In the beginning *Escherichia coli* assembled the proto-ring: an initial phase of division

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**SUMMARY**

Cell division in *Escherichia coli* begins by assembling three proteins, FtsZ, FtsA and ZipA to form a proto-ring at midcell. These proteins nucleate an assembly of at least 35 components, the divisome. The structuring of FtsZ to form a ring and the processes that effect constrictions have been explained by alternative but not mutually exclusive mechanisms. We discuss how FtsA and ZipA provide anchoring of the cytoplasmic FtsZ to the membrane and how a temporal sequence of alternative protein interactions may operate in the maturation and stability of the proto-ring.

How the force needed for constriction is generated and how the proto-ring proteins relate to peptidoglycan synthesis remain as the main challenges for future research.

**OVERVIEW OF SEPTATION**

Cell division is the last event in the bacterial cell cycle. It takes place by producing a rigid transversal septum after the growth processes fully duplicate the contents of the cell. Septation is always preceded by segregation of the duplicated nucleoids ensuring that each of the daughter cells contains a fully replicated chromosome. Specialized molecules control the positioning of the division machinery at midcell preventing any harmful fragmentation that septum progression could cause on unsegregated nucleoids.

The division machinery, the divisome, establishes a complex interacting network together with DNA, lipids and peptidoglycan components. It comprises a variable number of proteins, depending on the bacterial species. These proteins have redundant multiple functions and connections serving as safeguards to complete division and to guarantee the efficiency and versatility of the process. Altogether an efficient division process allows the proliferation of bacteria under a variety of conditions, being the reasons for their success in the environment. Free-living bacteria, as *E. coli*, have more elaborated divisomes, while those that need to grow as intracellular parasites, as *Mycoplasma genitalium*, have streamlined ones (1,2).

The structure of the *E. coli* divisome as revealed by immunofluorescence images of dividing cells is called division ring (3). For its assembly, three essential proteins, FtsZ, FtsA and ZipA, are first gathered at midcell forming a proto-ring attached to the inner membrane (Figure 1) (4). In the proto-ring structure the cytoplasmic FtsZ, a GTPase, is anchored to the membrane by its interaction with ZipA and FtsA. ZipA is a bitopic protein containing a transmembrane domain (5). FtsA is a protein of the actin family that associates to the membrane by a short amphiphatic helix (6,7). The assembly of the FtsZ ring is dependent on a continuous energy supply and. The FtsZ ring requires either ZipA or FtsA (8). Although it can be formed in the presence of any of them, division is affected unless both are present (9). A maturation period takes place after the proto-ring is assembled and before the rest of the divisome proteins become incorporated (10). During
maturation the proto-ring assembly is not stable (11). Once the full divisome is assembled, the motor force, likely exerted by the polymerization of FtsZ, results in membrane constriction followed by the production of a septum.

LOCALIZATION OF THE DIVISION SITE

Two negative regulatory systems, nucleoid occlusion and the Min system, have been described to determine the position of the division site at midcell. Their combined action inhibits polymerization of FtsZ blocking the assembly of the proto-ring at places other than the cell center. Nucleoid occlusion blocks septation across the nucleoid, therefore excluding a potentially harmful guillotine effect on the chromosome. In E. coli it is accomplished by SlmA, a DNA-associated division inhibitor directly involved in preventing the FtsZ polymerization in the vicinity of the nucleoid (12). A dimer of SlmA binds simultaneously to two molecules of FtsZ and to specific DNA sequences distributed all over the chromosome except at the Ter region. Prior to division, Ter localizes at midcell, coinciding with the site in which the FtsZ-ring is assembled, and it is the last chromosomal region to segregate (13,14).

The E. coli Min system comprises three proteins, MinC, MinD, and MinE. Together they inhibit the polymerization of FtsZ at the poles, and therefore prevent the production of non-viable anucleated minicells. Although with a low activity, MinC is sufficient to inhibit the assembly of FtsZ polymers (15). It is proposed that the carboxy-terminal domain of MinC interacts directly with FtsZ filaments preventing FtsZ-FtsZ lateral interactions and disrupting the interactions with FtsA and ZipA, which are essential for Z ring formation (15-17). MinD is an ATPase that activates and anchors MinC to the membrane. MinE regulates the localization of the MinCD complex by restricting it to the cell poles, thus allowing the FtsZ ring assembly only at midcell (for review (18)). However, even in the absence of MinE, those FtsZ rings located at the poles are more sensitive to MinCD- induced disassembly than those present at non-polar positions (19). In addition, the functionality of the Min system may be further enhanced at the poles, probably because MinCD senses membrane curvature (20,21). The ability of the MinCDE system to modify the placement of proteins is not limited to septation, as it can also influence the localization into polar foci of proteins unrelated to cell division as tryptophanase TnaA or chaperonin GroES (22).

THE PROTO-RING: THE SCAFFOLD OF THE DIVISOME

The FtsZ polymers need two more proto-ring components, the FtsA and ZipA proteins, both associated to the inner membrane, to localize in a ring in close proximity to it. Although non-essential for the division progress, the Zap proteins (FtsZ associated proteins) may be considered as accessory components of the proto-ring because they affect the assembly and dynamics of FtsZ.

Assembly, organization and stabilization of the FtsZ polymers in the proto-ring

The best-described and phylogenetically most conserved component of the proto-ring is the cytoplasmic GTPase FtsZ, a structural homologue of the eukaryotic tubulin (23-25). Highly dynamic protofilaments of FtsZ are reorganized in the vicinity of the cell membrane at the division site. Their continuous rearrangement during the cell division process is postulated to be the main force to drive membrane constriction, a process that starts when the divisome is fully assembled (reviewed in (26) and (27).

Available techniques still do not allow an exact visualization of the detailed arrangement of the FtsZ polymers in the proto-ring. Two models, ribbon or scattered, have been proposed to describe the arrangement of the FtsZ polymers in the ring. The ribbon model proposes that FtsZ would form sufficiently long filaments to coat the internal surface of the inner membrane and assemble side by side as a single band. A compact ring would then be maintained by means of lateral contacts between filaments. The proof to support this model is that in vitro conditions favouring the elongation and the lateral associations between protofilaments lead to the assembly of FtsZ into filaments longer than 500 nm (28). Such filaments have been observed when FtsZ is adsorbed onto mica surfaces, or when calcium is present or at low pH (<6.0) (29-31). Long FtsZ filaments are also formed under more physiological conditions in the presence of accessory proteins or crowding agents that mimic the in vivo conditions (32-34). In solution, long FtsZ protofilaments have also
been found assembled as ribbons on the outer surface of artificial membrane tubules (35).

The scattered model proposes that short protofilaments of FtsZ are arranged in a disperse and divergent manner. It is supported by images obtained in vivo using high-resolution microscopy. In Bacillus subtilis and E. coli, the half-time turnover of FtsZ molecules within the division ring when estimated by FRAP is 9 seconds, supporting that short protofilaments (45-90 monomers, about 200nm long) are formed during the recovery time (36). Using wide-field single molecule fluorescence imaging, the depolymerization of FtsZ from Caulobacter crescentus has been determined to occur in the nano-second timescale (37). FtsZ-ring images have been obtained from C. crescentus by electron cryotomography and by two- and three-dimensional super-resolution imaging, and from E. coli using photoactivated localization microscopy (PALM). In both cases they are compatible with a scattered arrangement of short filaments that accumulate within a limited space forming a loose ring assembly (37-39).

The assembly and stabilization of the FtsZ polymers in the proto-ring are regulated by the FtsZ-associated proteins (ZapA, ZapB, ZapC and ZapD), which exhibit functionally redundant roles in binding and bundling polymeric FtsZ (32,40-46). These proteins act at midcell promoting the transition of the FtsZ polymers from a helical band into a compact ring by cooperatively stimulating the lateral association of protofilaments (47,48). The Zap proteins bind to a conserved motif of 10-16 residues found within the C-terminal end of FtsZ (49-52). The motif mediates the binding to FtsA, ZipA, MinC and ClpX. MinC is a negative regulator of FtsZ polymerization and ClpX is the recognition moiety of the ClpXP protease. In consequence, a role as a central hub to integrate signals that modulate divisome assembly in E. coli has been assigned to this C-terminal end of FtsZ (53). In B. subtilis, where Zap proteins are not present, a positively charged variable region found downstream this conserved sequence mediates electrostatic interactions between FtsZ protofilaments. The equivalent region of the FtsZ E. coli protein is not charged, requiring then the Zap proteins to stabilize FtsZ lateral interactions (54).

FtsA and ZipA: the anchors of FtsZ polymers to the membrane

FtsA is an ATP binding protein, belonging to the actin/Hsp70 superfamily (55). The self-interaction of FtsA is essential for the E. coli division process (56,57). The ATPase activity of FtsA has not been firmly established, perhaps because its hydrolytic activity is low and an unidentified cofactor is required in vivo for its full activation. The ATP binding activity is, nevertheless, associated with polymerization. FtsA polymers are formed in vitro, either in solution or when attached to lipids, and in vivo when it is overproduced (58-60). When ATP is available, the Streptococcus pneumoniae FtsA interacts in a head-to-tail manner, growing bidirectionally to form protofilaments until no monomers are available (59). Given the negligible ATPase activity, FtsA polymers remain as helical protofilaments showing a tendency to form bundles. When attached to a lipid monolayer, the FtsA protofilaments from Thermotoga maritima undergo a conformational change forming straight tubular structures. A short C-terminal amphipatic helix in FtsA is responsible for attachment to the membrane (6) and also it diminishes self-interaction (56).

Gene constructs engineered in E. coli to produce FtsA-FtsA tandems result in the FtsZ polymers being retained at the cell poles after septation, suggesting that the dimerization or oligomerization of FtsA modifies the stability of FtsZ polymers and promotes Z-ring integrity (62). FtsZ is proposed to be involved in FtsA dimerization given that the FtsA crystallizes as a dimer when the FtsZ C-terminal peptide is bound to FtsA but a monomer if the FtsZ peptide is absent (55,60). These data suggest a cooperative interaction of three components, i.e. FtsZ, FtsA and the cytoplasmic membrane, to assemble in a stable proto-ring composed of FtsZ and FtsA polymers arranged in the correct orientation at the inner membrane (Figure 1a).

FtsA is also a key connector between the proto-ring components and some downstream proteins like FtsN. The 1C subdomain of FtsA is necessary and sufficient to interact with the cytoplasmic domain of FtsN (57,63,64) and it is also involved in the interaction between two FtsA molecules (57,59,60). It follows that both FtsA functions, self-interaction and recruitment of the late division
proteins, may be mutually exclusive (65). The oligomerization state of FtsA may then have a role in recruiting the late assembling divisome proteins.

An additional essential component of the gamma-proteobacteria proto-ring is ZipA, a transmembrane protein found in the E. coli cytoplasmic membrane as a monomer or homodimer. The monomer provides an attachment of FtsZ, its only known protein partner, to the membrane. The homodimeric form may activate the production or stability of the FtsZ polymers during the assembly of the division ring (66). ZipA may provide a stronger physical link of FtsZ to the membrane than FtsA, as both the interaction between ZipA and the membrane and between ZipA and FtsZ are stronger than the equivalent interactions provided by FtsA. The already mentioned phylogenetically conserved C-terminal peptide of FtsZ fits into a pocket located in the globular domain of ZipA and establishes a hydrophobic interaction (67), while the same FtsZ peptide, adopting a different conformation, interacts with the surface of the domain 2B of FtsA by means of weaker electrostatic force (60). The association of FtsA to the membrane would be also weaker as it is established through a short C-terminal amphipatic helix (6), while ZipA is physically anchored through a N-terminal transmembrane domain (5). The essential role of ZipA in septation can be replaced by FtsA*, a gain-of-function mutant (68,69). However, division driven by FtsA* is not completely normal suggesting that ZipA is to some extent relevant in the process. ZipA can protect FtsZ from degradation by ClpP, an activity that FtsA* cannot perform, and therefore it may play some role in division additional to the anchoring of FtsZ to the membrane (53).

PROTO-RING MATURATION

The proto-ring components remain assembled at the midcell during part of the cell cycle, 15-35% depending on the growth rate, before the recruitment of the rest of the divisome components (10). This is a surprisingly long time in particular when considering that at fast-growing rates the initial assembly of the proto-ring that will work in the division of one cell occurred in its mother (70).

During this period the proto-ring may be engaged in redirecting peptidoglycan synthesis to initiate transversal growth leading to the production of the septum. This period may also involve rearrangements in the stoichiometry or/and in the interactions of the proto-ring elements between themselves or with the membrane (10). After the addition of GTP, the polymerization of FtsZ bound to a ZipA protein attached to a lipid bilayer (Figure 1b) is not immediate needing an incubation interval to occur (29). Polymerization under these conditions is accompanied by a change in the fluidity of the membrane (71,72). In addition, FtsZ and ZipA, but not FtsA, are required for a pre-septal peptidoglycan synthesis, which is independent of FtsI (also called PBP3), an enzyme involved in the synthesis of the septum (68). Among the rest of the proteins involved in peptidoglycan synthesis and maturation none has been shown to be essential for preseptal peptidoglycan synthesis. However the role of ZipA in preseptal peptidoglycan synthesis in cells containing FtsA* is dispensable (68,69). FtsZ may then be the key actor, through its indirect association to the membrane, in effecting the preseptal peptidoglycan synthesis, although a molecular mechanism to describe this activity remains to be discovered (Figure 1b).

The second event occurring during proto-ring maturation is a rearrangement in the interactions of its components to allow the incorporation of the late divisome proteins. Two interactions between the proto-ring elements and the late proteins have been described, FtsZ with FtsE, and the 1C subdomain of FtsA with the cytoplasmic domain of FtsN (57,63,64). As both FtsZ and FtsA are likely to be present as oligomers, a mechanism to allow their alternative interaction with their late assembling divisome protein partners has to be invoked. Based on the detailed analysis of FtsA mutants that bypass the need of ZipA, Lutkenhaus and coworkers (65) have proposed a model in which the anchoring of FtsZ to the membrane is initially established through FtsA oligomers. Preliminary evidence (73), suggests that, upon an increase in the density of its association to lipid bilayers, ZipA undergoes a transition from a compact to an extended conformation in which its globular domain can explore a wider volume in its environment (Figure 1a). ZipA may then compete for the C-terminal tail of FtsZ found inside the FtsA-FtsZ hetero-oligomers at midcell. This would
produce a discontinuity in the FtsA oligomers at the sites where a ZipA molecule becomes inserted and at the same time would gradually replace the anchoring of FtsZ to the membrane provided by FtsA (Figure 1b). This would then liberate the 1C domain of FtsA from the self-interaction allowing it to engage in recruiting FtsN into the mature divisome (65) (Figure 1c). The absence of FtsN, the last protein that can be visualized forming a ring at the E. coli divisome, results in the disassembly of proto-rings, being the ZipA rings the most sensitive (11). Moreover, after FtsN deprivation new proto-rings do not assemble until the levels of FtsN are restored, indicating that the presence of FtsN is required for the proto-ring assembly (11). Interestingly, a phylogenetic correspondence between the presence of both genes zipA and ftsN has been observed in gammaproteobacteria and has been interpreted as an indication of their concurrent role in septation (74).

PROTO-RING STABILIZATION AND DIVISOME COMPLETION

Prior to the initiation of constriction, the remainder of the divisome proteins are recruited and the proto-ring becomes stabilized into the divisome (Figure 1d). Besides FtsN (11), a role to maintain the integrity of the divisome has been suggested for most of the late-assembling proteins as the number of rings, and even of proto-rings, diminishes in the absence of any of them (75). Several essential division functions have been adscribed to either individual divisome proteins or to their subcomplexes. FtsK is needed to resolve possible chromosome cointegrates resulting from replication (76,77). FtsEX, an ATP-binding cassette transporter-like complex, acts as a regulator of cell-wall hydrolysis at the division site. Upon its interaction with FtsZ, FtsE undergoes conformational changes mediated by ATP hydrolysis to modify the transmembrane FtsX component. The modification favours the recruitment of the amidase activator EnvC by FtsX in the periplasm (78,79). The small bitopic membrane proteins FtsQ, FtsB and FtsL form an independent subcomplex able to establish abundant interactions with other divisome proteins (80-82). In the absence of additional evidence it is possible that they may have either a regulatory or a structural role. FtsW belongs to the SEDS (shape, elongation, division and sporulation) family of transporters involved in the translocation of the lipid-linked peptidoglycan precursors across the cytoplasmic membrane (83). Together with FtsI, its linked class B PBP, FtsW forms a subcomplex specific for septal peptidoglycan synthesis (84) (Figures 1c and 1d). FtsI has a similar topology to FtsN, they contain a transmembrane region followed by a long linker spanning the periplasm and a C-terminal globular domain contacting the peptidoglycan layer. FtsI and FtsN interact with non-essential division proteins that participate in other late septation processes, i.e. with PBP1B (85) or MtgA (86) for peptidoglycan synthesis, with the Tol-Pal complex for the invagination of the cell wall during constriction (87,88), and with the AmiB and AmiC amidases to effect the hydrolysis of peptidoglycan needed to split the wall of the two daughter cells (89).

MEMBRANE CONSTRICTION AND SEPTUM PROGRESSION.

Once the divisome is completely assembled, all the layers of the cell envelope are connected and the constriction process, involving all of them, starts. As no contractile elements have been found outside the cell membrane, the constriction force seems to be exerted from the cytoplasm by pulling the envelope inwards (Figure 1d). The available evidence suggests that the main motor exerting the force required for constriction is FtsZ itself. Two mechanisms have been proposed to describe how FtsZ may exert a pulling force. They involve either bending or condensation of the FtsZ polymers and are not mutually exclusive. In the bending model, the motor force is exclusively exerted by FtsZ, in a cycle of polymerization, membrane attachment, conformational change, depolymerization and nucleotide exchange, all driven by GTP hydrolysis (38). Osawa et al. (90) found that a membrane-targeted FtsZ chimera, when self-internalized in flattened tubular liposomes can assemble into constricted areas that relax as GTP is hydrolysed. This observation suggests that the bending detected in the FtsZ rich regions of the liposomes is not stable and that further components of the septum, as peptidoglycan, are necessary to produce a stable structure as constriction progresses. In this model the hydrolysis of GTP would be involved in
modifying the curvature of the FtsZ polymers (91,92).

In the condensation model the constriction force would be generated by the lateral attraction between different FtsZ filaments that would interact to form a compact structure (93). Results from in silico modelling suggest that the FtsZ ring may undergo a transition from a low to a high-density state by condensation of protofilaments and not by recruiting more FtsZ monomers. In this model GTP hydrolysis would accommodate a gradual morphological change from a loose helix to a compact FtsZ ring by facilitating monomer turnover during condensation (94,95). Increasing the number of lateral contacts between protofilaments would compress them causing a shift from a lose spiral extending along the cell length to a compact ring located at midcell. The FtsZ-ring would further condense across the cell diameter to form a thicker annular structure with its diameter decreasing as constriction progresses. The FtsZ associated proteins, as ZapA or ZapB, have been suggested to assist in this mechanism (96). At the end of constriction the condensed narrow ring quickly disassembles and the septum closes (39). It is not known if, upon their disassembly, the components of the divisome are degraded or if they can be partially recycled.

FURTHER QUESTIONS.

Our knowledge on many aspects of cell division, as summarized in this review, provides a detailed account of the components of the divisome that includes the description of many of their interactions and their spatial and temporal assembly within the cell. However, only a partial picture is still available on their functions. In particular, a description of the detailed molecular mechanisms involved in septation, and moreover the connection between the biochemical properties of each divisome component and its role in bacterial proliferation has to be completed. A relevant question is how the divisome affects the membrane and modifies the direction of wall growth at the inception of septation. How the divisome components are disposed after the closure of the septum is another question that will also need additional research. Hopefully, techniques as high-resolution microscopy (97) and in vitro reconstruction procedures (98) are now available to investigate these questions. The replies will clarify both how the process of septation is initiated and how does it finish.

Finally, under ideal growth conditions, E. coli will assemble a divisome to constrict the envelope and after 20 minutes will divide and start again.

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**FOOTNOTES**

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FIGURE LEGENDS

FIGURE 1. A speculative model showing the proposed stages in the assembly of the *E. coli* protoring. The model focuses on interpretations derived from currently discussed evidence. Initial stage of proto-ring assembly (a) shows FtsZ attached by its carboxy-end to the membrane-associated FtsA protein. The C-terminal ends of FtsZ (red circles) oligomers formed with GTP (purple triangles) contact to the ATP-containing FtsA oligomers (in blue) (green triangles) bound to the membrane via its amphipatic helix. ZipA is shown in its compact conformation. Rearrangement of the proto-ring components (b) shows the disruption of the FtsA oligomer. This may involve a competition between ZipA and FtsA for the C-terminus of FtsZ in which ZipA may adopt its extended conformation. At this stage the FtsI-independent pre-septal peptidoglycan synthesis (highlighted in red), proceeds by an unknown mechanism requiring FtsZ and ZipA (or FtsA*). The interaction of the ZipA-FtsZ complex simultaneously increases the membrane fluidity (highlighted in red). After the disruption of the FtsA oligomer, its 1C domain is released from the FtsA self-interaction becoming free to recruit the late proteins (c): FtsN (green oval) and FtsI (purple circle). Cell constriction and its associated synthesis of septal peptidoglycan (d) may be initiated by the pulling of the membrane. The force, transmitted by ZipA and FtsA, may result from the FtsZ polymer dynamics. A transient membrane constriction may become stabilized by septal peptidoglycan (highlighted in purple) newly synthesized by FtsI.
Figure 1
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