Movement dynamics of divisome proteins and PBP2x: FtsW in cells of Streptococcus pneumoniae

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Bacterial cell division and peptidoglycan (PG) synthesis are orchestrated by the coordinated dynamic movement of essential protein complexes. Recent studies show that bidirectional treadmilling of FtsZ filaments/bundles is tightly coupled to and limiting for both septal PG synthesis and septum closure in some bacteria, but not in others. Here we report the dynamics of FtsZ movement leading to septal and equatorial ring formation in the ovoid-shaped pathogen, Streptococcus pneumoniae. Conventional and single-molecule total internal reflection fluorescence microscopy (TIRFM) showed that nascent rings of FtsZ and its anchoring and stabilizing proteins FtsW and FtsI move as a flow from mature septal rings coincident with MapZ rings early in cell division. This mode of continuous nascent ring movement contrasts with a failsafe streaming mechanism of FtsZ/FtsA/EzrA observed in a ΔmapZ mutant and another Streptococcus species. This analysis also provides several parameters of FtsZ treadmilling in nascent and mature rings, including treadmilling velocity in wild-type cells and FtsZ(GTase) mutants, lifetimes of FtsZ subunits in filaments and of entire FtsZ filaments/bundles, and the processivity length of treadmilling of FtsZ filament/bundles. In addition, we delineated the motion of the septal PBP2x transpeptidase and its FtsW glycosyl transferase-binding partner relative to FtsZ treadmilling in S. pneumoniae cells. Five lines of evidence support the conclusion that movement of the bPBP2x:FtsW complex in septa depends on PG synthesis and not on FtsZ treadmilling. Together, these results support a model in which FtsZ dynamics and associations organize and distribute septal PG synthesis, but do not control its rate in S. pneumoniae.

**Significance**
This study answers two long-standing questions about FtsZ dynamics and its relationship to septal peptidoglycan (PG) synthesis in Streptococcus pneumoniae. In previous models, FtsZ concertedly moves from midcell septa to MapZ rings that have reached the equators of daughter cells. Instead, the results presented here show that FtsZ, FtsA, and EzrA filaments/bundles move continuously out from early septa as part of MapZ rings. In addition, this study establishes that the movement of bPBP2x:FtsW complexes in septal PG synthesis depends on and likely mirrors new PG synthesis and is not correlated with the treadmilling of FtsZ filaments/bundles. These findings are consistent with a mechanism where septal FtsZ rings organize directional movement of bPBP2x:FtsW complexes dependent upon PG substrate availability.

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bacteria, such as the human respiratory pathogen, *Streptococcus pneumoniae* (pneumococcus). Newly divided ovococcus bacteria form prolate ellipsoid-shaped cells containing equatorial rings composed of FtsZ and other proteins (*SI Appendix, Fig. S1A, Bottom*) (17, 18). These equatorial rings become the mature septum at the start of division (19, 20). Mature FtsZ rings contain all of the proteins required for the stabilization and placement of FtsZ protofilaments and for PG synthesis during the next round of division (21). *S. pneumoniae* lacks conventional nucleoid occlusion mechanisms, and high-resolution microscopy shows that FtsZ protofilaments are distributed in nodal patterns around mature septal FtsZ rings that surround the undivided nucleoid marked by its origin of replication (*SI Appendix, Fig. S1A, Bottom*) (22–24).

To construct an ellipsoid shape, two modes of PG synthesis are organized by the septal FtsZ rings in *S. pneumoniae* (25). Septal PG synthesis mediated by class B PBP2x (bPBP2x) and other proteins closes inward to separate cells, whereas peripheral PG synthesis mediated by bPBP2b and other proteins emanates outward from midcells to elongate cells (*SI Appendix, Fig. S1A, Top*). Early in division, a ring composed of MapZ (LocZ) splits (*SI Appendix, Fig. S1*) and is moved by peripheral PG synthesis toward the equators of the daughter cells (26, 27), preceded by the origin of replication (*SI Appendix, Fig. S1A, Top*) (23). MapZ movement precedes migration of FtsZ, FtsA [FtsZ membrane anchor and transglycosylase PG regulator] (20), and EzrA [FtsZ assembly modulator in *B. subtilis* (28) and FtsZ assembly positive regulator in *S. pneumoniae*] to the equators (*SI Appendix, Fig. S4 B–F*). Middle to late-cell division, FtsZ, EzrA, and FtsA are observed at the closing septum as well as at both developing equators, resulting in a distinctive three-band pattern (*SI Appendix, Fig. S1A, Middle and SI Appendix, Figs. S3A and S4 B–F*). After FtsZ, EzrA, and FtsA relocate to equators, proteins involved in PG synthesis, including DivIVA (negative-curvature binding protein that determines cell shape) (29), GpsB (negative-curvature binding protein that determines cell shape) (30), GpsB (regulator that distributes septal and peripheral PG synthesis) (31), StkP (Ser/Thr protein kinase that regulates PG synthesis) (32), and bPBP2x (19) remain at the septum and migrate to equators right before cells divide (*SI Appendix, Fig. S4 G–L*).

Little is known about how FtsZ moves from the septum to the MapZ rings that have arrived near the equators of daughter cells. Current models postulate that FtsZ migrates en masse from the septum to the equatorial MapZ rings at a later stage in division (e.g., in current study) or en masse from the treading FtsZ ring of *Streptococcus mutans* (33), which is evolutionarily distant from *S. pneumoniae* (33). In this study, en masse streaming of FtsZ from septa to equatorial rings was detected in a minority (~7%) of dividing *S. mutans* cells (33). Here, we show that key proteins involved in FtsZ ring assembly and in septal and peripheral PG synthesis have different dynamics during pneumococcal cell division. We demonstrate and describe several parameters of FtsZ treading in *S. pneumoniae*. Furthermore, we report that nascent rings containing FtsZ, FtsA, and EzrA move out from mature septa guided by MapZ throughout the cell cycle. Streaming of EzrA was only observed in ΔmapZ mutants as a possible division failsafe mechanism. In contrast, several other proteins were confined to mature septa and showed little dynamic movement within the limits of conventional TIRFm. Finally, we show that bPBP2x interacts with FtsW and that both proteins show directional movement along mature septal rings, independent of FtsZ treading. Together, these findings reveal aspects about the movement and assembly of FtsZ/FtsA/EzrA filament/bundles in dividing *S. pneumoniae* cells and show that septal bPBP2x–FtsW complexes require PG synthesis for movement.

**Results**

Relocation of *S. pneumoniae* Cell Division and PG Synthesis Proteins Occurs in Three Stages and Is Dependent on pH. To compare the dynamics of pneumococcal cell division and PG synthesis proteins, we constructed and vetted a large set of fluorescent and HaloTag (HT) protein fusions expressed from single-copy genes at their native chromosome loci (*SI Appendix, Table S1*). Each protein fusion contains a linker region specified in *SI Appendix, Table S1*, but omitted in the text and figures to simplify designations. An uncapsulated derivative (Δcps) of serotype 2 strain D39 was used for these studies, because encapsulated D39 forms short chains (*SI Appendix, Fig. S1A*) that make microscopy more difficult, and capsule tends to mask morphology defects of constructs (34). None of the final fluorescent- and HT–protein fusions ostensibly altered growth or cell morphology, and each showed localization of labeled proteins at septa and new equators of dividing cells grown exponentially in C+Y liquid medium, pH 6.9 (5% CO₂) (*SI Appendix, Figs. S2 and S3*), consistent with previous localization studies (see below).

Demographics generated by MicrobeJ (35) from fields of exponentially growing cells supported and extended the conclusion that *S. pneumoniae* division and PG synthesis proteins relocate from the septa of single, early divisional cells (left side of demographics) to the equators of new daughter cells (right side of demographics) in three distinct stages (*SI Appendix, Figs. S2H and S4*). MapZ relocates early, before FtsZ, FtsA, and EzrA (*23, 26, 27*). Residual MapZ remained between new equatorial rings until the migration of FtsZ and its associated proteins, FtsA and EzrA (*SI Appendix, Fig. S4 A–F*), but a third septal ring of MapZ was observed (ΔmapZ) (33), which is evolutionary distant from these conditions (see also refs. 23 and 27). FtsZ, FtsA, and EzrA next relocate to new equators at approximately the same time, with residual EzrA and FtsA remaining at septa when most of FtsZ has migrated (*SI Appendix, Fig. S4 A–F*). Other cell division and PG synthesis proteins—including DivIVA, MltG, GpsB, StkP, bPBP2x, and FtsW—remain at septa after most FtsZ, FtsA, and EzrA have departed and move to the equators of daughter cells late in the division cycle (*SI Appendix, Figs. S2G and S4 J–L*). The localization of StkP, bPBP2x, and FtsW is diffuse away from septal and equatorial rings than that of the other proteins examined throughout the cell cycle (*SI Appendix, Figs. S2G and S4 J–L*). Western blot control experiments did not detect cleavage of the GFP or HT reporter domains from GFP-StkP and HT-bPBP2x (*SI Appendix, Fig. S5*). As shown later, diffusiveness in demographics corresponds to diffuse movement detected by TIRFm.

During these experiments, we unexpectedly noticed that the size and shape of wild-type ΔΔps cells depends on pH in C+Y liquid medium. At pH 7.6 (5% CO₂), which supports natural competence (36), pneumococcal cells are markedly longer and larger than at pH ~6.9 (5% CO₂), which is the physiological pH at the surface of epithelial cells in the human respiratory tract (*SI Appendix, Figs. S6A and B*) (37). Undercutting the effects of higher pH, strains expressing GpsB-GFP or the GFP-StkP showed morphological defects characteristic of reduced GpsB or StkP function, respectively, in C+Y at pH 7.6, but not at pH 6.9 (*SI Appendix, Figs. S2 and S6*). Effects of pH on cell length and aspect ratio of wild-type cells were not observed in brain–heart infusion (BHI) broth (*SI Appendix, Fig. S6A*), which we used in previous studies but cannot be used here because of autofluorescence.

**Dynamics of FtsZ in Nascent Rings That Form Parallel to Mature FtsZ Septal Rings.** TIRFm and epifluorescence microscopy showed that FtsZ filament motion was detected both inside and outside of mature septal rings in *E. coli* (13, 38) and *B. subtilis* cells (12). To determine the patterns of FtsZ movement in *S. pneumoniae* cells, we performed comparable TIRFm, which limits illumination to a 100- to 150-nm slice and removes out-of-focus background fluorescence light (39). TIRFm of cells was performed on agarose pads containing C+Y, pH 7.1 (no CO₂). Newly formed C+Y, pH 7.1 (no CO₂). Newly grown cells lack conventional nucleoid occlusion (*SI Appendix, Fig. S1A, Bottom*).
namt FtsZ ring detected slightly before the other ~50% of the time (e.g., Fig. 2A). We confirmed that this outward movement was not specific to FtsZ-sfGFP by using other FtsZ-tagged constructs (SI Appendix, Fig. S8). Nascent FtsZ rings move away from septal rings, add more filaments/bundles, and develop into early equatorial rings, in which directional velocities of FtsZ filaments/bundles are still detected (A in Fig. 1A and 2B). The diameters of equatorial rings became larger than those of residual septal rings, and the number of overlapping FtsZ filaments/bundles within new equatorial rings continue to increase (Figs. 1A and 2A). As the density of FtsZ filaments/bundles increases in new equatorial rings, motion is indicated by fluctuations in TIRFm kymographs (Fig. 1C, SI Appendix, Fig. S7E, and Movies S1 and S2). We show below that there is a correspondence between the position of nascent rings of FtsZ, FtsA, and EzrA and movement of the MapZ protein ring out from mature septal rings to the new equatorial rings of daughter cells.

Velocities of *S. pneumoniae* FtsZ-sfGFP filaments/bundles moving in either direction in nascent rings were determined from kymographs (Fig. 1A–D and SI Appendix, Fig. S9A). FtsZ filament velocities were similar in nascent (31.5 ± 13.0 nm/s; average ± SD) and early equatorial rings (34.4 ± 13.7 nm/s), with a combined average FtsZ filament velocity of 32.4 ± 13.3 nm/s in cells in C+Y, pH 7.1 (no CO2) (Fig. 1A–D). FtsZ filament velocities were comparable in cells in C+Y, pH 7.8 medium (no CO2) (33.0 ± 10.0 nm/s) (SI Appendix, Fig. S6D). The velocities of *S. pneumoniae* FtsZ filaments are similar to those reported previously for FtsZ filament/bundle movement in septal rings of *E. coli* (27.8 ± 1.7 nm/s) (13) and *B. subtilis* (32 ± 7.8 nm/s) (12). Other tags [-GFP or i-tag-HT (iHT)] on *S. pneumoniae* FtsZ resulted in a filament/bundle velocity of ~32 nm/s, similar to that of FtsZ-sfGFP, with the exception of FtsZ-HT, which moved about 28% faster at ~41 nm/s (SI Appendix, Fig. S9B). Of these constructs, FtsZ-sfGFP is the most functional, as this fusion causes minimal synthetic defects when combined with ΔmapZ (SI Appendix, Fig. S9C), similar to FtsZ-CFP published previously (23). We also analyzed the time between FtsZ-sfGFP filament appearances moving in the same direction (Fig. 1E). The relative frequency of appearance of FtsZ filaments moving in the same direction for the most part followed a random distribution.

quantitate FtsZ filament/bundle velocities by TIRFm in densely packed mature septal rings (Fig. 1B and C and Movie S1). FtsZ filament/bundle speeds in mature septal rings were determined by wide-field imaging of vertically oriented cells, as described below.

We detected the initial stages of formation of nascent FtsZ rings on either side of mature septal rings (Fig. 1A–C). FtsZ in nascent rings was detected as oblong spots moving in both directions parallel to mature septal rings (Fig. 1A–C and Movie S2). Nascent FtsZ rings first appear very close to mature septal rings, and this distance increases as the nascent FtsZ filaments move outward toward the equators of daughter cells, eventually resulting in the characteristic pattern of three parallel FtsZ rings in mid-to-late divisional *S. pneumoniae* cells (Fig. 1A and C). Summations of TIRFm images taken over 180-s movies indicate that the diameters of nascent rings start out approximately equal to those of mature septal rings (Fig. 1A and C). Kymographs through the long axis of cells show that nascent FtsZ rings form asynchronously on both sides of mature septal rings, with one
except between 28 and 37 s (Fig. 1E). The diameter of FtsZ-sfGFP rings in these live pneumococcal cells was determined by 2D-deconvolution epifluorescence microscopy to be 0.80 ± 0.06 μm, which corresponds to a circumference of ~2.500 nm. Thus, the frequency of FtsZ filament appearance at intervals of 28–37 s (peak at 32 s) cannot be caused by circumbacterial periodicity of FtsZ filaments moving at ~32 nm/s, but may be related to an averaged clocked initiation of new FtsZ filaments.

**FtsZ Filament/Bundle Dynamics and Processivity in Mature Septal Rings.** To determine the speed, processivity, and lifetime of FtsZ filaments/bundles in mature septal rings, individual *S. pneumoniae* cells expressing FtsZ-sfGFP were oriented vertically in a microhole device described previously (Fig. 3A and SI Appendix, Fig. S11, and Movie S3). Images were denoised, and kymographs were generated (Experimental Procedures and SI Appendix, Fig. S10) (12). Lengths and angles of ~600 FtsZ filament tracks from 29 cells were quantitated and used to compute FtsZ filament/ bundle speeds, processivity, and lifetimes (Fig. 3B–D). FtsZ filament/bundles move bidirectionally around *S. pneumoniae* mature septal rings at an average speed of 30.5 ± 9.3 nm/s, which is comparable to the average velocity of FtsZ filaments/bundles in nascent and equatorial rings (32.4 ± 13.3 nm/s) (Fig. 1D) and independent of cell diameter (SI Appendix, Fig. S11B). We conclude that the dynamic properties of FtsZ filaments/bundles in nascent and early equatorial rings match those of FtsZ filaments/bundles in mature septal rings. We also measured the total distance traveled by FtsZ filaments/bundles within septa. This gives a processivity distribution with an average of 515 ± 331 nm (Fig. 3C), meaning that an FtsZ filament typically traverses about one-fifth of the circumference of an *S. pneumoniae* cell. Related to processivity, the time that FtsZ filaments/bundles exist in tracks is distributed with an average of 17.1 ± 9.4 s (Fig. 3D).

**Fig. 3.** FtsZ moves bidirectionally around the mature-septal division plane with filament/bundle velocities similar to those determined in nascent FtsZ rings. IU9985 cells expressing FtsZ-sfGFP were immobilized vertically, and FtsZ-sfGFP dynamics (Movie S3) was determined as described in SI Appendix, Experimental Procedures. (A, Upper) Representative snapshot images of FtsZ-sfGFP septal rings with typical diameters used in analyses. Images in SI Appendix, Fig. S11 illustrate the range of cell diameters observed. (Lower) Kymographs around cell circumference showing multiple FtsZ-sfGFP filaments/bundles treadmilling in both directions. Time-lapse images of the ring circumference were unwrapped into lines (black arrow, Upper Left) to generate the kymograph rows. (Scale bars, 500 nm.) (B–D) Individual filament/bundle tracks in kymographs were quantified from 29 cells (SI Appendix, Fig. S10) to give distributions of FtsZ-sfGFP filament treadmilling speed (b; n = 605), processivity (C; n = 544), and lifetime (D; n = 544). Mean ± SD; speed = 30.5 ± 9.3 nm/s; processivity = 515 ± 544 nm; lifetime = 17.1 ± 9.4 s.

Treadmilling of FtsZ Filaments/Bundles in Mature and Nascent Rings and Dependence of FtsZ Filament Velocity on GTP Hydrolysis. Previous studies have shown that FtsZ filaments move by a treadmilling mechanism in *E. coli* and *B. subtilis* (12, 13). To demonstrate treadmilling of FtsZ filaments/bundles in *S. pneumoniae* mature and nascent rings (Fig. 1), we performed SM-TIRFm on two functional FtsZ-HT constructs, FtsZ-HT and iHT-FtsZ. A limiting concentration of HT substrate was added to approach SM detection of FtsZ-HT or iHT-FtsZ by TIRFm (40) (Fig. 4, red and SI Appendix, Fig. S12) in cells whose positions were delinied by bright-field microscopy. EzrA-mNG was used as a fiducial marker for the locations of rings (Fig. 4, green) in experiments using FtsZ-HT. As presented below, FtsZ and EzrA exhibit similar patterns of movement in nascent and mature rings. In mature septal, nascent, and equatorial rings in daughter cells, SMs of FtsZ appear as stationary foci that persist before disappearing (Fig. 4A, red spots, SI Appendix, Fig. S12C, and Movie S4). We interpret these transient, static foci of single FtsZ molecules as representing nonmoving FtsZ molecules within the cores of FtsZ filaments/bundles that are translocating by a treadmilling mechanism (Fig. 4B). The average lifetime of FtsZ-HT foci detected in mature and nascent rings was 11.9 ± 9.1 s, with some foci persisting for 15–20 s in the strain also expressing EzrA-mNG (Fig. 4C). The average lifetime of FtsZ-HT in the absence of EzrA-mNG, or of iHT-FtsZ was 12.7 ± 8.5 s or 16.8 ± 11.7 s, respectively (SI Appendix, Fig. S12A). The average length of a treadmilling filament is set by the subunit lifetime and average filament speed, because subunits bind to the plus end of a filament, and then depolymerize from the minus end (Fig. 4B). Thus, the estimated FtsZ filament length is 519 ± 399 nm for FtsZ-HT or 506 ± 420 nm for iHT-FtsZ (SI Appendix, Fig. S12B). This length is larger than the *E. coli* FtsZ filament cluster length determined by fluorescence microscopy in *E. coli* cells (41, 42), but matches the average *S. pneumoniae* FtsZ filament length reconstituted in-vitro (43). In addition, we confirmed that the velocity of *S. pneumoniae* FtsZ filament/bundle movement depends on GTP hydrolysis by FtsZ, as reported previously for other bacteria and in biochemical
FtsA and EzrA Form Nascent Rings with FtsZ in *S. pneumoniae*. We examined the movement of several proteins involved in FtsZ filament formation and stabilization (FtsA and EzrA) and in septal PG synthesis (MapZ, GpsB, StkP, bPBP2x, and FtsW) (see Introduction). Of this set, only FtsA and EzrA localize with FtsZ throughout the *S. pneumoniae* cell cycle (*SI Appendix*, Fig. S4 B–F) and form nascent rings in early divisional cells (Fig. 5 A and B). Kymographs along the long axis of cells show outward movement of EzrA and FtsA rings over a 9-min period, similar to that of FtsZ filaments (Fig. 2). Kymographs along the nascent and equator ring planes revealed that EzrA and FtsA traverse circumferentially, similarly to FtsZ (Fig. 5). The average velocity of EzrA-mNG (29.6 ± 15.3 nm/s), EzrA-GFP (33.6 ± 14.3 nm/s), and GFP-FtsA (33.3 ± 12.7 nm/s) in nascent/equatorial rings was similar to that of FtsZ filaments (Fig. 5 C and *SI Appendix*, Fig. S17 C). Another sandwich-fusion construct of FtsA (FtsA*-sIGFP-FtsA*) consistently moved ~31% faster (41.7 ± 16.2 nm/s) than most FtsZ or EzrA fusions in nascent rings (*SI Appendix*, Figs. S9B and S17 B and C). As noted above, an FtsZ-HT fusion also moved ~28% faster than three other FtsZ fusion constructs (*SI Appendix*, Fig. S9B). Overall, six FtsZ, EzrA, and FtsA fusions moved with approximately the same velocity of ~32 nm/s, suggesting that the slightly faster velocity of the FtsZ-HT and FtsA*-sIGFP-FtsA* constructs is anomalous. We conclude that FtsA and EzrA proteins associate with and stabilize FtsZ filaments throughout the *S. pneumoniae* cell cycle and have similar overall dynamics as FtsZ filaments/bundles, including nascent ring formation.

MapZ Location Corresponds to Positions of Nascent FtsZ and EzrA Rings in Early Divisional *S. pneumoniae* Cells. We wondered whether nascent ring formation of FtsZ, FtsA, and EzrA was coincident with movement of MapZ protein rings, which emerge from either side of mature septal rings concomitant with the start of peripheral PG synthesis (*SI Appendix*, Fig. S1A) and move perpendicular to the long axis of cells to the equators of the new daughter cells (Fig. 5, Figs. S1B and S18) (26, 27). In demographics and summations of movies, MapZ is localized primarily in mature septa or in two rings adjacent to septa, although a slight haze of MapZ remains between equatorial rings until FtsZ had fully exited from septa (*SI Appendix*, Figs. S4 A and C and Movie S8). No directional movement of MapZ or fluctuations of MapZ signal was observed in rings in kymographs (*SI Appendix*, Fig. S18 A and C), consistent with minimal MapZ movement reported previously for *S. mutans* MapZ (33). This conclusion was confirmed directly by SM-TIRFm of iHT-MapZ, which unlike HT-MapZ, did not cause cell morphology defects (*SI Appendix*, Fig. S18 A and B). SMs of iHT-MapZ that appeared in MapZ rings remained static for as long 60 s to >100 s before disappearing due to motion out of the TIRF plane or photobleaching (*SI Appendix*, Fig. S18 E and F and Movie S9).

In high-resolution 3D-structured illumination microscopy (SIM) images of cells coexpressing tagged MapZ and FtsZ, low amounts of FtsZ are detected in early divisional cells at positions corresponding to nascent rings observed by TIRFm (Fig. 1; arrowhead in Fig. 6 A, i and ii). These nascent FtsZ rings overlap with MapZ rings moving away from septa. Similarly, EzrA in nascent rings overlaps with the parallel MapZ rings adjacent to the septum in early divisional cells (boxed dotted box, Fig. 6 A, iii). In later divisional cells, EzrA remains at constricting septa surrounding segregating nucleoids, when all MapZ has moved to the equators of daughter cells, which also contain some EzrA (box, Fig. 6 A, iv). These results are consistent with MapZ acting as a guide for the nascent rings of FtsZ, FtsA, and EzrA that initially delivers some, but not all, of FtsZ, FtsA, and EzrA to the equators of daughter *S. pneumoniae* cells. If MapZ is a guide for formation of nascent rings, then we would expect aberrant movement of FtsZ/FtsA/EzrA filaments in *ΔmapZ* mutants. In the *ΔmapZ* genetic background, ∆mapZ mutants are viable and form nearly normal looking cells with some distortions and frequent misaligned division planes (23, 27, 45). However, some FtsZ-fusion constructs in ∆mapZ mutants exhibit a severe synthetic defect in growth and morphology that precludes their study in *S. pneumoniae* (23), but that was not commented upon in *S. mutans* (33). In contrast, FtsZ-sIGFP reactions (12, 13, 43). For these experiments, we constructed a *S. pneumoniae* mutant expressing FtsZ(G107S), which likely is defective in GTP binding based on homologs in other bacteria (*SI Appendix*, Fig. S13 A–C). The ftsZ(G107S) mutant is temperature sensitive for growth and lyzes at 43 °C (*SI Appendix*, Fig. S13 B). Following a shift from 32 °C to 42 °C, the ftsZ(G107S) mutant formed larger, more spherical cells than the ftsZ* parent strain, although the relative cellular amount of FtsZ(G107S) was comparable to that of FtsZ* in cells at 42 °C (*SI Appendix*, Fig. S13 C–E). Strains expressing FtsZ(G107S)-sFGFP are not viable. Therefore, we constructed a ftsZ(G107S)/βga4*:PftsZ–ftsZ-sfgfp merodiploid strain in which we expressed and tracked the movement of low levels of ectopically expressed FtsZ-sFGFP by adding limited concentrations (0.1/0.01 mM) of ZnCl2/MnCl2 at the still-permissive temperature of 37 °C (*SI Appendix*, Fig. S14). Under these conditions, the ftsZ*/PftsZ–ftsZ-sfgfp and ftsZ(G107S)/PftsZ–ftsZ-sfgfp strains show overall similar growth and FtsZ-sFGFP localization (*SI Appendix*, Fig. S14), although slightly aberrant cells with mis-localized FtsZ-sFGFP were occasionally observed for the ftsZ (G107S)/PftsZ–ftsZ-sfgfp strain. TIRFm of nascent FtsZ rings revealed that FtsZ-sFGFP filaments still move bidirectionally, but with significantly reduced velocity in the ftsZ(G107S) mutant compared with the ftsZ* parent strain (*SI Appendix*, Fig. S15 and Movie S5). Overexpression of another mutant allele, FtsZ (D214A) that is defective in GTPase activity, also severely decreases FtsZ filament/bundle velocity (see below) (*SI Appendix*, Figs. S24 A and S25 A and B). We conclude that *S. pneumoniae* FtsZ filament/bundle velocity produced by treadmilling is dependent on GTP binding and hydrolysis by FtsZ, consistent with previous studies in other bacteria (12, 13, 16).
Fig. 6. MapZ is present in nascent ring planes containing FtsZ and EzrA filaments. IFM (A) or TIRFm (B and C) was performed to characterize the role of MapZ relative to FtsZ/EzrA filament organization. (Scale bars, 1 μm.) (A) Representative images of 3D-SIM dual IFM colocalizing FtsZ-MyC and MapZ-L-FLAG3 (strain IU9090; i and ii) or MapZ-L-FLAG2 and EzrA-HA (strain IU9207, i and iv). Cultures were grown in BH at 37 °C in 5% CO2, and IFM and DNA staining with DAPI were carried out as described in SI Appendix, Experimental Procedures. The dotted yellow lines in the DAPI column are approximate outlines of cell shape. For cells (i), (ii), and (iii), arrows point to the nascent ring plane where FtsZ or EzrA filaments can be seen. For cell (iii), the nascent ring (dotted box) and whole cell (solid box) were rotated, while for cell (iv), only the whole cell was rotated (solid box). The experiment was performed twice with similar results. (B) Representative montages and accompanying kymographs of EzrA-GFP (green in montages) overlaid with bright field (blue outline, cells are black) in mapZΔ (IU10449) versus MapZ+ (IU10540), strains visualized by TIRFm (at one frame per second; see Movie S10). (Scale bars, 1 μm.) (C) No significant difference in the distributions of velocities of EzrA-GFP in the mapZΔ or MapZ+ strains was found by an unpaired two-tailed t test (GraphPad Prism).

and EzrA-GFP fusions in ΔmapZ mutants lack this severe defect and appear similar to ΔmapZ mutants (Fig. 6B and SI Appendix, Fig. S9C). TIRFm of EzrA-GFP movement in a ΔmapZ mutant indeed revealed aberrant, untimed streaming of EzrA, presumably in association with FtsZ filaments, from parent to daughter cells, often resulting in rings that are not perpendicular to the long axis of cells (Fig. 6B and Movie S10). Nevertheless, the rate of EzrA streaming was similar in ΔmapZ and mapZΔ strains (Fig. 6C). Altogether, these results are consistent with MapZ acting as a continuous guide for the orderly movement of FtsZ/FtsA/EzrA filaments from mature septal rings to new equatorial rings in daughter cells. However, in the absence of MapZ, a second streaming mechanism aberrantly distributes FtsZ/FtsA/EzrA filaments into daughter cells.

bPPB2x Is Dynamic Compared with Other Proteins That Mediate PG Synthesis. We next examined the motion of several other proteins involved in PG synthesis in S. pneumoniae. GpsB (regulator), DivIVA (regulator), MltG (endo-lytic transglycosylase), StkP (Ser/Thr kinase), bPPB2x (TP), and FtsW (GT) (see Introduction) remain at mature septa until late in the division cycle after FtsZ, FtsA, and EzrA have largely moved to the equatorial rings (SI Appendix, Fig. S2A and Movies S11–S15; see also Fig. 8A). In these mature septal and equatorial rings, fluctuation of GpsB or MltG signal indicative of ordered movement is not evident, whereas DivIVA and bPPB2x are actively moving, especially in equatorial rings (Fig. 7A and SI Appendix, Fig. S19 A–C), and bPPB2x motion is more diffuse around cells (Movie S15). In contrast, the motion of StkP is distinctively different from that of the other proteins examined. StkP locates in mature septal rings, where signal fluctuations are not readily apparent, but at the same time, StkP moves rapidly and diffusively throughout whole cells, which is captured as “clouds” of protein in summations of TIRFm movies (SI Appendix, Fig. S19D and Movie S14). Cells expressing GFP-StkP or HT-bPPB2x did not show obvious defects in growth or cell morphology (SI Appendix, Fig. S2), whereas HT-bPPB2x was labeled by an excess of HT-JF549 substrate. We conclude that at least one of these proteins, only FtsZ and its ring stabilizers, FtsA and EzrA, form nascent rings and that dynamics of the other proteins varies from minimally detectable by TIRFm to diffusive.

bPPB2x and Its Partner FtsW Move at the Same Velocities Along Septal Rings. SM-TIRFm experiments were performed to delineate the motion of bPPB2x relative to FtsZ in mature septal rings (Fig. 7B and Movie S16). SM-TIRFm detection of HT-bPPB2x was approximated by addition of a limited amount of HT-JF549 substrate (Fig. 7B, red) that gave the same rate of bPPB2x movement when titrated downward to where labeled FtsZ-sfGFP (Fig. 7B, green) and midcells determined from cell outlines (Fig. 7B; blue) were used as fiducial markers for the location of mature septal rings (Fig. 7A). Some bPPB2x molecules are detected as moving rapidly around cells in a sporadic fashion (Movie S16), consistent with TIRFm. The movement of these diffusive bPPB2x molecules was not analyzed further due to their lack of continuous tracks in SM-TIRFm. Other bPPB2x molecules attach onto mature septal rings and move directionally for at least 18 s (montage in Fig. 7B) and in some cases >30 s. The velocity of bPPB2x molecules in septal rings (21.9 ± 12.9 nm/s) is significantly slower than that of treading rings by FtsZ-sfGFP filaments (32.4 ± 13.3 nm/s) (Fig. 7C). Control experiments showed that the velocity of SMs of HT-bPPB2x is the same in strains that express FtsZ-sfGFP (Fig. 7C) or that express FtsZ+ (Fig. 7F).

To further demonstrate that the velocity of HT-bPPB2x is not dependent on the fusion construct, we tracked the dynamics of FtsW-HT in S. pneumoniae (Fig. 7F, SI Appendix, Fig. S20, and Movie S17). New results demonstrate that the biochemical GT activity of FtsW depends on its interaction with its cognate class B PBP (46). We confirmed this interaction in S. pneumoniae cells by: (i) colocalization of FtsW and bPPB2x as part of the septal synthesis complex (Fig. 7D; demographies in SI Appendix, Fig. S2G and quantification of paired widths in SI Appendix, Fig. S2H) and (ii) by communoprecipitation (co-IP) of FtsW with bPPB2x as bait in a 1:1 complex based on molecular mass (Fig. 7E). Consistent with a bPPB2x:FtsW complex, HT-bPPB2x and FtsW-HT move along mature septal rings at the same velocity in SM-TIRFm (Fig. 7F, SI Appendix, Fig. S20 A and B, and Movie S16), which is slower than that of FtsZ treading. In addition, both proteins display some level of sporadic movement throughout the membrane in TIRFm summations (Fig. 7A and SI Appendix, Fig. S20C), suggesting that their interaction may occur independently of divisome localization. Attempts to determine the velocity of FtsW-GFP by TIRFm (SI Appendix, Fig. S20D) and compare it with that of FtsW-HT (Fig. 7F and SI Appendix, Fig. S20 B and C) were not successful. Unlike FtsW-HT at septa after ∼2 h of saturated labeling (Fig. S20C), the density of FtsW-GFP was too dense at septa to distinguish circumferential velocities in TIRFm kymographs.

Movement of bPPB2x and FtsW Depends on PG Synthesis and Not FtsZ Treadmilling in S. pneumoniae. Finally, we examined whether the velocity of bPPB2x and FtsW is strongly correlated with FtsZ treadmilling, as was demonstrated in E. coli and B. subtilis (12, 13). We found that there is minimal correlation between the velocity of bPPB2x movement on septa and the rate of FtsZ
treadmill (Fig. 8A). To perform these experiments, we determined the velocity of bPBP2x at septa by SM-TIRFm in FtsZ (GTPase) mutants that slowed down FtsZ treadmill by ~2x [FtsZ(D1075S)] (SI Appendix, Figs. S21 and S22 and Movie S18) or ~10x [overexpression of FtsZ(D214A)] (SI Appendix, Figs. S23–S25 and Movies S19 and S20) and that lead to a percentage of cells with aberrantly placed division rings. Strikingly, reduction of FtsZ treadmill velocity by ~2x or ~10x does not reduce bPBP2x velocity or reduces it only slightly (~1.5x), respectively (Fig. 8A and SI Appendix, Figs. S24 and S25C). Notably, in the FtsZ(D214A) mutant, bPBP2x moves ~5x faster than the FtsZ filaments/bundles. Similarly, FtsW velocity is reduced by only ~1.4x in the FtsZ(D214A) mutant (SI Appendix, Fig. S25D). Finally, reduction of FtsZ treadmill velocity over this range does not affect the net level of PG synthesis, as determined by incorporation of FDAA label for 2.5 m (Fig. 8B). These results contrast sharply with those for B. subtilis, where inhibition of FtsZ treadmill significantly reduces FDAA labeling (12).

We next tested whether bPBP2xFtsW velocity depends on PG synthesis. In S. pneumoniae, there are two MurA (UDP-N-acetylglucosamine enolpyruvyl transferase) homologs that catalyze the first committed step of PG synthesis (47, 48). Deletion of murA1 (spd_0967; also called murD) does not significantly alter growth, cell morphology, or FtsZ treadmill velocity in C-Y, pH 7.1 (SI Appendix, Fig. S26C). However, the velocity of bPBP2x and FtsW is consistently reduced by ~1.5x in the ΔmurA1 mutant compared with the murA11 parent (Fig. S8C). We conclude that limitation of PG synthesis slows down bPBP2x:FtsW velocity without detectably affecting FtsZ filament/bundle velocity. Finally, we added the β-lactam mexitilcin at a concentration that inhibits most of bPBP2x TP activity almost specifically (22). Mexitilcin (lipid I biosynthesis-inhibitor) did not inhibit bPBP2x:FtsW velocity (Fig. 8C), but nearly completely stopped the movement of bPBP2x (Fig. 8D, SI Appendix, Fig. S27, and Movie S22). Together, these combined results indicate that movement of bPBP2x:FtsW complexes along septal rings depends on PG synthesis and is independent of the movement of FtsZ filaments/bundles.

Discussion

Partitioning of FtsZ filaments/bundles into daughter cells occurs in ovoid-shaped (ovococcus) bacteria, such as S. pneumoniae, by a mechanism that is fundamentally different from the Min and nucleoid exclusion systems present in rod-shaped bacteria (49). In S. mutans, MapZ forms a third ring at division septa (26), whereas in other laboratory strains and in the progenitor D39 background of most S. pneumoniae laboratory strains, this third ring is rarely detectable (23, 27), making it unlikely that it plays an obligatory role in S. pneumoniae genetic background (26, 27). Similarly, in one laboratory strain, MapZ forms a third ring at division septa (26), whereas in other laboratory strains and in the progenitor D39 background of most laboratory strains, this third ring is rarely detectable (SI Appendix, Figs. S2C, S4A, and S1B) (23, 27), making it unlikely that it plays an obligatory role in S. pneumoniae division.

The interesting conjecture was made that MapZ reaches the equators of daughter cells, it serves as a “beacon” for relocation of FtsZ from mature septal rings (26). A recent paper proposes a concerted streaming mechanism in which FtsZ moves late in division from septa to equators in some S. mutans cells (33). In contrast, here we report that FtsZ transport to equators in S. pneumoniae is a continuous process throughout the cell cycle (Fig. 9). Early in S. pneumoniae division, nascent filaments/bundles of FtsZ are detected near and moving parallel to mature septal rings (Figs. 1 and 9). In the ~10-min interval (one-third of a generation) between the initial movement of MapZ and the migration of most FtsZ to equators (SI Appendix, Fig. S4A and B), nascent FtsZ filaments/bundles move outward and become more dense until they reach equators, after which the remainder of FtsZ
migrates to form mature equatorial rings (Fig. 2). Progressive nascent ring formation was detected in both Δcpx derivatives and in the progenitor encapsulated cpx+ parent D39 strain. These nascent FtsZ rings also contain EzrA and FtsA, which bind to membrane anchor, and stabilize FtsZ filaments/bundles (Fig. 5 and SI Appendix, Fig. S16), but none of the other PG synthesis proteins analyzed in this study was detected moving in nascent rings. FtsZ, FtsA, and EzrA move at the same velocity in the major of tagged constructs (Results), indicative of formation of nascent FtsZ/FtsA/EzrA filament/bundles.

High-resolution microscopy and effects of a ΔmapZ mutation suggest that nascent FtsZ/FtsA/EzrA filaments/bundles use MapZ as a guide, as opposed to a beacon, to reach the equators of daughter cells. Three-dimensional SIM immunofluorescence microscopy (IFM) images detect FtsZ and EzrA together with MapZ in early and later nascent rings (Fig. 6A). Furthermore, in the absence of MapZ, orderly nascent EzrA rings are lost, and EzrA abruptly streams between daughter cells, often resulting in the aberrant ring orientation reported previously (Fig. 6B) (23). Streaming rarely was observed in wild-type S. pneumoniae cells (~1%) and represents a second mechanism for translocation of FtsZ and its associated proteins to daughter cells. In this respect, streaming is a “failsafe” mechanism that accounts for the lack of lethality of S. pneumoniae ΔmapZ mutations.

In S. mutans cells, continuous FtsZ nascent ring formation was not reported and streaming, which was detected in ~7% of cells, is proposed as the primary mechanism for FtsZ movement from septal to equatorial rings (figure 6 in ref. 33). A possible reason for this difference is that S. pneumoniae and S. mutans are evolutionarily distant Streptococcus species (33), and ovococcus bacteria exhibit differences in the relative timing of septal and peripheral PG synthesis (53). On the other hand, technical or strain differences may underlie the different results. In particular, the S. mutans cells in movies shown in ref. 33 appear to be in middle-to-late divisional stages and contain prominent equatorial rings, whereas movement of S. pneumoniae FtsZ was recorded in early-to-late stages of division in this study (Figs. 1 and 2). Taken together, our results indicate that treadmillng FtsZ/FtsA/EzrA filaments/bundles are components of migrating MapZ rings throughout the cell cycle in S. pneumoniae (Fig. 9), and may thus play an important role in assembly and organization of these rings, about which little is known. This transport mechanism also moves part of the cellular FtsZ population to

**Fig. 8.** S. pneumoniae bPBP2x and FtsW movement depend on PG synthesis and are not correlated with FtsZ treadmilling or FtsZ(GTPase) activity. Strains were grown in Cu-Y, pH 6.9 at 37 °C in 5% CO2 to OD600 = 0.1–0.2, at which point cells were labeled with FDA, washed, and fixed. Alternatively, cells were labeled with 120 pM HT-JF549 ligand, washed, and SM-TIRFm performed (at one frame per second) to track dynamics of HT-bPBP2x or FtsW-HT. (A) Mean HT-bPBP2x velocity is not correlated with FtsZ-stGFP treadmillng velocity. Average velocities ± SDs from two or three independent biological replicates are shown for strains: IU9985 (ftsZ-stgfp), IU13910 (ht-bp2x), IU14375 (ftsZ1075G bp2x), IU14508 (ftsZ1075G ht-bp2x); IU15181 [PftsZ-fzts(D214A)st-gf], and IU15041 [Pbps2x-fzts(D214A)ht-bp2x] (SI Appendix, Experimental Procedures and Table S1 and Movies S5 and S18–S20). (B) Box-and-whisker plots (whiskers, 5th and 95th percentile) of different FtsZ(GTPase) mutants showing that mean FDAA labeling of PG per cell is not reduced in FtsZ(GTPase) merodiploid mutants (middle compared with right strains). P values were obtained by one-way ANOVA analysis (GraphPad Prism, nonparametric Kruskal-Wallis test, where *P < 0.05; **P < 0.01; ***P < 0.001). Values are determined from two independent biological replicates (SI Appendix, Experimental Procedures). (C) HT-bPBP2x and FtsW-HT velocity is reduced in the absence of MurA1. Velocities were determined by SM-TIRFm in strains IU13910 (ht-bp2x), IU15039 (ΔmurA1 ht-bp2x), IU15096 (ht-bp2x), and IU15173 (ΔmurA1 ftsW-h1) as described in Fig. 7. Shown is the average velocity ± SD of n tracks. P values were obtained by one-way unpaired, two-tailed t tests (GraphPad Prism), where **P < 0.001. (D) HT-bPBP2x movement is inhibited when cells are treated with methicillin. A final methicillin concentration of 0.3 µg/mL was added on top of an agarose pad, after which IU13910 (ht-bp2x) cells were added as described in SI Appendix, Experimental Procedures. Cells were visualized by SM-TIRFm after 45–75 m of treatment with methicillin at 37 °C (Movie S22). A summation is shown of movie frames over 180 s with arrows pointing at septa where molecules of bPBP2x no longer move circumferentially, as indicated by the kymographs (n = 57). (Scale bar, 1.0 µm.) Numbers correspond to the arrows in the summation. The experiment was performed independently twice with similar results. The velocity of FtsZ-stGFP remained unchanged, consistent with FtsZ treadmilling, independent of PG synthesis (n = 84) (SI Appendix, Fig. S27 and Movie S21).

**Fig. 9.** Summary diagram of the movement dynamics of FtsZ, FtsA, EzrA, bPBP2x, FtsW, and regulators of PG synthesis in S. pneumoniae cells. The division cycle is simplified to three stages. In early divisional cells (I), the equator becomes the septum of dividing cells, and most divisome proteins locate to the mature septum, with the exception of Skp and bPBP2x, which also exhibit sporadic, diffusive movement throughout entire cells. bPBP2x and FtsW molecules attach to the mature septal ring and move in one direction or the other for as long as 30 s. After the start of peripheral PG synthesis, MapZ bifurcates into rings on both sides of the mature septal ring, and the MapZ rings start to move outward toward the positions of the equators in the daughter cells (I→II). Concurrently, nascent filaments/bundles consisting of FtsZ and its associated proteins FtsA and EzrA are detected in MapZ rings, suggesting that MapZ rings continuously guide a fraction of FtsZ/FtsA/EzrA to new equatorial rings, where FtsZ may nucleate the transport of remaining FtsZ from the mature septal ring later in division. Treadmilling velocity and dynamics of FtsZ and EzrA are the same in mature septal, nascent, and equatorial rings, whereas FtsA may move faster than FtsZ in nascent rings. FtsZ/FtsA/EzrA filaments/bundles accumulate as nascent MapZ rings move away from the mature septal ring and become early equatorial rings in daughter cells. Directional movement of or association with nascent rings was not detected for PG synthesis proteins bPBP2x, FtsW, DivIVA, StkP, and GpiB, and these PG synthesis proteins remain at constricting septa until migrating to new equatorial rings late in cell division, where bPBP2x and FtsW form a complex and move circumferentially in the direction [3]. The movement of bPBP2x/FtsW complexes in septal PG synthesis depends on and reflects new PG synthesis and is not correlated with the treadmilling of FtsZ filaments/bundles. See Discussion for additional details.
The equatorial rings, where the FtsZ filaments/bundles may serve to nucleate the transport of the remainder of septal FtsZ later in the division cycle (Fig. 9). This movement likely occurs mainly by depolymerization of FtsZ filaments/bundles and possibly by some new protein synthesis, because concerted streaming of FtsZ filaments/bundles was rarely observed in wild-type S. pneumoniae cells.

In the course of these studies, we also determined a set of basic parameters about FtsZ dynamics in S. pneumoniae. Filaments/bundles of wild-type FtsZ always move with a velocity of ~32 nm/s in rings at all stages of division and while streaming in ΔmapZ mutants (Figs. 1D, 3B, and 6C). The same velocity of FtsZ filament/bundle movement was determined by TIRFm (Fig. 1D) and independently by wide-field observation of vertically immobilized cells (Fig. 3). A treadmilling mechanism of FtsZ filament/bundle movement was confirmed directly by SM-TIRFm (Fig. 4 and SI Appendix, Fig. S12). SM-TIRFm and immobilized cell measurements indicated the lifetime of FtsZ subunits in filaments/bundles is ~15 s (Fig. 4C and SI Appendix, Fig. S12) and the lifetime of entire FtsZ filaments/bundles is ~17 s (Fig. 5D). Based on the average subunit lifetime and the velocity of filament/bundle movement, the average length of treadmilling FtsZ filaments/bundles in S. pneumoniae cells is ~500 nm (SI Appendix, Fig. S12B), which is similar to the length of dividing septal FtsZ filaments/bundles reconstructed in vitro (43). In addition, our measurements show that the processivity of treadmilling of FtsZ filaments/bundle is ~500 nm (Fig. 3C), indicating that FtsZ filaments/bundles traverse ~20% of the circumference of S. pneumoniae cells on average. Finally, as expected from previous precedents (12, 13), mutations that decrease GTP binding or the GTPase activity of FtsZ reduce the velocity of FtsZ treadmilling by as much as 10x (Fig. 8A). FtsZ (GTPase) mutations also disrupt the placement of division planes compared with wild-type cells (SI Appendix, Figs. S14, S21, and S23).

Besides FtsZ, FtsA, and EzrA, none of the PG synthesis proteins tested in this study was a member of translocating MapZ rings (SI Appendix, Fig. S16). Within the limits of standard TIRFm, some of these proteins showed minimal movement in mature septal and equatorial rings (i.e., GspB, MltG, and StkP) (Introduction and SI Appendix, Fig. S19), whereas DivIVA and bPBP2x showed obvious dynamic movements (Fig. 7A and SI Appendix, Fig. S19C). We therefore determined the role of FtsZ treadmilling on the motion of bPBP2x and its partner FtsW in mature septal S. pneumoniae cells. A new biochemical study reported that FtsW GT activity depends on interactions with the septal class B PBP (46). In support of this interaction in S. pneumoniae cells, bPBP2x and FtsW colocalize at all stages of S. pneumoniae division (Fig. 7D and SI Appendix, Fig. S2 G and H) and bPBP2x pulls down FtsW in a likely 1:1 complex (Fig. 7E). In addition, SM-TIRFm showed that bPBP2x and FtsW move at the same velocity on septa (Fig. 7F). Moreover, besides bPBP2x stimulating FtsW GT activity in S. pneumoniae cells, the TP activity of bPBP2x is required for septal PG synthesis, because a pbp2x (S537A) active-site mutant is not viable (54), suggests that bPBP2x is lacking septal closure (SI Appendix, Fig. S28). This result is consistent with the notion that both bPBP2x TP and FtsW GT activities are required to drive bPBP2x:FtsW movement in the septa of S. pneumoniae cells, as discussed next.

Five pieces of evidence support the conclusion that movement of the bPBP2x:FtsW complex in septa of S. pneumoniae cells depends on PG synthesis and not on FtsZ treadmilling. First, the velocity of bPBP2x and FtsW is slower than that of FtsZ treadmilling in wild-type S. pneumoniae cells (Fig. 7C). Second, the decreased velocity of FtsZ treadmilling in FtsZ(GTPase) mutants is not correlated with a decrease of FtsZ mobility (Fig. 5A). In fact, in the slowest mutant [FtsZ(D214A) overexpression], bPBP2x is moving about 5x faster than FtsZ treadmilling. Third, severe reduction in FtsZ treadmilling velocity does not markedly decrease PG synthesis indicated by FDAA incorporation (Fig. 8B). Fourth, a decrease in PG synthesis precursors caused by a Δmura1 mutation, decreases the velocity of bPBP2x and FtsW by the same amount, but does not decrease FtsZ treadmilling rate (Fig. 8C). Finally, addition of methicillin at a concentration that mainly inhibits bPBP2x TP activity stops the movement of bPBP2x, but does not decrease the velocity of FtsZ treadmilling (Fig. 8D).

These results strongly support the conclusion that the movement of the bPBP2x:FtsW complex in septal PG synthesis in S. pneumoniae cells depends on and likely mirrors new PG synthesis and is not correlated with the treadmilling of FtsZ filaments/bundles. In contrast, the velocities of the septal class B PBPs of B. subtilis and E. coli are coupled to and limited by FtsZ treadmilling, resulting in a correlation between septal bPBP and FtsZ treadmilling velocities (12, 13). The mechanisms underlying this coupling and its relationship to the rate of PG synthesis in B. subtilis and E. coli are not understood. On the one hand, FtsZ treadmilling is further coupled to and limiting for septal PG synthesis and the constriction of B. subtilis cells (12). On the other hand, the velocity of FtsZ treadmilling is not correlated with the rate of septum closure of E. coli cells (13, 41). These differences suggest that additional metabolic (e.g., PG precursor pools) and structural (e.g., PG width and outer membrane synthesis) constraints may influence the relative rates of FtsZ treadmilling, bPBP complex movement, and PG synthesis in different bacteria (55, 56).

Besides the sidewall rod complexes of rod-shaped bacteria (14), there is another precedent for the dependence of PBP movement on PG synthesis. Recent results show that septal PG synthesis continues to close division septa of S. aureus after FtsZ treadmilling is inhibited by addition of a drug (PC190723) (16). This finding is again consistent with an FtsZ treadmilling-independent mechanism by which PG synthesis itself drives for PBP motion (13, 41). Attempts to perform a similar experiment with S. pneumoniae were not successful, because S. pneumoniae is not inhibited by PC190723 (57). The dependence of bPBP2x:FtsW movement on PG synthesis can be rationalized by a model proposed for the dependence of PBP movement on PG synthesis in sidewall elongation of rod-shaped bacteria (14). It was proposed that MreB filaments form tracks that direct the linear motion of PBP complexes (14, 58). At any point in a track, the PBP complex has used substrate behind it to synthesize PG, and the utilization of available substrate in front of it drives its motion. In the case of S. pneumoniae bPBP2x:FtsW, it is possible that FtsZ filaments/bundles, or other proteins in septal FtsZ rings, provide tracks that couple movement to PG synthesis. In this model, FtsZ treadmilling acts to dynamically distribute filament tracks that allow the spatial organization of directional PG synthesis, possibly through indirect interactions, as suggested recently for E. coli FtsZ and FtsN by high-resolution microscopy (42). Future studies will test this and related models to provide an understanding about the relationships among FtsZ treadmilling, PBP complex movement, and PG synthesis rate and location in different bacteria.

Experimental Procedures

Detailed experimental procedures are described in SI Appendix, Experimental Procedures, including: bacterial strains and growth conditions, Western blotting, 2D-epifluorescence microscopy and demograph generation; growth and imaging of live cells by TIRFm; TIRFm image acquisition and processing; periodicity analysis of FtsZ filaments/bundles in nascent rings; culture growth and sample preparation for microhole immobilization of S. pneumoniae cells; image acquisition, processing, and data analysis of vertically oriented cells in microholes; SM TIRFm; TIRFm of DbzG107S, P21, and S23ΔmurA1 in an ftsZ(G107S) mutant; TIRFm of P21-ftsZ(D214A) or P21-ftsZ(D214A)-sgfp meridionoid strains; 3D-SIM IFM; co-IP of FtsW-GFP with bPBP2x-FLAG; labeling of FtsW(GT)ase mutant cells with FDAA; and TIRFm of methicillin-treated cells.

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