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New insights into the assembly and regulation of the bacterial divisome

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Abstract

The ability to split one cell into two is fundamental to all life, and many bacteria can accomplish this feat several times per hour with high accuracy. Most bacteria call on an ancient homolog of tubulin, called FtsZ, to localize and organize the cell division machinery (divisome) into a ring-like structure at the cell midpoint. The divisome includes numerous other proteins, often including an actin homolog (FtsA), that interact with each other at the cytoplasmic membrane. Once assembled, the protein complexes that comprise the dynamic divisome coordinate membrane constriction with synthesis of a division septum, but only after overcoming checkpoints mediated by specialized protein-protein interactions. In this Review, we focus on the most recent evidence for how divisome proteins of *Escherichia coli* assemble at the cell midpoint, interact with each other, and regulate activation of septum synthesis. We also briefly discuss the potential of divisome proteins as novel antibiotic targets.

To survive and proliferate, a bacterial cell has to coordinate many metabolic and physical tasks. One of the most daunting challenges it faces is the need to split itself in two after accurately duplicating and partitioning its genome, all while maintaining a cell wall capable of withstanding high turgor pressure. In the case of fast-growing cells such as *Escherichia coli*, this act of binary fission needs to happen every ~20 minutes, and requires perfect coordination among the inner membrane, a cell wall, and the outer membrane all at the correct location at midcell between newly replicated daughter chromosomes. The complex protein nanomachine that drives bacterial cytokinesis is often called the divisome.

After several decades of study, we now know many of the proteins that assemble into the bacterial divisome. How the divisome is targeted to the cell midpoint by spatial regulatory systems is reasonably well understood in a number of species, although it is becoming clear that mechanisms for centering the divisome are diverse. On the other hand, the temporal regulation of divisome activity and the signaling between its cytoplasmic and periplasmic protein machines has been illuminated only recently. This Review covers some of the recent

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significant advances in our understanding of how the divisome is assembled, kept in check, and ultimately activated, emphasizing major spatial and temporal regulatory mechanisms. We will focus mainly on the *E. coli* model system, about which most is known, but compare and contrast to other species when relevant. In particular we will outline the key roles of the tubulin-like FtsZ and actin-like FtsA proteins in divisome organization and activation. We also discuss recent advances in divisome reconstitution *in vitro*, potential roles of biomolecular condensates, and targeting divisome proteins with small molecules.

Conserved divisome proteins

FtsZ is the master cytomotive protein organizer of the divisome in most bacteria. It assembles into polymers at the division plane to form the "Z ring" [1], which is required for the localization of all other divisome proteins. These FtsZ polymers move directionally around the inner membrane by treadmilling, which involves subunits joining at one end of a polymer while leaving the opposite end, ultimately acting as a molecular motor [2,3]. However, FtsZ cannot attach to the membrane by itself, and needs to bind to membrane-associated proteins, through its conserved C-terminal tail, to organize into a Z ring. In *E. coli,* these membrane tethers include FtsA, one of several conserved bacterial actin homologs [4], and the less well-conserved ZipA, initially discovered as an FtsZ-Interacting Protein [5–7]. Together, FtsZ, FtsA and ZipA comprise the "proto-ring", which represents the initial cytoplasmic setup stage of the *E. coli* divisome [8] (FIG. 1A). The FtsE and FtsX proteins, which complex together, also arrive at this point [9].

After a distinct delay [10,11], other *E. coli* divisome proteins are recruited to the proto-ring in a second stage in the order FtsK-(FtsQ/FtsL/FtsB)-FtsW-FtsI-FtsN, as shown by immunofluorescent or GFP-tagged localization of each of these proteins in mutants in which other divisome proteins are inactivated or depleted [12]. Inactivation of any one of these proteins leads to a lethal cell division block, although division of cells lacking FtsE or FtsX is restored when grown in medium with high osmotic strength [13]. Two of these, FtsW and FtsI, are essential septal wall synthesis enzymes conserved in most walled bacteria [14] and work together, so they will be referred to as FtsWI. There are numerous ancillary divisome proteins, such as the Zap proteins [15], cell wall amidases, and their regulators such as EnvC, whose absence individually does not result in major cell division defects but are important in combination with other members of their group in facilitating normal cell division. These have been covered in several recent reviews, including a comprehensive review of *E. coli* cell division [16].

Centering the divisome

A key task for a rapidly dividing rod-shaped bacterium such as *E. coli* is to identify the next site of cell division, which is identified very early in the cell division cycle [17]. Several spatial regulatory systems contribute to Z-ring centering in *E. coli,* including the Min system and nucleoid occlusion (NO), both of which act as localized negative regulators of FtsZ polymerization [18,19]. When the Min system is inactivated, cells divide either properly at the central site or asymmetrically near the cell poles to form DNA-less minicells, whereas

mutants defective in NO display no defects unless other systems, such as the Min system itself, are disabled [20].

The *E. coli* Min system consists of three proteins, MinC, MinD, and MinE [21]. MinD, which is a deviant Walker ATPase [22], uses its amphipathic helix to bind to the membrane when ATP-bound and leaves the membrane after ATP hydrolysis, which is stimulated by its membrane-associated partner, MinE [23,24]. As a result, membrane-bound MinD-ATP is "chased" off the membrane by MinE as MinD-ADP until the regeneration of MinD-ATP allows it to rebind the membrane, repeating the cycle [25–27]. This behavior is essentially a Turing reaction-diffusion system [28], which has been reconstituted *in vitro* as traveling waves of MinD and MinE on planar lipid bilayers [29]. In the select group of rod-shaped species that harbor MinD and MinE, these waves organize into zones around cell poles that move periodically (and rapidly, within a few seconds) to the opposite cell pole, forming a bipolar concentration gradient (FIG. 1A).

Recent evidence suggests that this membrane-associated gradient may help gather polymerizing FtsZ toward the cell center [30,31]. Nevertheless, MinC is clearly important for spatially restricting the Z ring to midcell, as it binds directly to two domains of FtsZ and inhibits FtsZ polymerization [32] and mutants lacking only MinC still divide aberrantly. The key to Z-ring centering is that MinC associates with MinD and oscillates with it [27,33], thus inhibiting Z-ring formation mostly at the cell poles where MinD is present most of the time. This inhibition is visible as an oscillation that runs counter to the Min oscillation [34,35]. Cell geometry is crucial for the centering mechanism, as Min proteins seek the longer axis for their oscillations, even in spheroidal cells and compartments [36,37]. During the septation process, Min oscillation transitions into a double oscillation in each daughter cell compartment to identify future division sites [38]. There is evidence that MinC and MinD from *E. coli* and other species form copolymers in vitro [39–42], suggesting that copolymers of MinCD near cell poles may restrict FtsZ assembly there. However, so far there is no evidence that MinCD copolymers are important for Min function in *E. coli* cells [43].

The other major spatial regulator of the Z ring, NO, is mediated in *E. coli* by a DNA-binding protein called SlmA (originally isolated as a synthetic lethal allele of Min) [20]. Specific chromosomal SlmA binding sites (SBS) are biased away from the replication terminus (*Ter*) near midcell and towards the chromosomal replication origin (*OriC*) near the cell poles, resulting in a bipolar gradient of SlmA in the cell [44,45]. In addition, and crucial for its mechanism of action, SlmA also binds to FtsZ [46,47] and interferes with its assembly [48,49]. Consequently, SlmA tends to inhibit FtsZ assembly in the vicinity of the nucleoid, particularly near *OriC* (FIG. 1A).

SlmA, however, is not well conserved in other species that use NO. In *B. subtilis,* NO is implemented by Noc, a ParB homolog that is structurally unrelated to SlmA. Like SlmA, Noc forms nucleoprotein complexes at several specific binding sites on the chromosome [50]. Unlike SlmA, which binds to FtsZ and spreads on DNA flanking its binding sites [49], Noc uses an N-terminal amphipathic helix to spread on the membrane and recruit chromosomal DNA there, which indirectly discourages local Z ring formation [51], probably by inhibiting FtsZ subunit migration away from existing division septa [52].

Despite their different mechanisms of action, both Noc and SlmA have a strong tendency to form phase-separated biomolecular condensates at the membrane that recruit FtsZ, potentially sequestering FtsZ in a non-polymerized state [53,54] (FIG. 1A). These phase-separated states are reversible: addition of GTP to FtsZ-SlmA condensates triggers assembly of FtsZ polymers and dissolution of the condensates [53]. Analogously, addition of CTP to Noc promotes its phase separation, and addition of high levels of KCl can dissolve both Noc and SlmA condensates [54,55].

It remains to be seen how these phase transitions function in bacterial cell division. A fluorescent derivative of FtsZ, along with associated divisome proteins, frequently forms polar foci in quiescent *E. coli* cells, which dissolve upon resumption of growth conditions [56]. If these foci are biomolecular condensates, which has not yet been demonstrated conclusively but is supported by the ability of purified FtsZ to form condensates [57], they may sequester FtsZ monomers at low GTP concentrations during nutrient starvation and permit FtsZ to polymerize at higher GTP concentrations triggered by rapid growth. This has implications for the efficacy of antibacterials against bacterial persister cells, as a drug target inside a condensate may be more difficult for the drug to access [58]. Very recently, it was reported that a protein called PomY forms biomolecular condensates that promote assembly of the Z ring at midcell in *Myxococcus xanthus* [59] (FIG. 1C). Fortunately, new tools are rapidly emerging to identify and confirm condensates in bacterial cells [60]. For example, single particle tracking in combination with photobleaching recovery measurements can help determine whether a fluorescently tagged protein is forming condensates. In addition, fluorescently-tagged proteins with different tendencies to form condensates in bacteria can be used as key controls.

The Min system, FtsZ and a membrane tether have been used as a minimal system to reconstitute cell division *in vitro*, using lipid membranes as cell-like vessels. Following initial reports of FtsZ forming rings inside of liposomes and occasionally constricting them [61], and reconstitution of an oscillating Min system inside liposomes [62,63], two recent reconstitution studies expressed MinCDE along with FtsZ and a membrane tether inside cell-like liposomes using a cell-free transcription/translation system that optimizes protein folding and native stoichiometries [64,65]. These studies were able to generate robust Min oscillations that organized FtsZ polymers around the middle of the liposome, ultimately producing isotropic constrictions. The most recent study reconstituted FtsZ, its FtsA membrane tether, and MinCDE inside liposomes to generate mid-liposome constrictions, and showed that Min oscillations and the condensation of FtsZ polymers at mid-liposome reinforce each other [66] (FIG. 1B). These reconstitutions have made impressive progress towards understanding how primitive wall-less cells may have duplicated. However, complete abscission of liposomes with FtsA and FtsZ has not yet been reported, possibly because additional proteins are needed to destabilize FtsZ polymers and/or fuse membranes [67]. Future studies will also need to account for NO and other effects of the bacterial nucleoid, and should probably exploit spatial regulatory systems from other bacteria that lack Min or NO systems and may be simpler to implement.

Finally, several positively-acting spatial regulatory systems are known to center the Z ring in a number of species, including *E. coli* (FIG. 1C). For example, the MatP protein of

E. coli binds to multiple DNA sites flanking the chromosomal *Ter*, which localizes near midcell prior to Z ring assembly [68]. MatP also binds to ZapB, a protein that in turn binds to ZapA, which promotes bundling of FtsZ polymers [69–71]. Along with its ability to bind to the membrane [53] and organize the chromosome, these properties demonstrate that MatP links the Z ring with *Ter*, providing spatial as well as temporal regulation with respect to chromosome replication [72,73]. In other species, the Pom system in *M. xanthus* [74], mobile rings of MapZ in Streptococci [75], and SsgB protein in Streptomyces [76] act by positive regulation (FIG. 1C). PomXYZ constitute a MinD-MinE-like system that moves from the nucleoid to new division sites, whereas MapZ binds both peptidoglycan and FtsZ and helps to ferry FtsZ and proto-ring proteins towards new daughter division sites while the midcell septum is closing [77].

Stages of divisome assembly

Once the proto-ring is established, the remaining divisome proteins are recruited by multiple interactions. In *E. coli*, recruitment of FtsWI to midcell requires FtsK and the FtsQLB complex [78,79], which in turn require FtsA and ZipA [5,80,81] (FIG. 2). FtsK is a multi-pass transmembrane protein fused to a DNA motor that coordinates chromosome segregation with cell division [82–84]. Another DNA translocase, SftA, is recruited to the Z ring in *B. subtilis* and may be involved in similar coordination [85]. Only the N-terminal ~200 residues of FtsK (FtsKn), comprising all four transmembrane domains [86] are required for cell division [87–89]. FtsQ, FtsL and FtsB are all conserved bitopic membrane proteins with short cytoplasmic tails and large periplasmic domains that interact with each other in a fairly stable FtsQLB complex [79,90–92]. Recent genetic and structural studies, discussed more below, suggest that FtsB and FtsL interact directly with the periplasmic domains of FtsW and FtsI and trigger their activities [92–95].

The mechanism by which FtsA recruits FtsK and FtsQLB is becoming clearer. Recent data from pull down and affinity copurification assays suggest that FtsA binds to FtsKn [96] and FtsKn forms a tight bimolecular complex with FtsQ [95], likely reflecting how this recruitment pathway occurs *in vivo*. Moreover, coevolutionary sequence analysis suggests that the first periplasmic loop of FtsKn interacts with a beta strand within the periplasmic domain of FtsQ between residues Q96 and K113 [95]. A better mechanistic understanding of how FtsQLB is recruited by FtsA and FtsK will necessitate structural dissection of the FtsQ-FtsK complex and a potential FtsA-FtsK complex.

FtsA-FtsN interactions have been characterized in greater detail. FtsA directly interacts with FtsN's cytoplasmic tail, called $FtsN_{cyto}$ [97–99] (FIG. 2, 3A). However, FtsN's recruitment to the divisome also depends on interactions with other divisome proteins including FtsI [100], making FtsN the last essential divisome protein to be recruited. FtsN's periplasmic domain includes a short segment essential for FtsN function ($FtsN^{E}$) and a nonessential C-terminal SPOR domain [101,102] (FIG. 2). The latter binds specifically to denuded cell wall glycans whose stem peptides have been cleaved off by cell wall amidases generated during septum synthesis [103]. Consequently, increased septum synthesis by FtsWI, triggered by FtsN, results in more FtsN recruitment, which reinforces a positive feedback loop that drives septum

synthesis forward [101]. Divisome checkpoints, discussed in detail below, probably ensure that this feedback loop is not activated until the right conditions are met.

During early stages of cell division, treadmilling FtsZ polymers are needed to organize septum synthesis enzymes in diverse bacterial species [2,104–106] but are dispensable once septum synthesis is fully underway[107,108]. Consistent with this, a typical *E. coli* cell is ~ 1 μm in diameter, but as the septum closes and the membrane invaginates, FtsZ leaves first, starting at ~600 nm septal diameter (FIG. 1A), whereas later divisome proteins like FtsN start to depart after septal diameter decreases below 300–400 nm [72,109,110]. In support of these findings, rapid inactivation of FtsZ at later stages of septation does not affect the completion of division [111,112]. Later divisome proteins, whose domains mainly reside in the periplasm, also are spatially separated from FtsZ and other proto-ring proteins [113]. Therefore, FtsZ is needed only to start the septum building process, and once the septal template has been built, FtsWI will ensure its faithful continuation in the proper position without the need for an FtsZ guide. What triggers FtsZ release? Under slow growth conditions, cellular levels of FtsZ increase during Z ring assembly and then decrease during disassembly due to proteolysis [114]. However, the relatively rapid release of FtsZ from the constricting septum implicates other regulatory mechanisms. One may involve a recently discovered interaction between ZipA and the core domain of FtsZ [115], which is weaker than the well-known interaction between ZipA and FtsZ's C-terminal tail. This weaker interaction with the polymerizing domain of FtsZ hints at a more regulatory role than simple membrane anchoring.

Divisome checkpoints and the key role of FtsA

The first hint of a divisome checkpoint came from isolation of a hypermorphic missense allele of FtsA, called FtsA* ($FtsA_{R286W}$), that completely bypasses the requirement for ZipA and suppresses defects in other divisome proteins including FtsQ and FtsK [116,117]. Cells expressing FtsA* are ~20% shorter than WT cells, consistent with circumventing a checkpoint [118]. Subsequently, other single residue changes in FtsA were isolated that have FtsA*-like properties, and all map to the FtsA oligomer interaction interface (FIG. 3B) [119,120]. These locations as well as genetic assays indicated that FtsA*like variants have altered oligomerization properties [119]. Another hint of a checkpoint came from different variant, $FtsA_{E124A}$, that is able to bypass FtsN [121]. Unlike R286W, the E124A allele does not induce hyper-division, and maps to the 1C subdomain of FtsA instead of the 2B subdomain, farther from the longitudinal oligomer interface, suggesting a distinct regulatory mechanism (see below). In support of this idea, $FtsA_{R286W}$ poorly bypasses FtsN, $FtsA_{E124A}$ poorly bypasses ZipA, and neither can bypass the simultaneous loss of both ZipA and FtsN [121]. Interestingly, ZipA and FtsN seem to have coevolved [122]. This is consistent with their putative complementary roles in activating FtsA, and is further supported by the ability of either ZipA or FtsN to trigger preseptal peptidoglycan synthesis (independent of FtsWI) by activating the semi-redundant class A penicillin binding proteins PBP1A and PBP1B [123].

Recent biochemical studies have provided key new insights into the role of FtsA's oligomeric state as a potential divisome checkpoint. When purified *E. coli* FtsA is added to lipid membranes and visualized by negative stain electron microscopy, it forms striking

20 nm-wide, roughly planar ring-shaped oligomers [124] (FIG. 3A). In contrast, purified FtsA* variants assemble into broken curved oligomers (e.g. $FtsA_{R286W}$) and/or double stranded (DS) filaments ($FtsA_{G50E}$) instead of mini-rings [125,126]. Although the role of ATP in FtsA function is not yet understood, it is noteworthy that a well-known thermosensitive allele ($FtsA_{S195P}$) that maps to the ATP site and is defective in ATP binding/hydrolysis can be intragenically suppressed by FtsA*-like mutants, which also have higher intrinsic ATPase activity and undergo more rapid dynamics than WT FtsA [118,120,127]. Although it is not yet known what oligomeric structures $FtsA_{S195P}$ forms when inactive, and FtsA mini-rings have not yet been observed in cells, these data support the idea that “unlocking” the mini-rings switches FtsA to an active form that promotes septum synthesis. Furthermore, FtsA’s oligomerization activity can deform lipid membranes in vitro, which may help FtsZ to constrict the cytoplasmic membrane during septum synthesis [128].

As suggested by the ability of $FtsA_{E124A}$ (as well as another allele mapping to the 1C subdomain, $FtsA_{I143L}$) to bypass it [129] (FIG. 3B), FtsN seems also to be involved in a checkpoint. Indeed, FtsN was originally discovered through its ability to suppress several divisome defects when overproduced [130]. Although FtsN likely activates septum synthesis mainly through its periplasmic interactions with FtsWI, FtsN may also help to activate septum synthesis on the cytoplasmic side of the membrane by stabilizing the FtsA DS filament form. In support of this idea, adding a 5–10 fold molar excess of purified $FtsN_{cyto}$ ($FtsN_{1–32}$) to FtsA on a lipid membrane is sufficient to switch most minirings to antiparallel DS filaments [126] (FIG. 3A). The unique lateral associations between FtsA oligomers that generate the DS filaments can be detected *in vivo* by crosslinking and are mediated by adjacent 1C subdomains, which is also where $FtsN_{cyto}$ binds [97,126] (FIG. 3B). Importantly, the FtsN bypass variant $FtsA_{E124A}$ forms mini-rings *in vitro* like wild-type FtsA but switches to DS filaments at lower concentrations of added $FtsN_{cyto}$ compared with WT FtsA [126]. This suggests that the E124A residue change tips the balance towards DS filaments, perhaps by weakening interactions between mini-ring subunits, but may also rely on mini-rings to be unlocked by an earlier step. This is consistent with the aforementioned poor ability of $FtsA_{E124A}$ to bypass ZipA *in vivo* compared with FtsA* alleles.

Notably, FtsN-bound DS filaments of FtsA are curved in such a way that they can reinforce negative curvature on membranes to which they are attached [126]. FtsA DS filaments would thus have a strong tendency to orient along the curved short axis of the cell instead of the straight long axis, which may help to gather treadmilling FtsZ polymers within a more concentrated band. It should be emphasized that although the periplasmic $FtsN^E$ segment is essential for *E. coli* cell division, $FtsN_{cyto}$ is dispensable [98,131], indicating that the FtsA-FtsN interaction is only one of several pathways to activate septum synthesis [99,129,132] (see also below). In addition to FtsN’s role in promoting or stabilizing FtsA DS filaments, FtsA oligomerization state seems to dictate whether FtsN can bind to it [99,119]. For example, FtsA* interacts more efficiently with FtsN than WT FtsA and also earlier in the cell cycle [127,132,133]. This implies that $FtsN_{cyto}$ may bind more efficiently to free ends of unlocked FtsA than to mini-rings, which may also be the case for other divisome proteins recruited by FtsA such as FtsK and/or FtsQLB.

The FtsA-FtsN interaction is also interesting in an evolutionary context. Bacterial Type IV pili are anchored to the membrane by a protein called PilM that has remarkable structural similarity to FtsA, with similar subdomain organization [97,134]. Another similarity is that PilM also forms dodecameric rings on the inner surface of the cytoplasmic membrane [135,136]. One of several pilus proteins that interact with the PilM ring, PilN, has a bitopic topology like FtsN. Even the binding sites for PilM and FtsN on FtsA are similar, as the cytoplasmic tails of both target the cleft between the 1A and 1C domains (near the FtsA E124 bypass residue), although at distinct specific sites [126,134].

In addition to FtsN, ZipA and FtsX are both implicated in switching FtsA to an activated state, perhaps by unlocking mini-rings. In contrast to FtsN, whose main function is activating an already assembled divisome, both ZipA and FtsX act early to recruit later divisome proteins [13,81,137] and can be bypassed by hypermorphic alleles or overproduction of some divisome proteins [138], again suggesting that free FtsA oligomer ends in unlocked mini-rings are important for recruitment of later divisome proteins. ZipA and FtsX interact directly with FtsA at sites in the 2B and 1A domains, respectively [137,139], suggesting that their mechanisms of activation are distinct (FIG. 3B). Although the mechanism of FtsA unlocking by ZipA is not yet known, unlocking of FtsA by FtsX is regulated by the ATPase activity of FtsX's cytoplasmic binding partner FtsE (FIG. 3A). FtsX's periplasmic binding partner EnvC, which stimulates cell wall amidases important for splitting septal peptidoglycan, is also regulated by FtsX's ATPase activity [140,141]. Interestingly, Z rings are less condensed in EnvC mutants, suggesting that signaling within the divisome proceeds not only outward from FtsEX to the amidases but also inward from EnvC through FtsEX to FtsZ [142].

How might these oligomeric switches in FtsA confer checkpoint-like properties? One way is through effects on FtsZ. On lipid membranes *in vitro*, protofilaments of FtsZ are held apart by FtsA mini-rings, and only bundle in the presence of FtsA* derivatives [124,125]. Similar parallel but separated FtsZ filaments, which may represent the initial form of FtsZ prior to septum synthesis, have been observed in cells by cryo-EM tomography [143]. These results are consistent with FtsA mini-rings, assuming that they are confirmed to exist in cells, being in an initial locked state and support the idea that FtsA oligomerization state regulates FtsZ assembly state [144].

Although the molecular mechanisms that trigger FtsA's oligomeric switch or change FtsZ filament bundling state are not yet known, the switch to non-ring FtsA oligomers *in vitro* increases FtsA packing density on the membrane, which provides a denser array of membrane tethers for FtsZ to associate laterally and interact more dynamically [124,127] (FIG. 3A). As mentioned above, at least one part of the checkpoint likely involves increased bundling/condensation of FtsZ polymers. This bundling activity is enhanced by the binding of Zap proteins to FtsZ [15] and self-inhibited by FtsZ's disordered C-terminal linker [145–147], but is crucial for promoting septum synthesis [148].

As is postulated for FtsA-FtsN interactions, FtsA-FtsZ oligomeric states may rely on a self-reinforcing mechanism. A variant of FtsZ ($FtsZ_{L169R}$), named FtsZ* [149] has a strong tendency to laterally associate into protofilament pairs and ribbons *in vitro*, and like FtsA*

it can bypass ZipA and suppress some divisome defects in *E. coli*, including bundling defects caused by the lack of Zap proteins [149,150] (FIG. 3A). Importantly, these self-bundled FtsZ* protofilaments seem to promote unlocking of FtsA mini-rings *in vitro*, thus mimicking FtsA* [124]. As FtsA mini-ring disassembly *in vitro* promotes FtsZ bundling and vice-versa, these two processes may reinforce a positive feedback loop *in vivo* that irreversibly drives septation forward, especially if bundled FtsZ can act as a direct template for FtsA DS filaments (FIG. 3A). However, hyper-bundled FtsZ also confers a fitness cost, as cells with extra FtsZ* or FtsZ* combined with FtsA* have septal abnormalities and are less viable [125,149]. It should also be noted that other variants that increase FtsZ bundling *in vitro* such as $FtsZ_{E93R}$ [151] do not bypass ZipA, so the degree and type of bundling is likely to be important for activation.

Another key part of the FtsA checkpoint may involve FtsA curved DS filaments themselves and their ability to activate septum synthesis. A recent model proposed that DS filaments of FtsA, triggered by $FtsN_{cyto}$ binding, act as rudders that guide septal wall synthesis powered by FtsWI [126]. Such “surfing” cytoplasmic FtsA rudders would respond to the periplasmic activities of FtsWI directly through contacts with FtsN, which binds FtsA and FtsI, and by the FtsQLB complex, which binds and activates FtsW [93] and may also interact with FtsA [152] (FIG. 3C). This mechanism is roughly analogous to the membrane-associated rudders of the elongasome (cell wall elongation machinery) formed by DS filaments of MreB, which locally guide sidewall synthesis by PBP2 and RodA and are mediated by MreCD and RodZ [153,154]. These potentially analogous mechanisms underscore the evolutionary kinship between divisome and elongasome [126,155] and their competition for proteins and substrates [142,156,157].

Other divisome checkpoints

The FtsA-$FtsN_{cyto}$ interaction is clearly an important regulator of *E. coli* divisome activity. However, the isolation of hypermorphic variants of FtsL, FtsB, FtsI and FtsW, named FtsL*, FtsB* FtsI* and FtsW*, which all map to periplasmic domains in these proteins, indicate that crucial checkpoints operate in the periplasm (FIG. 2, 3C). Like FtsA*, some periplasmic hypermorphs exhibit strong hyper-division phenotypes consistent with constitutive activation of septum synthesis. For example, *ftsL** induces hyper-division and short cell length [158]. In addition, *ftsL** and *ftsB** can partially bypass FtsK or FtsN. These data suggest that FtsB* and FtsL* proteins mimic the interaction between an activating protein such as FtsN and the FtsQLBWI complex, switching it to an active form. The absence of known *ftsQ** alleles, along with evidence that FtsQ may interact directly with upstream divisome proteins such as FtsK and FtsA, suggest that it is the FtsBL portion of the complex that directly activates FtsWI. This idea is supported by genetic evidence pinpointing specific periplasmic domains in both FtsB and FtsL called CCD (constriction control domain), and a nearby domain in FtsL called AWI (activator of FtsWI) that harbor the hypermorphic alleles and activate the FtsWI septum synthases [159].

Moreover, recent structural studies combined with targeted mutations that decrease or increase function suggest that the activation signal travels from FtsB to FtsL to FtsI to FtsW [94], all via periplasmic interactions. The ultimate target of both checkpoints is probably the

fourth extracellular loop (ECL4) of FtsW, as FtsW* mutants of *E. coli* such as $FtsW_{E289G}$ mapping to this loop can bypass FtsN and other divisome defects, including dominant negative mutations in ECL4 itself. Similarly, FtsW* alleles in *Caulobacter crescentus* can override upstream divisome defects, and the strongest of these alleles is in the same ECL4 [161]. In addition to periplasmic activation of FtsWI via FtsQLB, genetic evidence suggests that FtsA acts directly on the cytoplasmic side of the *E. coli* divisome to activate FtsW through the latter's second cytoplasmic loop [93] (FIG. 3C). Consistent with a model of direct FtsA-FtsW signaling, FtsQ can be bypassed by coexpression of FtsA* along with a derivative of FtsW* that is artificially targeted to the divisome independently of FtsQ. Notably, *ftsL* and *ftsB* cannot be bypassed, at least so far, probably because conformational changes in FtsL and FtsB are essential for activating FtsWI.

In a different type of checkpoint, FtsK coordinates septum synthesis with chromosome segregation. This is particularly clear in *C. crescentus*, which, like other alpha-proteobacteria, lacks an obvious NO mechanism and instead uses a MinD-like multifunctional protein, MipZ, to couple chromosome segregation with Z ring placement [162]. Moreover, it seems that a divisome protein called FzlA binds directly to FtsZ and the translocase domain of FtsK, thereby coupling activation of FtsWI with chromosome segregation [163]. As it interacts with both proto-ring and late divisome proteins, FtsK probably acts similarly to coordinate divisome activity with chromosome segregation in *E. coli* [84], but the precise mechanism is not yet known. An interesting case of divisome-chromosome coordination operating in the opposite direction is the conserved CcrZ protein, which in *S. pneumoniae* uses its interaction with FtsZ at midcell to regulate the activity of DnaA, the key chromosome replication initiator protein [164].

Keeping up with treadmilling FtsZ

In addition to its checkpoint role, FtsA oligomers, along with ZipA, organize treadmilling polymers of FtsZ along the cell membrane [150,165] (FIG. 4A). *B. subtilis* FtsA oligomers seem to achieve this by transiently binding to treadmilling FtsZ subunits, which remain stationary in the middle of a treadmilling FtsZ polymer until they diffuse from the polymer end. This could achieve net translocation around the cell circumference (FIG. 4) by iterative diffusion and capture [104,166]. Another possibility is that FtsA tracks the ends of FtsZ polymers by a Brownian ratchet mechanism [106,167]. It is also possible that *B. subtilis* FtsA oligomers actually treadmill along with FtsZ polymers [3], but this requires that each FtsA polymer have a "minus" and "plus" end—i.e., polarity. This may be the case for *B. subtilis* FtsA, which forms polymers in parallel with FtsZ filaments [168]. However, it is certainly not for *E. coli* FtsA, as its closed minirings and antiparallel filaments would have no plus or minus ends required for polarized subunit gain and loss. While *E. coli* FtsA arcs could theoretically treadmill, they would literally go around in circles and could not maintain the ~30 nm/sec directional velocity observed for FtsZ [2]. Instead, FtsA oligomers—including mini-rings—probably keep up with FtsZ polymers in *E. coli* by disassembling and reassembling into new oligomeric structures that track the ends of FtsZ polymers. In support of this idea, turnover of fluorescently labeled FtsA in *E. coli* cells is nearly as rapid as FtsZ turnover [118] and the translocation velocities of fluorescent FtsA foci around the Z ring are similar to those of FtsZ [169] and of FtsA in other species [3,105], averaging ~30 nm/

sec. Moreover, single molecules of FtsN that lack the periplasmic region, and thus mainly interact with FtsA, are either stationary or move fast at ~30 nm/sec (FIG. 4B), consistent with the idea that FtsA structures track treadmilling FtsZ [167].

Although FtsA and ZipA are the primary FtsZ membrane tethers in *E. coli*, other proteins may provide this role in many other species. One of these is SepF, which is highly conserved in Gram-positive bacteria and archaea [170]. SepF has an amphipathic helix for binding the membrane, and assembles into curved polymers at the membrane that bind directly to FtsZ and promote protofilament bundling [171,172]. Moreover, SepF can functionally replace FtsA in *B. subtilis* [173] and regulates septum thickness [174], indicating that it shares FtsA's ability to activate and guide septum synthesis activity. Archaeal SepF lacks a conserved glycine crucial for polymerization, suggesting that polymerization may not be important for SepF function in archaea [170]. In addition to SepF, many Gram-positive bacteria express a protein with ZipA-like membrane topology called EzrA that binds to FtsZ and seems to act as a ZipA-like membrane tether. EzrA regulates FtsZ assembly, either negatively in *B. subtilis* or positively in *Streptococcus pneumoniae*, where it helps to condense Z rings [175,176]. Finally, it is notable that the actinobacteria, which includes important pathogens such as *Mycobacterium tuberculosis*, completely lack FtsA (and ZipA). Perhaps to replace FtsA's role, *M. tuberculosis* divisome proteins such as FtsW and FtsQ bind directly to FtsZ, at least *in vitro* [177,178], suggesting that FtsQ and FtsW act along with SepF as additional membrane tethers for FtsZ. However, it remains unclear why FtsZ needs multiple membrane tethers, unless they are involved in FtsZ's early release (see above) or some other type of regulatory mechanism.

The Rings of Power

Consistent with the spatial separation of different divisome proteins mentioned earlier, it was recently discovered that the *E. coli* divisome consists of both a fast track that includes FtsZ and FtsA, which, as mentioned above, collectively translocate around midcell at an average speed of ~30 nm/sec, and a separate slow track that includes FtsN and the FtsWI processive septal synthase complex that move at ~9 nm/sec (FIG. 4A–B). The fast track uses the GTPase activity of FtsZ to rapidly sample cellular space and optimize positioning of FtsZ polymers to guide septal synthesis. The slow track, on the other hand, reflects direct engagement of proteins in septal wall synthesis, is independent of FtsZ's GTPase activity, and persists throughout septum closure [167,179]. Individual subunits of *E. coli* FtsA within mini-rings or arcs probably remain transiently stationary as they interact with stationary FtsZ subunits within the interior of treadmilling polymers. Initial FtsN binding to individual FtsA subunits might also be transiently stationary until the concentration of FtsN reaches sufficient levels to convert most of the FtsA into DS filaments, which then could move along the membrane as a unit in response to FtsWI activity. Single molecule tracking suggests that FtsN's tracking of slow FtsWI movement occurs independently of FtsN-FtsA interactions [167,179], which is also consistent with FtsN interacting most strongly with the FtsWI complex. Whether FtsN remains bound to the FtsWI-directed FtsA DS filaments as septation continues is not clear. Overall, the transfer of proteins between tracks is reminiscent of a fast-moving ski gondola detaching from the main cable and transiently transferring to a slower-moving cable as it enters a lifthouse at the top or bottom of a ski

slope to pick up or drop off passengers, followed by its reattachment. It remains to be determined how this protein handoff occurs at the molecular level, including how many FtsA subunits switch from fast to slow tracks after septation has started.

Divisome proteins as potential drug targets

Our improved understanding of the inner workings of the divisome can help develop new inhibitors of bacterial cell division as potential antimicrobials, and their mechanisms of action can shed new light on divisome function. The vast majority of naturally-occurring and synthetic peptide and small molecule inhibitors target FtsZ, and have contributed to our understanding of FtsZ function. For example, the *B. subtilis* protein MciZ, which inhibits Z ring formation in the mother cell during sporulation, binds to the bottom of an FtsZ subunit, capping the growing filament and increasing bulk GTP hydrolysis [180]. This has provided important insights into the role of FtsZ's GTPase activity and how FtsZ filaments grow [181]. Similarly, the potent benzamide compound PC190723 binds to the interdomain cleft (IDC) of FtsZ (FIG. 5), freezing FtsZ monomers in a conformation that prevents treadmilling and thus inhibits cell division, at least in Gram-positive species [182]. Newer benzamide derivatives optimized for docking in the IDC can inhibit Gram-positive FtsZ with sub-micromolar affinity and can also inhibit *E. coli* defective for efflux pumps [183], suggesting strategies that combine the small molecule with an efflux pump inhibitor or outer membrane disruptor [184].

Despite these advances, the targeting of other conserved divisome proteins has been limited. For example, no successful FtsA inhibitor has been reported, although there is potential for interfering with FtsA's oligomeric state transitions. Another promising avenue is the targeting of protein-protein interactions within the divisome, which has been validated by isolation of an inhibitor of FtsQ-FtsB interactions [185]. A macrocyclic peptide derived from FtsB was modified to make a covalent interaction, which inhibited division of *E. coli* cells with membrane permeability defects. In addition to optimizing known divisome inhibitors, it is clear that FtsW and the related RodA protein, now established as peptidoglycan polymerases [14], are particularly rich targets to exploit for antimicrobials because of their size, high conservation, and multiple interaction domains.

Future challenges and questions

Impressive progress has been made in the last few years towards understanding the mechanisms underlying regulation of divisome activity, including the existence of a fast track in the cytoplasm for septal guidance that signals to activate a slow track coordinated with septal wall synthesis. A very recent cryo-EM structure of *Pseudomonas aeruginosa* FtsQLB complexed with FtsWI reveals a large conformational change in FtsWI between inactive and active states [95]. Moreover, another recent study based on structural predictions suggests that the essential role of $FtsN^E$ may be to directly tie FtsI and FtsL together in the periplasm [94].

In addition to learning more about how this switch is thrown in other model systems, there are several outstanding questions that remain. How do other parts of the divisome change

structurally upon activation, and can alterations in FtsA oligomeric state be observed in dividing cells? What is the mechanism by which hypermorphic alleles such as FtsL*, FtsB*, FtsI* and FtsW* hyperactivate division? What switches divisome proteins from tracking FtsZ to tracking FtsWI and vice versa? More broadly, recent structural breakthroughs cited in this review suggest that a high resolution cryo-EM structure of an entire divisome complex, including the proto-ring, may be possible. Such a structure would help to address the above questions, facilitate the design of better small molecule inhibitors, and get closer to the goal of an autonomously replicating synthetic cell. In addition, improvements in high resolution fluorescence imaging of growing bacteria will be crucial for understanding how the proto-ring assembles at midcell and which cell cycle cues regulate it. Finally, understanding more about the variations on the cell division theme in diverse bacteria should provide the breadth of insight needed to fully understand how the simplest of cells duplicate, and how the first cells evolved.

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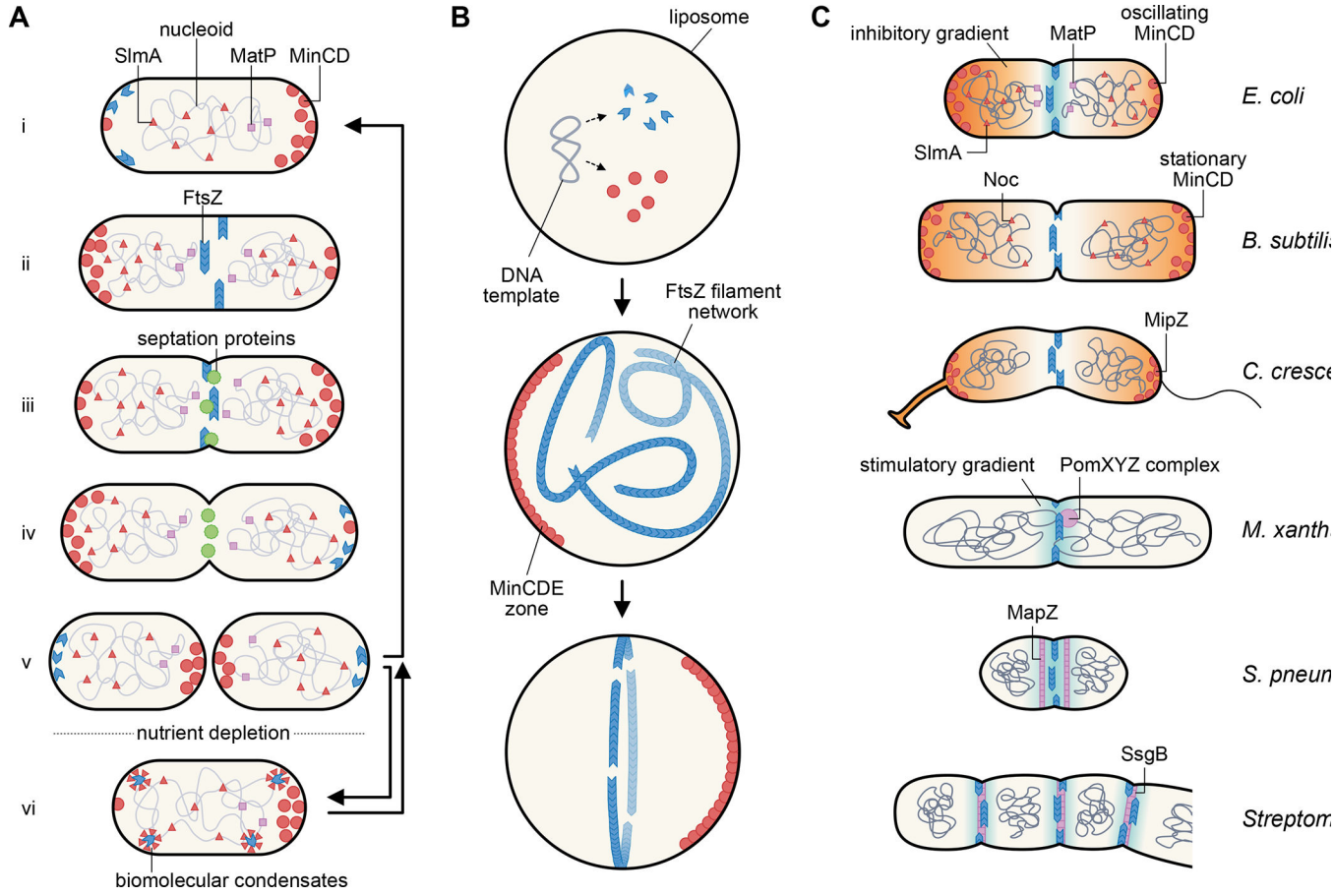

Fig. 1. Centering and organizing the divisome.

(A) *Escherichia coli* cell division cycle. Newborn cells (i) carry several spatial FtsZ positioning systems, including SlmA and MatP on the nucleoid and MinCD oscillating between the cell poles, that keep FtsZ from assembling into a Z ring at the cell poles or on top of the unsegregated nucleoid. As the replicated daughter nucleoids segregate, the Z ring assembles at midcell (ii) and recruits other septum-synthesis proteins (iii), which result in formation of the division septum. During septum synthesis, FtsZ leaves the division site prior to the other septation proteins (iv). The final step of cytokinesis results in two daughter cells (v), which begin the next cycle during growth conditions. Nutrient depletion or stress induces a quiescent state (vi) in which FtsZ localizes to putative biomolecular condensates that can revert back to polymer forms after growth restart. (B) Reconstitution of a centered proto-ring in lipid vesicles. After FtsZ, FtsA, and Min proteins are synthesized from a plasmid template or added as purified protein to lipid vesicles, they self-organize into membrane-associated polymers or gradients. Oscillation of MinCDE within the liposome helps to corral FtsA-tethered FtsZ polymers into a focused ring, mimicking in vivo behavior. (C) Theme and varations of Z ring positioning systems in diverse bacterial species, showing three examples of negative spatial regulators (orange gradients) and three examples of positive spatial regulators (blue gradients) and the proteins involved.

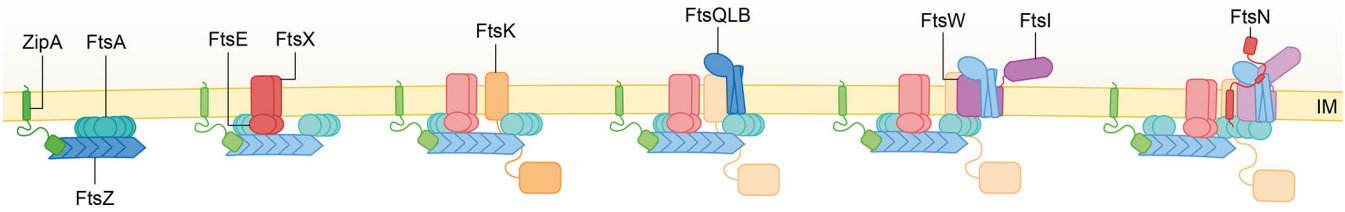

Fig. 2. The *E. coli* divisome is built in stages.

FtsZ is tethered to the membrane by FtsA and ZipA, forming the proto-ring. This is accompanied by the recruitment of the ABC transporter-like FtsEX complex and FtsK, which contains an N-terminal membrane bound domain required for cell division connected to a cytoplasmic DNA motor domain via a long linker. The next complex to be recruited is FtsQLB, which is important for recruitment and activation of the septum-specific transpeptidase (FtsI) and glycosyltransferase (FtsW). FtsN binds to and activates FtsWI enzymatic activity and is present at high concentrations at later stages. FtsN's four distinct domains, the short cytoplasmic tail ($FtsN_{cyto}$), transmembrane domain, essential periplasmic domain ($FtsN^{E}$), and SPOR domain, are highlighted in the cartoon. The recruitment stage for each protein is highlighted by more intense coloring.

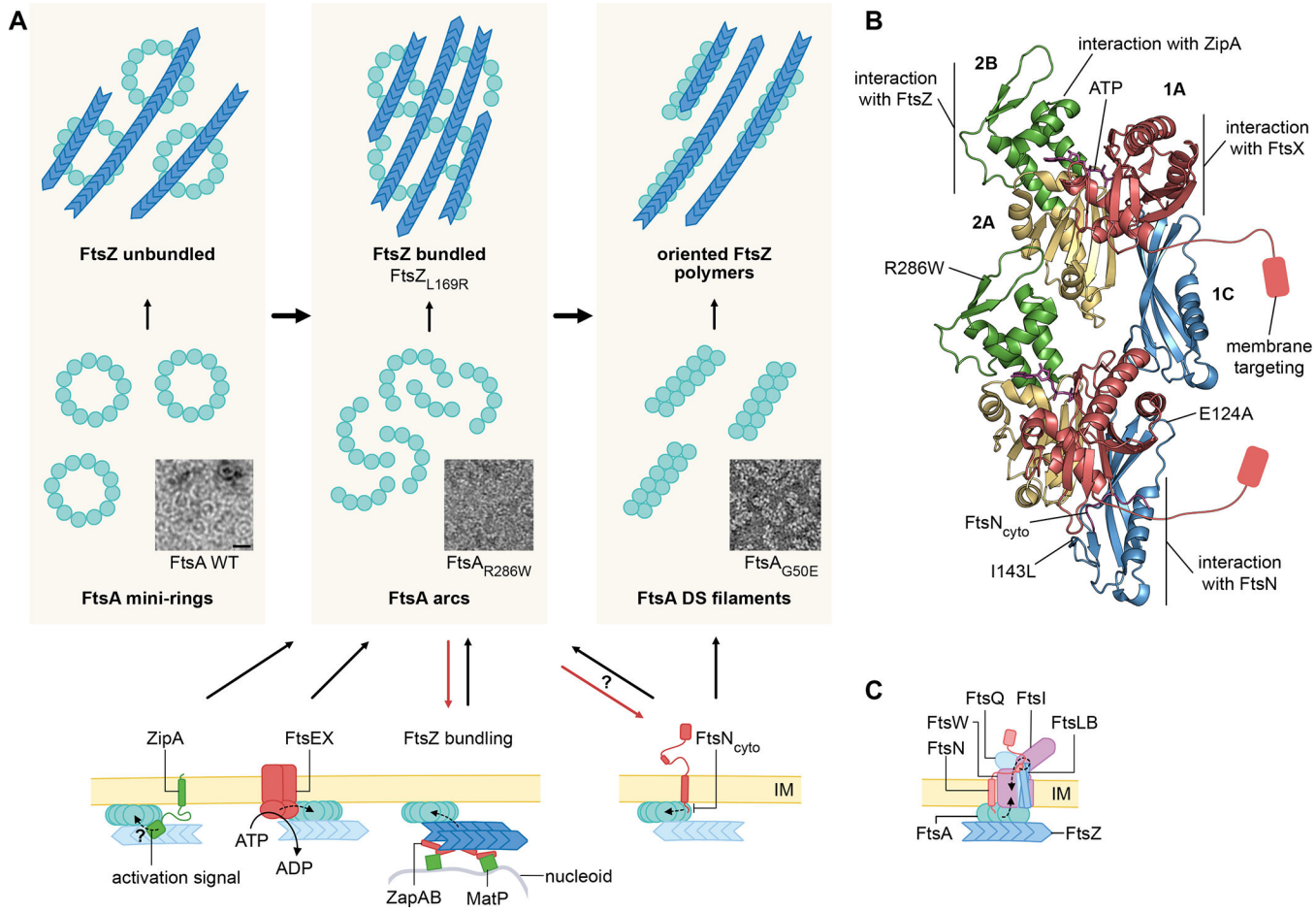

Fig. 3. FtsA oligomerization state regulates divisome activation.

(A) Distinct oligomeric states of FtsA and their effects. WT FtsA initially forms mini-rings on lipid membranes that tether and align separated FtsZ polymers. Although mini-rings have not yet been visualized *in vivo*, unlocking of FtsA mini-rings by ZipA, FtsX, FtsN (arrows), or by mutations, would be expected to form open FtsA oligomers (arcs) or DS antiparallel FtsA filaments. *In vitro*, both of these unlocked FtsA states seem to induce tethered FtsZ polymers to laterally associate into bundles, possibly by increasing membrane packing density. FtsA*-like variants bypass several divisome defects *in vivo* and bypass the mini-ring form *in vitro*, instead assembling mostly into arcs (e.g. R286W) or DS filaments (G50E). FtsZ polymer bundling may be another divisome activation signal, as the self-bundling $FtsZ_{L169R}$ (FtsZ*) variant bypasses several divisome defects in vivo and may convert FtsA into an unlocked, active form, suggesting a positive feedback loop (red and black arrows). In addition to FtsN's ability to unlock FtsA mini-rings, unlocked mini-rings may facilitate binding of FtsN (red and black arrows). (B) FtsA interactions, variants and activities. FtsA interacts with $FtsN_{cyto}$ through subdomain 1C, FtsZ and ZipA through interactions in subdomain 2B, FtsX through subdomain 1A, another FtsA subunit through several interfaces, and the cytoplasmic membrane via a membrane-targeting amphipathic helix. FtsA*-like substitutions such as R286W likely weaken monomer-monomer interactions that result in unlocking of mini-rings *in vitro*. FtsN bypass alleles (I143L, E124A) map to

subdomain 1C, potentially mimicking $FtsN_{cyto}$ binding. (C) The changes in FtsA oligomeric state signal the later divisome proteins such as FtsW to switch to the activated form (upward arrow). In addition to the cytoplasmic activation signal from FtsA, conformational changes in FtsQLB in the periplasm, along with FtsN's essential domain, are also crucial for activation of FtsWI (downward arrow).

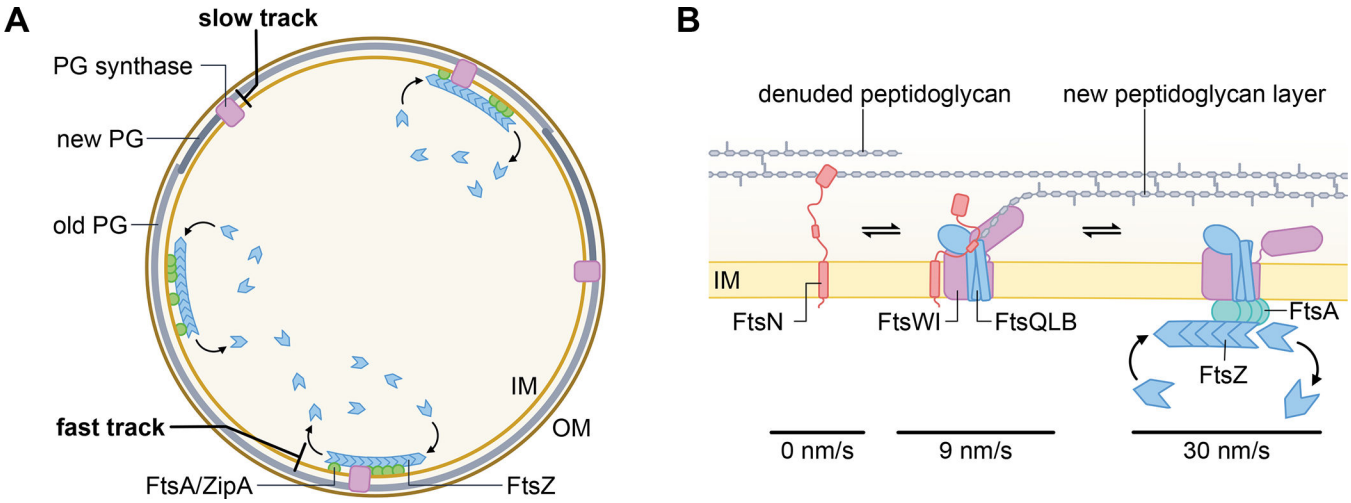

Fig. 4. Divisome proteins build the septum in two tracks.

(A) Cross section of a dividing *E. coli* cell depicting several processive, FtsZ treadmilling complexes that move rapidly along the cytoplasmic face of the inner membrane to organize septum synthesis, in coordination with several independently moving, slower track of proteins that actively engage in septal peptidoglycan (PG) synthesis in the periplasm. (B) Detail of complexes comprising the different speed modes during septum formation. A subset of FtsN proteins are transiently stationary, possibly because they are bound to denuded septal PG resulting from amidase activity. Another subset of FtsN proteins is on the slow track (~9 nm/s) when in complex with FtsQLB and FtsWI as they synthesize septal PG, activated by FtsN. On the right is a putative fast moving (~30 nm/s) complex containing FtsZ and FtsA that spatially guides FtsQLB and FtsWI so the latter are properly placed to be handed off to the slow track for septum synthesis.

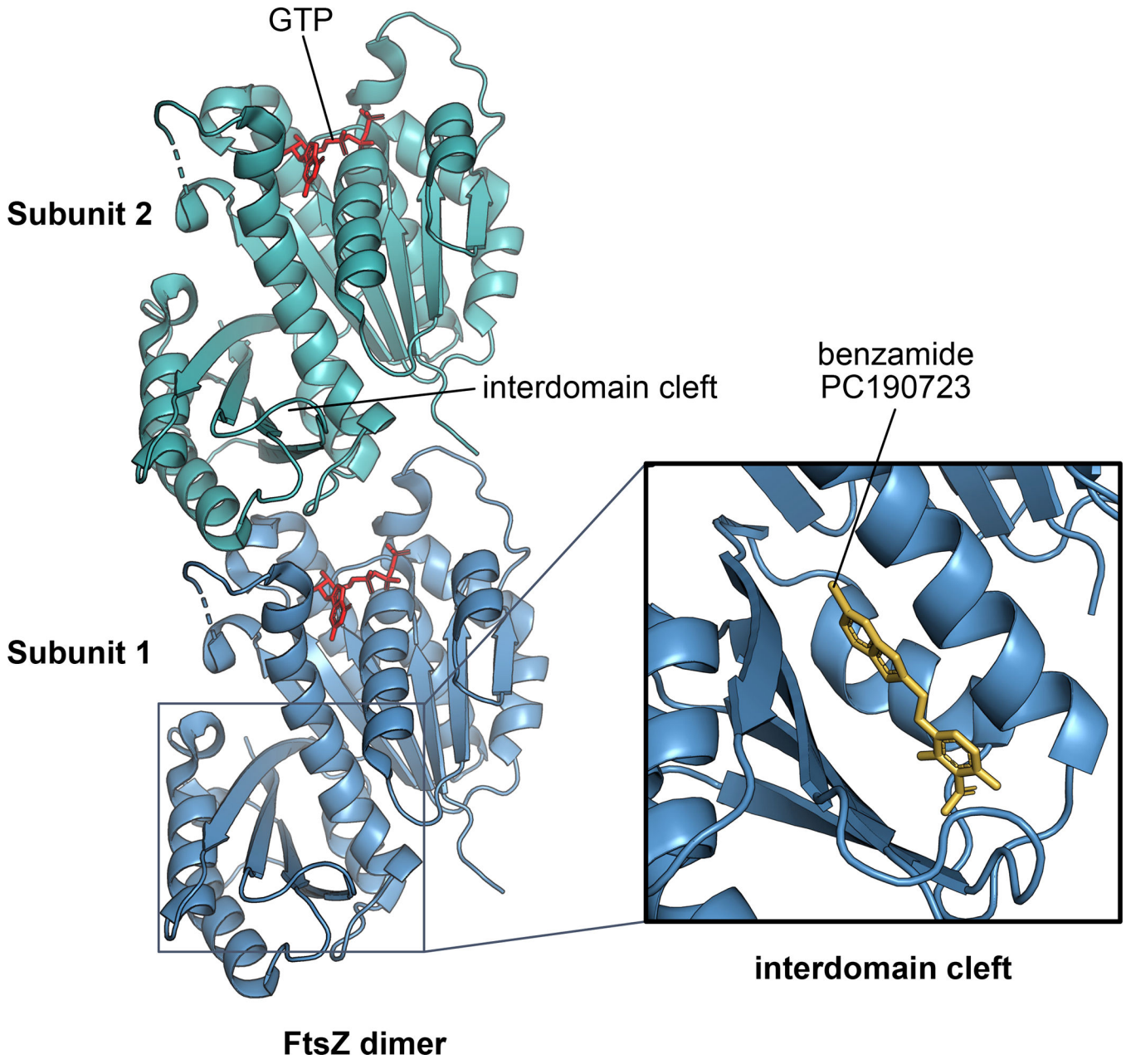

Fig. 5. Binding of a small molecule inhibitor to the FtsZ interdomain cleft.

A dimer of the core polymerizing domain of FtsZ is shown, highlighting the interdomain cleft that connects the N-terminal GTP binding domain with the C-terminal domain. Many small molecule inhibitors of FtsZ, including the benzamide PC190723 and its derivatives, insert into the interdomain cleft (inset) and inhibit conformational changes within the FtsZ subunit required for its ability to dynamically treadmill.