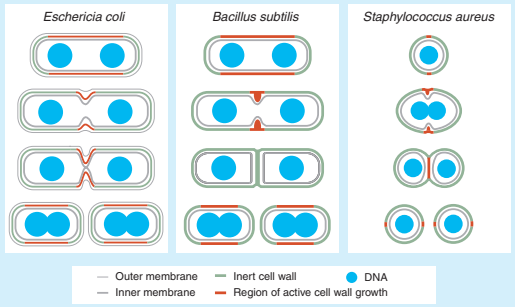
FtsZ and to the deletion of either DivIVA, a protein required to localize MinCD in this organism, or another Z-ring effector protein, EzrA [21,22]. EzrA, which is also non-essential, is conserved among low GC-content gram-positive bacteria and interacts directly with FtsZ to inhibit Z-ring assembly [23]. Loss of EzrA in *B. subtilis* leads to the formation of ectopic Z-rings, particularly at the cell poles [24]. Finally, SpoIIE is specifically required to stabilize polar Z-ring assemblies during the polar division required for sporulation in *B. subtilis* [25]. Consistent with this activity, it has been shown to interact with FtsZ in yeast two-hybrid assays [26].

Recently, photobleaching was used to investigate the role of several of these proteins in modulating the dynamic behavior of FtsZ-rings. In *B. subtilis*, the deletion of either of the FtsZ-destabilizing proteins, EzrA and MinCD, or of the assembly-promoting protein ZapA had only minimal effects on the turnover rate of FtsZ-rings. In *E. coli*, the effect of a MinCDE deletion was only somewhat greater, slowing FtsZ turnover twofold [17]. This lack of effect is somewhat surprising. However, given the fact that deletion of these proteins still allows for midcell division, this may make sense, particularly if the effect of these proteins is localized, as is the case with MinCD.

Site Selection

Prior to the actual division, it is critical for the cell to establish the site at which division should take place. Determining the division site primarily depends on FtsZ-ring placement, which is governed by two overlapping processes (Figure 3A). The first, termed

Box 1. Cell wall, cell shape and the division machinery.



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While some components of the cell division machinery are conserved and present in nearly all bacteria, others have diverged significantly. Moreover, the actual set of cell division proteins in each species varies widely. This variation presumably reflects the diversity of bacterial shapes and envelope structures.

For example, bacteria of the genus *Mycoplasma* lack a cell wall and only require a system to constrict their single membrane layer. Their genome harbours only one conserved cell division gene, the ring forming FtsZ. Typical bacteria, however, need to divide a murein cell wall and possess a majority of the conserved cell division proteins. Gram-negative bacteria presumably must also be able to cope with splitting their outer membrane.

Simple spherical cells such as *S. aureus* synthesize new cell wall material as a single equatorial band (shown in red). Gradual constriction of this band ultimately generates the two new poles of the daughter cells. After division, a new equatorial band is formed at the midcell of the daughter cells and the process starts again. In this way, continuous cell wall synthesis at midcell is sufficient to drive growth as a sphere. Rod-shaped bacteria, on the other hand, alternate between two modes of cell wall synthesis. During the elongation phase, new cell wall material is inserted diffusely along the lateral walls. At the appropriate point in the cell cycle, cells switch to a division specific mode in which cell wall incorporation is limited to the nascent septum. Hence, the evolution of a rod shape has necessitated a mechanism to temporally regulate the onset of division specific cell wall synthesis. Interestingly, when elongation specific cell wall synthesis is impaired, the normally rod-shaped *E. coli* grow as spheres.

Finally, the division event itself also differs between species — even among different rod-shaped bacteria. *B. subtilis*, a gram-positive rod, synthesizes a septal cross wall between the nascent daughter cells. Once this septum is formed, the two cells possess their own separated membranes, but remain connected by septal murein. They are ultimately liberated by autolysins that specifically degrade the connecting murein. By contrast, the gram-negative rod *E. coli* divides by coordinated constriction of all three layers of its cell envelope.

'nucleoid occlusion', has been proposed to explain the tendency of Z-rings to form in regions containing little or no DNA [27–29]. Nucleoid occlusion theoretically limits potential division sites to the midcell and cell poles. The second pathway is responsible for suppressing Z-ring formation in the DNA-free regions at the poles, a task carried out by the Min proteins. Deletion of these proteins in both *B. subtilis* and *E. coli* results in the formation of polar Z-rings and the production, through polar division, of DNA free minicells [30,31]. Hence, in combination the two pathways ensure that a FtsZ-ring only forms in the DNA free region left at midcell after chromosome segregation. Due to their overlapping functions, however, cells defective in only one of the two pathways remain viable.

MinC and MinD are widely conserved in bacteria and together negatively regulate FtsZ polymerization. Overexpression of the two Min proteins together prevents Z-ring formation, resulting in a cell division block [32]. MinC acts as a dimer and is the primary FtsZ destabilizing agent, binding to and disrupting FtsZ polymers. MinD binds cooperatively to the membrane via a carboxy-terminal amphipathic helix in a manner that requires oligomerization and binding of ATP [33–36]. MinD recruits MinC in bulk to the membrane, apparently increasing the local concentration of MinC, which is critical for its function [22]. In addition, MinD also contributes to specific activation of MinC and its recruitment to septal assemblies [22]. Consequently, in the absence of MinD, MinC remains in the cytoplasm and is unable to inhibit Z-ring formation.

MinD is a member of a family of bacterial ATPases that includes the ParA ATPases involved in plasmid segregation. ParA-like proteins polymerize *in vivo* and *in vitro* and exhibit complex dynamics, similar to eukaryotic cytoskeleton proteins [37,38]. Perhaps not surprisingly, MinD itself exhibits several cytoskeletal characteristics. In *E. coli*, MinD oscillates from pole to pole with a period of about 40 seconds [39]. Recent evidence indicates that MinD travels along a spiral-like path, suggesting that polymerization of MinD into a helical filament underlies this dynamic behavior [40].

The spatial regulation of MinCD requires a third protein, MinE. In the absence of MinE, MinCD localizes uniformly to the membrane and prevents Z-ring formation throughout the cell [30,41–44]. By competing with MinC for MinD binding, MinE effectively displaces MinC from the complex [35,45,46]. MinE then stimulates the ATPase activity of MinD, ultimately triggering the release of MinD from the membrane [33,47]. MinD then reassembles on the membrane at the opposite pole where MinE concentrations are presumably lowest. As a result of this oscillation of MinD, MinC is maintained at a time-averaged concentration that is highest at the poles and lowest at midcell, which in turn leads to pole-specific FtsZ-depolymerization as first proposed by Raskin and de Boer [39,42].

The oscillation of MinD has been simulated *in silico.* These simulations, based purely on the biochemical properties of the Min proteins and FtsZ, demonstrate the ability of MinCDE and FtsZ to self-organize into an oscillatory system in the absence of any pre-existing spatial information [48–52]. The most recent iteration of this approach has incorporated the observed polymerization dynamics of MinD [53]. Consistent with the ability to self-organize, recent work indicates that MinCDE from the Gram-negative coccus *Neisseria gonorrhoeae* are capable of establishing oscillations within the rod-shaped *E. coli* [54]. Moreover, in a set of elegant experiments in round mutants of *E. coli*, MinCDE were capable of establishing an oscillation

Site selection	MinC	FtsZ inhibitor
	MinD	ParA family ATPase, Min oscillator
	MinE	Min topological regulator
	SlmA	Nucleoid occlusion
Z-ring associated	ZapA	FtsZ positive regulator
	FtsZ (Z)	Tubulin homologue, forms Z-ring scaffold
	ZipA	Stabilizes Z-rings at membrane
	FtsA (A)	Actin superfamily member, ATPase, stabilizes Z-rings at membrane, recruits downstream components
'Late' division proteins	FtsEX	Similar to ABC transporter, unknown function
	FtsK (K)	Chromosome segregation
	FtsQ (Q)	Unknown function
	FtsL (L)	Unknown function
	FtsB (B)	Unknown function
	FtsW (W)	SEDS family member, unknown function
	FtsI (I)	Division specific transpeptidase
	FtsN (N)	Contains murein binding domain, unknown function
	AmiC	Amidase required for cell separation

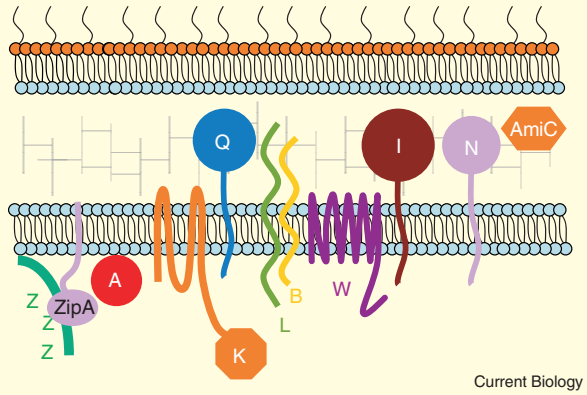

Figure 2. Cell division proteins of *E. coli*.

Cell division in *E. coli* requires at least ten proteins (bold) and many more are implicated. Due to the complexity of the division process, it is not surprising that this list includes cytoplasmic, periplasmic and membrane-embedded proteins; however, no components of the outer membrane have been specifically linked to cell division.

along the long axis of the cell, sensing small asymmetries in the spherical cells that are generated during division and leading to alternating division planes as is seen in *Neisseria* [55]. Hence, MinCDE do not sense a fixed physical structure, but are capable, on their own, of establishing the long axis of the cell, and in so doing define the next generation of poles.

In marked contrast to the dynamic behavior in *E. coli*, MinCD are anchored to both poles in *B. subtilis* by means of a polarly localized protein, DivIVA [56]. Why *E. coli* maintains such an apparently extravagant oscillating system compared to *B. subtilis* is unclear as is the question of which type of behavior is more prevalent among different bacteria.

Recently, potential factors underlying nucleoid occlusion have been identified in *B. subtilis* and *E. coli* [28,57,58]. In *B. subtilis*, it was noticed that the deletion of a small, uncharacterized ORF, *yyaA*, renamed *noc*, is lethal in combination with mutations affecting the Min system. At the same time, in *E. coli* a synthetic lethal screen based on the hypothesis that mutations in both the MinCD and nucleoid occlusion pathways would be lethal, pulled out the relatively uncharacterized gene *ttk*, renamed *slmA*. In cells lacking the Min system, the loss of these genes resulted in the frequent formation of FtsZ structures atop nucleoids in addition to the normal internucleoid Z-rings. In contrast, overproduction of these proteins in wild-type cells inhibited division. The two proteins also are required for an anti-guillotine checkpoint, which prevents midcell division in cells defective in DNA replication. Cells that cannot replicate their chromosome accumulate a single DNA mass at midcell, and division, if it does occur, normally occurs asymmetrically leaving the chromosome intact. In *slmA* and *noc* mutant cells, however, the Z-ring can form directly over this central DNA mass, resulting in a division event that cuts the chromosome. Finally, SlmA was shown to affect FtsZ polymerization *in vitro*, resulting in the bundling of FtsZ polymers in a manner comparable to other FtsZ binding proteins, suggesting that the effect of these proteins may be direct. Interestingly, although both Noc and SlmA show homology to DNA-binding proteins, they are unrelated to each other. This indicates that either the precise molecular mechanism of nucleoid occlusion may differ across bacterial species or that diverse proteins have been co-opted to perform the same function.

FtsZ Binding Proteins Tether Z-rings to the Membrane

Two FtsZ-stabilizing proteins, FtsA and ZipA, are essential for cell division and critical for the stability of Z-rings in *E. coli*. Mutation of either protein alone prevents cell division, but has little effect on the frequency of Z-ring formation *in vivo* [59–61]. If, however, both proteins are deleted, cells are unable to form Z-rings [59]. Both proteins bind to a conserved carboxy-terminal extension of FtsZ and are able to localize to the Z-ring independently [62–64]. Several studies have indicated that the ratio of ZipA and FtsA to FtsZ in the cell is critical for ring assembly. Overexpression of either protein is toxic to cells, whereas simultaneous overexpression of FtsZ rescues this phenotype, presumably by restoring the appropriate stoichiometric ratio [65,66]. Both proteins also play a role in tethering FtsZ to the inner face of the cytoplasmic membrane. ZipA contains an amino-terminal membrane anchor that is connected to a cytoplasmic carboxy-terminal FtsZ-binding domain by a long flexible linker [62,67]. FtsA, although a cytoplasmic protein, contains a carboxy-terminal amphipathic helix that targets FtsA to the membrane [68]. In either case, promoting FtsZ polymerization at the membrane is critical for proper Z-ring formation.

An Overview of the Late Cell Division Proteins

Once the Z-ring has been established, the remaining essential division proteins are recruited, all of which are either single-pass or multi-spanning membrane proteins (Figure 2). Most of the latter contain only a very small cytoplasmic domain, which, with the exception of FtsL, is completely dispensable for function [69–71]. Two proteins, FtsQ and FtsN, require only

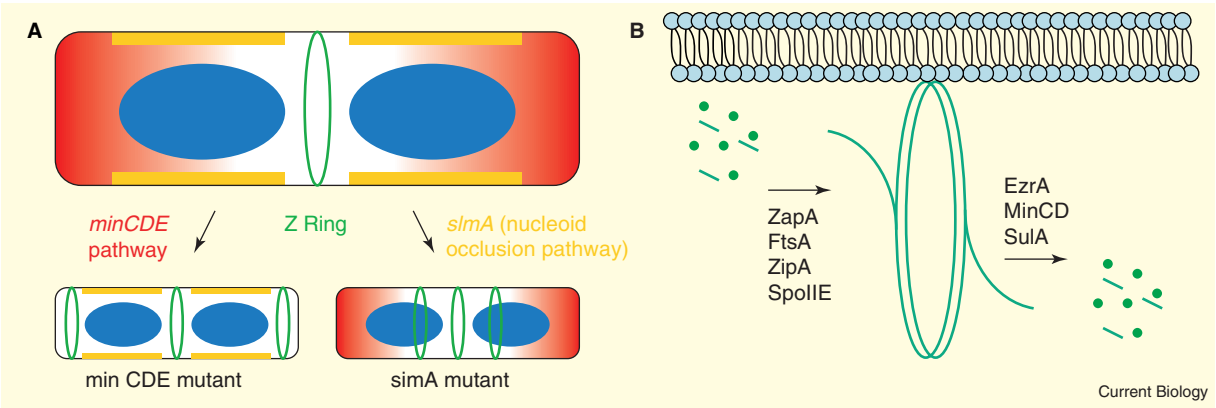

Figure 3. Pathways and factors regulating Z-ring positioning and stability.

(A) MinCDE (red) and SlmA (yellow) combine to confine Z-ring formation to midcell by inhibiting FtsZ polymerization at the poles (MinCDE pathway) or over the nucleoid (nucleoid occlusion pathway). Cells defective in only one pathway exhibit ectopic Z-ring formation, but cells divide successfully often enough to remain viable. (B) In addition, several positive and negative factors have a role in regulating FtsZ polymerization into Z-rings.

their periplasmic domains. Despite significant efforts, little is known about the function of these proteins in cell division.

FtsK belongs to a family of proteins involved in chromosome partitioning. It consists of a smaller amino-terminal domain containing four transmembrane segments and a large carboxy-terminal cytoplasmic domain connected by an unstructured linker region. The carboxy-terminal domain of FtsK acts as a DNA motor, utilizing ATP to translocate DNA in a sequence-directed fashion [72–74]. Deletion of this domain, despite having a modest effect on DNA segregation, does not interfere with cell division. In contrast, the amino-terminal domain of FtsK is both necessary and sufficient for division [75,76]. Interestingly, the amino-terminal domain of the *B. subtilis* FtsK homologue SpoIIIE is involved in the final membrane fusion events required to separate the membranes of the forespore and mother cell during sporulation [77,78]. The amino-terminal domains of FtsK and SpoIIIE, however, show no sequence similarity [79]. Moreover, SpoIIIE does not appear to be required for septation in *B. subtilis*, leaving open the question of whether the role of SpoIIIE in facilitating the final membrane fusion in sporulation is at all related to the role of FtsK in cell division.

FtsQ, FtsL and FtsB form a broadly conserved complex of cell division proteins that lies in the pathway between the assembled Z-ring scaffold, consisting of FtsZ and FtsZ binding proteins, and the septal peptidoglycan synthesis machinery. FtsQ is a bitopic membrane protein with a large periplasmic domain of 225 amino acids. FtsL and FtsB consist of little more than a transmembrane segment and a coiled-coil leucine zipper-like domain. Beyond their interactions with the divisome, no function has been attributed to these proteins so far.

Ingrowth and separation of the bacterial cell wall are expected to require extensive synthesis, remodeling and degradation of peptidoglycan. *E. coli* contains a large number of penicillin binding proteins (PBPs), which are generally involved in peptidoglycan synthesis (reviewed in [80]). Strikingly, the only PBP clearly essential for cell division is FtsI (PBP3), one of two class B high molecular weight (HMW) PBP transpeptidases in *E. coli*. The transpeptidase activity of FtsI is confined to the division site and its catalytic activity depends on the division status of the cell [81–83]. The precise transpeptidase reaction carried out by FtsI is also specific to the septum. Replacing its active site with the similar active site of PBP2, the class B HMW-PBP transpeptidase required for cell elongation, disrupts FtsI function. It is thought that these two transpeptidases differ in substrate specificity, with FtsI exhibiting a preference for tripeptide side chains as the acceptor and PBP2 for pentapeptide side chains [83,84].

Much less is known about the role of FtsW, which is a member of a family of SEDS (Shape, Division and Sporulation) proteins [85]. Genes encoding SEDS proteins are found invariably in the proximity of genes encoding class B transpeptidases and are often co-transcribed with them. Consistent with a functional relationship, mutation of either gene in a given pair of SEDS proteins and transpepdidases results in a similar phenotype. *E. coli* possesses two such gene pairs, one encoding FtsW and FtsI, which are required for division, and another encoding RodA and PBP2, which are required for cell elongation. Even though no function has been demonstrated experimentally, some have speculated about a role of these proteins in transporting peptidoglycan precursors to the periplasm where they can be utilized by their cognate transpeptidase [86].

FtsN has proved to be a rather enigmatic member of the cell division machinery. It is found only among gamma-proteobacteria [6] and is the last known essential protein to be recruited to the septum [87,88]. FtsN was identified as a multi-copy suppressor of a thermosensitive mutation in *ftsA* [89]. Overexpression of FtsN also suppresses thermosensitive mutations in *ftsK*, *ftsQ* and *ftsI*. FtsN exhibits an amidase-like fold at its carboxyl terminus that has been demonstrated to bind murein in several *in vitro*

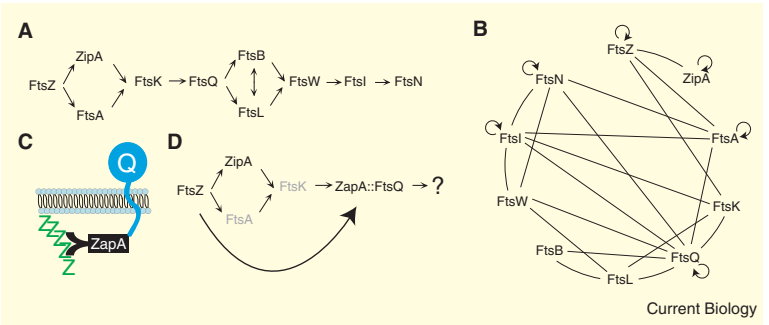

Figure 4. Hierarchies and interactions of bacterial cell division proteins.

(A) Bacterial cell division proteins localize according to a defined hierarchy or pathway. (B) Bacterial two-hybrid assays detect many putative interactions between the proteins of the divisome, consistent with a model in which protein–protein interactions play a role in this assembly pathway. Lines indicate a positive signal between two proteins in at least one two-hybrid assay, while circular arrows indicate potential self-interaction. (C and D) A premature targeting method allows for bypassing of the normal recruitment requirements for a given protein. In this scheme, the FtsZ-binding protein ZapA (black) is fused to the division protein FtsQ (blue). The ZapA moiety renders the localization of FtsQ independent of FtsA and FtsK (shown in grey). If a protein relies on FtsQ, but not FtsA or FtsK, it should be recruited by the ZapA-FtsQ fusion and localize normally in strains deficient in either of the upstream proteins. If it does not, this indicates that the protein in question requires additional information to localize. Systematic analysis in this manner should allow for the determination of the specific signals required for each recruitment step. (Portions of this figure adapted and/or reproduced with permission from [105].)

experiments [90,91]. Although this finding suggests that the protein may be involved in peptidoglycan remodeling, no murein hydrolase activity was detected. Moreover, deletion of the amidase-like domain has no obvious cell division defect.

Assembly of the Divisome

In *E. coli*, the majority of cell division proteins, with the exception of FtsZ and ZipA, are present at levels (~ 40–300 molecules per cell) that are insufficient to form autonomous ring-like structures. Rather, it is likely that these proteins assemble into discrete complexes that are attached, perhaps via FtsA, to the Z-ring.

Studies on the localization of cell division proteins in conditional mutants revealed that they localize according to a defined and strikingly linear hierarchy of dependence (Figure 4). In this hierarchy, a given protein requires the presence of all upstream proteins to localize to midcell and is in turn required for the localization of all downstream proteins (reviewed in [5]). Several models can be envisaged for how these proteins are recruited to midcell. The recruitment hierarchy necessarily demands only that the protein or proteins immediately upstream of a given protein be localized. Each step in the hierarchy can be explained by a number of mechanisms, including a simple, direct protein–protein interaction, the recognition by the localizing protein of the assembled divisome complex at midcell or by the production of a substrate recognized by the localizing protein, such as a particular peptidoglycan modification. Moreover, the hierarchy theoretically allows for a complex or subcomplex of proteins, which can form independently of and be recruited *in toto* to the division site. In an alternative model, proteins only associate once they arrive at the division site.

Some of these models could be distinguished based on the temporal order of arrival of the proteins at the septum. It is important to note that the recruitment hierarchy reflects dependence relationships and does not necessarily reflect a temporal order. There is some evidence for a time lag between Z-ring formation and localization of the late proteins (FtsQ, FtsW, FtsI and FtsN) based on the relative frequency of cells exhibiting FtsZ localization and those with detectable localization of the other proteins [92]. However, as FtsZ is at least 50-fold in excess, it remains possible that the observed differences in localization frequency could, in part, be due to variation in the detection of these proteins at midcell. Beyond this, there has been no comprehensive direct determination of the actual temporal order of arrival of these proteins at midcell. In recent work, the major effort has been to study the potential interactions among these proteins and the molecular events that drive their association at the division site. Several approaches have been used in these studies, including co-purification of proteins, bacterial two-hybrid systems, classical genetics and two new cytology based methods to directly assess protein recruitment. These studies have provided insight into several key steps of divisome assembly.

The FtsQLB Complex

The recent identification and characterization of a complex of three cell division proteins, FtsQ, FtsL and FtsB, provides an example of the synergy that results from applying multiple approaches in diverse bacteria. In *E. coli*, FtsQ localizes in the absence of FtsL and FtsB, but is required for the localization of both of the latter proteins. All three proteins are, in turn, required for the recruitment of FtsW and FtsI [93–95].

The initial characterization of the cellular role of FtsB (originally YgbQ) yielded two observations, which were consistent with a putative cell division subcomplex. First, FtsL could not be detected in cells depleted of FtsB, which suggests that FtsB stabilizes FtsL. Second, FtsL and FtsB require one another for proper localization [93]. Subsequently, using *E. coli* membrane extracts, FtsL and FtsB could be co-immune precipitated along with FtsQ, which was shown to be required for efficient co-precipitation [96].

At the same time, several groups analyzed all the potential interactions among the cell division proteins using various bacterial two-hybrid approaches ([97, 98] and M. Gonzalez and J. Beckwith, unpublished; Figure 4B). These approaches yielded a remarkably high number of potential interactions between cell division proteins. Many proteins exhibited interactions

with multiple partners, suggesting a surprising degree of interconnectivity within the divisome. However, one must approach these results with some degree of caution. First, they rely on overexpression of the reporter protein fusions, which could result in magnifying potentially weak interactions and lead to false positives. Second, the two-hybrid analyses are performed in *E. coli* where the fusion proteins are likely to be incorporated into the divisome. In this presumed complex, a positive two-hybrid result may reflect proximity within the divisome and not direct interaction. Nonetheless, all approaches identified interactions between FtsQ, FtsL and FtsB, consistent with the results of the co-precipitation experiments.

FtsQ, FtsL and FtsB homologues have also been studied in two Gram-positive bacteria and the results have yielded a similar picture. In *B. subtilis,* the FtsQ, FtsL and FtsB homologues are dependent on one another for localization. $FtsL_{Bs}$ appears to be an intrinsically unstable protein, which requires the FtsQ and FtsB homologues, $DivIB_{Bs}$ and $DivIC_{Bs}$, respectively, for its own stability. $FtsL_{Bs}$ is, in turn, required for the stability of the $DivIC_{Bs}$ [99–101]. There is, however, some debate as to whether or not these proteins interact directly [102,103]. Using a co-purification approach in *S. pneumoniae*, $DivIB_{Sp}$ can be shown to associate with an artificially constrained heterodimer of the periplasmic domains of $DivIC_{Sp}$ and $FtsL_{Sp}$ in a 1:1:1 manner [104]. Taken together, these data provide evidence for a conserved subcomplex of cell division proteins.

The ability to detect such a complex in *E. coli* provided the means to test whether assembly of these proteins occurs independently of other proteins in the cell or requires the proper assembly of all upstream divisome components (FtsZ, ZipA, FtsA and FtsK). Initial results showed that the formation of this subcomplex did not require FtsK, a protein required for the localization of all three members of the subcomplex [96]. This issue was explored further using a premature targeting approach, in which FtsQ was targeted to the Z-ring via fusion to the FtsZ binding protein ZapA (Figure 4C,D). Using this approach, it was shown that recruitment of FtsL and FtsB to the midcell by FtsQ does not require FtsA or FtsK [105].

Regarding the potential interpretation of the localization hierarchy discussed above, these various approaches strongly indicate that, at least in *E. coli*, FtsQLB proteins form a stable complex that is independent of other known division proteins and of the division status of the cell. This complex can then be targeted to the division site by the action of FtsQ. Hence, the localization of FtsL and FtsB is not driven by the recognition of either a protein complex unique to the septum or a specific septal substrate as may have previously been imagined. Taken together with the data from *B. subtilis* and *S. pneumoniae*, this supports a central and conserved role of the FtsQLB complex in organizing the divisome.

Evidence for Non-Sequential Assembly

The cell division hierarchy is striking because of its linearity. The results mentioned above, however, indicate that proteins within the pathway are capable of assembling into complexes despite an apparent break in the hierarchy — the ZapA–FtsQ fusion protein readily recruits FtsL and FtsB in the absence of two upstream proteins FtsA and FtsK. Interestingly, expression of the ZapA–FtsQ fusion effectively recruits FtsK to the septum in cells deficient for FtsA, a condition in which FtsK would not normally be localized [105]. This back recruitment suggests an interaction, though not necessarily a direct one, between FtsQ and FtsK, consistent with bacterial two-hybrid results. If localized FtsK activity was required to recruit FtsQ to midcell, one would not expect such back-recruitment to be possible. Hence, this provides further evidence that the assembly pathway does not necessarily reflect a series of temporally ordered events.

The Role of FtsA and ZipA in the Recruitment of Late Proteins

There is evidence that FtsA plays a direct role in the recruitment of downstream cell division proteins. It is a member of the actin superfamily, but differs in the possession of a unique domain, IC, that is responsible for the ability of FtsA to interact with and recruit downstream cell division proteins [106–109]. Bacterial two-hybrid approaches have provided evidence for interactions between FtsA and other essential cell division proteins, including FtsZ and several downstream proteins [97,98,107]. In contrast, in similar assays, ZipA interacts only with FtsZ [98]. Hence, we have a situation in which ZipA does not interact with downstream components of the divisome, yet is nonetheless required for their localization. This requirement persists despite the presence of FtsA, which localizes to the Z-ring independently of ZipA and interacts with the same components. Hence, a model in which FtsA binds to and recruits downstream components is not sufficient to explain the observed localization dependency relationships. The recent identification of a mutant in the *ftsA* gene*, ftsA*,* that allows growth of a strain lacking *zipA* indicates that FtsA possesses at least the potential to perform the function of ZipA, a fact suggested by the observation that, in contrast to *ftsA*, *zipA* is not widely conserved [110]. Interestingly, the *ftsA** mutation maps to a domain that is not involved in recruitment of downstream components. One possible interpretation of these data is that ZipA induces a specific conformation of the Z-ring or the FtsZ–FtsA complex that is required for recruitment of downstream components. Determining the mechanism by which FtsA* substitutes for ZipA should help to decipher the precise signals that allow for the assembly of the divisome on the Z-ring scaffold.

Mechanisms for Localization of a Division Specific Transpeptidase

Because of the substrate specificity of its transpeptidase activity, one mechanism to localize FtsI activity would be to spatially regulate the availability of its transpeptidation substrates. Since FtsW is required for proper localization of FtsI, it was suggested that FtsW might play a role in the regulation of substrate

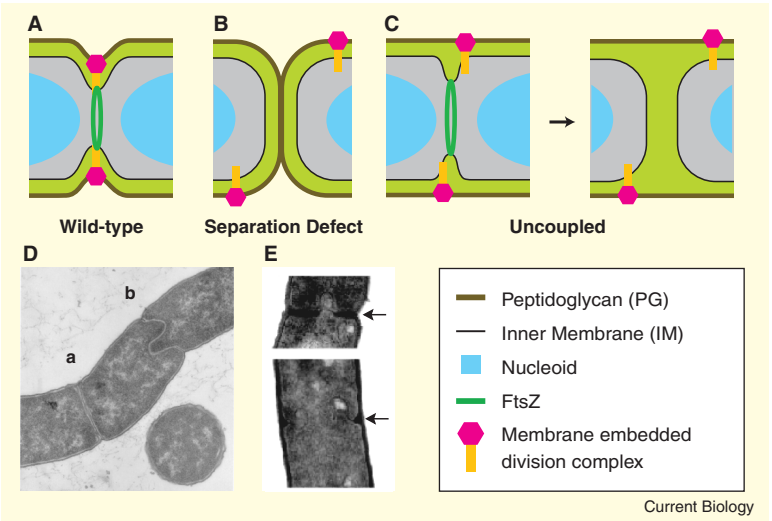

Figure 5. Decoupling invagination of the cell envelope.

(A) In wild-type cells, constriction of the inner membrane and septal cell wall synthesis normally occur together. (B) In cells with a separation defect, the septum forms normally, but the septal murein cannot be hydrolyzed to form two separated poles. (C) In mutants with uncoupled invagination, membrane invagination occurs normally, presumably driven by FtsZ ring constriction. Septal cell wall ingrowth, however, fails. It is unknown whether the division specific cell wall synthesis machinery follows the Z-ring or is left behind. (D) In *E. coli*, a mutant lacking several murein hydrolases yields chains of cells connected by two types of septa. In most cells, a septum is synthesized, but the cells remain attached by septal murein, marked 'a'. In some cases, however, the inner membrane appears to divide normally, but septal peptidoglycan synthesis is defective, giving rise to a septum like the one marked 'b' (reproduced with permission from [121]). (E) *B. subtilis* mutants lacking PBP2x also show a phenotype consistent with decoupling (reproduced with permission from [126]). Membrane invaginations are evident at the leading edge of aborted peptidoglycan ingrowth (arrows).

availability. This, however, does not appear to be the case. Specifically, FtsI localizes normally when its active site is inactivated by acylation with various antibiotics (M. Wissel and D. Weiss, personal communication). Moreover, the transmembrane segment of FtsI is both necessary for localization and by itself sufficient to direct a GFP fusion protein to the division site [71,111]. Hence, the localization of FtsI would be most easily explained by a direct physical interaction with FtsW via the transmembrane domain of FtsI.

In contrast to the situation in *E. coli*, there is evidence that the division specific FtsI homologues in Gram-positive cocci require localized substrates for their own localization. In *Staphylococcus aureus*, the localization of the FtsI homologue, PBP2, is disrupted by treatment with antibiotics that either block peptidoglycan precursor production or directly inactivate the enzyme's active site [112]. This result, however, begs the question of how *S. aureus* spatially regulates substrate availability. One possible mechanism comes from *S. pneumoniae*, where deletion of a small carboxypeptidase, PBP3, thought to be responsible for generating tripeptide transpeptidation substrates for the *S. pneumoniae* FtsI homologue, PBP2x, leads to frequent mislocalization of PBP2x such that it no longer co-localizes with FtsZ and its cognate SEDS protein FtsW [113]. Together, these studies support a model in which a class B transpeptidase is recruited by a localized pool of transpeptidation substrate, the distribution of which may depend, in turn, on the spatially regulated activity of additional PBPs.

This model, according to which a localized substrate is used to target an enzyme of the divisome to midcell, does not directly apply to FtsI. However, the existence of such a mechanism in the division process of other bacteria implies that we ought not rule out such a model for *E. coli* as it remains possible that other cell division proteins may use such a mechanism.

FtsN Recognizes Diverse Signals at Midcell

The septal signals that direct FtsN have been difficult to elucidate. Some have speculated that the closeness of FtsI and FtsN in the recruitment pathway and the putative murein-binding domain in FtsN suggest that FtsN recognizes a product made by FtsI. However, when FtsI activity is inhibited by acylation with FtsI-specific beta-lactams or by missense mutations, FtsN still localizes normally (M. Wissel and D. Weiss, personal communication). Moreover, as discussed above, this murein binding domain is apparently not essential — mutants missing it readily support cell division — and hence cannot be the sole localization determinant [90].

Alternatively, FtsN could be specifically recruited via a direct interaction with FtsI. Two independent bacterial two-hybrid experiments demonstrate an FtsI–FtsN interaction [97,98] and work by Wissel and Weiss identified several point mutations in *ftsI* that disrupt FtsN localization [114]. These mutations map to a small region of the n-PB domain, which is thought to be important in associating with other cell division proteins [4]. Both findings are at least consistent with FtsN directly recognizing FtsI at the septum.

Experiments using the premature targeting approach, however, indicate that the story may be more complicated. In FtsA depleted cells, when FtsQ is targeted to the Z-ring via fusion to ZapA, not only are FtsL and FtsB being recruited, but also FtsW and FtsI. Strikingly, FtsN failed to be recruited under these circumstances [105]. Hence, the mere presence of FtsI at the septum is insufficient for proper FtsN localization.

Thus, the most reasonable explanation for FtsN localization is that it recognizes multiple signals at the division site. Several findings support this view. Bacterial two-hybrid results show that, in addition to FtsI, FtsN also interacts with FtsA and FtsQ [89,97,98], which is consistent with the genetic interactions between *ftsN* and *ftsA, ftsK, ftsQ and ftsI.* Corbin *et al*.

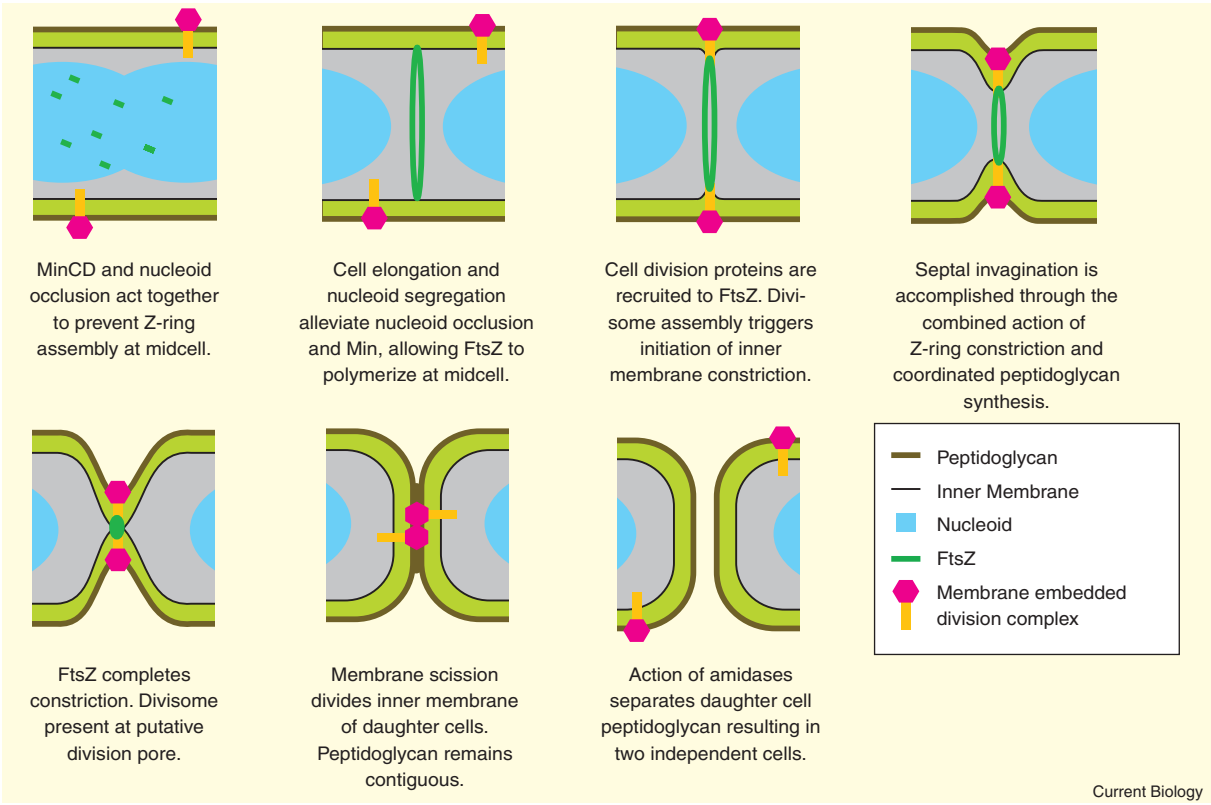

Figure 6. A schematic overview of the major events during one cycle of bacterial cell division.

[107] recently described a cytology-based two-hybrid method in which FtsA, when targeted to the pole via fusion to the polar-localized protein DivIVA, recruits FtsI and FtsN. This suggests that FtsA may play a critical role in facilitating the localization of these proteins [107]. Both FtsI and FtsN, however, do not require their cytoplasmic domains for function and FtsN functions normally when the periplasmic domain alone is fused to an unrelated transmembrane domain [69,71], making it unclear by what mechanisms they could interact directly with FtsA in the cytoplasm.

Obviously, further work will be required to define these localization requirements. However, given this complex behavior and the position of FtsN at the end of the recruitment hierarchy, it is tempting to speculate that FtsN may be able to integrate a variety of signals from the divisome, including both protein conformations and peptidoglycan structure and in doing so may act to regulate division initiation.

The Role of Non-Division Proteins in Cell Division

Localization of Non-Division PBPs to the Septum

E. coli and *B. subtilis* each possess only a single PBP that plays a specific and essential role in cell division. Depletion of this protein, the division specific class B HMW–PBP (FtsI/PBP2b), results in normal lateral cell wall synthesis, but an inability to divide. In contrast, mutations in other PBPs do not lead to such a typical cell division defect. Recently, however, some of these proteins have been implicated in formation of the division septum.

A systematic study of the localization of non-division PBPs in *B. subtilis*, indicates that many of them, including all class A PBPs, several class B PBPs and several low molecular weight PBPs are localized to the septum [115]. One of these, PBP1, is required for proper septum formation and its localization at the septum is dependent on assembly of the divisome, including DivIB, DivIC and the septum specific PBP2b [116,117]. In *E. coli*, PBP1a, PBP1b, PBP2 and PBP3 (FtsI) can be co-purified along with several lytic murein hydrolases, supporting the notion of a ‘peptidoglycan factory’ in which the activities of these PBPs are coordinately deployed [118,119]. This model, however, remains controversial. One of these non-division PBPs, the elongation-specific class B HMW-PBP, PBP2, also exhibits localization to the lateral walls and the midcell [120]. This is consistent with a role for this protein in cell division. With the exception of PBP1 in *B. subtilis*, however, no specific effect of these proteins on cell division has been demonstrated, leaving their involvement unclear.

Murein Hydrolases in Cell Separation

E. coli possesses a large number of murein hydrolases, the roles of which have been difficult to determine due to a large degree of apparent functional redundancy. Cells containing deletions of single or multiple hydrolases are completely viable [121]. Deletion of one class of hydrolases, the murein amidases AmiA, AmiB and AmiC, does, however, show a prominent cytological defect, suggesting a primary role in cell separation

[122]. Cells missing these amidases form chains in which the cytoplasmic membrane between cells is divided and septal murein is synthesized normally. They are, however, unable to cleave the septal murein to completely separate the two daughter cells (Figure 5). Consistent with a role in cell separation, one amidase, AmiC, localizes specifically to the division site in an FtsN-dependent manner [123].

Coordinating Cell Wall Ingrowth

An *E.coli* strain deleted for the three amidases also exhibits a second unusual phenotype, which is exacerbated by deletion of additional hydrolases. A small proportion (up to 5%) of the chain forming cells shows normal division of the cytoplasmic membrane, without accompanying septal peptidoglycan ingrowth [121]. This suggests that constriction of the membrane is initiated, but becomes decoupled from septal peptidoglycan synthesis (Figure 5). This is in marked contrast to *fts* mutants, which generally exhibit smooth aseptate filaments, with no evidence of membrane invagination.

In Gram-positive organisms, there is also evidence that membrane invagination and cell wall ingrowth can be decoupled. Electron microscopy of the septum of dividing *S. pneumoniae* shows that membrane invagination precedes cell wall ingrowth even in wild-type cells [124]. Consistent with this observation, there are some data indicating that Z-ring constriction begins prior to constriction of FtsW and the FtsI homologue, PBP2x [125]. It is unknown, however, whether constriction of the Z-ring can be initiated in the absence of these proteins as would be expected if they were truly uncoupled. In *B. subtilis*, depletion of PBP2b leads to the production of aborted septa, which in some cases show normal membrane constriction, suggesting that the requirements to initiate membrane invagination and for ongoing peptidoglycan synthesis are distinct (Figure 5) [126]. Finally, in *Mycobacterium tuberculosis*, a resuscitation factor, RPF, which is required to allow re-initiation of growth and division of latent bacteria, shows characteristics of a lytic glycosylase [127]. Hence, peptidoglycan modification may be linked to the cell cycle, perhaps via the regulation of the availability of substrates required for resumption of cell wall growth.

Although not at all conclusive, this variety of evidence provides reason to investigate a model in which the initiation of cell division and Z-ring constriction is linked to some form of peptidoglycan checkpoint, either the presence of a unique set of divisome components or a unique characteristic of the septal murein. The observation of penicillin-insensitive peptidoglycan synthesis in *E. coli*, which requires FtsZ but is independent of late cell division proteins, could be linked to such a checkpoint as well (reviewed in [128]). Based on this observation, it has been speculated that some septal murein synthesis takes place at the onset of cell division following Z-ring assembly, independently of and prior to the assembly of the remainder of the division machinery, and thereby marking the septal murein for further remodeling.

Concluding Remarks

The last decade or so has seen the identification of novel proteins involved in cell division within bacteria and of the localization of these proteins to their site of action at midcell. We now realize that the assembly of the Z-ring at the nascent division site is both highly regulated and highly dynamic. *In vitro* analysis has described many of the molecular events responsible for these early events. Study of the later events in cell division has proceeded more slowly. Nonetheless a picture of the divisome complex is beginning to emerge and together these insights have provided an increasingly detailed picture of the major cell division events (Figure 6). There remains, however, much left to explore. Identification of the precise function of these late cell division proteins remains a major goal in understanding divisome function. We also still know very little about the events that occur between the assembly of cell division proteins and the completion of division. This includes the mechanism of constriction, a picture of the peptidoglycan modifications involved and an understanding of the membrane scission step that occurs at the final stages of cell division. Finally, although significant progress has been made in several organisms, particularly *Caulobacter crescentus*, we are only beginning to understand how division is integrated into the bacterial cell cycle (see [129]).

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