BIOCELL
2022 46(11): 2343-2351

Tech Science Press

How does FtsZ's treadmilling help bacterial cells divide?

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Key words: FtsZ, Bacterial cell division, sPG synthesis

Abstract: Most bacteria assemble a ring-like macromolecular machinery scaffolded by the essential cytoskeletal protein FtsZ for cell division. Studies have broadly explored how FtsZ could polymerize at the correct place and time. Recently, the FtsZ-ring was found to exhibit dynamic treadmilling along the circumference of the division site, driven by GTP hydrolysis. This apparently directional motion of FtsZ seems to drive the movement of septal cell wall synthesis enzymes and to play an important role in modulating cell envelope constriction and septum morphogenesis. However, the relationship between FtsZ's treadmilling dynamics and cell wall synthesis varies in different bacteria. More importantly, the biophysical and molecular mechanisms governing these dynamic processes are unclear. In this viewpoint, we will focus on some new and exciting studies surrounding this topic and discuss potential mechanisms that underlie how FtsZ's treadmilling dynamics might regulate septal cell wall synthesis and cell division.

Introduction

FtsZ is a prokaryotic tubulin homolog (Nogales *et al.*, 1998) that plays a central role in regulating bacterial cell division. Thirty years ago, Bi and Lutkenhaus (1991) discovered that FtsZ molecules can form a ring-like structure (termed the "Z-ring") at the future division site and determined this ring to be the first known prokaryotic cytoskeleton. Similar to tubulin, FtsZ self-polymerizes upon GTP binding (Bramhill and Thompson, 1994) and subsequently hydrolyzes GTP molecules, inducing its depolymerization (Chen and Erickson, 2005; de Boer *et al.*, 1992). The Z-ring recruits more than thirty cell division proteins, many of them essential and involved in cell wall synthesis, to assemble a macromolecular machinery collectively called the **divisome** (Du and Lutkenhaus, 2017).

Given that FtsZ is broadly conserved among eubacteria and archaea, we wondered whether there exists a universal mechanism for prokaryotes to control and regulate the cell division process via the Z-ring. One well-accepted function of the Z-ring is to act as a scaffold for the recruitment of other divisome proteins, especially cell wall synthases and remodeling enzymes (Egan *et al.*, 2020; McQuillen and Xiao, 2020). Another potential function of the Z-ring was proposed to generate a mechanical constricting force based on the homology to eukaryotic microtubes. In this model, FtsZ-ring utilizes the energy from GTP hydrolysis by its constitutive monomers to pull the cell envelope inward and lead to cell constriction (Erickson *et al.*, 2010). This idea was strongly supported by the impressive experiments by Osawa *et al.* (2008) showing that FtsZ can polymerize to rings that bend and constrict liposomes *in vitro*. While this mechanism is intriguing, it raises questions such as: What is the exact physical model in which the Z-ring generates a homogeneous contractile force? Is the force strong enough to deform both the inner membrane and peptidoglycan cell wall against turgor? For insight into these questions, I refer the readers to the extensive discussion in the review by McQuillen and Xiao (2020).

The FtsZ force-generation model was challenged by Coltharp *et al.* (2016) who found that the cell constriction rate of *E. coli* cells does not depend on FtsZ's intrinsic GTPase activity (originally proposed to govern the force-generation). Instead, the rate-limiting step of *E. coli* cell division was identified as the cell wall synthesis rate. This result was later confirmed by labeling the nascent sPG using fluorescent d-amino acids (FDAAs) (Kuru *et al.*, 2012): the amount of newly synthesized (*E. coli*) cell wall was not correlated with FtsZ's GTPase activity (Yang *et al.*, 2017). These results are consistent with previous studies in which FtsZ GTPase mutants can still complete cell division with deformed or abnormal septa (Bi and Lutkenhaus, 1992; Lutkenhaus *et al.*, 2012). Considering the fact that the

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Received: 21 February 2022; Accepted: 22 April 2022

Doi: 10.32604/biocell.2022.022100

Z-ring constantly hydrolyzes GTP, it is important to understand why bacterial cells continuously consume GTP if the potential mechanical force is not essential.

FtsZ Dynamically Treadmills During Cell Division

Later, FtsZ's GTP hydrolysis was found to drive an unseen type of dynamic behavior of FtsZ: treadmilling (Bisson-Filho *et al.*, 2017; Loose and Mitchison, 2014; Yang *et al.*, 2017). FtsZ subunits in the Z-ring are known to exchange constantly with monomers in the cytoplasmic pool (lifetime ~10 sec) (Stricker *et al.*, 2002), where the exchange rate depends on the GTPase activity. This behavior and the corresponding kinetic mechanism were thoroughly discussed in a classic review article by Erickson *et al.* (2010). They also proposed that one possible scheme of FtsZ dynamics could be treadmilling, a behavior that widely exists in eukaryotic cytoskeletal filaments.

Margolin's group was the first to observe rapid dynamics and oscillations of the Z-ring back in 2004 (Thanedar and Margolin, 2004), but the treadmilling behavior of FtsZ was not determined until total internal reflection fluorescence microscopy (TIRFm) was introduced to image reconstituted FtsZ filaments *in vitro* and the Z-ring *in vivo*. FtsZ was found to form chunks or clusters in the Z-ring using super-resolution fluorescence microscopy (Buss *et al.*, 2015; Fu *et al.*, 2010; Strauss *et al.*, 2012). With much less phototoxicity, TIRFm allowed researchers to monitor FtsZ clusters for a long period of time with a high spatial-temporal resolution. Interestingly, those FtsZ clusters move directionally around the ring while individual FtsZ monomers stay immobile. This behavior is a hallmark of treadmilling (Bisson-Filho *et al.*, 2017; Buss *et al.*, 2013; Loose and Mitchison, 2014; Niu and Yu, 2008; Yang *et al.*, 2017): FtsZ monomers are added on one end (polymerization) and lost on the other (depolymerization), thus the filament appeared to be moving forward (Fig. 1).

The apparent directional movement of FtsZ clusters implies an energy input to the system. Unlike the bacterial sidewall building system (the **elongasome**) that utilizes the peptidoglycan (PG) synthesis reaction to power its directional motion (Dominguez-Escobar *et al.*, 2011; Garner *et al.*, 2011; van Teeffelen *et al.*, 2011), the FtsZ's treadmilling speed is only correlated with its intrinsic GTPase activity but not with the FtsZ regulators or sPG synthesis (Bisson-Filho *et al.*, 2017; Caldas *et al.*, 2019; Perez *et al.*, 2019; Ramirez-Diaz *et al.*, 2018; Squyres *et al.*, 2021; Whitley *et al.*, 2021; Yang *et al.*, 2017). Additionally, *in vitro* fluorescence and atomic force microscopic studies suggested that treadmilling dynamics also rely on FtsZ's densities and the type of surface tethers (Gonzalez de Prado Salas *et al.*, 2014; Loose and Mitchison, 2014; Marquez *et al.*, 2019; Mateos-Gil *et al.*, 2012; Ramirez-Diaz *et al.*, 2018).

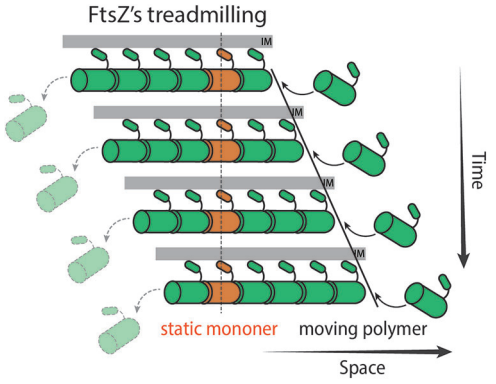

FIGURE 1. Diagram demonstrating FtsZ's treadmilling. FtsZ monomers polymerize on the right end of the filament while the last subunit depolymerizes from the left end. The filament thus moves to the right with immobile FtsZ subunits (orange).

The treadmilling dynamics were later confirmed in other bacterial species such as *S. mutans* (Li *et al.*, 2018), *S. aureus* (Monteiro *et al.*, 2018), and *S. pneumonia* (Perez *et al.*, 2019). Given the robustness of the treadmilling dynamics in living cells, it is natural to reason that the energy from GTP molecules is harvested to maintain FtsZ's treadmilling which further assists the sPG synthesis and cell constriction. Indeed, the treadmilling was showed to be essential for successful and efficient cell division: In *B. subtilis*, abolishing FtsZ's treadmilling by a small molecule inhibitor PC190723 stops cell division (Bisson-Filho *et al.*, 2017). In *E. coli*, the *fts*ZD212G mutation with a slow treadmilling speed causes abnormal division and filamentous cells (Bi and Lutkenhaus, 1990; Stricker and Erickson, 2003; Yang *et al.*, 2017).

FtsZ's Treadmilling Drives the Directional Motion of sPG Synthesis

Back when Bi and Lutkenhaus discovered the Z-ring, they speculated that "*formation of the ring would be the key point at which temporal and spatial control over division are exerted… perhaps by interacting directly with septal-specific peptidoglycan biosynthetic machinery at the leading edge of the invagination*" (Bi and Lutkenhaus, 1991). This speculation turns out to be visionary even though it was not possible to monitor Z-ring dynamics or sPG synthesis in living cells at that time.

With novel single-molecule imaging and labeling techniques, we are now able to track single protein molecules in living cells and measure the spatial distribution of PG synthesis (Cho *et al.*, 2016; Grimm *et al.*, 2015; Kuru *et al.*, 2012; Lee *et al.*, 2016; Yu *et al.*, 2006). The first tracked essential sPG synthase was the monofunctional transpeptidase (TPase) FtsI in *E. coli* (PBP2B in *B. subtilis*). These enzymes were observed to move processively along with the Z-ring (Bisson-Filho *et al.*, 2017; Yang *et al.*, 2017). Unlike their counterparts PBP2 (in *E. coli*) and PBP2A (in *B. subtilis*) in the elongasome, whose movement is driven by PG synthesis (Dominguez-Escobar *et al.*, 2011; Garner *et al.*, 2011; van Teeffelen *et al.*, 2011), the average speeds of FtsI and PBP2b are highly correlated with FtsZ's treadmilling speed. This exciting result indicated that FtsZ may convert the chemical energy in GTP into kinetic energy by treadmilling to drive the directed motion of these sPG enzymes. In other words, FtsZ could use its polymer dynamics to function as a linear motor to deliver cargos (sPG synthases) at target sites to initiate local septum synthesis.

This hypothesis is supported from pulse-labeling experiments showing that the newly synthesized septum is

uneven and clustered, akin to the discontinuous, clustered Z-ring structure in *E. coli*, *B. subtilis*, and *S. pneumoniae* (Bisson-Filho *et al.*, 2017; Perez *et al.*, 2021; Yang *et al.*, 2017). Such a discrete sPG pattern suggests that sPG synthases are not efficient in building the whole septum all at once, but that they, instead, work 'locally' producing 'patches' of new sPG around the ring. They therefore require regulators to modulate their spatial-temporal distribution to evenly construct the whole septum over time. As FtsZ clusters treadmill around the future septum, they likely guide enzymes to circle around the cell. Indeed, experiments have shown that the new septum becomes uneven or abnormal in FtsZ mutation strains with slow treadmilling speeds (Bisson-Filho *et al.*, 2017; Perez *et al.*, 2021; Yang *et al.*, 2017). Furthermore, the cell division rate in *B. subtilis* was shown positively correlated with FtsZ's treadmilling speed, indicating FtsZ's treadmilling may guide both the distribution and activity of sPG synthases (Bisson-Filho *et al.*, 2017).

However, the results from the gram-negative bacterium *E. coli* speaks to the contrary: FtsZ mutants with decreased GTPase activity and slower treadmilling only impact the septum morphology but do NOT alter the overall sPG synthesis or the constriction rate in most cases (Coltharp and Xiao, 2017; Yang *et al.*, 2017). These results suggested that FtsZ only regulates the spatial-temporal distribution but not the enzymatic activity in *E. coli*. This discrepancy grew more puzzling after several other findings revealed that FtsZ may be dispensable for sPG synthesis under specific conditions.

sPG Synthesis Can be Independent of FtsZ's Treadmilling

Soon after the discovery of FtsZ's treadmilling dynamics, Pinho's group monitored the septum closure process in *S. aureus* when FtsZ's treadmilling was inhibited. Surprisingly, they found that FtsZ's treadmilling is no longer required for sPG synthesis and cell constriction at a later stage of cell division, once the entire divisome is assembled (after the recruitment of the Lipid II lipase MurJ) (Monteiro *et al.*, 2018).

In *S. pneumonia*, the Winkler lab then found that bPBP2x, the essential sPG TPase, moved directionally yet in an FtsZ independent manner (Perez *et al.*, 2019). GTPase-defective FtsZ mutants did not decrease the moving speed of bPBP2x as that in *E. coli* or *B. subtilis*. Rather, inhibiting sPG synthesis slowed bPBP2x. Moreover, the sPG synthesis level of FtsZ mutant strains remained the same as that of wild-type cells, while the pattern of nascent sPG became irregular (Perez *et al.*, 2021; Perez *et al.*, 2019), very similar to the case of *E. coli* (Yang *et al.*, 2017).

More recently, Kevin *et al.* from the Holden lab re-examined how inhibition of FtsZ's treadmilling would disrupt cell division at different stages in *B. subtilis*. Similar to *S. aureus*, they found that a fraction of cells that have already proceeded to a later cell division stage continued to divide even when FtsZ's treadmilling was inhibited (Whitley *et al.*, 2021). Other early-stage cells could not constrict or build septal cell walls, as previous results (Bisson-Filho *et al.*, 2017). The authors also carefully measured the septum closure rate under different cell growth rates, finding that they are highly correlated. Given the insignificant change in the FtsZ's treadmilling speed under those conditions, there might be other regulatory factors of sPG synthesis besides FtsZ in *B. subtilis*.

In fact, FtsZ-independent cell constriction has been found years ahead. Soderstrom *et al.* (2014) determined the sequence of division proteins leaving the septum and found that the FtsZ-ring disassembled before the end of cytokinesis in *E. coli* (Soderstrom *et al.*, 2014). This result showed that FtsZ (and its treadmilling) is also dispensable in the last part of division in *E. coli*. All the above observations complicated the understanding of how FtsZ's treadmilling regulates sPG synthesis and cell division.

Rethink and Unify the Function of FtsZ in Cell Division

On one hand, in all bacteria tested thus far, FtsZ treadmills at approximately 30 nm/s regardless of gram-positive or gram-negative, rod or ovoid cell shapes, and even in artificial rectangular or heart-shaped cells (Table 1). Considering the significant differences in divisome composition and cell physiology among those bacteria, FtsZ's treadmilling seems to be highly conserved. It is possible that this dynamic property of FtsZ (or the Z-ring) serves as a basic and robust mechanism in regulating the cell division process.

On the other hand, bacteria live in drastically different environments and have evolved for billion years, developing different cell shapes, sizes, cell wall thicknesses and PG synthesis/hydrolysis enzymes. It is not unreasonable to assume that they also have very different ways to build the septum and constrict the cell envelope.

1. FtsZ's treadmilling condenses the divisome to the mid-cell

As the master regulator, FtsZ must first recruit and scaffold for other proteins to the future cell division site (i.e., the middle of the long axis in most rod shape bacteria). Recently, Squyres *et al.* (2021) from the Garner lab found that the Z-ring is not only simply positioned to mid-cell with sPG synthases. The FtsZ ring condenses along the progression of division, which facilitates the assembly of cell wall synthases such as PBP2b and FtsW in *B. subtilis* and enables them to function correctly. The reason might be that the enzymes have low concentrations inside the cell. Their functions often rely on other partners or activators (such as FtsQLB and FtsN in *E. coli* (Liu *et al.*, 2015; Tsang and Bernhardt, 2015)). A condensed Z-ring can generate a confined volume trapping downstream divisome components and thus raise their local concentration.

At the same time, Whitley *et al.* (2021) showed that FtsZ's treadmilling facilitates the aggregation of a narrow and matured Z-ring in the early division stage. After the tight ring formed, treadmilling becomes less important. It is worth mentioning that treadmilling was shown to facilitate transient FtsZ assemblies to localize to the correct cell division site (Walker *et al.*, 2020). These studies suggested that the first regulatory mechanism of FtsZ's treadmilling is to enable FtsZ filaments to dynamically encounter each other, locate at the mid-cell and condense to a "narrow ring" by lateral interactions. The condensed Z-ring subsequently helps other components assemble and trigger sPG synthesis complex formation (Fig. 2A).

TABLE 1

FtsZ's treadmilling speed in different bacterial species

Bacteria	FtsZ's treadmilling speed (nm/s)	Reference	Notes
Escherichia coli	27.8 ± 17.1[a]	(Yang *et al.*, 2017)	TIRFm
Escherichia coli	23 - 30[b]	(Soderstrom *et al.*, 2018)	Cells transformed to square, heart, or large circles shapes by drug Wide-field imaging of vertical cells in microholes.
Bacillus subtilis	32.0 ± 7.8	(Bisson-Filho *et al.*, 2017)	TIRFm
Bacillus subtilis	27[c]	(Whitley *et al.*, 2021)	Mature/early Z-rings Wide-field imaging of vertical cells in microholes
Streptococcus mutans	34.7 ± 16.6	(Li *et al.*, 2018)	TIRFm
Streptococcus pneumoniae	32.4 ± 13.3	(Perez *et al.*, 2019)	Nascent and equatorial Z-rings. TIRFm
Streptococcus pneumoniae	30.5 ± 9.3	(Perez *et al.*, 2019)	Mature Z-rings Wide-field imaging of vertical cells in microholes

Note: a. Standard deviation b. The average speed of FtsZ treadmilling in cells of different shapes c. The median speed.

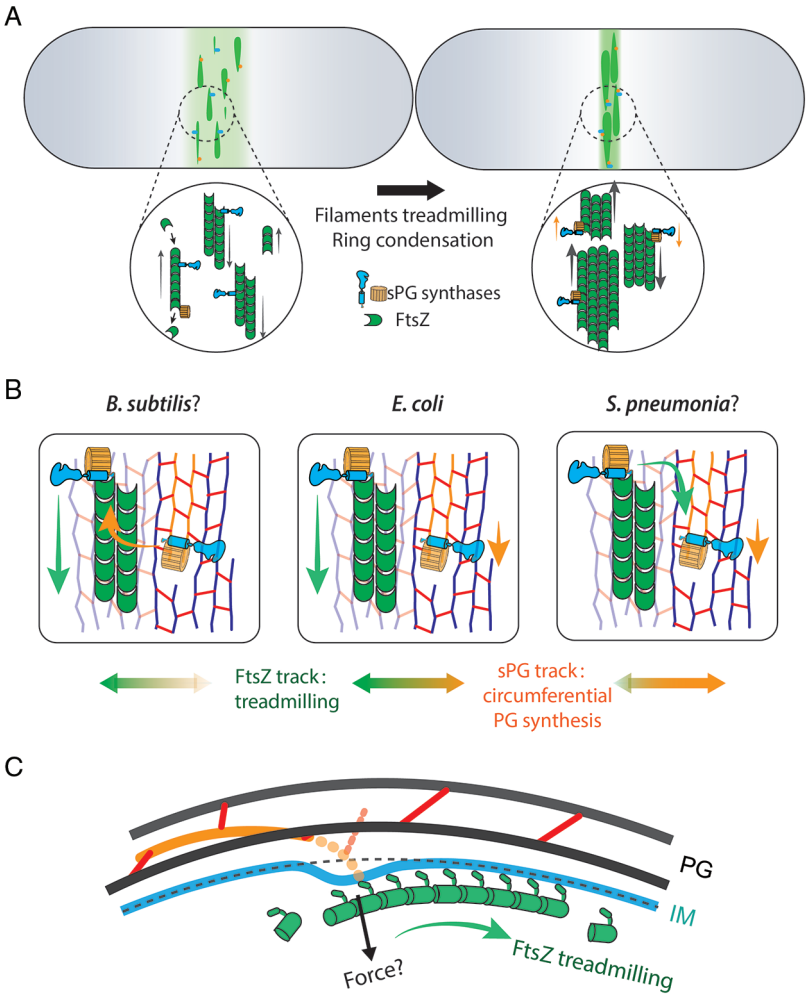

FIGURE 2. Potential models of FtsZ's treadmilling regulates the cell division in three different dimensions. A. FtsZ filaments (green) travel around the cell circumference via treadmilling (left). The treadmilling dynamics facilitate the formation of a narrow and mature Z-ring along the long axis (right) that concentrate and activate the sPG synthases (blue and orange parts) (Squyres *et al.*, 2021; Whitley *et al.*, 2021). B. The treadmilling direction is approximately along the glycan strands in septum. sPG synthases can be transported by FtsZ' treadmilling to different positions (green arrow) or synthesize new sPG processively in a different speed (orange arrow). In *B. subtilis*, synthases tend to associate more on the Z-track (Bisson-Filho *et al.*, 2017) (left) while enzymes in *S. pneumoniae* were found to move with PG synthesis (Perez *et al.*, 2019) (right). *E. coli* synthases move on both tracks (Yang *et al.*, 2021) (middle). C. FtsZ may generate contractile force radially toward the cell center. The deformation of the inner membrane could displace the synthesis complexes thus build the new septal cell wall inside the old one (Nguyen *et al.*, 2019).

2. FtsZ's treadmilling regulates the spatial distribution of sPG synthase complex along the ring

The next step of cell division is to synthesize new sPG material along the circumference of the cell. In rod-shaped bacteria, such as *E. coli* and *B. subtilis*, the glycan strands of PG are arranged circumferentially, while the peptide stems are along the long axis of the cell (Holtje, 1998). If the glycan strands are perfectly aligned and spaced, sPG

synthesis could use the old PG strand as a template, at least along the circumferential direction. Unfortunately, the glycan chains are not perfectly organized, as visualized by atomic force microscopy (AFM) (Pasquina-Lemonche *et al.*, 2020; Turner *et al.*, 2018). Since FtsZ filaments constantly treadmill around the cell's circumference and scaffold sPG synthases, it is natural to reason that FtsZ keeps the enzymes moving (perhaps synthesis too) along the ring. Three supporting observations were obtained: 1) FtsI and PBP2b molecules move along the ring at a similar speed as FtsZ's treadmilling; 2) the PG synthesis pattern is clustered like that of the Z-ring; and 3) new septa in some ftsZ mutation strains exhibit helical shapes similar to the Z-rings (Bisson-Filho *et al.*, 2017; Yang *et al.*, 2017).

However, it is difficult to comprehend how enzymes or enzymatic complexes can travel directionally along the Z-ring while every single FtsZ protein is static. The Liu and Xiao labs proposed a Brownian ratchet model in which minus end-attaching enzymes (i.e., FtsI) on a treadmilling filament will fall off while the last FtsZ subunit dissociates (Fig. 3). Although falling FtsZ monomers likely diffuses away in the cytoplasm, the sPG synthase is confined to the membrane and cannot escape the surrounding zone in a short time window. It could catch up and associate with the FtsZ subunit on the new 'minus-end' of the filament again. As such, the enzyme follows the FtsZ filament and moves forward (McCausland *et al.*, 2021). In this way, FtsZ transports sPG synthases to different septal sites and generates new clustered sPG. Even in *S. pneumoniae*, whose PG synthases move slower than FtsZ's treadmilling, the nascent sPG still exhibits a clear clustered pattern along the ring (Perez *et al.*, 2021; Perez *et al.*, 2019), indicating that FtsZ still determines the spatial distribution of PG synthases (perhaps in a passive way). Using a reconstituted lipid bilayer system, Baranova *et al.* (2020) observed the collective co-immigration of the cytoplasmic portion of FtsQ and FtsN with FtsZ. Even though the truncated proteins did not display a processive motion, the results demonstrated that the late divisome proteins could be redistributed by FtsZ's treadmilling.

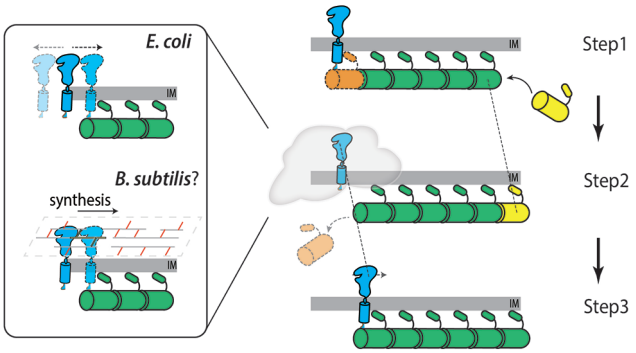

FIGURE 3. Diagram showing the Brownian ratchet model that FtsZ's treadmilling guides sPG synthases' directional movement. Step 1. The sPG synthase (blue) binds with the FtsZ subunit on the minus end of an FtsZ filament (orange). Step 2. The last FtsZ subunit dissociates from the filament leaving the synthase diffuses on the membrane (in *E. coli*, top left) or synthesizes sPG shortly (in *B. subtilis*, bottom left). Step 3. The synthase rebinds to the new end of FtsZ filament thus moves forward to the right at the same speed as that of the FtsZ filament (dashed lines).

3. FtsZ's treadmilling-associated synthase complexes may or may not be active

In addition to the spatial distribution, it is also important to understand whether FtsZ's treadmilling determines the sPG synthesis rate. On this aspect, the results seem to differ among bacteria. Between the completely dependent case of *B. subtilis* and the independent case of *S. pneumoniae*, we found that the motion of *E. coli* sPG synthases partially depends on FtsZ's treadmilling. These enzymes split into two populations: one goes with FtsZ, while the other was driven by the active sPG synthesis (Yang *et al.*, 2021). The results suggested a two-track model in which FtsZ filaments (clusters) carry enzymes (or synthase complexes) from one place to another along the ring until these enzyme molecules stochastically dissociation and/or encounter activators such as FtsN (Lyu *et al.*, 2021). The enzymes could then synthesize new sPG for ~200 nm in a highly processive manner. After the complexes terminate on the PG (stochastically or being inactivated), they are able to catch on a treadmilling FtsZ filament again (Fig. 2B middle, Fig. 3). Thus, treadmilling FtsZ filaments act as trains to collect free synthases and keep them on track to synthesize hot spots along the ring. This "two-track model" might explain the different behaviors in bacteria. Enzymes in *S. pneumoniae* might have low binding affinities to FtsZ and therefore cannot follow treadmilling for a sufficient time to be detected in experiments; only molecules processively synthesizing sPG were observed (Fig. 2B, right). In contrast, *B. subtilis* may have enzymes with fast sPG synthesis rates but not very processive, showing Z-track related motion only (Fig. 2B, left). Mechanistically, *B. subtilis* sPG synthases may rapidly proceed a couple of synthesis cycles (a few nanometers) after leaving the Z-track and departing from PG. FtsZ filaments then carry them until the next synthesis 'hot spot' (Fig. 3). This hypothesis might be able to explain the correlation of the sPG synthesis rate and FtsZ's treadmilling speed in *B. subtilis* (Bisson-Filho *et al.*, 2017). More simulations and experiments are required to reveal the true lying mechanism. For example, it would be interesting to examine the motion of the synthases and sPG synthesis pattern when FtsZ's treadmilling is abolished in *B. subtilis* and *S. aureus*.

4. Does FtsZ's treadmilling generate a constriction force to reduce the radius of the new sPG?

The synthesis of sPG is intrinsically different from that of the lateral cell wall. The new sPG material must be inserted inside of the old PG template to reduce the septal radius gradually, which means that the synthesis complex must break the symmetry and add new glycan strands biased to the cell's center. However, as introduced above and discussed in a detailed review article (McQuillen and Xiao, 2020), the FtsZ-ring might not be able to generate enough force to counter the turgor pressure and bend the cell wall mechanically. A possible mechanism is that the sPG synthases (transmembrane proteins) are pulled inside together with the inner membrane, which is deformed inward by FtsZ (Fig. 2C). Subsequently, the newly crossed linked sPG could lie under the old PG and gradually

constrict the cell envelope. In *C. crescentus* cells, sPG can grow as bulges instead of invagination at division sites with FtsZ's C-terminal linker (CTL) truncated variants, which might affect the transduction of the force to the inner membrane. (Sundararajan *et al.*, 2015),

Although it remains unclear how much force FtsZ produces in living cells, Schwille's group found that treadmilling FtsZ filaments could deform lipid tubes *in vitro* and wall-less *E. coli* cells (Ramirez-Diaz *et al.*, 2021). The authors were able to measure the force (1–2 pN/μm) that is partially coupled with GTP hydrolysis (or treadmilling). However, FtsZ GTPase defective mutation could still bend the liposome. This finding is consistent with the results that treadmilling-inhibited *B. subtilis* cells can still divide yet at a slower rate (the static ring generating less force) (Whitley *et al.*, 2021). The force is orders of magnitude smaller than the expected value (~5 nN/μm) to synthesize the new sPG on the inner side of the old PG against the turgor pressure from inside (Nguyen *et al.*, 2019). We speculate that either FtsZ filaments can generate greater force *in vivo* or there are unknown factors supporting the new sPG in the inward position, relieving the tension from the turgor. Undoubtedly more theoretical and experimental works need to be done to predict and measure the true mechanical force generated by FtsZ filaments or the treadmilling dynamics.

Future: Figure Out the Unknowns of FtsZ's Treadmilling in Bacterial Cell Division

From the limited number of organisms tested, the master cytoskeleton protein FtsZ presents almost identical properties in organization, GTPase activity, and treadmilling dynamics. A general model starts to emerge: the treadmilling Z-ring acts as a dynamic scaffold to assemble sPG synthases and keep them 'on track' on both the long axis and along the circumference. At least in some periods (aka, the late division stage after the whole divisome is established), FtsZ and its treadmilling become less important. In the gram-negative bacterium *E. coli*, for instance, FtsN was shown to form a separate ring structure and might serve as a scaffold in the later cell division stage (Lyu *et al.*, 2021; Soderstrom *et al.*, 2018). It is likely that different types of bacteria evolved their PG synthesis strategies by altering the affinity of PG synthases to FtsZ or sPG, enzymatic activity and processivity, lipid II substrate concentration, and the types of activators. Thus, cells can divide robustly with different cell sizes, growth rates, and environmental conditions. Current studies are primarily based on model bacteria that have similar cell dimensions and division rates. To further explore the dynamics and functions of FtsZ's treadmilling, more studies on other bacteria with unconventional shapes and different division rates are needed. Considering the complexity of sPG synthesis and consequently septal cell wall remodeling during growth and division, careful studies of how PG enzymes are regulated and work upon different cell wall structures, lipid II concentrations, and different activation or inhibition pathways are required to understand the full picture of FtsZ and sPG synthases and the septal cell wall.

We still do not know how FtsZ filaments arrange and treadmill on a molecular level in living cells yet. FtsZ protofilaments may organize into diverse structures *in vitro*, such as bundles, sheets, and other high-order structures (Gonzalez de Prado Salas *et al.*, 2014; Lu *et al.*, 2000; Mateos-Gil *et al.*, 2012; Sundararajan and Goley, 2017). *In vivo* super-resolution microscope revealed heterogenous cluster-like structures (Buss *et al.*, 2015; Coltharp *et al.*, 2016; Fu *et al.*, 2010; Holden *et al.*, 2014; Rowlett and Margolin, 2014) not in agree with the well-aligned continuous or short filaments from Cryo-EM images (Li *et al.*, 2007; Szwedziak *et al.*, 2014; Yao *et al.*, 2017). Resolving the organization of FtsZ inside divisome is critical to understand the fundamental physical mechanism of the treadmilling dynamics. New imaging techniques such as MinFLUX combined with functional FtsZ labeling methods might be a promising method to depict the molecular organization of FtsZ in cells (Balzarotti *et al.*, 2017; Moore *et al.*, 2017; Schmidt *et al.*, 2021).

Another amazing fact not explored in this article is that the cells could manage to maintain FtsZ in a steady and robust treadmilling state while reconstituted FtsZ requires a proper protein density and certain membrane tethers to trigger the treadmilling (Loose and Mitchison, 2014; Ramirez-Diaz *et al.*, 2018). Recent studies suggested that FtsA and ZipA may be with complicated structures and functions to regulate FtsZ filaments rather than simple membrane-attachments (Conti *et al.*, 2018; Krupka *et al.*, 2017). The cooperative treadmilling of multiple FtsZ filaments was shown enhanced by FtsZ binding protein ZapA *in vitro* (Caldas *et al.*, 2019). This FtsZ "crosslinker" has been found important to keep a narrow Z-ring *in vivo* (Buss *et al.*, 2013), indicating treadmilling itself might not be enough to create the tight ring mentioned in the previous section. To understand the molecule level mechanism of treadmilling regulation, computational modeling could reinforce our toolbox to explore a broad range of parameters that are difficult to test by experiments. In fact, simulations based on structural information and kinetic measurements have already provided valuable insights (Mateos-Gil *et al.*, 2019).

How much force can be generated by FtsZ's treadmilling in living cells is the other major question difficult to answer. New techniques or methods such as genetically encoded force sensors (Wang *et al.*, 2011) are needed to be carefully designed to measure the amount of constriction force generated by FtsZ during cytokinesis in real time. The force measurement companying with structural studies and computational simulations, we imagine, could complete one important piece of the puzzle.

As FtsZ's treadmilling was shown to regulate the sPG synthesis, it may also participate to coordinate the remodeling and reconstruction the outer membrane in gram-negative bacteria. A recent study had shown that FtsZ or some early division proteins can recruit the essential outer membrane protein folding complex, BAM (Consoli *et al.*, 2021). Whether FtsZ' treadmilling directly regulates the outer membrane remodeling in time and space like the sPG should be further studied.

Last but not the least, given its essential role in cell division, FtsZ has attracted a lot of attention as a target for new antibiotic development. Many potent molecules were

discovered or designed to inhibit the GTPase activity, polymerization, or depolymerization property of FtsZ (Pradhan *et al.*, 2021; Ur Rahman *et al.*, 2020). We hope this viewpoint article could provide some new angles for antimicrobial developers to think about targeting FtsZ's conserved treadmilling behavior or the regulatory pathways. In fact, some small molecules such as PC190723 have been shown to abolish the treadmilling and become a powerfully tool in the studies mentioned above (Bisson-Filho *et al.*, 2017; Monteiro *et al.*, 2018; Whitley *et al.*, 2021).

Acknowledgement: We thank Dr. Jie Xiao, Dr. Ryan McQuillen and Joshua McCausland for their critical reading and valuable advices on the manuscript.

Availability of Data and Materials: Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Author Contribution: The authors confirm contribution to the paper as follows: draft manuscript and prepare figures: X. Y. and R. L. All authors reviewed the final version of the manuscript.

Ethics Approval: Not applicable.

Funding Statement: This work is supported by the start-up funding by the University of Science and Technology of China KJ2070000083 (X.Y) and KY9100000035 (X.Y).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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