Cell PRESS

Special Issue – Synthetic Cell Biology

Towards a bottom-up reconstitution of bacterial cell division

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The components of the bacterial division machinery assemble to form a dynamic ring at mid-cell that drives cytokinesis. The nature of most division proteins and their assembly pathway is known. Our knowledge about the biochemical activities and protein interactions of some key division elements, including those responsible for correct ring positioning, has progressed considerably during the past decade. These developments, together with new imaging and membrane reconstitution technologies, have triggered the 'bottom-up' synthetic approach aiming at reconstructing bacterial division in the test tube, which is required to support conclusions derived from cellular and molecular analysis. Here, we describe recent advances in reconstituting *Escherichia coli* minimal systems able to reproduce essential functions, such as the initial steps of division (proto-ring assembly) and one of the main positioning mechanisms (Min oscillating system), and discuss future perspectives and experimental challenges.

The strategy of constructive (bottom-up) synthetic biology of bacterial cell division

The aim of synthetic biology is to dissect biological entities into functional modules (macromolecular assemblies, cells, tissues, organisms, and their networks), to modify them rationally, or to engineer entirely new ones with the goal of implementing novel biomimetic functionalities [1,2]. The bottom-up synthetic biology approach has strong physicochemical foundations; it is interdisciplinary and has a rigorous quantitative character. It aims at constructing biological parts, devices, and systems with increasing levels of complexity toward a minimal cell-like scaffold [3–5]. The 'top-down' approach relies rather on biotechnology and systems biology, seeking primarily the programming of novel gene circuits within an electronic and computational engineering framework [6,7].

In the context of bottom-up approaches, the *in vitro* reconstitution of minimal bacterial cell division machinery is an obvious and attractive goal. Considering the highly dynamic and membrane-associated nature of this essential machine, and the difficulty of assaying its functional reactions, it is a challenging prospect. However, in the past few years, new technologies for imaging and functional protein

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Keywords: constructive synthetic biology; cytomimetic biochemistry; macromolecular interactions; FtsZ; Min system; *Escherichia coli*.

Glossary

Amphitropic proteins: proteins that can exist as either soluble cytoplasmic or membrane-attached forms depending on physiological conditions of activation. Their reversible interaction with membranes seems to be mediated by specific lipids. This concept was proposed by Paul Burn (1988).

Atomic force microscopy (AFM): an imaging and measurement tool that provides a 3D map of a sample's surface. It allows the collection of topography images with resolution in the nanometer scale and, at the same time, maps other interesting properties of the samples. With the capacity to observe and manipulate biological surfaces under physiological conditions, AFM can be used to explore biological structures at the single-molecule level.

Division ring: a multiprotein complex whose presence at mid-cell is derived from the coincident location of several proteins (e.g., FtsZ, FtsA, ZipA, FtsN), because they are independently visualized as rings in dividing cells [8].

Divisome: the complete macromolecular machinery able to effect division in the living cell. All the proteins that form the division ring at mid-cell are part of the divisome. Other components such as periplasmic and outer membrane proteins and the cell envelope may also be integral parts of the divisome. The term divisome was proposed by Nanne Nanninga (1998).

Fluorescence recovery after photobleaching (FRAP): the observation and measurement of FRAP allows scientists to investigate the diffusion and motion of fluorescently labelled biological macromolecules. The fluorescently tagged biomolecule of interest is visualized at low light intensity followed by photobleaching of a user-defined region of interest with high-intensity light, causing the fluorophores to bleach in the selected region. The measure of the rate of recovery of fluorescence allows one to infer how fast macromolecules move.

Fluorescence resonance energy transfer (FRET): a technique for measuring interactions between two molecules. It relies on the ability to capture fluorescent signals from the interactions of labeled molecules in single living or fixed cells. If FRET occurs, the donor channel signal will be quenched and the acceptor channel signal will be sensitized or increased. This allows investigation of the colocalization of the donor- and acceptor-labeled probes and the molecular associations at close distances.

Minimal divisome: the theoretical minimal set of elements capable of effecting division. It can be defined genetically, when using mutants defective in some of the divisome elements, or biochemically when using artificial cell envelope assemblies in the test tube.

Membrane-targeting sequence (mts): among other amphitropic proteins, FtsA and MinD present a highly conserved C-terminal sequence motif that it is thought to form an amphipathic helix that mediates the direct interaction of these proteins with lipid bilayers. The mts from MinD was incorporated into chimeric FtsZ forms to bypass the need for membrane-anchoring proteins.

Proto-ring: the natural initial subset of the division ring able to be anchored to the cytoplasmic membrane. In *E. coli*, it is formed by FtsZ, FtsA, and ZipA and it is assumed to be the initial step of divisome assembly [10].

Total internal reflection fluorescence microscopy (TIRFM): a powerful fluorescence microscopy technique that allows imaging but also the measurement of dynamics and single-molecule events taking place in a thin section (~100 nm) in the contact surface between the specimen and the glass slide. An evanescent field propagates into the specimen on total reflection of the incident beam at the liquid/glass interface. In this way, only the fluorophores close to the surface are excited, thus reducing background fluorescence due to out-of-focus molecules.

Z-ring: a structure present at mid-cell of dividing cells and visualized by microscopy techniques using FtsZ-specific reagents, such as fusions to fluorescent proteins or antibodies against FtsZ. It is postulated to be a ring of variable diameter formed by the accumulation of FtsZ-containing complexes [8]. The Z- ring has been assumed to provide a scaffold on which the remaining division elements assemble due to their ability to interact directly or indirectly with FtsZ. The term Z-ring was proposed by Joe Lutkenhaus (1991).

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reconstitution in membranes have put this goal within reach. Today, we know the main elements, their biological function, and a large part of their biochemistry; therefore, it seems not unreasonable to expect that the application of this bottom-up synthetic strategy to construct minimal divisomes (see Glossary) in the test tube is feasible and will yield functional assemblies. This constructive synthetic approach will help us to support conclusions already derived from cellular and molecular analysis and, therefore, to complete our understanding of how the bacterial division machinery works.

The bacterial cell division machinery

Bacterial cell division is mediated by a multiprotein machine whose elements interact dynamically at mid-cell to form the division ring, a structure that drives cytokinesis, forming part of the divisome [8,9]. The position of the ring is highly regulated by two negative control systems that inhibit its incorrect formation (see below). In the case of *E. coli*, the division ring is formed by at least ten essential proteins, most of them integrated at the membrane. Their assembly pathway has been mostly derived from the behavior of mutants in which divisome elements have been

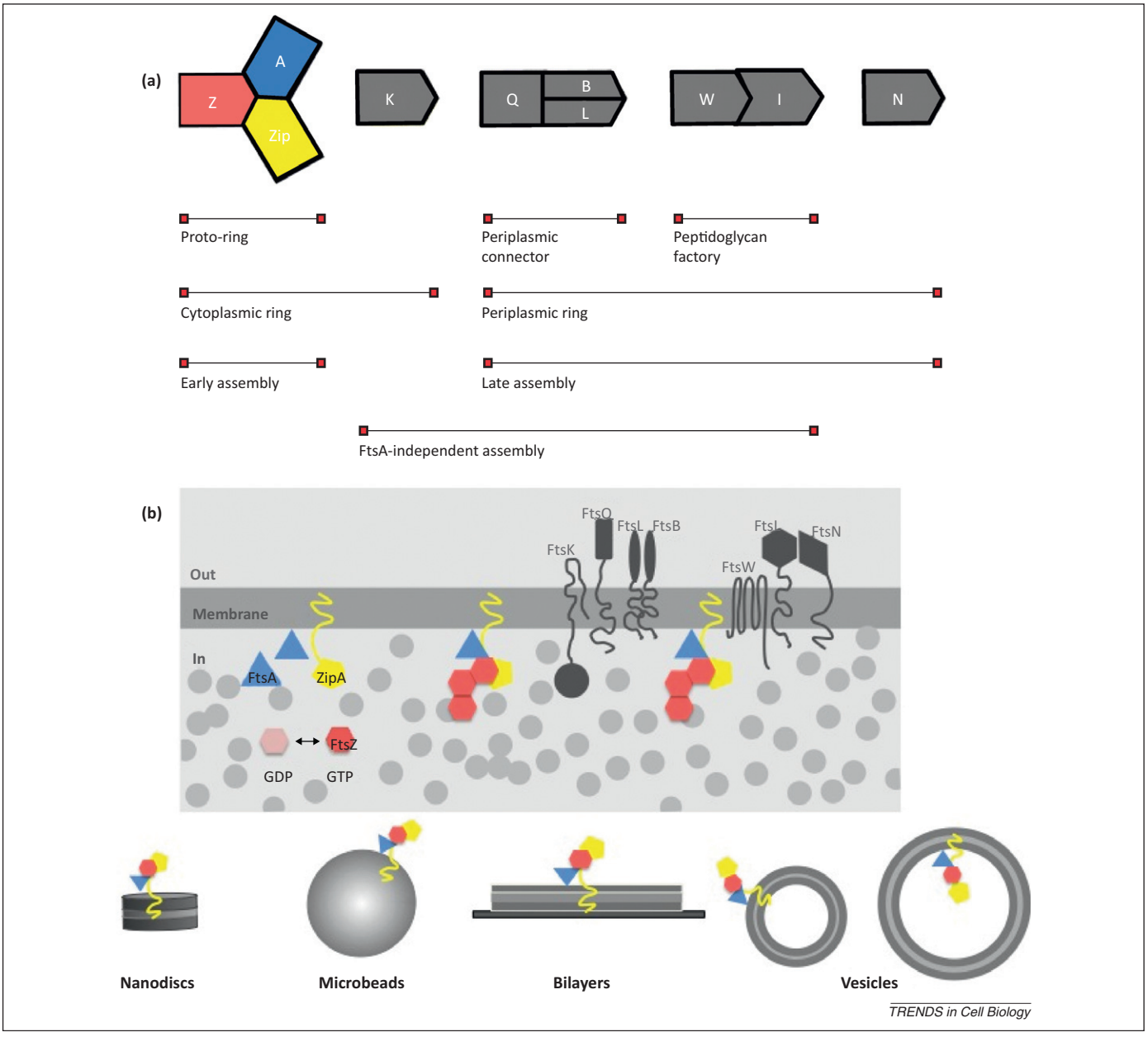

Figure 1. Assembly of the essential components of the *Escherichia coli* division ring. (**a**) First row: FtsZ interacts with FtsA and ZipA at the cytoplasmic membrane forming the initial assembly of the division machinery, the proto-ring. For the sake of simplicity other division proteins (such as FtsE/X or Zap proteins) are not shown in this scheme. FtsK is then added sequentially, followed by a group formed by FtsQ, B and L. FtsW and I (≡PBP3), two proteins involved in septal peptidoglycan synthesis, are then added together to the ring, which is completed by addition of FtsN. The rest of the rows describe the functionality and localization of the ring components. Republished with permission from [10]. (**b**) Proto-ring in cells and reconstruction of proto-rings in membrane systems. Diagram illustrating the position of essential divisome elements in the living cell and reconstruction of proto-rings in biomimetic membranes: nanodiscs, microbeads, supported lipid bilayers, and vesicles. The proto-ring components are colored; other elements of the divisome are shown in dark grey. The light grey circles in the upper part of the diagram represent macromolecules that contribute to the crowding of the cytoplasm. Vesicles show the two alternative orientations of the reconstructed proto-ring relative to the vesicle lumen.

genetically altered (Figure 1a) [10]. The first macromolecular assembly of the divisome is the proto-ring, comprising three proteins – FtsZ, FtsA, and ZipA – that associate at the cytoplasmic membrane forming the scaffold structure into which the rest of the essential division proteins are incorporated in a sequential and concerted manner (Figure 1a).

Central to assembly of the division machinery is FtsZ (40 kDa), the best-known prokaryotic division protein, which is widely conserved in bacteria and the ancestor of eukaryotic tubulin [11,12]. Although the basic unit of FtsZ is a monomer and the tubulin protomer is a heterodimer, both proteins bind and hydrolyze GTP and form polymers in a GTP-dependent manner, making FtsZ polymers very plastic and polymorphic. The GTP-dependent FtsZ assembly/disassembly cycle it is thought to be essential for Z-ring function [12–14]. Current research on FtsZ is aimed at describing the polymerization and force-generation mechanisms and evaluating the roles of nucleotide exchange and hydrolysis [11,15]. FtsZ is cytosolic and attaches to the membrane though interactions with FtsA or ZipA. FtsA (45 kDa) belongs to the actin family and contains a short amphipathic helix that it is thought to mediate its association with the membrane [16]. ZipA (35 kDa) contains an amino-terminal domain that is integrated into the membrane and connected to a cytoplasmic FtsZ-interacting domain via a flexible linker [17,18]. The attachment of FtsZ to the membrane requires either ZipA or FtsA, but in the simultaneous absence of both, no localization of FtsZ occurs. Gain-of-function mutants of FtsA, such as FtsA*, can bypass the need for ZipA for recruitment of downstream proteins to form a division ring [13,19]. Addition of FtsA* to FtsZ should be sufficient, in principle, to form an active, membrane-anchored proto-ring without ZipA. However, there is no evidence suggesting that the behaviors of FtsA* and native FtsA are the same.

Spatial modulators of divisome assembly – the oscillating Min CDE system

In *E. coli*, the positioning of the division ring at mid-cell at the end of the cell cycle is regulated by two independent mechanisms, the Min system and nucleoid occlusion, that cooperate to target the formation of the ring at the right place [9,14,20,21]. The control exerted by the bacterial nucleoid (bulk chromosomal DNA), largely due to crowding/electrostatic effects and the action of the protein SlmA [22], is nevertheless not able to block the formation of polar Z-rings, suggesting that the task of restricting septation to mid-cell might significantly rely on the Min system.

The Min system comprises MinD, E, and C proteins, which oscillate repeatedly from one pole of the cell to the other (Figures 2 and 3) [23,24]. A description of the biochemical and molecular details that account for the interchange between membrane-bound and cytoplasmic locations of the Min proteins are reviewed elsewhere [25,26]. Briefly, MinC inhibits FtsZ assembly [24,27,28]. MinD, a membrane-bound ATPase member of the Walker

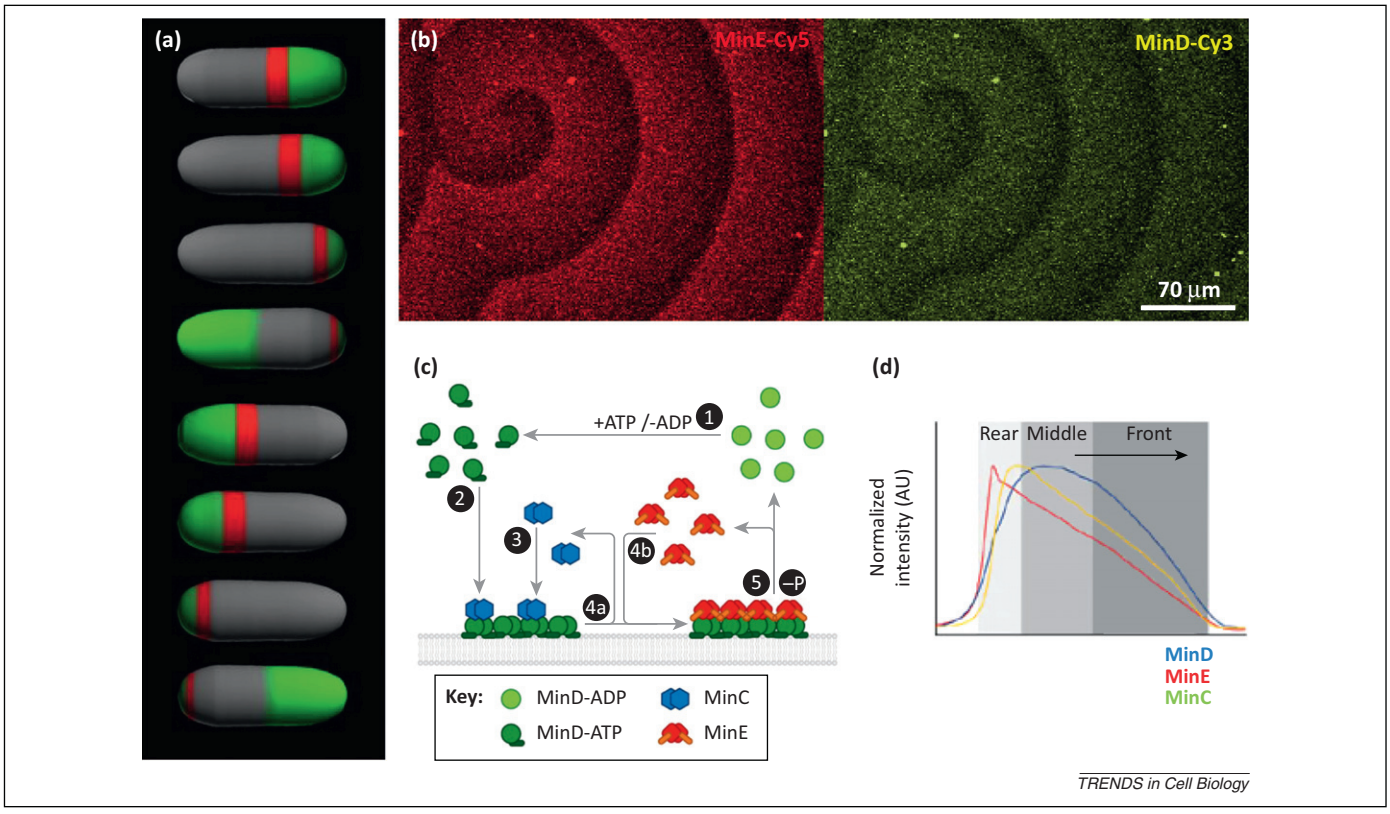

Figure 2. Min oscillating system. (**a**) MinE fluorescence (red) is observed as it pushes the green fluorescent signal of MinD (yellow). Scheme from [78]. (**b**) Confocal fluorescence micrographs showing spiral waves of MinD and E in the presence of ATP on a supported lipid bilayer *in vitro*. (**c**) Simplified scheme of the biochemical reactions underlying the oscillating behaviour. ATP-bound MinD binds to the membrane as dimers. Both MinC and MinE can bind to membrane-bound MinD, but MinE is able to displace MinC from MinD. MinE stimulates the ATPase activity of MinD and, on hydrolysis of ATP, MinD and MinE are released from the membrane [26]. (**d**) Normalized fluorescence intensity profiles of Min D, E, and C corresponding to waves equivalent to the ones shown in (b). Intensity profiles reveal the relative position of the Min proteins within the wave. Black arrows indicate the traveling direction of the protein waves.

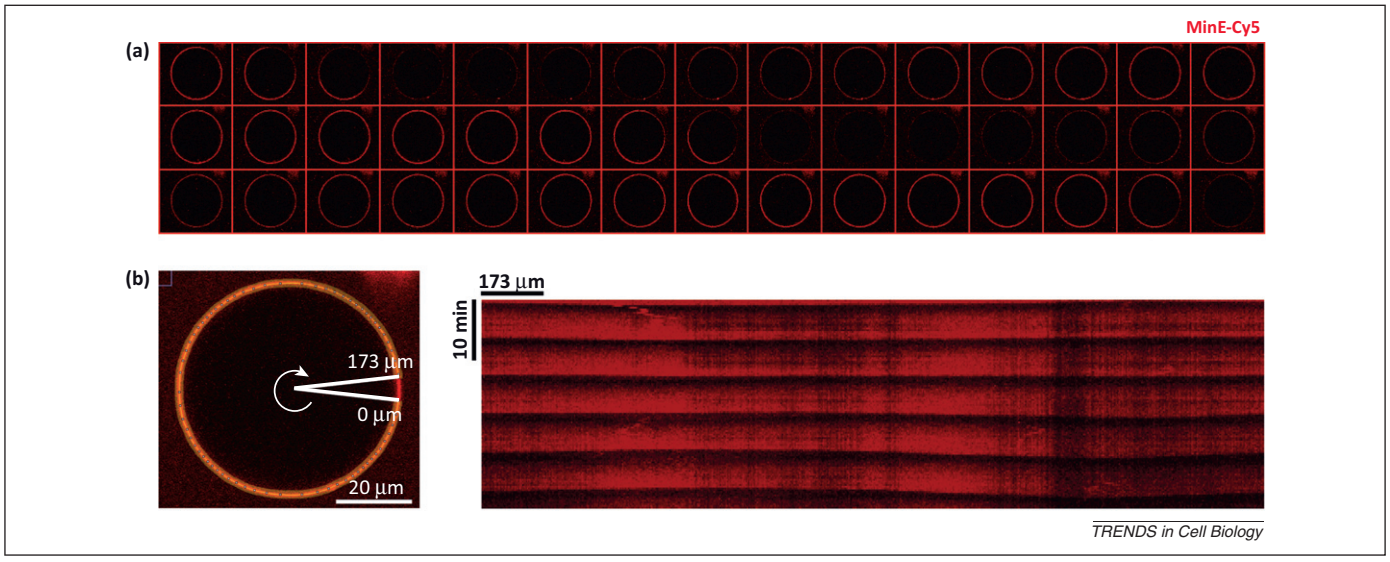

Figure 3. MinD/E waves propagating on the outer surface of giant unilamellar vesicles (GUVs). (**a**) Sequential confocal images of the equatorial plane of a GUV in which MinD/E oscillations are observed on its outer surface are shown (images starting on the upper left toward the right side of the panel; taken every 5 s). The waves appear as an alternate increase and decrease in the fluorescence intensity of Cy5-labeled MinE. (**b**) Kymograph of the GUV shown in (a). The rim of the vesicle is analyzed as a straight line and its fluorescence intensity represented over time. This reconstruction closely resembles the kymographs of the waves previously observed in supported lipid bilayers.

A cytoskeletal ATPase (WACA) family, powers the oscillation by ATP consumption [25,29,30]. It activates MinC's inhibitory activity and recruits it to the membrane, while MinE activates MinD's ATPase to detach MinD from the membrane and drive the oscillation [31]. Their combined action results in FtsZ assembly into a ring at mid-cell, where the local time-averaged concentration of MinC inhibitor is the lowest. Fusions of the Min proteins with fluorescent proteins allow following of these pole-to-pole oscillations *in vivo*. In this way, another prominent feature of the Min system can be observed, the so called 'E-ring', comprising a sharply defined band of MinE that travels behind MinD and MinC [23].

The use of biomimetic membranes to reconstruct minimal divisome assemblies

One major advantage of *in vitro* reconstitution experiments in biomimetic membranes is that both biochemical and biophysical parameters can be controlled precisely, providing an opportunity to investigate the complex spatial and temporal dynamics of membrane-based processes under defined conditions (Figure 1b and Box 1) [32–34]. Supported membrane structural organization (e.g., the presence of membrane microdomains) can be studied in detail by fluorescence microscopy, but are also perfectly suited to be studied by surface probe techniques such as atomic force microscopy (AFM), to yield nanoscopic resolution. Employing total internal reflection fluorescence (TIRF) as an excitation scheme, the dynamics of single lipid and protein molecules in membranes can be visualized and dynamic interactions of soluble proteins with membranes can be resolved [35]. Another model membrane system useful in reconstitution experiments is giant unilamellar vesicles (GUVs), which are closed lipid bilayers with diameters well above the optical resolution limit, from several to hundreds of micrometers. Due to their large size, they are perfectly suited to investigations by optical microscopy, and the behavior of macromolecules (divisome proteins) trapped in the GUV interior can be studied by microspectroscopy techniques [36]. Finally, coated microbeads, comprising lipid bilayers deposited on solid, spherical surfaces, are a useful novel membrane model to reconstitute macromolecular assemblies. They offer a robust procedure to generate lipid surfaces with uniform curvatures and cell-like sizes, in which both lipid composition and the material of the bead can be modified to obtain the desired properties of the system [3,32].

Nanodiscs, formed by a membrane scaffold protein encircling a phospholipid mixture [37], are a promising novel tool to study assembly on a lipidic surface while the system under study remains soluble. When a target membrane protein is included in the mixture, each nanodisc self-assembles and incorporates a single target molecule, preserving its natural structural and functional properties. Targets may be single proteins (e.g., ZipA) or even a protein complex (e.g., ZipA or FtsA plus FtsZ). Due to their small size, nanodiscs remain soluble, allowing the use of solution biochemical and biophysical tools [38,39] to characterize the embedded protein and to monitor and measure its interactions with other proteins.

Reconstruction of a minimal divisome in the test tube, step-by-step

For successful reconstitution of bacterial cell division, two essential phenomena observed in intact cells need to be achieved by a minimal protein machinery: (i) the assembly of the divisome components at discrete regions along the cell length (this occurs usually at mid-cell in normal bacterial division, but might vary in mutants such as those defective in the Min system); and (ii) the function of the force-generating elements that initiate constriction of the membrane. We will first discuss approaches toward reconstituting the Min positioning system and then touch on the perspectives of realizing a contractile proto-ring.

Box 1. The membrane as an element of the divisome

In the bacterial cell, it is likely that membrane component redistributions during cell division may create lipid domains [79] and regions highly concentrated in specific components that can trigger membrane deformations (favouring curved structures) [80,81] that might contribute to the accurate selection of the division site and to the force generation that drives constriction. In addition, membrane fluidity might also modulate bacterial division, as suggested by recent observations that increasing fluidity favours cytokinesis of L forms [82] and FtsZ polymers modify the viscoelastic properties of *E. coli* lipid monolayers to become more fluid and plastic [83,84]. The composition of a bilayer defines its mechanical properties [85]. Three major lipids constitute the inner membrane of *E. coli*: phosphatidylethanolamine (74%), phosphatidylglycerol (19%), and cardiolipin (3%). This mixture confers a net negative charge to the membrane at physiological pH of 7.5. The relevance of the lipid composition takes place at two levels, modulating the mechanical properties of the membrane and offering specificity as recruiting factors. First, enriched domains on cardiolipin were detected at both the cell poles and the septal region of dividing *E. coli* cells [86]. This conical phospholipid tends to form high-curvature rather than bilayer structures, indicating that its accumulation might provoke mechanical destabilization of the bilayer thus facilitating morphological transformations of the membrane. Second, its anionic character, together with its marked distribution in key areas of the membrane during cell division, make cardiolipin a suitable target for amphitropic proteins such as FtsA [16]. These differences in the distribution of cardiolipin in the membrane along the cell suggest an active role of this lipid in cell division. In addition, MinD has been shown to bind preferentially to domains of anionic phospholipids on the inner membrane [87]. Related to this, it has been reported that membrane potential might be relevant for the binding of MinD and FtsA to the membrane [88].

The high protein content present on the inner membrane of *E. coli*, where the lipid/protein ratio is 0.4, is notable [89]. This is likely to have a major effect on the physical properties of the membrane, because imposition of physical constraints on its plasticity would make it less prone to change. In summary, the restoration of the membrane can also be considered a strategy towards the reconstruction of bacterial division. Accordingly, the manipulation of membrane composition in membrane models would contribute to revealing its relationship with the constriction process.

Reconstituting Min oscillations in vitro

The Min system has been described as a reaction–diffusion system in which, starting from a homogeneous distribution, the oscillatory patterns emerge by self-organization on dynamic instability powered by the hydrolysis of ATP. In fact, these dynamics largely depend on collective membrane binding and unbinding. Thus, a crucial step toward a minimal system for Min oscillations was achieved by the reconstruction of *in vivo* cycling behaviour on artificial bilayers mimicking the inner membrane of *E. coli* [26,40]. In the presence of ATP, protein waves emerged on *E. coli* lipid bilayers on incubation of purified and fluorescently labelled Min proteins at physiological ratios [41,42] (Figure 2). In the absence of spatial boundaries, these waves propagated in arbitrary directions across the bilayer. Using confocal and single molecule TIRF microscopy, all three proteins were simultaneously imaged and the order of events during wave propagation could be determined (Figure 2).

Furthermore, this *in vitro* system reproduces some relevant features of the *in vivo* oscillations [40], such as a similar speed, about 0.5 μm/s, which depends on the concentrations of MinD and MinE [26]. In addition, the obtained distribution profiles are compatible with the dynamic behaviour displayed *in vivo*, such that MinE accumulates following MinD and MinC toward the rear of the wave, resembling the E-ring in cells. It was confirmed that MinC is not required for the cycling, but assumes the role of a passenger. Interestingly, the wavelength of the *in vitro* pattern is 10 times larger than that in an *E. coli* cell, which has been proposed to be explained by crowding effects or higher reaction rates [26], although neither has been confirmed experimentally yet.

According to single-molecule imaging of Min dynamics [26], the patterns emerge from an isotropically distributed carpet of MinD on the membrane [40]. A difference in the membrane residence times between MinD and MinE, with MinE being attached longer than MinD, originates small fluctuations in the MinD/MinE relative ratio, such that accumulation of MinE initiates a dominant wave of protein detachment, leading to faster detachment of MinD toward the end of the wave. Fluorescence resonance energy transfer (FRET) experiments on these supported bilayers confirmed that MinE binds to MinD as a dimer [40].

Regarding a plausible explanation for the formation of the E-ring *in vivo*, evidence from several experiments points in favor of persistent binding of MinE [43–45] rather than formation of higher-order complexes [46]. The difference in residence times between MinD and MinE, together with a decrease in the diffusion coefficient of MinE by a factor of two in the back of the wave, may explain the accumulation of MinE in the rear and therefore the E-ring *in vivo* [40]. Fluorescence recovery after photobleaching (FRAP) experiments demonstrated that incorporation of MinE starts from the front of the wave, further corroborating accumulation – in contrast to cooperative binding – of MinE [26]. Experiments using MinE C1, a mutant unable to bind to the membrane, revealed that transient membrane binding of MinE could be partially involved in the reported persistent binding [43,47,48]. Unlike wild-type MinE, the fluorescence intensity profiles of the much less regular waves based on MinE C1 do not show its accumulation at the rear, nor displacement of MinC from membrane-bound MinD [40].

The mechanistic picture that emerges from the data obtained by reconstruction of a minimal Min system is summarized in Figure 2. Recently, it has been verified that the Min patterns can be equally well established on vesicle membranes (Figure 3). The obvious next step is now to encapsulate the Min protein dynamics into a closed compartment and convert the propagating waves – which do not allow directing Z-ring assembly to a specific site – to true oscillations with a clear minimum in Z-ring assembly inhibition.

Minimal bacterial proto-rings in vesicles

The activity, interactions, and assembly properties of the proto-ring elements can be quantitatively characterized in minimal membrane systems structured as nanodiscs, coated-microbeads, lipid bilayers, and, finally, vesicles (Figure 1b). The first example of this synthetic approach [49] demonstrated the assembly of a purified variant of FtsZ ring inside unstable vesicles that spontaneously changed in shape over time, suggesting that FtsZ is able to at least partly constrict the region of the liposome in

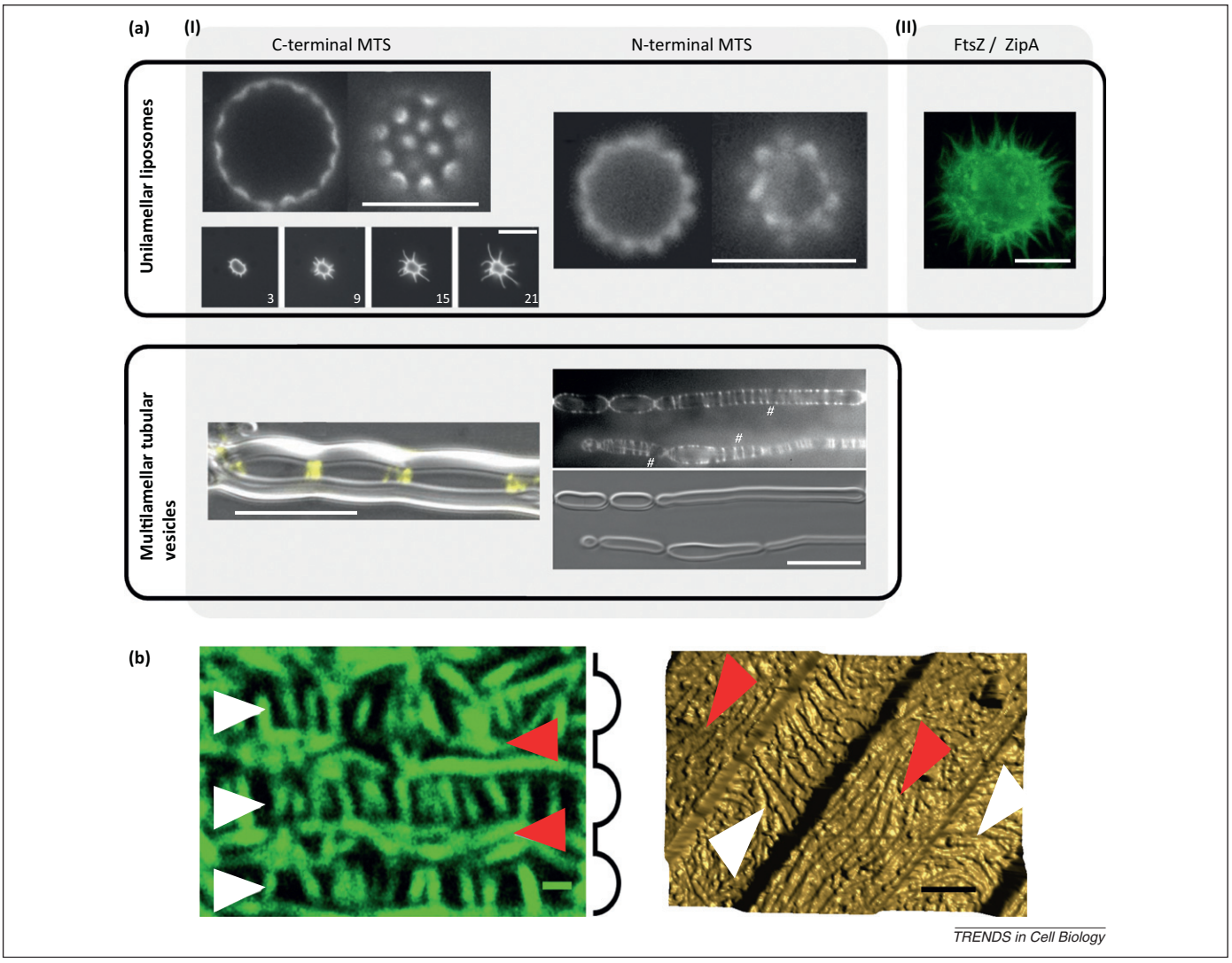

Figure 4. Reconstruction of the contractile FtsZ element. (**a.I**) Reconstruction of chimeric FtsZ (C-terminal or N-terminal mutants) in unilamellar liposomes and tubular vesicles. FtsZ is able to narrow the region of the liposome in which it is located. Composed image of liposome contour (from phase contrast microscopy) and fluorescence of chimeric FtsZ colored in yellow [49]. In unilamellar liposomes, chimeric FtsZ deforms the bilayer according to the location of the mutant construct, suggesting that FtsZ presents an intrinsic angle of polymerization. In tubular vesicles, FtsZ is able to condense into several rings, narrowing the region in which it is located. Adapted from [49]. (**a.II**) Membrane tubulation of giant vesicles containing ZipA driven by native FtsZ polymers as demonstrated by confocal microscopy using Alexa 488-labeled FtsZ (A. Martos *et al.*, unpublished). Scale bars = 10 μm. (**b**) (Left panel) Fluorescence image of copolymers of YFP-mts-FtsZ and FtsZ assembled on glass substrates having curved grooves. The profile of the surface is shown on the right. (Right panel) Atomic force microscopy image of filaments assembled on a similar substrate. Red arrows show filaments on planar surfaces and white arrows show filaments on the curved grooves. Scale bars = 1 μm [57].

which it is located (Figure 4a.I). The authors used an artificially membrane-targeted FtsZ variant (membrane-targeting sequence [mts]-FtsZ) to tether it to the liposome in the absence of physiological membrane-anchoring proteins. The cylindrical geometry of the tubular vesicles seems to be important, because no Z-rings were found inside spherical liposomes. The incorporation of mts-FtsZ polymers into the outside of liposomes produced visible deformations (liposome tubulation) [50], an observation recently confirmed using a vesicle reconstruction containing natural FtsZ and ZipA (Figure 4a.II) (A. Martos *et al.*, unpublished). FtsZ polymer bending (associated with the natural curvature of FtsZ) is thought to be a relevant feature in generating the force that promotes membrane deformation, because the geometry of the distortion depends on the FtsZ terminal region at which the membrane-targeting sequence is attached. These experiments proved that FtsZ polymers alone are capable of generating force to promote lipid vesicle deformation, although the biological implications of this force within the (much more challenging) task of full membrane constriction and cell division remain unclear [49–51].

The next step towards proto-ring reconstruction has been achieved by incorporating the native FtsZ form (instead of the mts-FtsZ variant) together with FtsA inside giant vesicles obtained from bacterial inner membranes (Figure 5). Being more complex in composition than artificial lipid vesicles, they maintain a distribution of proteins and lipids resembling more closely the native membrane, and contain the essential membrane-anchoring factor ZipA naturally inserted. FtsZ polymerization alters the spatial distribution of the proto-ring complexes inside the vesicle, resulting in dislodging of FtsA from the membrane to associate with FtsZ fibers in the vesicle lumen, showing that, under assembly-promoting conditions, the interaction between FtsZ monomers is stronger than the binding

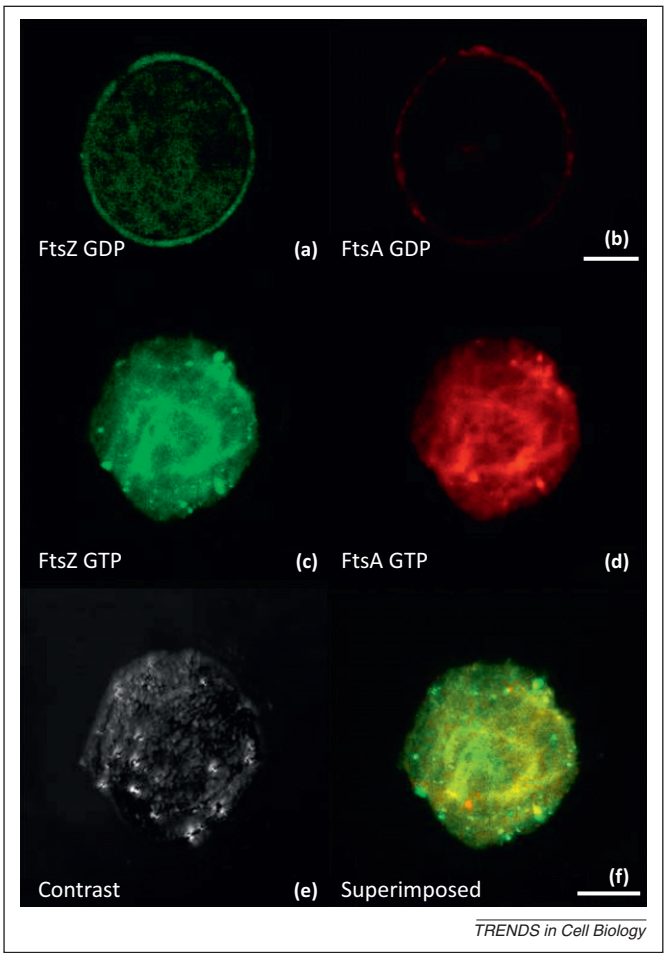

Figure 5. Reconstruction of proto-ring elements in giant unilamellar vesicles (GUVs). The figure shows the spatial distribution of FtsZ and FtsA inside GUVs made from inner bacterial membrane. Confocal images of Alexa 488-labeled FtsZ (green; **a** and **c**) and Alexa 647-labeled FtsA (red; **b** and **d**), formed in the absence (**a** and **b**) and in the presence (**c** and **d**) of GTP. The superposition of (c) and (d) can be observed in (**f**). Differential interference contrast image of the giant unilamellar inner membrane vesicle (**e**). Scale bars = 10 μm. Republished with permission from [52].

strength of FtsA to the membrane [52]. These findings suggest that in addition to the known role in the association of FtsZ with the membrane during division, FtsA may have other roles related to its amphitropic character, which may involve signalling of proto-ring assembly completion or the activation of late divisome functions. Interestingly, FtsA interacts with FtsN [53], a late-assembling protein essential in septation, because its absence results in destabilization of the already assembled divisome, even affecting the proto-ring elements [54]. These results show that the interaction between elements of the divisome is far more complex than what could be deduced from the visual inspection of the assembly of rings in a living cell. These approaches can help to compare the *in vivo* and *in vitro* systems to obtain new knowledge on the process of division.

Structural features of minimal proto-rings on supported and free-standing lipid bilayers

The structural and dynamic organization of FtsZ polymers has been studied by AFM on mica and on lipid bilayers containing a soluble variant of ZipA, suggesting that they are very plastic [55,56]. FtsZ polymers anchored to lipid bilayers through ZipA form dynamic 2D networks that retain their capacity to dynamically restructure, to fragment, to anneal, and to condense laterally [56]. In a recent approach [57], microstructured substrates to support membranes were employed to investigate the curvature-dependent membrane attachment, and thus curvature preference, of FtsZ rings. It could be shown that Z-rings comprise pre-curved FtsZ filaments, which preferentially orient themselves along curvatures resembling the native inner diameter of *E. coli* cells. FtsZ filaments seem to avoid smaller diameters (resembling constriction stages during cell division), which can be evidenced by the slanted orientation of filaments in membrane-coated grooves of greater curvature (Figure 4b). This speaks for the requirement of additional factors to facilitate constriction during cell division and against FtsZ filaments or rings being the sole force-generation system. However, conclusive direct measurements of forces exerted by FtsZ filaments with or without additional divisome proteins, similar to motor protein assays, are still lacking.

Biochemical features of proto-ring proteins in coated beads and nanodiscs

Lipid- and membrane-coated beads allow measurement of the binding affinities of soluble proteins to membrane structures. A recent example is the incorporation of FtsA in these structures [58], showing that the apparent affinity of FtsA to the inner membrane is tenfold higher than to the *E. coli* lipids (without integral proteins). The reconstitution of the three proto-ring elements in coated beads is feasible, because these membrane systems appear to retain their capability to interact with FtsZ (Monterroso *et al.*, unpublished). Beads encoded with gold/silver sensor particles before membrane coating are being exploited to design assays to monitor interactions between proto-ring elements [59].

The nanodisc technology has recently been exploited to elucidate biochemical features of elements of the bacterial division machinery and their interactions under well-defined lipid environments [38]. A single copy of the full-length ZipA protein from *E. coli* has been incorporated in phospholipid bilayer nanodiscs. ZipA in nanodiscs binds FtsZ oligomers (GDP forms) and polymers (GTP forms) equally with moderate affinity, which is also similar to the binding strength of a soluble form of ZipA to FtsZ, suggesting that the transmembrane segment of ZipA has no role in complex formation. These findings have provided new insights into the role of ZipA as a passive anchoring device, supporting the notion that FtsZ tethering to the membrane has enough plasticity to allow the remodelling of the architecture of the division ring during constriction.

Outlook: experimental challenges

The bottom-up synthetic approaches described here, to test the properties of the elements of the division machinery and their interactions in well-defined and controllable environments, have helped us to gain new mechanistic insights into their functions. However, regarding possible reconstitution of full bacterial cell division in minimal systems, many more experimental challenges await us. First, the true origin of a constriction force will have to be

elucidated. A model of action of FtsZ (and the potential influence of companion division proteins) needs to be completed to reconcile the FtsZ force-generating properties *in vitro* [60] and the FtsZ dynamics *in vivo* [12], with their implication on division. Moreover, the mechanical role of the cytoplasmic membrane (Box 1) and cell wall synthesis during septum formation are far from being understood. Second, the correct positioning of the division site by the Min protein system will have to be verified, by introducing the Min system as well as the required proto-ring elements into deformable closed containers, preferably vesicles. These goals will require the successful introduction of functional proteins into the vesicles at physiological salt conditions without disrupting them (which has proved to be rather complicated at large scales so far) and the identification of physical preconditions to convert traveling Min waves into steady oscillations. Progress in current research toward the optimization of procedures to encapsulate protein complexes with high yield inside vesicles in a controlled manner using permeable membranes and microfluidic technologies will be crucial [61–64]. Moreover, lipid chemistry and microsystems technology will probably be required to establish vesicle size and shape as tunable parameters in reconstitution experiments. In a recent study, [65] it was demonstrated that Min waves and patterns already respond quite strongly to the structure of membranes. In a 2D assay with patterned membranes, it was found that the waves could be directed by sensing the dimensions of membrane patches, without any confinement in 3D being required. This raises the interesting question of whether the inner surface, rather than the volume of bacterial cells, provides the spatial cue for division-related processes.

The nucleoid, a highly charged structure that occupies a large volume of the intracellular space, may significantly influence protein interaction networks in the cytoplasm and at the membrane; for example, it has been recently shown that large DNA molecules act as an exclusion zone for actin fibers within a microsphere [66]. Therefore, future reconstitution approaches may need to use environments that faithfully reproduce natural crowding and its associated electrostatic/excluded-volume effects when attempting to assemble functional divisomes (Box 2). Research toward a better understanding of the physicochemical and biochemical properties of highly concentrated heterogeneous mixtures of proteins and/or other macromolecules [67,68] and the production of artificial organelles [69] will be essential. Along the same lines, the reconstitution of fortifying shells around membranes, mimicking the peptidoglycan layer, appears to be a highly attractive, albeit complicated, mosaic stone in a full mechanical understanding. However, cell division studies on so-called L forms – bacterial cells deprived of their cell walls – argue against a decisive role of this feature in cell division [70]. Their division, which does not require FtsZ, is far from being understood.

In addition to these fundamental questions, nearly all of the subsystems discussed above, such as the Min and Fts proteins, deserve a more quantitative study of their biochemical properties to better understand and modulate their functional reactivity. Of particular interest are their specific ways of interacting with the membrane as one key template for all division-related processes. New and improved biophysical methods need to be introduced to bacterial cell biology, to allow for a better understanding of membrane binding and dissociation, such as super-resolution and single-molecule imaging, dynamic techniques like fluorescence correlation spectroscopy, and plasmonic methods invoking the potential of nano-optics [71,72].

Box 2. Numbers in *E. coli* cell division

Proto-ring elements

It is estimated that each *E. coli* cell contains 4000–15 000 FtsZ [90,91], 600–3000 FtsA [90], and 1000–1500 ZipA [90,92] molecules. In the case of FtsZ, this would correspond to an average concentration of approximately 3–10 μM (0.1–0.4 mg/ml). Non-essential division proteins (e.g., Z-ring-stabilizing elements) are estimated to be less than 5% of FtsZ numbers [93]. Because FtsZ assembly follows a cooperative mechanism, it would be distributed into soluble and polymeric forms whose relative abundance would be modulated in the cell by several parameters, such as protein concentration, other companion proteins, physiological ligands such as magnesium, potassium or the ratio of GTP to GDP [51,94], and nonspecific electrostatic and crowding effects (see below). Taking all these factors together, the amount of FtsZ that is part of the Z-ring is estimated not to be over two-thirds of the total cellular FtsZ [12,94], which is enough to encircle the cell.

Min proteins

The abundance of MinE molecules per cell has been estimated to be 1100–1400; MinD is present at 2000 [42] and MinC at about 400 [41].

The crowded bacterial interior

It is estimated that 20–30% of the bacterial cytoplasm is occupied by macromolecules, among them 200–300 mg/ml protein, 75–120 mg/ml RNA, and 11–15 mg/ml DNA [95,96]. These macromolecules inside the cell seem to be (transiently) clustered in replicative, structural, and metabolic networks rather than homogeneously distributed [97]. The abundance of macromolecules in bacteria causes volume exclusion effects, both in solution and in membranes. These effects substantially increase the effective concentration and reactivity of the proteins and may modify the extent and rate of protein–protein and protein–membrane interactions [68,98]. Because crowding leads to phase separation, it may also modify the spatial arrangement of macromolecular networks involved in essential processes [99].

Concluding remarks

To conclude, the strategy discussed in this review together with complementary bottom-up synthetic approaches using either cell-free expression systems inside giant vesicles [73–75] or bacteria with simplified genetic content will provide the best chance for making progress in the synthetic approach toward studying bacterial division. It is fair to say that, with optical technology entering the submicroscopic regime [76], there are many fundamental discoveries to be made on bacterial systems that take us a step closer to the phenomenon of life in its minimal representation. However, the most important steps toward its fundamental understanding will probably still have to come through bottom-up reconstitution [77].

Acknowledgments

We thank Professor Miguel Vicente (CNB-CSIC) for useful comments and especially for sharing with us unpublished material and contributing the cell cycle definitions in the Glossary. We also thank the anonymous reviewers for useful remarks. Work in the authors' laboratories is supported by Human Frontiers Science Program through grant

RGP0050/2010-C102 (to G.R. and P.S.), by the Spanish Government through grant BIO2011-C03-03 (to G.R.), by the European Commission though contract HEALTH-F3-2009-223432 (to G.R.), and by the DFG Leibniz Prize (to P.S.). We apologize to the authors whose work we were not able to include in this review owing to space limitations.

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