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The bacterial divisome: ready for its close-up

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Bacterial cells divide by targeting a transmembrane protein machine to the division site and regulating its assembly and disassembly so that cytokinesis occurs at the correct time in the cell cycle. The structure and dynamics of this machine (divisome) in bacterial model systems are coming more clearly into focus, thanks to incisive cell biology methods in combination with biochemical and genetic approaches. The main conserved structural element of the machine is the tubulin homologue FtsZ, which assembles into a circumferential ring at the division site that is stabilized and anchored to the inner surface of the cytoplasmic membrane by FtsZ-binding proteins. Once this ring is in place, it recruits a series of transmembrane proteins that ultimately trigger cytokinesis. This review will survey the methods used to characterize the structure of the bacterial divisome, focusing mainly on the *Escherichia coli* model system, as well as the challenges that remain. These methods include recent super-resolution microscopy, cryo-electron tomography and synthetic reconstitution.

1. First sightings

Cell-division genes of *Escherichia coli* were originally designated *fts* genes, because thermosensitive mutants of these genes conferred a filamentous temperature-sensitive phenotype. At the non-permissive temperature (usually 42°C), *fts* mutant cells continue to elongate without dividing, forming filaments that can be longer than 150 µm in rich growth medium. As newborn *E. coli* cells are approximately 3 µm long by 1 µm wide, this represents at least 50 mass doublings, all the while continuing to extend the cell wall and membrane continuously, as well as replicate and segregate their nucleoids as visualized with DAPI staining. These multinucleate filaments indicated that the *fts* genes were involved specifically in cytokinesis, but electron microscopy of thin sections of *E. coli* (and many other bacteria) could never reveal any type of structure in normal dividing cells visible by negative staining. The isolation of *min* mutants of *E. coli* that made anucleate minicells at the cell pole suggested that the divisome-centring mechanism could be disrupted, but again no specific structures within *min* mutant cells could be discerned.

The first breakthrough came in 1991 when Erfei Bi and Joe Lutkenhaus used immunogold labelling to identify FtsZ, a product of the final *fts* gene in a highly conserved segment of cell wall and cell-division genes called the dcw (division and cell wall) cluster. Their work showed that gold particles clustered exclusively at the site of division at midcell in thin sections of *E. coli*, indicating that FtsZ was a structural component of the divisome and formed what is now called the Z ring [1]. Soon thereafter, new methods adapted for bacterial cell biology would set the stage for direct visualization of divisome structural proteins in intact cells.

The first of these methods, immunofluorescence microscopy (IFM), had been used for many years in eukaryotic cells but was first adapted for *Bacillus subtilis* cells by Liz Harry and Kit Pogliano in Rich Losick's laboratory. In addition to cell fixation and incubation with a primary antibody followed by a fluorescent secondary antibody, the key step involved cell permeabilization by limited lysozyme treatment, allowing the antibodies to enter the bacterial cells [2]. Using IFM, Arigoni *et al.* [3] found that the sporulation phosphatase

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SpoIIE localized to the asymmetric septum that separates the *B. subtilis* mother cell from the developing spore. IFM was quickly adapted for use in *E. coli* and other species, and was used to confirm that FtsZ strongly localized to the divisome at midcell, between segregated daughter nucleoids [4,5]. A series of papers from several groups then used IFM to demonstrate that other known products of *fts* genes, including FtsA, FtsQ, FtsW and FtsI, also localized sharply to division sites, provided that FtsZ was there [6–9]. Using a combination of the *fts* mutants and IFM, a new *fts* gene called *ftsN* was found to localize only when the other *fts* genes were intact, indicating that it needed preassembled ring components for recruitment and probably acted late in cell division [10]. This use of both cytology and genetics enabled the first rough understanding of a recruitment dependency, which in turn suggested a temporal hierarchy. This would have been very difficult to dissect with genetics alone.

At nearly the exact same time that IFM for bacteria was developed, green fluorescent protein (GFP) was rediscovered as a genetically encodable fluorescent tag [11]. As with IFM, eukaryotic cells were the first application of this exciting new technology, but the Losick laboratory soon adapted GFP for use in bacteria and used it to localize proteins in specific cell compartments during *B. subtilis* sporulation [12]. Shortly thereafter, our laboratory used FtsZ–GFP fusions to visualize FtsZ and FtsA for the first time in living cells [13]. With help from David Ehrhardt, who applied a computationally intensive method called deconvolution, or wide-field optical sectioning, originally developed by John Sedat's group [14], we reported the first three-dimensional view of the Z ring. GFP tagging now enabled the localization of any protein, without the need for specific antibodies or for cell fixation. This technology ushered in further breakthroughs that would be impossible with IFM alone, as described in §2.

However, tagging with fluorescent proteins comes with risks, including perturbation of the target protein by the tag [15,16]. Indeed, *E. coli* FtsZ tagged with GFP is not fully functional and as with other GFP-tagged proteins, artefacts can result, including dominant negative effects and spurious aggregation [13]. Fortunately, when FtsZ–GFP is produced at low levels in cells containing native FtsZ, it seems to behave consistently with IFM data. Moreover, other divisome proteins that bind directly to FtsZ, such as ZipA and ZapA in *E. coli*, and EzrA in *B. subtilis*, among others, can mimic FtsZ localization quite faithfully without perturbing the system much and thus serve as proxies for FtsZ [17–19].

2. Casting call: towards a low-resolution structure of the divisome

The use of IFM and fluorescent protein tags, ideally in combination, soon resulted in a fairly comprehensive understanding of the low-resolution structure of the divisome in several model organisms, including *E. coli*, *B. subtilis* and *Caulobacter crescentus*, and enabled researchers to demonstrate localization of similar homologues in other diverse bacterial species. As a result, we learned that many diverse bacteria, euryarchaea, plant plastids and even a few primitive mitochondria harbour Z rings, and that perturbing localization of the rings results in cytokinesis defects. Moreover, fluorescence microscopy has been crucial in screening for Z ring binding proteins [20–23], particularly as many of these proteins would be very hard to identify by genetic or biochemical methods because of transient interactions and/or modest phenotypes when inactivated.

To understand more about divisome structure, several avenues have been taken. One successful approach identified which divisome proteins could still properly localize after removal of other divisome proteins. As knockouts of divisome protein genes are generally lethal, these types of experiments have mostly been done in model systems that have sophisticated genetic tools such as thermosensitive mutants, regulatable promoters or suicide plasmids that can rapidly induce the depletion of a specific protein from the cell. Because filamentous cells of many rod-shaped bacteria such as *E. coli* can remain viable for several generations, IFM or fluorescent protein-tagged divisome proteins were then used to determine whether a given divisome protein depended on another. For example, in *E. coli*, the divisome proteins FtsQ, FtsL and FtsI fail to localize to the divisome in the absence of FtsZ, FtsA or FtsK but can localize in the absence of FtsN [24]. This demonstrates that FtsQ, FtsL and FtsI depend on FtsZ, FtsA and FtsK but not on FtsN, placing them in the middle of the recruitment order. This dependency order was roughly consistent with the actual timing of their detectable accumulation at the Z ring, with the more dependent proteins arriving later [25].

Another approach towards a divisome structure has been to define the binary protein–protein interactions within the divisome (figure 1). The most powerful genetic approach has been bacterial two-hybrid assays based on two fragments of the *Bordetella pertussis* adenylate cyclase [26]. These fragments normally do not interact efficiently, but if each is attached to a protein that interacts with another protein *in vivo*, they bring the two adenylate cyclase fragments together, and cAMP is synthesized. In a *cya*$^{-}$ cell incapable of synthesizing cAMP endogenously, the production of cAMP results in the induction of the *lac* operon, which in turn can be measured by colorimetric substrates such as X-gal or MacConkey dye (figure 1*a*). In addition, although adenylate cyclase is cytoplasmic, the enzyme itself is anchored to the membrane and thus provides a membrane-bound environment similar to most divisome proteins. The studies using this assay have identified numerous binary interactions between divisome proteins [27–29]. However, bacterial two-hybrid analysis, like any two-hybrid assay, is prone to false positives and negatives and should be used mainly as a genetic screen and to generate hypotheses that can be tested more rigorously using other methods (see below).

In contrast to the sequential recruitment order of divisome proteins suggested by genetic experiments, the bacterial two-hybrid studies hint at a more integrated network of overlapping interactions. In addition to the known interactions among FtsZ and FtsA, ZipA and the Zap proteins in *E. coli*, some additional interactions among later recruits to the divisome have been confirmed using purified proteins. For example, it has been known for some time that FtsQ, FtsL and FtsB form a defined subcomplex [30], and the most attractive model to date is that they are in a 2 : 2 : 2 stoichiometry. As each of these proteins is present at approximately 200–400 molecules per cell [31], there may be approximately 100–200 FtsQLB complexes per divisome. More detailed interaction data for FtsQLB comes from photo-cross-linking of these proteins using unnatural amino acids placed in strategic positions [32]. The advantage of this method is that it can be

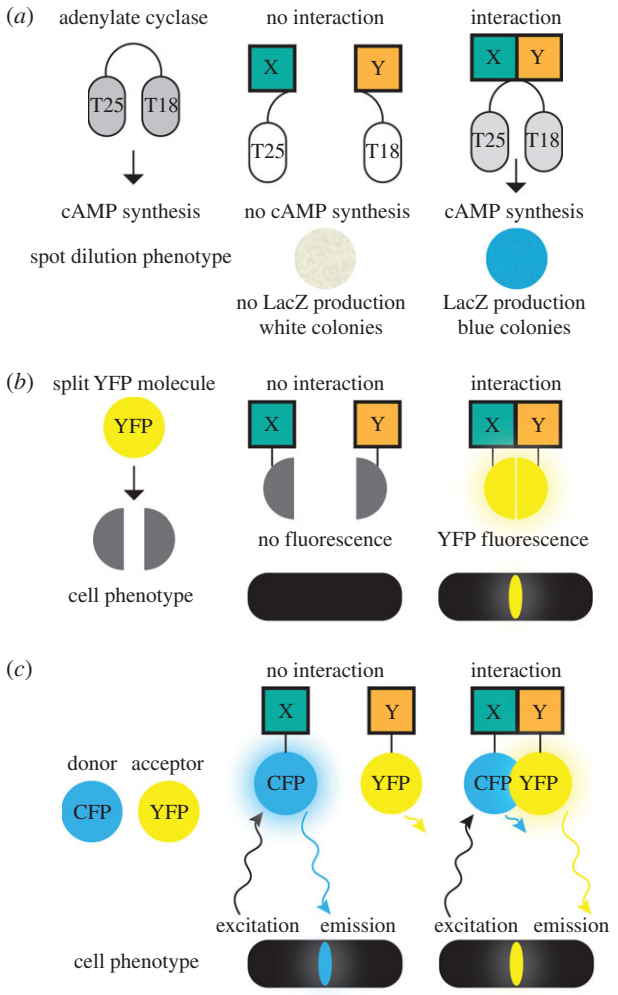

Figure 1. Schematic of protein–protein interaction methods. Bacterial two-hybrid assay: domains of adenylate cyclase are fused to two divisome proteins, X and Y (*a*). Interaction results in cAMP synthesis and production of β-glalctosidase (LacZ) in colonies. Bimolecular fluorescence complementation: two parts of a YFP molecule are fused to two divisome proteins, X and Y (*b*). Interaction results in YFP fluorescence at the divisome. Förster resonance energy transfer: CFP and YFP are fused to two divisome proteins, X and Y (*c*). Interaction results in an increase in fluorescence of the acceptor fluorophore, YFP.

done in growing cells in the natural membrane environment [33]. The cross-linking study pinpointed several regions within the periplasmic domain of FtsQ that are important for interacting with FtsB and/or FtsL. Another well-established interaction in the divisome is between FtsA and FtsN [34]. This was at first surprising, as FtsA is an early recruit to the Z ring, while FtsN is mainly a late recruit [10]. However, it was found that a small amount of FtsN molecules are recruited early by FtsA [35], which initiates a positive feedback loop that involves septal wall synthesis [36], consistent with FtsN's ability to contact peptidoglycan directly [37]. The result is a robust localization of FtsN to the divisome at late stages.

Two other methods have been used to measure protein–protein interactions and the location of those interactions in *E. coli* cells. One of these, called bimolecular fluorescence complementation or BIFC, relies on expressing the N-terminal and C-terminal fragments of GFP or derivatives such as yellow fluorescent protein (YFP). Normally, these fragments are non-fluorescent. However, if they are brought in close proximity via a strong protein–protein interaction, they will anneal to reconstitute the fluorescent protein and thus display a fluorescent pattern that mimics the interaction [38] (figure 1*b*). BIFC was used to confirm interactions between FtsZ and ZipA, as well as ZapB with itself [39]. In addition, BIFC identified new putative ZipA–ZapA and ZipA–ZapB interactions. BIFC has several advantages. It can pinpoint a close interaction in a live cell, and is sufficiently stable that even transient interactions can be marked. It can be used with standard microscope set-ups and requires no complex image processing. However, the disadvantage is that the reconstitution is relatively inefficient, so false negatives will be high. Moreover, the reconstitution of intact GFP/YFP is irreversible, making the BIFC fluorescence signal also irreversible; therefore, BIFC cannot be used to monitor off rates.

The other *in situ* method is Förster resonance energy transfer (FRET). FRET is the transfer of energy from a donor fluorophore, whose emission wavelength overlaps the excitation wavelength of a higher-wavelength receptor fluorophore. This transfer only occurs efficiently when the donor and acceptor fluorophores are in close contact, for example, between 1 and 8 nm apart. In this range, the FRET signal rises sharply with proximity. FRET can be measured as an increase in donor emission upon bleaching of the acceptor, as the donor retains the energy normally transferred to the acceptor when donor and acceptor are close together (figure 1*c*). Alternatively, a decrease in donor emission and/or an increase in acceptor emission can be detected. The power of microscopic FRET is that like BIFC, it can measure as well as pinpoint protein–protein interactions in living cells. As a result, it has been used for several applications in bacteria. For example, protein–protein interactions and dynamics within chemoreceptor clusters have been demonstrated by FRET, using protein fusions to the donor cyan fluorescent protein (CFP) and acceptor YFP [40,41].

In the first use of FRET to investigate protein–protein interactions in the bacterial divisome, Tanneke den Blaauwen's group measured interactions among FtsZ, ZapA, FtsQ, FtsW, FtsI and FtsN of *E. coli* [42]. Using the mKO fluorescent protein as donor and mCherry as acceptor fusion tags, they detected strong FRET between FtsZ and FtsZ, ZapA and ZapA, and between FtsZ and ZapA, as expected. They also found that the later divisome proteins FtsW, FtsI and FtsN interacted, and FtsN interacted with itself. More surprisingly, they measured significant FRET for ZapA–FtsN and ZapA–FtsI. These results, along with direct FtsA–FtsN interactions demonstrated biochemically [34] indicate that early and late divisome proteins, while temporally separated, are not necessarily spatially segregated; moreover, as mentioned above, a small proportion of late divisome protein FtsN arrives early [35]. Further biochemical studies will need to be done to confirm these FRET results, as it is always possible that FRET can lead to false positives if fluorescent proteins are in proximity but their attached divisome proteins do not directly interact. Likewise, false negatives can arise if two proteins interact but their attached fluorescent proteins are sterically hindered and/or not properly oriented for optimum FRET.

A powerful combination of FRET and fluorescence lifetime imaging, or FLIM, was used to assess the interactions between FtsZ and two positive spatial regulators of FtsZ positioning, SsgA and SsgB, in *Streptomyces* [43]. The advantage of FRET–FLIM over FRET alone is that measuring changes in the short lifetime of the fluorophores upon close

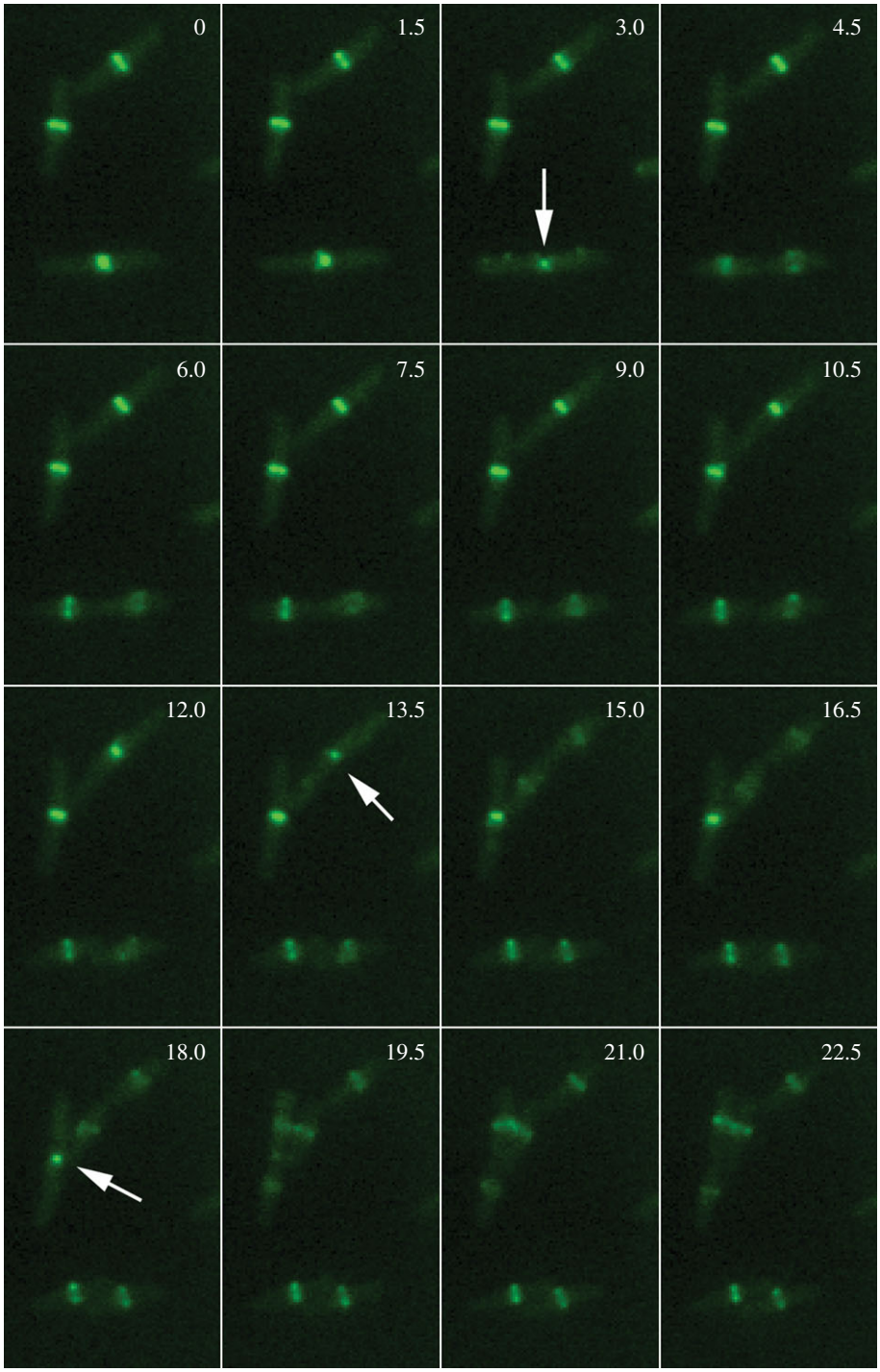

Figure 2. Dynamics of FtsZ in dividing *E. coli* cells. Low levels of FtsZ–GFP were produced in otherwise wild-type *E. coli* cells to monitor the localization of FtsZ during growth in time lapse on an agar pad under the microscope. Elapsed time is shown in minutes. Arrows point to constricting Z rings just prior to septum completion and cell separation for the three cells in the original field. Note that FtsZ–GFP fluorescence rapidly relocalized to future division sites.

interactions overcome some of the limitations of FRET, including background (non-FRET) donor fluorescence in the acceptor channel and vice versa.

3. Visualizing rapid dynamics of cell-division proteins and regulators in living cells

As mentioned in §2, the real power of tagging with fluorescent proteins lay in being able to follow protein dynamics in live cells over time. The first example of this was the demonstration that FtsZ–GFP in *E. coli* assembles into Z rings with some discontinuities but a clear lumen, which then constricted down to a diffraction-limited spot before disappearing [44] (figure 2). We now know that most of the FtsZ leaves the constriction zone prior to other Fts proteins such as FtsK and before complete septum formation [45,46]. During constriction, a portion of FtsZ–GFP can be seen to spiral out of the closing ring, and non-ring FtsZ (as FtsZ–GFP) seems to move rapidly in spiral patterns [47,48]. Such FtsZ spirals, also visible by IFM, have also been observed in *B. subtilis* cells during the transition from medial to asymmetric septation as part of the sporulation pathway, as well as during vegetative septation [49–51]. However, in other

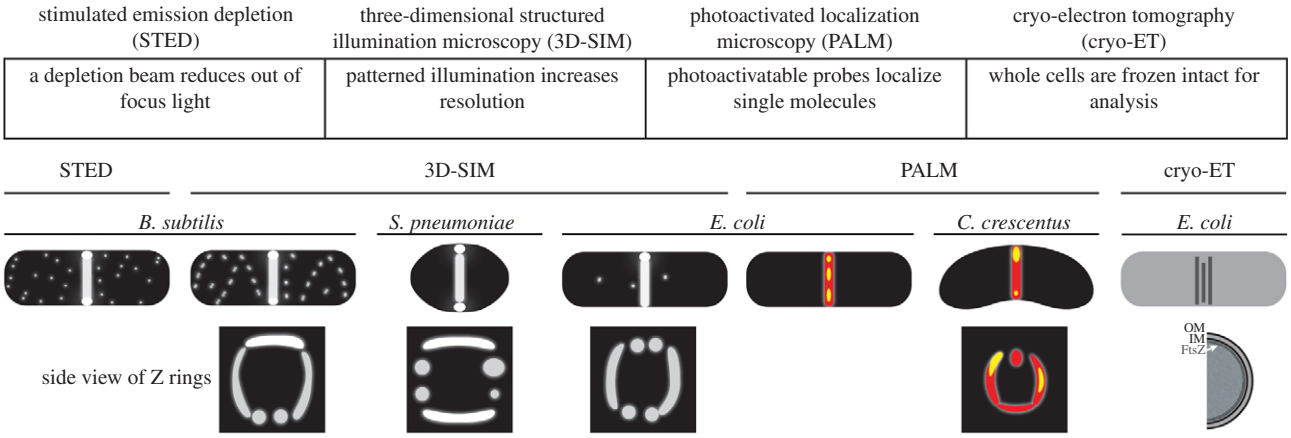

Figure 3. Examples of Z ring localization using different methods of super-resolution microscopy. The main principle of each method is listed in the table. The side views of Z rings represent a rotated image of the cell above them. For the PALM images, yellow areas represent areas of higher signal intensity. Cartoons from left to right were drawn based on Z-ring localization reported in [66–72].

species, FtsZ often forms mobile foci prior to forming the Z ring [52,53].

Advanced microscopy techniques have also been used to measure how quickly FtsZ subunits turn over in what appears to be a fairly static Z ring structure. The Erickson laboratory used fluorescence recovery after photobleaching (FRAP) to monitor the time it took for a bleached section of a Z ring decorated with FtsZ–GFP molecules to become fluorescent again. As photobleaching with a laser permanently ablates the fluorescence of an FtsZ–GFP molecule in the ring, the only way that the bleached part of the ring can recover fluorescence is if unbleached FtsZ–GFP molecules from elsewhere in the cell (ring or non-ring FtsZ) replace the bleached molecules. If this recovery did not occur, it would indicate that the FtsZ–GFP molecules in the Z ring are static, with little turnover. However, recovery within the Z ring reproducibly occurs within approximately 10 s, demonstrating that there is rapid interchange between FtsZ molecules in the Z ring and elsewhere in the cell [54]. This turnover is slower in cells carrying a mutant of FtsZ that has lower GTPase activity, consistent with the connection between GTP hydrolysis and FtsZ assembly [55]. Similar rapid turnover dynamics have been observed in active Z rings of *B. subtilis* and *Streptomyces*, indicating that fast subunit exchange is a widely conserved property of FtsZ [43,54]. GFP-tagged FtsA had similar dynamics as FtsZ [56], consistent with their proposed coassembly at the membrane.

Time-lapse analyses of GFP fusions were essential for uncovering the mechanism of Z ring centring in *E. coli*. It has been known for some time that MinC and MinD were cell-division inhibitors and MinE somehow antagonized them to centre the Z ring [57]. But the breakthrough came when David Raskin in Piet de Boer's laboratory discovered that the bulk of GFP–MinD oscillated from pole to pole, forming large, transient arrays at the membrane [58]. GFP–MinC followed essentially the same pattern, whereas MinE–GFP formed a mobile ring at the edge of the MinD array [58–61]. These observations, in combination with biochemical characterization of Min protein activities, led to a plethora of mathematical models, still being refined today, that accurately simulate the oscillations and Z ring centring [62]. None of these models could have been developed or tested without the ability to monitor movement of Min proteins in cells in real time.

More evidence in support of Min protein oscillations and their effects on the Z ring came from more recent studies of GFP-tagged divisome protein dynamics. Although FtsZ by IFM or FtsZ–GFP in live cells forms a sharp band at midcell, a considerable amount of FtsZ, accounting for at least half of the total cellular FtsZ, is also present outside the ring [63]. It follows that other FtsZ-binding proteins such as ZipA, ZapA, ZapC and ZapD in *E. coli* will also be in significant numbers outside the ring. Interestingly, bulk FtsZ, ZipA and ZapA proteins, as visualized with fluorescent protein tags, all oscillate in response to Min oscillations [48,64,65]; when MinCD is at one pole, most of the divisome proteins are at the opposite pole because of the transient initiation of FtsZ assembly away from MinCD.

4. Super-resolution of the divisome: is the Z-ring a patchy toroid?

As the bacterial divisome is essentially invisible by electron microscopy, the advent of methods to resolve fluorescent tags at resolutions lower than the diffraction limit of light (approx. 200–300 nm) has led to significant insights into our understanding of its fine structure. Several new super-resolution technologies have paved the way (figure 3), although more refinements will surely follow [73]. One of these, three-dimensional structured illumination microscopy (3D-SIM), spatially patterns the excitation laser light to resolve two fluorescent point sources that are as little as 100 nm apart in the plane of the cover glass (*XY*), although the resolution is lower in the *Z* plane [74,75]. Although it requires a sophisticated instrument and software, the advantage of 3D-SIM is that it does not need to detect single molecules and thus can be used with fluorescent protein tags in multiple colours. The speed of this method allows the use of time-lapse imaging of live cells, although laser illumination during acquisition of multiple images needed to make the reconstruction can result in significant photobleaching, which can cause reconstruction artefacts. Another method that does not require single molecule detection but can achieve high resolution is stimulated emission depletion (STED), which also requires a specialized microscope/laser system [76]. STED can theoretically provide much higher resolution than 3D-SIM,

because the technique is based on physically masking the out of focus light from a point source. However, this is technically challenging at present, and image acquisition is slow.

The first super-resolution images of the bacterial divisome came from IFM of *B. subtilis* FtsZ (STED), *B. subtilis* cells producing FtsZ-GFP as the only copy of FtsZ in the cell (3D-SIM), and *Staphylococcus aureus* cells producing FtsZ-GFP as a label with native FtsZ present (3D-SIM) [66,68,76]. These studies revealed that FtsZ formed a patchy, discontinuous-appearing structure, with clear areas of low fluorescence around the ring circumference. Such discontinuities confirmed a pattern suggested previously by lower-resolution deconvolution studies [44], but the sharp 3D-SIM images removed any doubt. Moreover, the rapid image acquisition permitted by 3D-SIM showed that *B. subtilis* FtsZ–GFP patches in the ring moved around, suggesting that these assemblages move along the cytoplasmic membrane [68]. The unevenly distributed Z ring detected by this method suggested a model for how increased FtsZ density might contribute to force generation during constriction [68,77]. In *E. coli*, FtsA and ZipA also formed patches that often, but not always, colocalized [67]. This supports the idea that FtsZ and its membrane anchors assemble together in clusters. In agreement with previous conventional microscopic studies, the super-resolution images showed that FtsZ often formed a spiral structure at the midpoint of cells that were not yet actively constricting. 3D-SIM was also used to show the existence of double rings of a cell-division protein in *B. subtilis*. Unlike the Min system of *E. coli*, the *B. subtilis* Min system lacks MinE and instead MinCD are recruited, via another protein called MinJ, to rings that lie to either side of the Z ring. These rings, which are difficult to discern by conventional fluorescence microscopy but visualized distinctly by 3D-SIM, are formed by a protein called DivIVA, and recruitment of MinCD to these rings keeps the MinCD inhibitor away from the active Z ring while preventing spurious Z rings from assembling nearby [78].

Another class of super-resolution methods has been used that can locate single molecules to a very small region. Instead of patterning the excitation light to increase the resolution of a large number of fluorescent molecules, these methods, called PALM (photoactivated localization microscopy) or STORM (stochastic optical reconstruction microscopy) rely on fluorescent tags that are in the off (often dark) state until turned on by a specific excitation light. PALM and STORM were originally named for separate applications but describe essentially the same single molecule technology. The position of a single molecule can then be measured by calculating the centre of the diffraction-limited fluorescent spot that is spread among multiple pixels. Controlled photoactivation of hundreds of individual tagged molecules and calculation of their positions within the cell results in nanoscale resolution, better than anything currently achievable by 3D-SIM or STED. Using photoswitchable fluorescent proteins such as mEos fused to FtsZ for PALM of live cells, Jie Xiao's laboratory has found that the *E. coli* Z ring is approximately 110 nm wide and, in agreement with the other super-resolution studies, is a patchy assemblage of FtsZ filaments [79]. A 3D-PALM study of the *Caulobacter* Z ring using FtsZ-Dendra fusions supports these results, concluding that the Z ring in normal cells is patchy [72]. Interestingly, the PALM studies of *E. coli* indicate that FtsZ polymers within the Z ring are not all aligned circumferentially, but are often positioned at odd angles, sometimes parallel with the cell's long axis [69]. A study using fluorescence polarization found a similar lack of circumferential FtsZ polymer orientation in *E. coli* and *B. subtilis*, although the polymers were more aligned circumferentially in *Caulobacter* [80]. It is likely that there are significant species-specific differences in Z ring organization and ultrastructure, depending on the mode of cytokinesis and other factors. For example, most Gram-positive bacteria and many Gram-negative bacteria including *E. coli* form a clear septum, whereas some alpha-proteobacteria such as *C. crescentus* divide solely by constriction [81] and not by a combination of septation and constriction like *E. coli*. These differences are reflected not only in the intrinsic behaviour of FtsZ polymers, but also in the FtsZ-associated factors that modify FtsZ polymer shape, length and turnover.

In addition to refining what we know about FtsZ polymer density and orientation, super-resolution microscopy has also helped visualize how far the Z ring extends into the cell. In *E. coli*, the divisome consists of many periplasmic proteins such as the Tol/Pal complex, which extends all the way to the outer membrane and helps coordinate outer membrane invagination with Z ring constriction and formation of the division septum [82]. Combining molecular biology methods with PALM, the Xiao laboratory recently provided evidence that FtsZ, ZapA, ZapB and a protein called MatP form a protein network that extends from the cytoplasmic membrane inward to the chromosomal replication terminus, with which the DNA-binding protein MatP interacts directly. Deletion of the *matP* gene speeds the rate of Z ring constriction, indicating that the entire network is important for proper regulation of the divisome and normally helps to keep it in check [83]. A different type of super-resolution method using an astigmatic point spread function in combination with wide-field imaging found that the Z ring in *Caulobacter* is quite thick. Taken together, these results suggest that the Z ring is actually a patchy toroid instead of a ring [84,85].

Finally, super-resolution methods have been recently used to define divisome complexes in smaller bacterial cells that previously stretched the limits of conventional fluorescence microscopy. As mentioned above, 3D-SIM was used to visualize a patchy Z ring in *S. aureus*. In addition, 3D-SIM was used to localize divisome components of *Streptococcus pneumoniae*, which grow and divide as small ovococci. Using 3D-SIM for IFM, Malcolm Winkler's group found that penicillin-binding proteins Pbp1A and Pbp2x localize to division septa like FtsZ, but form rings clearly outside the Z ring and remain at maturing division septa even after FtsZ has migrated to future division sites [70]. Moreover, at a certain point in the *S. pneumoniae* division cycle, the Pbp1a ring is detectably outside the Pbp2x ring, suggesting that these proteins form concentric layers. Although it was possible to detect ZapA and FtsZ outside an inner ZapB ring in dividing *E. coli* cells by conventional resolution [86], clearly the new methods are poised to refine our views of divisome ultrastructure in intact cells. Moreover, 3D-SIM was recently used to distinguish the effects of MapZ, an FtsZ positioning regulator, on Z ring structure in *S. pneumoniae* [87].

5. Viewing native divisome structures by cryo-electron tomography

Yet another relatively new and powerful microscopic method has made inroads in understanding nanoscale protein

assembly structure in bacteria: cryo-electron tomography (cryo-ET) (figure 3). Unlike conventional negative-stain transmission EM, for which cells are fixed, often sliced into thin sections, and then stained with uranyl acetate to reveal protein structures, specimens for whole-cell cryo-ET are plunge-frozen without further staining. The resulting images are low, by contrast, but can be computationally enhanced, and do not suffer from fixation, labelling or staining artefacts. Although the small size of bacteria is a disadvantage for most light microscopy, thin cells are actually required for sufficient image contrast. As a result, species that generate thin cells, such as *Rhodobacter sphaeroides* and *C. crescentus*, make it possible to visualize protein complexes if they are sufficiently concentrated. *E. coli* and other larger rod-shaped cells are difficult to image because they are too thick, although tiny minicells of *E. coli* and other species have recently been exploited to visualize surface protein assemblies [88–90].

Caulobacter crescentus is particularly suitable for cryo-ET analysis of the divisome as it divides solely by constriction, thus making a very thin bridge between daughter cells that is optimal for this technique. An initial cryo-ET study of the *C. crescentus* Z ring visualized long polymers of presumably FtsZ, which were periodically anchored to the cytoplasmic membrane [91]. Such an arrangement is consistent with known interactions between FtsZ and its membrane anchor proteins such as FtsA (ZipA is absent in *C. crescentus*), which are lower in abundance than FtsZ and should periodically tether FtsZ polymers to the membrane. A more recent cryo-ET study also visualized long polymers, and more controls were done that suggested these polymers were FtsZ itself [71]. A major conclusion of this study was that the Z ring is comprised at least partially of continuous long FtsZ polymers, which seems to be at odds with the patchy nature of Z rings visualized by 3D-SIM and PALM. It is possible that some long FtsZ polymers or polymer bundles in the Z ring might be difficult to detect by 3D-SIM if they are sparse (although they would be easier to detect with PALM). As mentioned in §1, the lack of complete function of FtsZ fusions to fluorescent proteins could result in these fusions not assembling into continuous polymers *in vivo*; however, cells producing native FtsZ also displayed patchy Z rings by IFM, ruling this out as a significant problem [67,79]. Future studies with different high resolution methods, including correlated light and cryo-EM that can match a structure with a specific protein or proteins [92], will be needed to clarify these results.

6. Reconstitution of the divisome on membranes *in vitro*

Perhaps the most striking advance in the field of bacterial cell division has been the recent successes in reconstituting parts of the divisome on membranes. In this 'bottom-up' approach, individual proteins can be manipulated, along with cofactors, to understand mechanistic details that are too difficult to parse in a whole-cell system. There are three notable examples of reconstitution with a cell-division phenotype. The first is the interaction between FtsZ and liposomes, investigated by Harold Erickson's group. Initially using a version of FtsZ fused to an artificial membrane anchor, they found that FtsZ polymers could assemble into rings inside liposomes and pinch them in the presence of GTP [93]. More recently, they found that purified FtsZ together with FtsA*, a variant of FtsA that can divide an *E. coli* cell in the absence of ZipA, could form a ring and divide a small proportion of liposomes to apparent completion in the presence of GTP for FtsZ and ATP for FtsA* [94]. These experiments suggested that FtsZ and FtsA were sufficient to exert a significant inward pinching force on a membrane and could be doing so in the cell. Cryo-EM of inside-out Z ring complexes on liposomes displayed long parallel bundled filaments on the membrane [71,95], supporting the *in vivo* cryo-ET data.

The second type of reconstitution comes from Martin Loose and Tim Mitchison, who investigated the interplay between purified FtsA and FtsZ on supported lipid bilayers (SLBs) [96]. By placing FtsA on top of an SLB and then adding a fluorescently tagged FtsZ, they found that these two proteins self-organized into dynamic polymer networks upon addition of GTP and ATP [97]. These networks often organized into ring-like structures on the flat surface of the membranes, indicating that curved polymers of both FtsZ and FtsA are favoured. The nature of the dynamics suggested that FtsZ polymers were moving by treadmilling, which can be described as subunits adding onto the front of a polarized polymer while simultaneously sloughing off the rear. This type of dynamics would be analogous to the treadmilling observed in eukaryotic actin filaments and microtubules.

The third notable example of reconstitution, from Petra Schwille's group, is the use of SLBs to mimic the size and shape of bacterial cell membranes. Addition of purified MinD and MinE labelled with fluorescent tags resulted in the formation of migrating waves on flat SLBs, with MinE lagging behind MinD just like in the cell [98]. More recently, SLBs have been fashioned in bathtub-like containers. When MinE and MinD are added to these, they oscillate from one end to the other [99]. Most impressively, when MinC and FtsZ are added to the mix, FtsZ ends up being positioned at midcell. Although there are clearly other regulatory factors involved in Z ring positioning, these reductionist experiments show that the basic patterns of cytokinesis can be reconstituted *in vitro* from a minimal set of components. Maxicells, which are *E. coli* cells devoid of chromosomes, also contain an oscillating Min system and a centred Z ring, supporting the idea that cell membrane geometry can position a Z ring without the need of the bacterial nucleoid [100,101].

7. Concluding remarks

The recent advent of super-resolution imaging methods has made a significant impact on the field of bacteriology by increasing the precision with which protein localization can be viewed in the smallest cells. Combining the power of super-resolution microscopy methods with genetic and biochemical approaches will further our understanding of how the divisome functions to divide bacterial cells and shed light on the protein–protein interactions that regulate this process.

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