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Current Opinion in Microbiology

The divisome at 25: the road ahead

Tanneke den Blaauwen[1], Leendert W Hamoen[1] and Petra Anne Levin[2]

The identification of the FtsZ ring by Bi and Lutkenhaus in 1991 was a defining moment for the field of bacterial cell division. Not only did the presence of the FtsZ ring provide fodder for the next 25 years of research, the application of a then cutting-edge approach—immunogold labeling of bacterial cells—inspired other investigators to apply similarly state-of-the-art technologies in their own work. These efforts have led to important advances in our understanding of the factors underlying assembly and maintenance of the division machinery. At the same time, significant questions about the mechanisms coordinating division with cell growth, DNA replication, and chromosome segregation remain. This review addresses the most prominent of these questions, setting the stage for the next 25 years.

Addresses
[1] Swammerdam Institute for Life Sciences, University of Amsterdam, 1081 HZ Amsterdam, The Netherlands
[2] Department of Biology, Washington University in St. Louis, St. Louis, MO 63130, USA

Corresponding authors: den Blaauwen, Tanneke (t.denblaauwen@uva.nl), Hamoen, Leendert W (l.w.hamoen@uva.nl), Levin, Petra Anne (plevin@wustl.edu)

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As a field, bacterial cell division has been defined by a series of breakthrough discoveries that resulted in new hypotheses followed by the steady addition of data providing molecular support for or against these hypotheses (Figure 1). Among these discoveries are the identification of conditional alleles in *E. coli* cell division genes [1••], the identification of *ftsZ*, the tubulin-like cell division gene that serves as the basis for assembly of the cytokinetic machinery in bacteria and archaea [2••], and the identification of the first set of proteins involved in the spatial regulation of cytokinesis [3••].

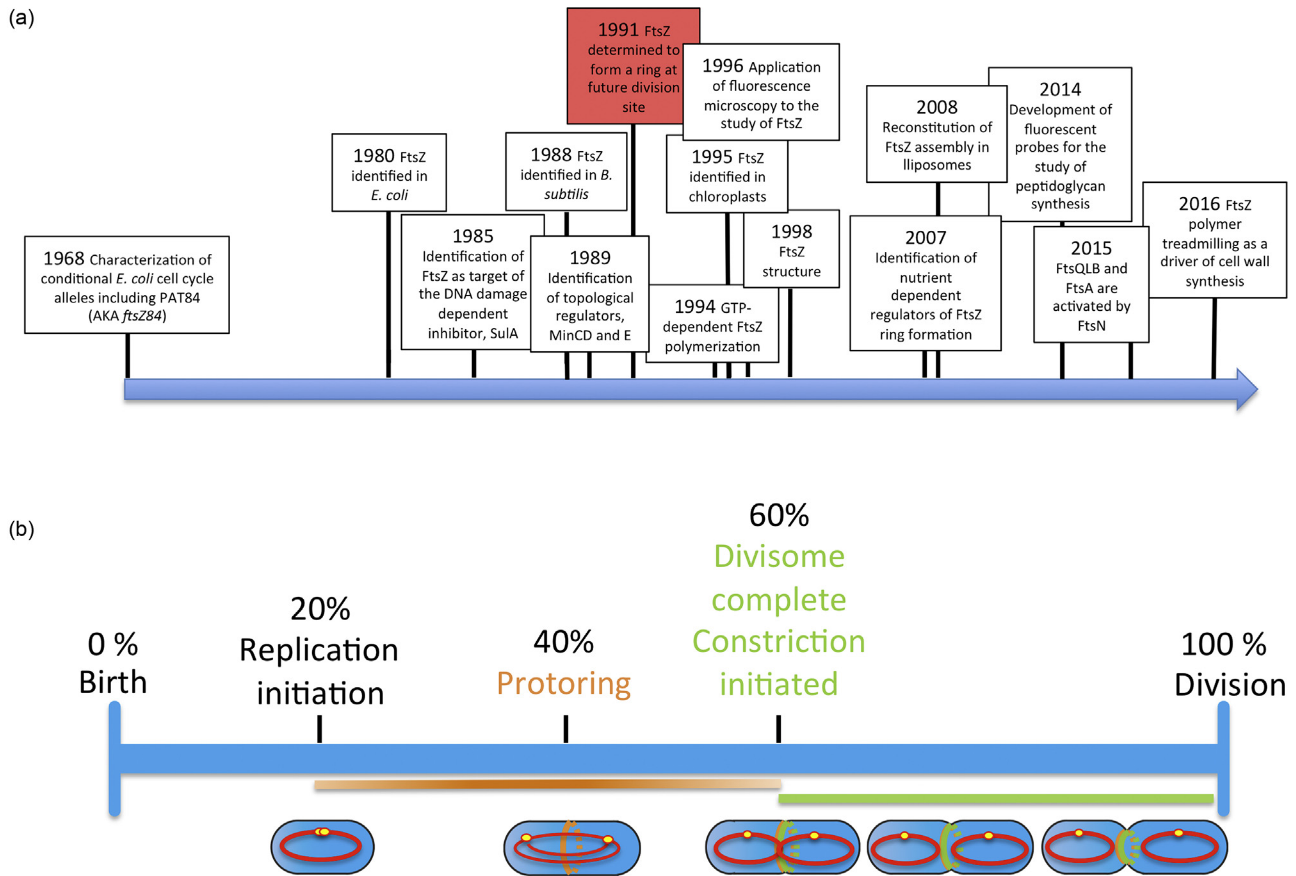
The most outstanding of all such breakthroughs, however, is undoubtedly the 1991 report that FtsZ forms a ring at the nascent division site [4••]. Utilizing cryo-EM in conjunction with immunogold staining, Bi and Lutkenhaus determined that FtsZ forms a ring-like structure at the future site of cell division in *Escherichia coli*. The identification of the FtsZ ring had an immediate and profound impact on the field. Most importantly, the presence of the FtsZ ring suggested bacteria are not so different from eukaryotes with regard to the use of cytoskeletal proteins for morphogenesis—a somewhat radical idea at the time.

In the intervening 25 years, a flurry of work focused on cloning additional cell division genes and characterizing their relationship with FtsZ. These efforts led to the conclusion that the FtsZ ring serves as a scaffold for assembly of the division machinery, a complex macromolecular structure composed of over 20 known proteins in *E. coli* and a similar number in the Gram-positive model organism *Bacillus subtilis* (although not all conserved). Together these proteins—collectively termed the divisome—coordinate cell envelope invagination during cytokinesis. Other research focused on illuminating factors contributing to the temporal and spatial regulation of FtsZ assembly and ensuring that division is coordinated with chromosome segregation [5,6,7••,8,9•].

Biochemical studies revealed the GTP-dependent formation of FtsZ polymers and determined that FtsZ assembly into single stranded polymers is a cooperative process [10•,11]. FtsZ's status as the first bacterial cytoskeletal protein was capped by solution of its structure in 1998, which revealed remarkable similarity with tubulin (solved in the same year) despite extremely limited sequence conservation [12••,13]. Like tubulin, FtsZ monomers can bind GTP on their own, but dimerization is required for formation of a shared, GTPase active site [3••,12••].

Most recently, advances in microscopy including single molecule tracking and structured illumination revealed the 'FtsZ ring' to be a discontinuous structure composed of short single stranded polymers held together via lateral interactions [14••]. Fluorescence recovery after photobleaching (FRAP) experiments illuminated the dynamic nature of the ring, demonstrating that monomer turnover within the ring occurs on average once every 9 s [15••]. Finally, two new landmark studies indicate that FtsZ polymers serve as treadmilling platforms for the septal

Figure 1



(a) Bacterial cell division time-line of discovery. **(b)** Division cycle progression time-line. Note that timing of division-related events are based on work in MC4100 cells cultured in minimal glucose medium [37,102]. Initiation time was calculated based on an 80 min mass doubling period using the CCSim program available at https://sils.fnwi.uva.nl/bcb/cellcycle/ [103].

peptidoglycan synthesis machinery, countering the long held view that constriction of the FtsZ serves as a force generating mechanism to drive cytokinesis [16••,17••] (Figure 2).

Despite these great strides, our picture of the molecular forces underlying the assembly and activity of the cell division machinery is far from complete. In particular, significant questions remain about the mechanisms controlling localization of the cell division machinery and coordinating its assembly and activation with cell growth, DNA replication, and chromosome segregation. Below we outline the most prominent of these, sketching a road map of sorts for the future of this field.

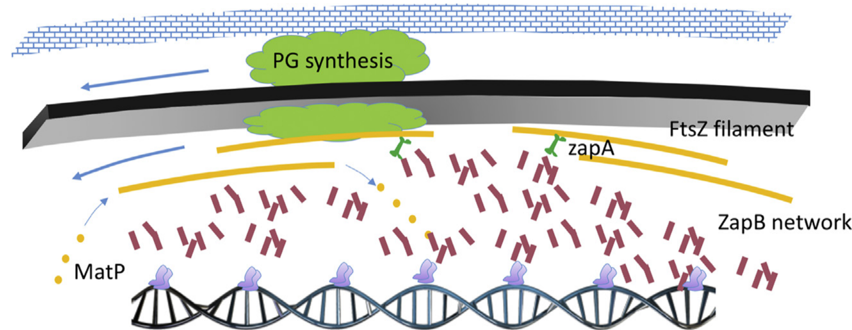
FtsZ recruitment to the nascent division site

Despite species-specific variations in its physical location, the selection of the nascent division site is a highly precise affair. In both *E. coli* and *B. subtilis* assembly of the division machinery takes place within ~2% of the cell's geographical middle generating two identical daughter cells [18•,19,20]. This level of precision suggests a multi-layered process involving both positive regulation—in the form of factors that promote FtsZ assembly at the nascent septal site—and negative regulation—in the form of factors that prevent FtsZ assembly at aberrant subcellular positions such as close to cell poles or over unsegregated chromosomal material (AKA the nucleoid). The mechanisms by which different bacteria solve this problem appear to differ substantially.

Division site selection in *B. subtilis*: ready-set-go

Data from the Wake and Harry laboratories strongly implicate the initiation of DNA replication in the positional regulation of FtsZ in *B. subtilis* [18•,21]. Bacterial DNA replication precedes assembly of FtsZ and is initiated by the binding of the AAA+ ATPase DnaA to the origin of replication (*oriC)* at midcell. DnaA binding results in open complex formation, permitting the replication machinery to load and replication fork elongation to proceed. In *B. subtilis*, blocking replication at initiation results in elongated cells with single, medially positioned

Figure 2



FtsZ polymers serve as GTP-dependent treadmills for the cell wall synthesis machinery in *E. coli* and *B. subtilis*. The peptidoglycan synthesis machinery (green) is tethered to short FtsZ filaments (yellow). FtsZ-dependent GTP hydrolysis stimulates treadmilling in which GTP bound monomers are added to the putative (+) end of the filament and GDP bound monomers are released from the (–) end. Treadmilling leads to the processive insertion of cell wall material at the septum. In *E. coli*, the positive regulators of division, ZapA (green) and ZapB (red) help organize FtsZ polymers within the divisome. ZapB helps coordinate division with DNA replication via interactions with the terminus binding protein MatP (gray).

chromosomes [18•]. The FtsZ ring is off-center in these cells, immediately adjacent to the unsegregated nucleoid. Strikingly, when DNA replication initiation and open complex formation is allowed to proceed, but replication fork elongation is blocked, FtsZ assembly shifts to midcell.

Based on these observations, Harry and Moriya proposed a 'ready-set-go' model in which assembly of the DNA replication initiation machinery at the origin, readies (or 'potentiates') midcell for FtsZ assembly [21]. Although the molecular mechanism underlying the ready-set-go model remains elusive, the idea that medial division site selection requires a handoff between the DNA replication machinery and FtsZ is appealing. Not only does it provide a satisfying link between the two pillars of the cell cycle, DNA replication and cytokinesis, but it also explains why increases in intracellular levels of FtsZ has a very modest impact on the timing and frequency of medial FtsZ ring in *E.* coli and *B. subtilis* formation [22•,23•] (for an excellent review of the factors coordinating division with DNA replication and chromosome segregation see Ref. [24]).

Division site selection: positive regulation by 'marker' proteins

While similar localization determinants have yet to be identified in *E. coli* and *B. subtilis*, in a wide range of organisms, FtsZ assembly at the nascent septum depends on the activity of a regulatory protein that marks the location of the future division site. In *Myxococcus xanthus*, PomZ, is recruited to midcell before FtsZ and promotes FtsZ assembly at this position [25]. PomZ is a homolog of ParA, an ATPase implicated in plasmid and chromosome partitioning, supporting a connection between DNA replication and cell division. MapZ (also known as LocZ), forms a ring at midcell in the Gram-positive pathogen *Streptococcus pneumoniae*, before FtsZ recruitment, where it serves to drive assembly of the division machinery [26]. After division, the MapZ ring splits in two and moves to the future division site in the newborn daughter cells. Although FtsZ is dispensable for hyphal growth in *Streptomyces coelicolor* [27], it is absolutely required for sporulation, which requires the transformation of long syncytial filaments into individual exospores. During this transformation, SsgA localizes to internucleoid spaces, recruiting first SsgB and then FtsZ to this position to initiate assembly of the cytokinetic machinery [28•]. In all these cases, how these 'marker' proteins recognize the nascent septal site remains an open question.

Corralling FtsZ

FtsZ assembly is subjected to multiple layers of regulation before, during, and after establishment of the nascent division site to promote efficient assembly of the cell division machinery ensure orderly progression through the cell cycle. Regulatory proteins include EzrA, which inhibits aberrant FtsZ assembly along the longitudinal axis of the cell in *B. subtilis*, and MatP and ZapB, which coordinate interactions between the division machinery and the terminus of DNA replication during chromosome segregation in *E. coli* [29a•,b,30], Most prominent among such regulatory factors are the Min proteins, which inhibit FtsZ assembly at aberrant positions, particularly cell poles, and DNA-associated nucleoid occlusion proteins (NO), SlmA in *E. coli* and Noc in *B. subtilis*, which help prevent FtsZ assembly over the unsegregated chromosomes [8,9•]. Importantly, cells defective in both Min and NO are still capable of establishing a medial division site with remarkable precision, counter to the oft suggested, yet erroneous idea that they play a primary role in medial division site selection [31••,32,33]. Division is overwhelmingly medial in *B. subtilis min* mutants, and septation is more or less equally distributed between medial

and polar positions in *E. coli* cells defective in *min* gene function [33–35]. NO in particular, appears to be primarily an insurance policy as NO mutants are essentially indistinguishable from wild type cells except under conditions in which DNA segregations is severely perturbed or in the absence of *min*. At the same time, while not essential for establishment of a medial division site in *E. coli* or *B. subtilis*, NO in *Vibrio cholerae* is strongly involved in timing and position of its Z-ring reinforcing the value of studying essential processes in multiple organisms [36].

Recruitment of the 'late' division proteins

Assembly of the division machinery is a multi-step process involving two sets of factors: the so-called 'early and late' division proteins. The early proteins include FtsZ and its membrane anchor FtsA, both of which are highly conserved among the bacteria, as well as other less well conserved factors including ZipA, EzrA, and the Zaps. The first to assemble at the division site, the early division proteins form what is collectively termed the 'proto-ring' (also known as the Z-ring). Proto-ring formation is followed by a time delay that can occupy up to 20% of the cell cycle, after which the 'late division proteins' assemble [22•,37•]. The late genes include the transpeptidase FtsI, and FtsW [3••,38•], both of which are required for synthesis of the septal wall [39,40]. The precise function of FtsW is somewhat unclear. Significant data support a role for the enzyme as a transporter of Lipid II linked cell wall precursors from the cytoplasm to the periplasm [41]. At the same time, FtsW shares a limited amount of homology with the putative elongation-specific transglycosylase RodA, raising the possibility it might serve a similar function during synthesis of septal peptidogylcan [38•].

As with FtsZ, the mechanisms controlling late protein recruitment to the proto-ring are poorly understood. Overproduction of FtsZ in *B. subtilis* accelerates proto-ring formation somewhat, yet does not alter the timing of late cell division protein recruitment [22•]. In *E. coli*, overproduction of FtsN—which interacts with both the proto-ring and the late division proteins—stabilizes the divisome but does not reduce the time between early and late cell division protein localization to midcell [37•,42]. Like FtsZ, the concentration of late proteins is more or less constant over the course of the cell cycle, suggesting recruitment is governed at the level of assembly [43].

It is possible that initial invagination of the cell membrane driven by the proto-ring serves as a temporal and topological marker for assembly of the late proteins. However, there is conflicting data about the potential for the proto-ring to initiate constriction in the absence of the late proteins [44]. On the one hand, purified FtsZ and FtsA or purified FtsZ fused to the membrane binding amphipathic helix of FtsA are sufficient for GTP-dependent constriction of liposomal membranes [45,46]. On the other hand, insertion of amphipathic helices in between lipids is known to strongly deform liposomes [47]. and the amphiphathic helix of FtsA is no exception (H. Strahl & LH, unpublished). It is thus debatable how well these *in vitro* studies reflect the situation *in vivo*, all the more so as these studies do not take into account the contribution of membrane potential, which is essential for FtsA function [48•]. Significantly, cryo-EM work has failed to find evidence for membrane invagination by the proto-ring [49], arguing against a role for local membrane curvature in triggering recruitment of late cell division proteins.

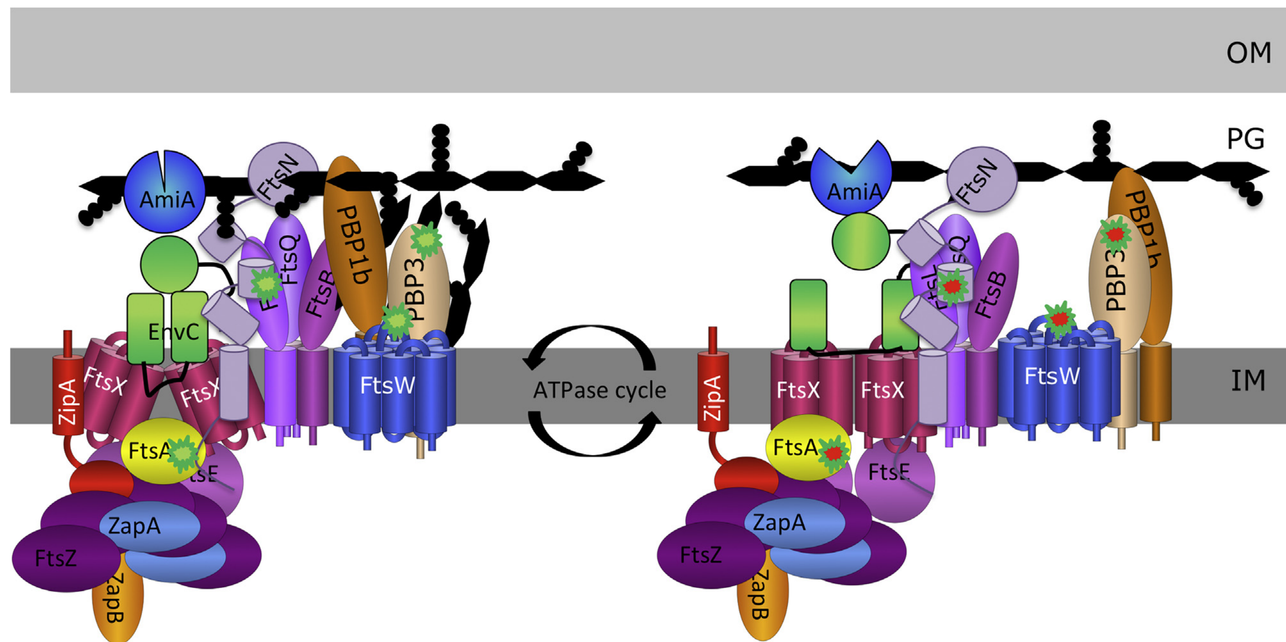
Instead, recent work suggests that cooperative assembly of late proteins is stimulated in response to interactions between FtsA and the late protein FtsN that alter FtsA's conformation and stabilize assembly of three conserved bitopic transmembrane proteins; FtsQ, FtsL and FtsB (DivIB, FtsL, and DivIC in *B. subtilis*) [50•,51,52•]. Lacking any apparent enzymatic function, FtsQLB localize as a group and stability of all three proteins depends on direct interactions between themselves and other late cell division proteins, particularly FtsW, FtsI and FtsN, all of which are recruited to the division site subsequent to FtsQLB [53–56]. Recent genetic data suggest that in *E. coli* the ABC-transporter-like complex, FtsEX, plays an important role in the timing of late protein recruitment, mediating the interaction between FtsA and FtsN, driving FtsA into the 'on' conformation and stimulating interaction with other components of the division machinery including FtsQLB [50•].

Triggering cytokinesis

Formation of the septal wall requires the transpeptidase FtsI and its putative cognate transglycosylase FtsW, as well as a bifunctional transpeptidase-transglycosylase, PBP1b. While FtsI and FtsW are essential, PBP1b is dispensable in the presence of the normally elongation-specific penicillin binding protein, PBP1a, suggesting the two PBPs are functionally interchangeable [57]. Allelic variants of *ftsI* and *ftsW* support a rate-limiting role for both in septal wall formation. In *E. coli*, a heat-sensitive FtsI variant, FtsI23, dramatically reduces the rate of septal wall synthesis while gain-of-function mutations in *C. crescentus* FtsW(A246T and F145L), and FtsI (I45V) significantly reduce cell size, consistent with accelerated division [44,58].

Despite their critical role in cytokinesis, recruitment of FtsI and FtsW to the nascent division site is insufficient to drive cytokinesis. Instead, a growing body of evidence supports a role for FtsN as a trigger for cytokinesis in part via direct interactions with FtsI and FtsW, but also through its role as an activator of FtsA mediated recruitment of FtsQLB [42,59–63] (Figure 3). In support of this idea, gain-of-function mutation in *E. coli* FtsA, FtsA* (R268W) and FtsL, FtsL* (E88K), appear to accelerate maturation of the divisome and bypass the essential

Figure 3



The *E. coli* divisome consists of two sets of factors: the early proteins (FtsZ, FtsA, ZipA, and ZapB in this figure), which constitute the proctoring, and the late proteins, whose recruitment is subsequent to and dependent upon the early proteins. FtsN bridges the early and late proteins, interacting with FtsA to stabilize the FtsQLB complex in the periplasm, and with FtsI/PBP3 and FtsW to stimulate cell wall synthesis (the latter interaction is not shown). Green starbursts indicate pre-activation state of FtsA, FtsN, FtsW and FtsI/PBP3 while green and red starburst indicates activated state. Additionally, FtsEX mediated ATP hydrolysis stimulates amidase activity (AmiA in this figure), thereby coordinating cell wall synthesis with hydrolysis to facilitate daughter cell separation. The model is not meant to reflect actual interaction stoichiometries, because they have yet to be determined. In addition, it is not yet clear if the amidases remain in complex with EnvC as drawn or if this interaction is also regulated. See text for details.

functions of FtsN, as well as another essential cell division protein FtsK, suggesting that FtsN's stimulatory role can be mediated solely through FtsA and FtsQLB under certain conditions [64•,65,66•].

Significantly, although FtsA, FtsQLB, FtsI and FtsW are widely conserved, FtsN is limited to Gram-negative organisms, suggesting that other bacteria utilize different mechanisms to activate division. Consistent with this idea, cell division proteins are phosphorylated in several bacterial species, including *B. subtilis*, *S. pneumoniae* and *Mycobacteria*—but not *E. coli*, suggesting a potential additional route for activation (for a review see Ref. [67]).

Divisome ultrastructure and the role of the 'bundlers'

Super resolution imaging indicates that the FtsZ ring is a discontinuous structure, appearing as larger nodes of high concentration separated by thinner regions of low concentration in both *E. coli* and *B. subtilis* [14••,68,69]. The ring is similarly discontinuous in *Caulobacter crescentus* [70] and FtsZ forms patchy foci in *Streptococcus pneumoniae* [71]. Wide field and confocal microscopy of longitudinally dividing symbiotic bacteria that grow while attached with one pole on the skin of marine nematodes can initiate constriction using a discontinuous Z-elipse [72•]. In these organisms constriction can also initiate from a single pole, utilizing an arc-like FtsZ structure instead of a ring [73]. Coupled with FtsZ's strong tendency to form lateral interaction alone *in vitro* [74], and the large number of proteins identified as 'FtsZ bundlers,' (for a review of this class of proteins see Ref. [75]) these observations supported a model in which the ring is composed of short FtsZ polymers held together in part via lateral interactions between single stranded protofilaments.

How lateral interactions relate to recent work indicating that FtsZ polymers 'treadmill' *in vivo*-depolymerizing from one end (−) and polymerizing from the other end (+) is unclear. Treadmilling results in rapid rearrangement of single stranded polymers, effectively moving them in one direction at a speed of about 20–40 nm/s depending on the bacterial species [16••,17••]. Treadmilling had previously been observed *in vitro* but its physiological significance was unclear before these studies [76]. Lateral interactions between protofilaments inhibit subunit turnover and reduce FtsZ's innate GTPase activity. Bundling of FtsZ should thus reduce the treadmilling speed of the protofilaments. However, in *E. coli* treadmilling speed seems to be independent from

the Zaps (ZapA, ZapB, ZapC, ZapD), all of which promote ring-formation *in vivo* and lateral interactions *in vitro*, as well as the spatial regulators, SlmA and MatP [16••,75].

A lack of impact on treadmilling dynamics argues against the Zaps and other proteins that promote lateral interactions *in vitro*, doing the same *in vivo*. Based on an average speed of 30 nm/s of treadmilling, the GTPase activity of *E. coli* and *B. subtilis* FtsZ should be approximately 0.3–0.6 mol GTP/mol FtsZ/s at 21°C [17••], which is much slower than the *in vitro* GTPase activity of 4.8 mol Pi/mol *E. coli* FtsZ/s at 30°C [77,78], but similar to the 0.8 mol Pi/mol *B. subtilis* FtsZ/s at 37°C under non-bundling conditions. Therefore, the *in vivo* speed of treadmilling does not exclude bundling. One possibility is that "bundling" proteins play a different role *in vivo*, ensuring that FtsZ filaments are maintained within the plane of the nascent septal site. For example, this large class of proteins might be important for filling in gaps between FtsZ protofilament clusters to maintain FtsZ's circumferential orbit. Consistent with this model, ZapA and ZapB molecules are visible by super resolution microscopy between FtsZ clusters [79].

The role of FtsZ's C-terminal linker domain and conserved C-terminal peptide in establishing and maintaining the ultrastructure of the FtsZ ring is another outstanding question. The flexible C-terminal linker is critical for FtsZ assembly dynamics both *in vitro* and *in vivo* [80•,81•]. This intrinsically disordered region averages ~50–60 residues in length in all but the alpha-proteobacteria where it can be over 200 residues. For example, in *C. crescentus* the C-terminal linker is ~150 residues in length and appears to mediate interactions between FtsZ and the cell wall synthesis machinery in addition to playing a role in FtsZ assembly [82]. The C-terminal peptide —a highly conserved set of approximately a dozen residues at the very end of the FtsZ polypeptide—has been implicated in interactions between FtsZ and a wide range of modulatory proteins (*e.g.*, [83–85]). At the seame time, its high degree of evolutionary conservation contrasts strongly with the lack of conservation among modulatory proteins, raising the possibility that the C-terminal peptide may help mediate longitudinal interactions between FtsZ subunits, and along with the C-terminal linker, contribute to the cooperative nature of FtsZ assembly [86].

Membrane fusion and daughter cell separation

Despite occupying a respectable portion of the division cycle, we have yet to identify the factors required for the last step in division: the membrane fusion event that generates two independent daughter cells. Like the assembly of the divisome, its disassembly is a time consuming event that occupies approximately 15% of the cell division cycle [87,88•]. In *E. coli* FtsZ and its membrane tethers FtsA and ZipA have left the closing septum well before the daughter cells are separated in two different compartments. The cytoplasm is compartmentalized before the periplasm [89] and a subpopulation of FtsN molecules together with FtsK, ZapB, and MatP all remain at mid cell after FtsI and the FtsQLB complex have left [87]. FtsK and MatP remain at midcell even after the other five proteins have moved away (TdB unpublished). By virtue of its role as a large integral membrane complex involved in translocating DNA trapped by the invaginating septum, and its persistence at the division site, FtsK is a good candidate for driving closure of the cytoplasmic membrane similar to the activity of SpoIIIE during sporulation in *B. subtilis* [90]. Since the essential membrane binding domain of FtsK can be bypassed by the FtsL* and FtsA* mutants [91], it seems unlikely to be the only protein involved in membrane closure. Also, the periplasmic part of FtsK is very small and unlikely to play a significant role in the closure of the peptidoglycan layer and outer membrane. An alternative for this is the Tol-Pal system, which bridges the entire cell envelope (inner membrane, cell wall, and outer membrane). Tol-Pal is involved in the coordination of concerted outer membrane and cell wall invagination [92] and possibly in lipid retrogade transport [93]. TolB follows the dynamics of FtsN (TdB unpublished) consistent with a role in the later stages of division. Cell wall cleavage between newly formed daughter cells is governed by the peptidoglycan hydrolases that are activated by FtsEX [94–97]. In agreement, the amidase AmiC localizes at midcell until daughter cell separation is complete [98,99] (TdB unpublished). In *Streptococcus pneumoniae* the peptidoglycan hydrolase PcsB appears to serve the same role as *E. coli* amidases. PcsB activity is stimulated by the divisome proteins FtsEX, which transverses the plasma membrane to activate PcsB on the outer surface of the cell envelope. Once activated, PcsB monomers interact with their counterparts in the opposing cell to 'unzip' the intervening peptidoglycan linking the two daughter cells [100,101].

Conclusion

The study of bacterial cell division and cell cycle regulation has come a long way since Yukinori Hirota and Antoinette Ryter attempted to make sense of a collection of conditional *E. coli* mutants in the laboratory of François Jacob [1••] (It is fitting that the field of bacterial cell division has its origins in this lab, as it was Jacob who said "Le rêve d'une bactérie doit devenir deux bactéries." *The dream of a bacterium is to become two bacteria*). Important as it was, Bi and Lutkenhaus' discovery that FtsZ forms a ring was only a beginning [4••], raising a host of questions that have kept many laboratories including our own busy for a quarter century. While a good number of such mysteries have been solved, many exciting questions—including those highlighted above—remain unanswered.

Recent advances in imaging and image analysis have made some of the questions tractable for the first time. Others, particularly those that involve analysis of essential and nearly essential factors, will require significant creativity and industry to solve. Despite these challenges, the compelling nature of the subject matter coupled with the well-known power of microbial genetics gives us confidence that the field will continue to thrive and grow for many years to come.

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