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Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division

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The mechanism by which bacteria divide is not well understood. Cell division is mediated by filaments of FtsZ and FtsA (FtsAZ) that recruit septal peptidoglycan synthesizing enzymes to the division site. To understand how these components coordinate to divide cells, we visualized their

movements relative to the dynamics of cell wall synthesis during cytokinesis. We found that the division septum was built at discrete sites that moved around the division plane. FtsAZ filaments treadmilled circumferentially around the division ring~~,~~ and drive~~ing~~ the motions of the peptidoglycan synthesizing enzymes. The FtsZ treadmilling rate controlled both the rate of peptidoglycan synthesis and cell division. Thus, FtsZ treadmilling guides the progressive insertion of new cell wall, by building increasingly smaller concentric rings of peptidoglycan to divide the cell.

In most bacteria, cell division involves the inward synthesis of peptidoglycan (PG), creating a septum that cleaves the cell in two. The location of the septal PG synthases is regulated by filaments of the tubulin homolog FtsZ, which associates with the cytoplasmic side of the membrane via the actin-like FtsA and other factors. FtsZ forms membrane-associated filaments with FtsA (FtsAZ) (*1*, *2*). Together, they form a dynamic structure, the Z ring, which encircles the cell at the future division site (*3*) and recruits PG synthases and other proteins involved in cytokinesis (*4*). Once the division machinery is mature, the Z ring constricts, while the associated synthases build the septum that partitions the cell in two.

Comment [BEPO1]: We hyphenate three-syllable words with like.

Comment [BEPO2]: If time (meanwhile) is meant, remove comma before "while.". Otherwise "and" may be better.

We do not have a clear understanding of how the components of cell division interact in space and time to carry out cytokinesis, as we have been unable to observe the dynamics of each component relative to each other or to the structure they build: The organization and dynamics of FtsZ filaments within the Z ring remain ill-defined~~;~~ it is not known how FtsAZ filaments control the activity of PG synthases~~;~~ and the dynamics of septal PG synthesis have never been directly observed. To gain insight into how these components work together to divide bacteria, we visualized the dynamics of septal PG synthesis in relation to the movements of FtsAZ filaments and the septal PG synthase Pbp2B in the Gram-positive *Bacillus subtilis*.

To assess the dynamics of septal PG synthesis, we sequentially pulse-labeled growing cells with different colors of fluorescent D-amino acids (FDAAs) (table S1), which are incorporated into PG (*5*) by the D,D-transpeptidation activity of penicillin-binding proteins (PBPs) (*6*). Three-dimensional (3D) structured illumination microscopy (3D-SIM) showed that sequential three-color FDAA pulse-labeling resulted in bull's-eye patterns at the division plane (Fig. 1A), demonstrating that the septum is progressively synthesized inward from the cell surface. Short, sequential pulses of two FDAA colors resulted in discrete spots or arcs distributed around the septum, with the colors more offset ~~compared~~ than for~~to~~ cells pulsed simultaneously (Fig. 1, B and C, and fig. S1, A and B). Thus, PG synthesis occurs at discrete sites that move around the division plane.

We next observed how discrete sites of PG synthesis develop into a complete division septum by labeling cells with FDAAs and using increasing pulse durations. Both the total amount of labeling and the area of labeled regions increased with pulse duration (Fig. 1D, left, and fig. S2, A to C). Following short pulses, septa contained discrete spots or arcs (fig. S1C). As pulse duration increased, these arcs elongated, gradually transitioning into complete rings at longer pulses (Fig. 1D, right). As expected, PG synthesis inhibitors reduced FDAA incorporation (fig. S2D). To explore the location of the PG synthases relative to newly incorporated PG, we followed short FDAA pulses with Bocillin, which labels active PBPs (*7*) while inactivating them. The Bocillin signal was offset from the newly-synthesized PG (Fig. 1, C and E), suggesting that the PG synthases also move around the division plane.

Comment [BEPO3]: I suggest "and gradually transitioned"

We next examined the motions of the division-specific PG synthases and their associated cytoskeletal polymers. Total internal reflection fluorescence microscopy (TIRFM) of a functional mNeonGreen-FtsZ fusion expressed from the native locus (fig. S3, A to E, and tables S2 and S3) revealed directional movements within newly assembled Z rings (Fig. 2A and movie S1). Furthermore, in almost every cell, we observed small mNeonGreen-FtsZ filaments outside the Z ring moving directionally around the cell at the same rate as within Z rings, similar to oscillations previously observed in *Escherichia coli* (*8*). A functional FtsA-mNeonGreen fusion showed motions identical to FtsZ (Fig. 2B and movie S1), and two-color imaging confirmed that FtsA and FtsZ colocalized and moved together (Fig. 2C) (*9*). Overexpression of a second *ftsAZ* operon (FtsA, mNeonGreen-FtsZ) resulted in many more directionally moving filaments outside the Z ring without affecting fitness (Fig. 2D and fig. S3F). To resolve FtsZ motion in dense, actively constricting Z rings, we vertically immobilized bacteria in agarose microholes, orienting the division plane parallel to the objective (fig. S4). This revealed multiple FtsZ filaments moving in both directions around the constriction site over a wide range of ring diameters (600 to 1000 nm) (Fig. 2E, fig. S4E, and movie S2). The movement of multiple FtsZ filaments around the Z ring may explain the heterogeneous structures and complex "patch" dynamics observed via super-resolution microcopy (*10*–*12*), as well as the fast turnover of FtsZ subunits (*13*).

We next asked if the division-associated transpeptidase Pbp2B moves with FtsAZ. At native expression levels, mNeonGreen-Pbp2B moved directionally along the Z ring; this became more apparent with reduced expression (fig. S5, A and B). To observe the motions of single Pbp2B molecules, we labeled HaloTag-Pbp2B expressed from the native locus with low concentrations of HaloLigand-JF549 (*14*). TIRFM revealed two types of Pbp2B motion: 1(i) directional motion around the cell width, always localized to Z rings, and 2(ii) diffusion on the membrane, not localized to Z rings (Fig. 2F; fig. S5, C to E; and movie S3). We did not observe diffusive Pbp2B motion along Z rings at any acquisition rate. In some cases, we observed multiple Pbp2B molecules moving directionally within the same ring, sometimes in opposite directions, indicating that the Z ring contains multiple, independent synthetic sites. FtsZ, FtsA, and Pbp2B all moved at similar velocities (Fig. 2G and fig. S5, D and E), suggesting that their motions are associated.

We investigated the mechanism driving FtsAZ/Pbp2B motion, first testing if, similar to MreB (*15*), Pbp2B inactivation would halt FtsAZ motion. However, FtsAZ motion was unaffected by multiple PG synthesis inhibitors (Fig. 3, A and H, and movie S4) or depletion of Pbp2B (Fig. 3, B and H, and movie S5).

We next tested if directional FtsAZ motion arose from filament treadmilling, as observed in vitro (*16*). Consistent with treadmilling, sparse labeling of FtsZ or FtsA in cells demonstrated that single molecules of both proteins were immobile within moving filaments (*17*) (Fig. 3C; fig. S5, F to J; and movie S6). Because treadmilling requires nucleotide hydrolysis, we assayed FtsA motion as we modulated the guanosine triphosphatase (GTPase) activity of FtsZ. Exogenous expression of FtsZ(D213A), a mutant in which Ala replaces Asp^{213} that has been shown to have greatly reduced GTPase activity in *E. coli* (*18*), gradually reduced FtsAZ velocity, stopping motion at high inductions (Fig. 3, D and G, and movie S7A). Likewise, addition of PC190723, an inhibitor of FtsZ guanosine triphosphate (GTP) hydrolysis (*19*) halted FtsZ movement (Fig. 3E and movie S8A). Conversely, addition of MciZ, a Z ring antagonist that, at low levels, increases FtsZ GTPase activity (*20*), increased FtsZ velocity (Fig. 3, F and H, and movie S7B). We next tested whether FtsZ treadmilling dynamics affected Pbp2B movement. PC190723 or

Comment [BEPO4]: Filaments that…moved without affecting OR moving filaments that did not affect fitness. I think the former is better; please improve the sentence.

Comment [BEPO5]: What is suggesting? I think the similarity suggested.

overexpression of FtsZ(D213A) caused Pbp2B molecules to become immobile while the remaining FtsZ colocalized (Fig. 3, D and E, and movie S8B). Pbp2B velocity scaled with FtsZ treadmilling velocity under various perturbations (fig. S5K). Thus, FtsZ treadmilling is required for the directional motions of both FtsAZ filaments and septal PG synthases.

Given the mobile nature of septal PG synthesis, we reasoned that the directional movements of FtsAZ~~/~~ and Pbp2B around the division plane could be coupled to septal PG synthesis. To test this, we labeled cells with FDAAs as we altered FtsZ dynamics. Overexpression of FtsZ(D213A) created long, slowly ~~-~~growing FtsA-mNeonGreen spirals, which incorporated FDAAs along their entire length (Fig. 4A and movie S9). Likewise, long PC190723 treatments resulted in fragmented patches of both FtsZ and FDAA incorporation (fig. S6A), indicating Pbp2B activity is constrained by FtsAZ location. However, these strong inhibitions of FtsZ dynamics required much longer pulses to achieve FDAA labeling, suggesting that FtsZ treadmilling limits PG synthesis. To test this, we altered FtsAZ velocity as we pulse-labeled cells with FDAAs. This revealed that both the total amount and total area of PG synthesis within the ring are modulated by FtsAZ velocity: Conditions that slowed dynamics decreased both the total amount and area of FDAA labeling (Fig. 4B and fig. S6, B and C). Conversely, increasing FtsAZ velocity (with MciZ) increased both the total amount and area of labeling. Thus, in *B. subtilis*, both the amount and spatial distribution of septal PG synthesis are directly coupled to, and limited by, the rate of FtsZ filament treadmilling.

Comment [BEPO6]: Doesn't the longer time or length of time suggest? Maybe ", and this suggests" or ". This indicates…."

Because FtsAZ dynamics control the rate of septal synthesis, we asked whether the rate of cytokinesis depended on FtsAZ treadmilling. We modulated treadmilling velocity by ~~1~~(i) introducing mutations affecting GTP hydrolysis into FtsZ at the native locus, ~~2~~ (ii) titrating exogenous FtsZ(D213A), ~~3~~(iii) expressing MciZ, and ~~4~~(iv) other perturbations (table S4). This revealed that the cytokinesis rate scaled with FtsZ treadmilling: Division was slower when velocity was decreased~~,~~ and faster when velocity was increased (Fig. 4C; fig. S7, A to C; and movie S10). Even under the strongest perturbations, these decreased rates of cytokinesis did not alter the rate of cell elongation (fig. S7, D to F). Thus, in *B. subtilis*, FtsAZ treadmilling is both coupled to, and limiting for, septal PG synthesis and cell constriction.

Our results indicate that cell division occurs by the action of discrete enzyme-filament complexes that, driven by FtsZ treadmilling, move around the division plane, building new PG during their transit (Fig. 4D). FtsZ treadmilling creates long~~-~~range order from the local activity of the PG synthases, linking circumferential enzyme motion to the insertion of cell wall. This tight coupling may yield uniform insertion of new material around the division plane, building the septum inward in progressively smaller concentric rings. Cell division slightly differs in *E. coli*, where FtsZ treadmilling also distributes PG synthesis around the ring (*21*), perhaps because the rate of PG synthesis is limiting relative to FtsZ treadmilling (*10*). This difference may arise from different levels of cell wall precursors between the two organisms~~,~~ or ~~that~~ because *E. coli* must also couple PG synthesis to outer membrane insertion.

The coupling between FtsZ treadmilling and PG synthesis can unify previously conflicting models of cell division. FtsZ filaments have been proposed to generate force to bend membranes (*2*, *22*)~~,~~ and to scaffold PG synthesis (*10*, *23*). If FtsZ filaments deform membranes, coupling their movement to PG synthesis would allow each deformation to be reinforced ~~via~~ by means of synthesis of PG (*24*). Thus, multiple sites of local deformation and coupled reinforcing synthesis moving around the division site would iteratively build the invaginating septum.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S7

Tables S1 to S4

Movies S1 to S10 ~~available at http://garnerlab.fas.harvard.edu/FtsZ/~~

~~Code available at https://bitbucket.org/garnerlab/bisson-2016~~

References (*25*–*45*)

26 September 2016; accepted 20 January 2017

Fig. 1. Septal PG synthesis occurs at discrete, mobile sites. (**A**) Sequential FDAA labeling (HADA, 7-hydroxycoumarin-3-carboxylic acid–3-amino–D-alanine; BADA, BODIPY FL-D-alanine; TADA, tetramethylrhodamine 3-amino–D-alanine) of division septa shows outside-in synthesis (arrowhead). (Right) 90° rotations of septa. (**B**) Sites of PG synthesis move around the septum. (**C**) Correlation coefficient of overlap between colors in (B) and (E). Line~~÷~~, mean~~;~~ box~~÷~~, SD. ****$P < 0.00001$. (**D**) FDAA labeling proceeds from puncta to complete rings. (Left) Area of FDAA features increases with pulse length. Lines~~÷~~, mean, r~~÷~~, correlation coefficient of the means. (Center) Blinded classification of FDAA features at various pulse lengths. (Right) Representative images of septal PG structures. (**E**) Sites of PG synthesis are offset from synthetic enzymes. Colored bars indicate time course of FDAA labeling. All images were taken with 3D-SIM. Scale bars~~÷~~, 0.5 µm.

Fig. 2. FtsAZ and Pbp2B move directionally around the division site. (**A**) mNeonGreen-FtsZ (bAB185) shows directional motion inside (left) and outside (right) the Z ring; montage at 8-s intervals. (**B**) FtsA-mNeonGreen (bAB167) shows directional motion inside (left) and outside (right) the ring. (**C**) mNeonGreen-FtsZ and FtsA-HaloTag-JF549 (bAB229) colocalize and move together. , yellow. (**D**) FtsAZ overexpression [~~(~~100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) in bAB221~~)~~] creates increased FtsZ filaments showing directional motion outside the Z ring. (**E**) Vertically immobilized cells (*Bacillus* sp. SH41) show multiple, independent mNeonGreen-FtsZ filaments moving in both directions around the division site. Cropped rings and radial kymographs in early (left), middle (center), and late (right) divisional stages. Blue arrows indicate directional FtsZ tracks. (**F**) Single molecules of Pbp2B (bGS31, 15-min incubation of 50 pM JF549) move directionally around the division site. Blue to yellow indicates trajectory time. Kymographs drawn at yellow arrows. Scale bars, 0.5 µm. (**G**) Velocity distributions of FtsA filaments, FtsZ filaments, and single Pbp2B molecules. ~~Kymographs drawn at yellow arrows. Scale bars = 0.5 µm.~~

Fig. 3. Directional FtsAZ motion is driven by treadmilling, independent of cell wall synthesis, and is required for Pbp2B motion. (**A**) FtsZ continues to move directionally after treatment with penicillin G. bAB185 imaged 5 min after addition of 3 µl of 10 mg/ml penicillin G to an agarose pad. (**B**) FtsZ continues to move directionally after Pbp2B depletion. Before imaging, bGS31 was grown without IPTG until no divisions were observed (3 hours). (**C**) Single molecules of FtsZ and FtsA are immobile within the division site. Single molecules were obtained by growing bAB219 with no IPTG (left) or bAB229 with 15 min of 250 pM JF646 (right). (**D**) Overexpression of GTPase-deficient FtsZ stops FtsZ and Pbp2B motion. bAB217 (left) and bGS90 (right) were imaged after induction of FtsZ(D213A) (100 µM IPTG, 1 hour). (**E**) Directional motion of FtsZ and Pbp2B is stopped by PC190723. bAB185 (left) and bGS31 with 15-min incubation of 50 pM JF549 (right) 5 min after addition of 10 µM PC190723. (**F**) FtsZ velocity increases ~~following~~ after exposure to MciZ. bAB185 was imaged ~~following~~ after addition of 1 µM MciZ in a microfluidic device. (**G**) Velocity distributions of FtsZ filaments (bAB217) at different levels of FtsZ(D213A) induction. (**H**) Velocity distributions of FtsZ filaments under different perturbations.

Fig. 4. Cytokinesis is controlled by directional motion of FtsAZ filaments. (**A**) FtsZ(D213A) overexpression (1 mM IPTG in bAB217) produces slowly growing FtsA spirals with spiral FDAA insertion. (Top) Montage of growing FtsA spiral acquired with spinning-disk confocal imaging. (Bottom) Sequential FDAA labeling and FtsA localization imaged with 3D-SIM in a

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fixed cell. (**B**) Altering FtsZ velocity changes the amount and total area of FDAA incorporation. (left) PY79 with 1 μg/ml PC190723~~,~~ for 10 min, (middle) bAB217 with 20 μM IPTG for 1 hour, (right) AH93 with 50 mM xylose~~,~~ for 5 min. ~~Following~~ After treatment, cells were incubated with TADA for the indicated time, fixed, then imaged with 3D-SIM. (Far right) Total intensity and area of septal FDAA incorporation. Lines~~:~~, mean. (**C**) Cytokinesis scales with FtsZ treadmilling velocity. For each condition, pairs of strains were used to measure ~~1~~(i) FtsZ velocity (using mNeonGreen-FtsZ) and ~~2~~(ii) septation rates (using mNeonGreen-Pbp2B). (Top) Kymographs of constricting Pbp2B rings in different FtsZ backgrounds (line marks start of constriction). (Bottom) Plot of constriction time versus~~.~~ FtsZ velocity under~~in~~ different conditions. Treadmilling velocity and septation rates were acquired in identical conditions, save for measurements with MciZ (see SOM). Error bars~~:~~, SD of the mean. (**D**) Model for treadmilling-coupled cell division. (Top) The Z ring contains multiple FtsAZ filaments that treadmill around the division plane, pulling associated PG synthases. (Bottom) FtsZ treadmilling both regulates and distributes the activity of the PG synthases, building sequentially smaller uniform arcs of PG to divide the cell.