Organization of Peptidoglycan Synthesis in Nodes and Separate Rings at Different Stages of Cell Division of *Streptococcus pneumoniae*

Amilcar J. Perez[a†], Michael J. Boersma[a†], Kevin E. Bruce[a†], Melissa M. Lamanna[a], Sidney L. Shaw[a], Ho-Ching T. Tsui[a], Atsushi Taguchi[b], Erin E. Carlson[c], Michael S. VanNieuwenhze[d], and Malcolm E. Winkler[a#]

[a]Department of Biology, Indiana University Bloomington, Bloomington, IN 47405 USA

[b]Department of Microbiology, Harvard Medical School, Boston, MA 02115 USA

[c]Department of Chemistry, University of Minnesota, Minneapolis, MN 55455 USA

[d]Department of Chemistry, Indiana University Bloomington, Bloomington, IN 47405 USA

Running title: Microscopy of Vertically Oriented Pneumococcal Cells

Keywords: septal and peripheral peptidoglycan synthesis; penicillin-binding proteins (PBPs); FtsX; fluorescent D-amino acids (FDAAs); high-resolution 3D-SIM

[†]Contributed equally to this work. Author order was determined by random draw.

[#]Corresponding author:

Malcolm E. Winkler
Department of Biology
Indiana University Bloomington
1001 East Third Street
Bloomington, Indiana USA 47405
Phone: 812-856-1318
Email: winklerm@indiana.edu

ABSTRACT

Bacterial peptidoglycan (PG) synthesis requires strict spatial and temporal organization to reproduce specific cell shapes. In the ovoid-shaped, pathogenic bacterium *Streptococcus pneumoniae* (*Spn*), septal and peripheral (sidewall-like) PG synthesis occur simultaneously at midcell. To uncover the organization of proteins and activities that carry out these two modes of PG synthesis, we examined *Spn* cells vertically oriented onto their poles to image the division plane at the high lateral resolution of 3D-SIM (structured-illumination microscopy). Using fluorescent D-amino acid (FDAA) probes, we show that areas of new transpeptidase (TP) activity catalyzed by penicillin-binding proteins (PBPs) separate into a pair of concentric rings early in division, representing peripheral PG (pPG) synthesis (outer ring) and the leading-edge (inner ring) of septal PG (sPG) synthesis. Fluorescently tagged PBP2x or FtsZ locate primarily to the inner FDAA-marked ring, whereas PBP2b and FtsX remain in the outer ring, suggesting roles in sPG or pPG synthesis, respectively. Short pulses of FDAA labeling revealed an arrangement of separate regularly spaced "nodes" of TP activity around the division site of predivisional cells. Control experiments in wild-type and mutant strains support the interpretation of nodal spacing of TP activity, and statistical analysis confirmed that the number of nodes correlates with different ring diameters. This nodal pattern of FDAA labeling is conserved in other ovoid-shaped species. Tagged PBP2x, PBP2b, and FtsX proteins also exhibited nodal patterns with spacing comparable to that of FDAA labeling. Together, these results reveal a highly ordered PG synthesis apparatus in ovococcal bacteria at different stages of division.

SIGNIFICANCE

The spatial organization of PBPs and their TP activity at division septa is not well understood. In some bacteria, TP activity and PBP localization seem to be nodal (also called punctate), whereas in other bacteria, discrete foci of PBP activity are infrequently or not observed. Here we report two basic properties of the organization of PBPs and TP activity in the ovoid-shaped bacterium *Spn*. First, there is distinct spatial separation of the sPG machine, including FtsZ, from the pPG synthesis machine at the midcell of dividing *Spn* cells. Second, in predivisional cells, PBPs and TP activity are organized heterogeneously into regularly spaced nodes, whose number and dynamic distribution are likely driven by the PG synthesis of PBP:SEDS complexes.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus; *Spn*) is a Gram-positive, commensal bacterium of humans and a major opportunistic pathogen that causes life-threatening illnesses, including pneumonia, bacteremia, and meningitis (1, 2). *Spn* is a prolate ellipsoid-shaped bacterium, referred to as an ovococcus (3). Its distinct ovoid shape is maintained by a thick peptidoglycan (PG) wall that surrounds the entire cell (4). PG consists of glycan sugar chains of alternating units of β-(1,4) linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). Glycan chains are crosslinked together by PG peptides attached to MurNAc, thereby forming a mesh-like network (5, 6). As in most eubacteria, *Spn* PG synthesis is essential for normal growth and cell division and determines normal cell shape and chaining, which impact colonization and virulence (4, 7, 8). Besides protecting cells from turgor stress, *Spn* PG serves as a scaffold for surface-

3

attached virulence factors, including capsule, sortase-attached proteins, and wall teichoic acids (4).

In *Spn*, PG synthesis is organized initially by an FtsZ ring at the midcell equator perpendicular to the long axis of newly divided cells (Fig. S1). PG synthesis is carried out by distinct septal PG (sPG) and peripheral PG (pPG) modes (9-11). sPG synthesis produces the cross wall that separates daughter cells (12-14), whereas concurrent pPG synthesis elongates daughter cells from midcell to form ovoid-shaped cells (Fig. S1) (11-14). pPG synthesis in *Spn* functionally resembles preseptal PG elongation that occurs briefly at the beginning of division of rod-shaped bacteria (15, 16). However, pPG elongation in *Spn* cells remains confined to the midcell region, in contrast to lateral PG elongation organized by MreB-containing Rod complexes moving over the body of rod-shaped cells (5).

The location of these two modes of PG synthesis within the same midcell region requires significant coordination and organization. Previously, we used dual-protein 2D-immunofluorescence microscopy (IFM) and high-resolution 3D-SIM to determine the relative localization patterns of pneumococcal division and PG synthesis proteins at different stages of division (10-12). Stages of cell division were assigned retrospectively based on static "snap-shot" images of cells from non-synchronized exponential cultures. These studies showed that in newly divided, predivisional (stage 1) daughter cells, division and PG synthesis proteins locate to an FtsZ-organized, midcell equatorial ring (Fig. S1). As nearly simultaneous sPG and pPG synthesis begin (stage 2) (17), the equatorial ring begins to constrict and becomes the new septal ring. Some FtsZ and associated proteins EzrA and FtsA move outward continuously with MapZ from the septal ring toward the positions of the future equatorial rings in daughter cells (13). As

4

constriction and midcell elongation continue, FtsZ amount decreases at the midcell of middle- to late-divisional cells and begins to accumulate at the equators of daughter cells. Meanwhile, PG synthesis proteins remain at the midcell septal ring.

At the resolution of standard 2D-epifluorescence microscopy (2D-EFM), essential class B bPBP2x transpeptidase (TP), which interacts with the FtsW SEDS glycosyltransferase (GT) to carry out sPG synthesis (13, 18), locates in an apparent inner ring within an outer ring containing the class B bPBP2b TP (11), which interacts with the RodA SEDS GT to carry out pPG synthesis (Fig. S1) (19, 20). Several other proteins implicated in pPG synthesis locate to this apparent outer ring, including MreC that organizes and may activate bPBP2b (21), class A aPBP1a that has both GT and TP activities (12), MltG endo-lytic transglycosylase that cleaves glycan chains (21), and StkP serine/threonine kinase that mediates cell division (11, 12). Near the end of cell division (stage 4), remaining PG synthesis proteins that have not moved to developing equatorial rings converge to a small spot between the daughter cells (Fig. S1) (11), before finally moving to the equatorial rings of the daughter cells (13).

Additional evidence for physical separation of the sPG and pPG synthesis machines midway through cell division was obtained by staining with fluorescent vancomycin (FL-Vanco), which labels PG pentapeptides in areas of PG synthesis (22), with fluorescent D-amino acids (FDAAs), which label PG in regions of active PBP TP activity (23, 24),and with an activity-based β-lactone fluorescent probe (7FL) specific for bPBP2x (25, 26). FL-Vanco labeling combined with IFM of mid-divisional cells indicated localization of bPBP2x inside of regions of nascent PG synthesis that were surrounded by bPBP2b and other pPG synthesis proteins (11). Long pulses of FDAAs for 5 min followed by 3D-SIM showed

5

119   labeling of a midcell ring surrounding a prominent central dot of FDAA labeling,

120   resembling a "Saturn-like" pattern, in mid-divisional cells (11). Specific inhibition with the

121   β-lactam antibiotic methicillin and protein depletion experiments indicated that the central

122   dot of septal TP activity is attributable to bPBP2x (11). Likewise, these central dots of

123   FDAA labeling disappeared in rings of non-constricted septa in cells depleted for the

124   GpsB regulatory protein (10). Last, spatial separation of bPBP2x was confirmed with a

125   bPBP2x-specific, activity-based β-lactone fluorescent probe (7FL, Lac(L)-Phe-FL), whose

126   labeling recapitulated a "Saturn-like" labeling pattern, indicating that although most active

127   bPBP2x moves toward the center of the septum, some still remains in the outer peripheral

128   ring (25, 26).

129   Limitations of these previous fluorescent-protein and -probe studies are the low

130   resolution (≈250 nm) of conventional 2D-EFM and of rotated 3D-SIM images (27). Due to

131   their ovoid shape, *Spn* cells predominantly lie sideways along their long axis during

132   imaging on a flat surface. Imaging of septal rings and associated structures in horizontal

133   cells by 3D-SIM is limited by the Z-axial resolution of 250-300 nm of the system, which is

134   much lower than the 100 nm resolution in the XY lateral plane (27). This axial resolution

135   limitation remains when reconstructed image volumes of horizontally oriented cells are

136   rotated by 90° to visualize septal rings as circles. To overcome this obstacle, we devised

137   a simple method to reliably orient a small, but sufficient, number of *Spn* cells vertically

138   onto their poles (i.e., "on their heads") for 3D-SIM imaging, thereby enabling high lateral

139   resolution of division rings. Using this approach, we were able to detect FDAA-labeled

140   intermediate concentric rings of sPG synthesis TP activity in strains expressing

141   fluorescent PG synthesis proteins. This approach located the proteins to the outer

6

peripheral or inner septal rings and showed that these proteins are organized as nodal structures distributed regularly around these rings in early divisional cells. We further found that short pulses of FDAA labeling also revealed nodal distributions of TP activity that were disrupted in division mutants. Together, these results reveal a highly uniform organization of coordinated PG synthesis and distinct separation of the sPG and pPG synthesis machines at the midcell of dividing ovococcal bacteria.

RESULTS

"Snap-shots" of vertically oriented *Spn* cells labeled with FDAAs reveal constriction of concentric septal and peripheral rings. To circumvent the lower resolution during rotation of 3D-SIM images, we devised a simple method to capture a sufficient number of vertically oriented, fixed *Spn* cells to image division planes at high ≈100 nm × ≈100 nm XY lateral resolution. Wild-type (WT) D39 Δ*cps* cells were first labeled with a blue FDAA (HADA) for several generations and then with a red FDAA (TADA) for 2.5 min to label sites of new PG synthesis (Fig. 1A and 1B). Cells were fixed with formaldehyde and resuspended in a small amount of hardening anti-fade solution pipetted onto small, round glass coverslips onto which slides were placed. The anti-fade suspension medium minimized photobleaching, and its viscosity and the large number of cells in samples trapped some cells in a vertical orientation. This procedure led to a reliable, but small (≤1%), percentage of cells suspended on their poles, which could be spotted as circles (Fig. 1A and 1C), among many horizontally oriented cells (Fig. 1B) in each field.

7

164   Vertically oriented *Spn* cells were captured at different stages of the division cycle

165   (Fig. 1A and 1C). In predivisional cells, red TADA labeling appears as a single ring around

166   the division site (diagrammed in Fig. 1D). Remarkably, as division precedes, TADA

167   labeling is detected in a pair of intermediate concentric rings, which are designated as the

168   outer (peripheral) and inner (septal) rings. The inner ring represents the TP activity at the

169   leading-edge of the closing septum annulus that eventually converges down to a dot,

170   surrounded by the outer ring to form a "Saturn-like" pattern reported before, where the

171   dot results from bPBP2x TP activity (11). In very late divisional cells, all remaining TADA

172   labeling at midcell converges to a single dot at the division site, while new TADA rings

173   appear at the equators of daughter cells. The regions of TP activity, including their

174   relationship to membrane invagination, are summarized in Figure 1D. We conclude that

175   PG synthesis occurs at two distinct, separate locations at the midcell of dividing *Spn* cells.

176   **bPBP2x and FtsZ primarily locate to the constricting inner septal ring, whereas**

177   **bPBP2b and FtsX locate to the outer peripheral ring.** We reasoned that the spatial

178   separation observed in vertically oriented *Spn* cells could be used to assign PG synthesis

179   proteins to the sPG and/or pPG synthesis machine(s). We labeled strains expressing

180   isfGFP-bPBP2x or sfGFP-bPBP2b with TADA (125 µM) for a moderate time (2.5 min),

181   and then observed TADA and GFP fluorescence in vertically oriented cells (Fig. 2 and

182   S2). isfGFP-bPBP2x or sfGFP-bPBP2b was expressed from its native chromosomal

183   locus, and cells expressing these constructs exhibited minimal discernible growth or cell

184   morphology defects (Fig. S3A and S3B) (13). Western blotting with native antibody

185   showed a single species of each fusion protein (Fig. S3C). Nevertheless, isfGFP-bPBP2x

186   cellular amount is moderately increased (169%) compared to unlabeled WT bPBP2x,

8

whereas sfGFP-bPBP2b is underproduced to only 12-15% of the WT amount, without causing ostensible defects in cell growth and shape in BHI broth (Fig. S3). We characterized two other strains that express fusion constructs without causing defects in growth and morphology. In one strain, iHT-bPBP2x is expressed at ≈164% or ≈88% of WT bPBP2x level when grown in BHI broth or C+Y medium, respectively (Fig. S2C, S2E, and S3 A-C). In the second iHT-bPBP2b//P_{Zn}-iHT-bPBP2b merodiploid strain grown in BHI broth containing 0.3 mM Zn^{2+}/0.03 mM Mn^{2+}, iHT-bPBP2b is expressed at ≈WT bPBP2b level (≈107%; Fig. S2D and S3C).

In predivisional cells grown in BHI broth, TADA treatment for 2.5 min labels equatorial rings fairly uniformly, whereas sfGFP-bPBP2x and sfGFP-bPBP2b appear as nodes distributed regularly around the circumference of cells (Fig. 2A and, S2A, row 1). Independent labeling of WT bPBP2x with the 7FL β-lactone probe specific for bPBP2x corroborated a nodal organization in predivisional equatorial rings of daughter cells (Fig. S2F). As division progresses, isfGFP-bPBP2x moves inward resulting in formation of the inner septal ring (Fig. 2A and S2A, row 2), with some bPBP2x remaining in the outer ring, as was also observed for labeling with the 7FL (Fig. S2F) (25, 26). Displacement between the single TADA ring and the inner isfGFP-bPBP2x ring (Fig. 2A and S2A, row 2) likely reflects the continued movement inward of isfGFP-bPBP2x during the time required to wash away unincorporated TADA and fix cells. As the TADA-labeled inner septal ring becomes more prominent, isfGFP-bPBP2x condenses into a dense, concentric ring largely lacking detectable nodes at this resolution, with some isfGFP-bPBP2x remaining in the outer ring (Fig. 2A and S2A, row 3).

9

209   A similar pattern of localization was observed for slightly overexpressed or

210   underexpressed iHT-bPBP2x in cells grown in BHI broth or C+Y medium, respectively

211   (Fig. S2C and S2E), including residual iHT-bPBP2x remaining in the outer ring. In iHT-

212   protein fusion experiments, the blue FDAA HADA replaced TADA due to spectral overlap

213   of the red HT-JF549 ligand with TADA. Nodes of iHT-bPBP2x (Fig. S2C and S2E) were

214   often less distinct and regular than those of intrinsically fluorescent isfGFP-bPBP2x (Fig.

215   2A and S2A), likely because of non-optimal labeling with the extrinsic HT substrate.

216   By contrast, as isfGFP-bPBP2x progresses to the inner septal ring during division (Fig.

217   2A and S2A, row 3), sfGFP-bPBP2b expressed at a low level compared to WT bPBP2b

218   remains confined to the outer peripheral ring, with little, if any sfGFP-bPBP2b detected in

219   the inner ring marked by TADA (Fig. 2A and S2B, row 3). Likewise, iHT-bPBP2b

220   expressed near the WT bPBP2b level remains as nodes in the outer peripheral ring of

221   pre- and early divisional cells (Fig. S2D, row 1), but is not detected in the constricting

222   inner septal ring (Fig. S2D, row 2). Again, extrinsically labeled iHT-bPBP2b nodes vary

223   more than those of intrinsically fluorescent sfGFP-bPBP2b. Together, these results

224   identify intermediate states of spatial separation of the bPBP2x-containing sPG and

225   bPBP2b-containing pPG synthesis machines during *Spn* division (see Fig. 1D). The nodal

226   localization of bPBP2x and bPBP2b in the midcell rings of predivisional cells is taken up

227   below in the context of FDAA labeling patterns.

228   We extended this approach to a protein that had not been localized before. FtsX is a

229   polytopic membrane protein that forms a complex with the cytoplasmic FtsE ATPase and

230   the extracellular PcsB PG hydrolase in *Spn* (28-31). The FtsEX:PcsB complex is essential

231   for *Spn* growth due to its role in hydrolytic remodeling during PG synthesis (28, 32, 33).

10

Depletion of FtsEX:PcsB results in the formation of chains of spheroid cells (31, 33), characteristic of defective pPG synthesis (11, 28). To test this idea, we localized an FtsX'-isfGFP-FtsX' sandwich fusion in vertically oriented *Spn* cells (Fig. S2G). This FtsX sandwich fusion is expressed from the normal chromosomal locus and does not cause cell defects, even though about 60% seemed to be proteolytically cleaved once in the extracellular ECL1 domain near the fusion point (Fig. S3C). Throughout the cell cycle, FtsX-isfGFP-FtsX' appears as nearly evenly spaced nodes around the outer peripheral ring demarked by TADA labeling and was not detected in the inner septal ring later in division (Fig. S2G). Finally, we determined that FtsZ-sfGFP tracks with TP activity at the leading-edge of the closing septum annulus (Fig. S2H). We conclude that PG remodeling by the FtsEX:PcsB complex is likely confined to pPG synthesis in *Spn*, and that FtsEX and pPG synthesis is not associated with an FtsZ ring during much of the *Spn* cell cycle (see Fig. 1D). In addition, this example illustrates the utility of using FDAA labeling as a fiducial marker to distinguish proteins involved in sPG and/or pPG synthesis.

New PG synthesis is organized as a series of regularly spaced nodes around the midcell of predivisional *Spn* cells. At a labeling time of 2.5 min with 125 µM TADA, we observed apparent nodal variation of TADA intensity in the outer and inner rings of dividing *Spn* cells (Fig. 1 and 2). As noted above, we also observed nodal positioning of bPBP2x, bPBP2b, and FtsX in these rings (Fig. 2 and S2). To study these patterns further, we labeled WT *Spn* cells with a lower concentration of TADA (45 µM) for a very short pulse (17 s) before imaging vertically oriented cells (Fig. 3). To maximize the spacing between nodes, we confined our analysis of TADA labeling patterns to predivisional cells that have the largest diameters by inspection and that were relatively plentiful in fields of

11

255 non-synchronized cultures. Very short pulses resolved TADA labeling into a series of

256 regularly spaced nodes distributed around the equators of predivisional *Spn* cells (Fig.

257 3A).

258   To quantify the pattern of these nodes, we developed a custom vertical image analysis

259 graphical user interface (VIMA-GUI) using MATLAB (see *Experimental Procedures*). After

260 manually designating individual nodes in each division ring, the program accurately

261 determines the location of the ring, the diameter of the cell, the number of FDAA nodes,

262 and the arc distance between adjacent nodes. TADA pulse-labeled and fixed WT Δ*cps*

263 D39 cells have an average diameter of 0.84 μm ± 0.05 (SD), indicating that the selected

264 cells are at the same predivisional stage (Fig. 3A; Table S3). WT Δ*cps* D39 cells contain

265 an average of 9.8 ± 1.0 (SD) individual TADA-labeled nodes, with an average arc distance

266 between nodes of 0.27 μm ± 0.07 (SD) (Fig. 3B; Table S3). The consistency of these

267 measurements strongly supports the notion of an ordered placement of TP activity sites

268 early in *Spn* division.

269   **The pattern of FDAA-labeled nodes is not caused by image processing.** To

270 confirm that the regular nodal TADA-labeling pattern was not caused by the processing

271 steps required for rendering structured illumination images, we examined the raw data

272 and data-process parameters for TADA-pulse-labeled WT Δ*cps* D39 cells (Fig. 1 and 3).

273 Nodal labeling was still observed at the equators of pulse-labeled predivisional *Spn* cells

274 using conventional, low-resolution (≈250 nm) widefield 2D-EFM without image

275 deconvolution (arrows, Fig. 4A). Deconvolution improved the clarity of the nodes in these

276 low-resolution widefield images, but the images remained blurry compared to 3D-SIM

277 images (Fig. 3A). To examine the influence of the major user-defined data smoothing

12

278 function in SIM reconstruction, we processed 3D-SIM data by adjusting the Wiener filter

279 setting stepwise between 0.001-0.02 (34). Higher filter settings (above 0.01) resulted in

280 clear "honeycomb" patterning effects on the sample and in background regions. We found

281 no discernable patterning or grouping (e.g., over-separation of intensity into nodes) of

282 signal with settings below 0.006 (Fig. 4B, row 2) and set the Wiener filter at 0.001 for the

283 data presented in this work (Fig. 4B, row 1). Finally, we asked if the level of fluorescence

284 signal in the pulse-labeled cells was potentially contributing to an artificial separation of

285 signal into nodes. To this end, we determined the maximal pixel peak intensities in lines

286 drawn through nodes in cells labeled for 17s (Fig. 3) and through the nearly contiguous

287 labeling rings of cells labeled for 2.5 min (Fig. 1). Background offsets were determined

288 from regions lacking cells for the two labeling conditions, and these backgrounds were

289 subtracted from the mean maximal pixel intensities. The mean maximal pixel intensity of

290 nodes (425 A. U. ± 105) was similar to that of nearly contiguous rings (461 A. U. ± 169),

291 indicating that the SIM processing can produce nearly contiguous rings and separate

292 nodes at about the same intensity level. This equivalence argues that the observed nodes

293 are not simply being created by grouping together regions of low fluorescence signal. We

294 conclude that the 3D-SIM processing parameters used in these analyses did not create

295 the TADA nodal patterns observed in vertical *Spn* cells.

296 In support of this conclusion, the nodal pattern of TADA labeling becomes irregular in

297 mutants defective in FtsZ ring assembly or in the regulation of PG synthesis (next

298 section). This irregularity is not consistent with an image reconstruction artifact. In one

299 experiment, FtsZ was expressed from a Zn^{2+}-inducible promoter in a $\Delta ftsZ$//P$_{Zn}$-*ftsZ*$^+$

300 merodiploid strain grown in BHI broth containing sufficient Zn^{2+} to allow growth

13

301   comparable to the WT strain. The merodiploid strain was shifted to BHI broth lacking Zn^{+2}

302   for 2.5 h, which resulted in enlarged spheroid cells with increased (≈1.4-fold) diameters

303   (Fig. 5; Table S3). The FtsZ-depleted and WT FtsZ$^+$ strain were pulse-labeled (17 s) with

304   TADA, and vertically oriented cells were imaged by 3D-SIM. WT cells show a regular

305   nodal pattern in ≈94% of vertical cells, whereas the FtsZ-depleted cells with larger

306   diameters show a regular nodal pattern in only ≈40% of cells (Fig. 5A). The remaining

307   ≈60% of FtsZ-depleted cells show irregular nodal arrangements, often with large gaps

308   between nodes (arrow, Fig. 5A). "Regular" and "irregular" nodal patterns were initially

309   distinguished by visual heuristic criteria, where "regular" refers to nodal patterns that

310   appear by eye to be relatively evenly distributed, whereas "irregular" refers nodal

311   distributions that contain one or more large gaps with spacing estimated to be at least

312   twice that in the regular nodal pattern. Subsequent measurements of arc lengths

313   generally matched the initial visual criteria. FtsZ-depleted cells with regular patterns

314   contained more TADA-labeled nodes than WT or FtsZ-depleted cells with irregular

315   patterns (Fig. 5B, middle; Table S3). The mean distance between nodes was greater for

316   FtsZ-depleted than WT cells, with the variability of gap distances in FtsZ-depleted cells

317   with irregular patterns reflected by a high standard deviation (Fig. 5B, right; Table S3).

318   Taken together, this evidence strongly argues that the FDAA nodes are not an artifact of

319   3D-SIM imaging and that FtsZ is required to maintain the WT arrangement of FDAA nodes

320   in predivisional *Spn* cells.

321   **The number of regularly spaced FDAA-labeled nodes is correlated with the**

322   **diameter of predivisional *Spn* cells.** The increase in the number of nodes in FtsZ-

323   depleted cells with regular spacing (Fig. 5B, middle; Table S3) was suggestive of a

324 regulatory mechanism for node placement. To explore this idea, we determined labeling

325 patterns in five additional division and PG synthesis mutants that were pulsed with TADA

326 for 17 s and imaged vertically (Fig. 6). Amino acid changes in FtsZ(G107S) (divisome ring

327 organizer protein) (13), EzrA(T506I) (FtsZ-ring modulator in Gram-positive bacteria) (35),

328 and GpsB(K96N) (regulator of the balance between sPG and pPG synthesis) (10, 36)

329 cause temperature sensitivity (TS) at 42°C. TS mutants expressing EzrA(T506I) or

330 GpsB(K96N) were isolated for this study (Table S1). At the semi-permissive temperature

331 of 37°C in BHI broth, each mutant grows slower and forms cells with enlarged diameters

332 (Fig. S4A; Table S3). The TADA-labeling pattern of each mutant was similar to that of the

333 FtsZ-depleted strain. The majority (53% to 66%) of mutant cells show irregular nodal

334 patterns with gaps and greater arc distances, whereas cells with regular labeling patterns

335 contain an increased number of nodes spaced similarly to WT (Fig. 6A, 6B, and S4B;

336 Table S3).

337 We extended this analysis to Δpbp1a and ΔkhpA mutants that have significantly

338 smaller diameters than WT cells in BHI broth (Fig. S4A, Table S3). Δpbp1a mutants lack

339 aPBP1a and produce skinny, slightly elongated cells (21, 37), while ΔkhpA mutants lack

340 a regulatory RNA-binding protein and form smaller cells with the same aspect ratio as

341 larger WT cells (38). The nodal pattern in the Δpbp1a or ΔkhpA mutant is regular in >90%

342 of cells with an average arc distance similar to that of WT (Fig 6A and 6B; Table S3);

343 consequently, the mutant cells have fewer nodes per cell (Fig. S4B). When plotted, these

344 data show that the number of regularly spaced nodes, when present, increases linearly

345 with ring diameter (Fig. 6C), consistent with a mechanism that maintains WT spacing

346 between sites of PG synthesis in predivisional Spn cells.

Spacing of bPBP2x and bPBP2b nodes is similar to that of FDAA nodes produced by short pulse labeling. The number and arc distance of nodes of isfGFP-bPBP2x and sfGFP-bPBP2b in the midcell rings of predivisional cells (Fig. 2A, S2A, S2B, and 7A) were measured and found to be similar to those of the FDAA nodes in WT cells (Fig. 2B and 7C; Table S3). Notably, the spacing of the TADA (red) and sfGFP-bPBP2b (green) nodes determined in the same cell is similar, but displaced in most cells (Fig. 7A), providing further support that the nodal patterns are not a microscopy artifact. To quantitate the relative distributions of these patterns, we calculated correlation coefficients for the number and spatial distribution of TADA and sfGFP-bPBP2b nodes in 48 cells (Fig. S5A and S5B). As expected by the number of nodes (Table S3), there is a positive correlation between the number of sfGFP-bPBP2b and TADA nodes (Fig. S5A). In contrast, spatial correlation coefficients were distributed around 0, indicating no strong interdependence of the positions of the TADA and sfGFP-bPBP2b nodes (Fig. S5B).

We performed two additional labeling protocols to gain information about these patterns. First, we performed tandem short-pulse labeling of WT cells with TADA (17 s), which labels regions of active TP activity, followed by Boc-FL (21 s), which labels all active PBPs (Fig. 7B; Table S3). Displaced two-color patterns of TADA and Boc-FL nodes were produced, similar to those of TADA-labeled sfGFP-bPBP2b cells (Fig. 7A). Again, there was a positive correlation between the number of TADA and Boc-FL nodes (Fig. S5C), but no spatial correlation of the positions of the two kinds of nodes in 18 cells (Fig. S5D). The regular nodal pattern after Boc-FL pulse-labeling pattern did not match what would be expected from an earlier study done at long labeling times (39). We reprised this earlier study and obtained different results (*Appendix SI, Additional data*; Fig. S6), consistent

16

370 with those reported here (Fig. 7B). Last, we tandemly pulse labeled WT cells with three

371 different colors of FDAAs (green, then red, then blue) (Fig. S7). We again observed

372 regular, displaced nodal patterns for each color of FDAA probe (arrows, Fig. S7),

373 supporting the conclusion that there is a distributive, regular nodal pattern of PBP

374 localization and TP activity at the midcell ring of predivisional *Spn* cells.

375 **FDAA labeling is organized in nodes in other ovoid-shaped bacterial species.**

376 To determine if this regular, organized pattern of TP activity by PBPs is present in other

377 ovococcal species, we labeled *Streptococcus mitis* and *Enterococcus faecalis* with short

378 (17 s) pulses of TADA and imaged vertically oriented cells by 3D-SIM (Fig. 8). *S. mitis* is

379 a close evolutionary relative that exchanges DNA with *Spn* by natural competence, while

380 *E. faecalis* is more evolutionarily distant in the same *Lactobacillale* order (40-42). Both

381 species demonstrated a pattern of TADA-labeled nodes at midcell with an average arc

382 distance indistinguishable from that of WT *Spn* (Fig. 8B; Table S3). Thus, the regular

383 organized pattern of PBP TP activity in predivisional cells is widely distributed in

384 ovococcal species.

385

386 **DISCUSSION**

387 Whether the sPG and pPG synthesis machines of *Spn* are distributed contiguously in

388 a single midcell ring ("plum pudding" model) or separate from each other during septal

389 constriction has been a point of contention (11, 43-45). The demonstration of concentric

390 intermediate rings of TP activity reported here provides strong support for the separation

391 hypothesis (Fig. 1D and 9A). Localization of bPBP2x to the inner ring, which corresponds

392 to the constricting leading edge of the septal annulus, further supports closure driven by

17

393   PG synthesis by the bPBP2x:FtsW complex (13, 18). Some bPBP2x remains in the outer

394   ring of mid-to-late divisional cells when iHT-bPBP2x is expressed at near WT level in C+Y

395   medium (Fig. S2E) or when WT bPBP2x is itself labeled with the 7FL probe (Fig. S2F)

396   (25). Thus, active bPBP2x is both at the constricting leading edge of the septal annulus

397   and at its outer edge, suggesting expansion of the annulus in both places. The constricting

398   FtsZ ring also tracks with sPG synthesis at the leading edge of the septal annulus ring,

399   implying that FtsZ is not detectable in the outer pPG synthesis ring (Fig. S2H).

400   In contrast, bPBP2b expressed at 12% or at WT levels primarily localizes to the outer

401   pPG synthesis ring (Fig. 2A, S2B, S2D, and S3C). These results indicate that only about

402   12% of bPBP2b cellular amount is sufficient for normal growth and *Spn* cell morphology

403   (Fig. S3A and S3B), implying that bPBP2b is in excess in cells in this culture condition.

404   An implication of confinement of bPBP2b to the outer midcell ring is that the presumed

405   circumferential movement of the bPBP2b:RodA complex during pPG synthesis is guided

406   by a structure that lacks FtsZ filaments/bundles. *Spn* does not encode the actin-like MreB

407   protein that mediates PG elongation of rod-shaped bacteria (4), and determination of *Spn*

408   elongasome composition and organization is an area of active research.

409   FtsX is also confined to the outer pPG synthesis ring (Fig. S2G). FtsX is an essential,

410   polytopic membrane protein that forms a complex with the cytoplasmic FtsE ATPase and

411   the extracellular PcsB PG hydrolase in *Spn* (28-32). Depletion of essential FtsX, FtsE, or

412   PcsB results in chains of spherical cells, similar to those caused by depletion of bPBP2b

413   (11, 28, 30, 31, 33, 46). An FtsX'-isfGFP-FtsX' fusion was detected in the outer PG

414   synthesis ring, but not in the inner septal ring (Fig. S2G), consistent with a role for

415   FtsEX:PcsB in pPG synthesis, analogous to that played by FtsEX:CwlO in sidewall PG

18

416 synthesis in *Bacillus subtilis* (*Bsu*) (47, 48). Thus, *Spn* FtsX is in proximity to FtsZ and

417 FtsA in the nascent divisome in early predivisional cells (Fig. S2G); however, in contrast

418 to *E. coli* FtsX (49), as division proceeds, *Spn* FtsX physically separates from FtsZ and

419 FtsA, which are at the leading edge of the septal annulus (Fig. S2H). This result raises

420 the possibility that a PG remodeling hydrolase other than FtsEX:PcsB mediates sPG

421 synthesis. Based on these examples, dual labeling of vertical cells can be applied to

422 assign other PG synthesis, divisome, and regulatory proteins to the sPG or pPG synthesis

423 machines of *Spn*. This approach may also explicate PG stress responses, such as

424 possible roles of Class A PBPs in imparting resistance to an exogenously added PG

425 hydrolase following exposure of *Spn* laboratory strain R6 to a β-lactam antibiotic that

426 inhibits bPBP2x (and DacA (PBP3)) (14).

427 We noted that bPBP2x and bPBP2b are distributed in regular nodal patterns at the

428 equators of predivisional cells (Fig. 2, 7A, and S2 A-F). As the inner ring constricts, this

429 nodal pattern becomes more compact and difficult to resolve (Fig. 2A); hence, we

430 confined this study to the large equators of predivisional cells (Fig. 3A). By shortening the

431 FDAA pulse time down from 2.5 min to ≈17 s, we observed that TP activity also is

432 distributed in a regular nodal pattern with ≈10 nodes separated by an average arc length

433 of 0.27 ± 0.07 µm in WT cells (Fig. 3B). A comparable nodal pattern of FDAA pulse

434 labeling was detected in *S. mitis* and *E. faecalis* (Fig. 8), indicating a common organization

435 of TP activity in predivisional ovococcal cells.

436 Several controls support the conclusion that the nodal pattern was not caused by

437 processing steps required to generate structured illumination images (see *Results)*. Most

438 importantly, the regular nodal pattern of FDAA labeling was disrupted by gaps in a

19

439   majority of cells depleted for FtsZ (Fig. 5) or in temperature-sensitive *ftsZ*(G107S),

440   *ezrA*(T506I), and *gpsB*(K96N) mutants grown at the semi-permissive temperature of 37°C

441   (Fig. 6). Each of these growing TS mutants formed enlarged cells with increased

442   diameters compared to WT (Fig. S4). Between 34%-47% of these larger mutant cells still

443   showed regular nodal patterns of FDAA labeling with more nodes per ring spaced at the

444   WT arc distance (Fig. 6). Conversely, Δ*pbp1a* or Δ*khpA* mutants have smaller diameters

445   than WT with fewer FDAA nodes spaced at the WT arc distance (Fig. 6). Together, these

446   data are consistent with a mechanism that maintains a regular spacing of PG synthesis

447   in predivisional cells, irrespective of equator diameter (Fig. 6C and 9B).

448   The average arc distance was similar between nodes of FDAA labeling, isfGFP-

449   bPBP2x, isfGFP-bPBP2b, and Boc-FL (Fig. 2, 3, and 7). However, the positioning of

450   nodes in two-color FDAA/sfGFP-bPBP2b or FDAA/Boc-FL experiments showed low

451   correlation (Fig. S5), as did nodes sequentially pulse-labeled with three different colors of

452   FDAAs (Fig. S7). The displacement of FDAA nodes relative to bPBP2b or Boc-FL, may

453   reflect the time (≈1 min) that it takes to wash away unincorporated FDAA. It is also

454   possible that PBPs other than bPBP2b are active in predivisional cells. Altogether, these

455   results indicate that the placement of PBPs and TP activity is not fixed at single positions

456   on equators of predivisional cells, but rather, is dynamic and distributive.

457   The localization of PG synthesis and PBPs in early divisional cells varies among

458   bacterial species that use different modes of septum formation and division. Similar to the

459   patterns reported here for *Spn* and other ovococcal species (Fig. 2, 8, and S2), labeling

460   *Eco* for short pulses, but not for long times, with an FDAA results in nodal (also called

461   punctate) patterns, which were not quantitated at high resolution (50). Consistent with

20

462 nodal pattern formation, high-resolution microscopy of vertically oriented *Eco* cells

463 revealed a pattern of "discrete densities" of mCit-FtsI (bPBP3) distributed around the

464 entire septal ring of dividing *Eco* cells (51).

465   Different FDAA pulse-labeling patterns were reported for *S. aureus* (*Sau*) and *Bsu*

466 compared to those in *Spn* and *Eco*. Discrete foci of PG synthesis were not observed in

467 *Sau* cells labeled with short FDAA pulses and viewed by 3D-SIM, in an experiment similar

468 to Figure 3, or labeled with a pulse of other D-amino acid probes and viewed by

469 localization microscopy (52). However, while not commented upon, SIM images of sfGFP-

470 PBP1 in some vertically oriented *Sau* cells do appear nodal around closing septal rings

471 (53), and heterogenous localization of *Sau* GFP-PBP2 has also been reported (54).

472 Labeling *Bsu* with short, sequential pulses of two colors of FDAAs or an FDAA followed

473 by Boc-FL (55) gave a different pattern from the regular nodal pattern in *Spn* (Fig. 7 and

474 S7). At the lower resolution of rotated 3D-SIM images, sequential labeling with two colors

475 of FDAAs gives a pattern suggestive of a limited number of PG synthesis complexes

476 moving in both directions around the septum. Labeling *Bsu* with an FDAA followed by

477 Boc-FL again suggests a surprisingly limited number of active PBP complexes labeled by

478 Boc-FL adjacent to newly synthesized PG marked by the FDAA (55). We conclude that

479 nodal PG synthesis at septa occurs in different patterns in some bacteria and is

480 apparently absent in others, possibly reflecting different mechanisms of sPG synthesis

481 and cell separation. In this regard, *Spn* and *Eco* simultaneously close division septa while

482 separating daughter cells (13, 50), whereas septa formation by PG synthesis occurs

483 before cell separation in *Sau* and *Bsu* (52, 53, 55).

484   The mechanism that causes the regular nodal distribution of PBPs and their TP activity

485   in predivisional cells of *Spn* remains to be determined (Fig. 9B). Recent notable studies

486   have demonstrated heterogeneous supramolecular localization of membrane proteins

487   generally and at septa of *Sau* cells, including interacting enzymes that catalyze

488   phospholipid biosynthesis and the MreD regulator of PG synthesis (56, 57). This

489   heterogenous localization results in punctate patterns of these proteins at division septa,

490   resembling the localization of *Spn* PBPs reported here (Fig. 2 and S2). Polytopic MreD

491   plays a role in supramolecular localization of the phospholipid biosynthesis enzymes, and

492   heterogeneous punctate patterns are lost upon protein overexpression (57). A favored

493   explanation for punctate supramolecular organization is that membrane protein

494   complexes distort local curvature on membranes and thereby perturb diffusion, resulting

495   in distribution patterns for the majority of membrane proteins (57). Whether membrane

496   protein distribution, the size and composition of PG synthesis complexes, an unknown

497   scaffolding complex, or a combination of these mechanisms causes the nodal distribution

498   of PBPs and TP activity in predivisional *Spn* cells remains to be determined.

499   Finally, single molecules of bPBP2x and its interacting partner FtsW move

500   circumferentially around the septa of dividing pneumococcal cells in either direction at

501   ≈21 ± 8 (SD) nm/s (13). Based on data in that paper, runs extend for ≈28 ± 15 (SD) s (n

502   = 106 cells). In contrast, bPBP2x and FtsW not bound to septa move diffusively

503   throughout the cell membrane (13, 58). In *Spn*, movement of the bPBP2x:FtsW complex

504   at septa is driven by PG synthesis itself and not by treadmilling movement of FtsZ

505   filaments/bundles (13). In the static images here, we do not precisely know how many

506   molecules form the bPBP and FDAA-labeled nodes; but, a reasonable assumption is that

22

507 each node consists of multiple PBPs and regions of PG labeling. With this in mind,

508 circumferentially moving bPBP2x:FtsW complexes likely move linearly in both directions

509 across nodal regions driven by the PG synthesis echoed by FDAA labeling. Aggregate

510 movements of these heterogenous nodal complexes with time would then account for the

511 distributive, non-correlated labeling patterns in two-color pulse labeling experiments (Fig.

512 7B and S7). This and other hypotheses about the composition, organization, and dynamic

513 movement of these nodal PG synthesis complexes in *Spn* and other ovococcal bacteria

514 await future testing.

515

516 **EXPERIMENTAL PROCEDURES**

517 Detailed experimental procedures are described in *SI Appendix, Experimental*

518 *Procedures*, including bacterial strains (Tables S1 and S2) and growth conditions; growth

519 curve analysis; 2D-epifluorescence and phase-contrast microscopy of *Spn* cells;

520 saturating labeling with Boc-FL and Ceph-CT; FDAAs used; quantitative western blotting

521 of relative cellular amounts of WT and fusion proteins; localization in vertical *Spn* cells of

522 the following probes after the indicated labeling times: HADA (≈100 min) then TADA (2.5

523 min), TADA (2.5 min) in cells expressing isfGFP-bPBP2x, sfGFP-bPBP2b, or FtsX'-

524 isfGFP-FtsX', HADA (2.5 min) in cells expressing iHT-bPBP2x or iHT-bPBP2b, TADA (17

525 s); localization of bPBP2x with 7FL in WT *Spn* and a Δ*pbp1b* mutant; localization in

526 vertical *Spn* cells of the following probes after the indicated labeling times: TADA (17 s),

527 TADA (17 s) followed by Boc-FL (7 s), and BADA (40 s) followed by TADA (40 s) followed

528 by HADA (40 s); image acquisition by 3D-SIM; analysis of FDAA node distributions using

23

529 a custom MATLAB GUI; widefield fluorescence microscopy; and bPBP2x and bPBP2b

530 protein purification and antibody production.

531

547

548 **REFERENCES**

549 1. A. J. Loughran, C. J. Orihuela, E. I. Tuomanen, *Streptococcus pneumoniae*: invasion
550 and inflammation. *Microbiol Spectr* **7**, 10.1128/microbiolspec.GPP3-0004-2018
551 (2019).

552 2. J. N. Weiser, D. M. Ferreira, J. C. Paton, *Streptococcus pneumoniae*: transmission,
553 colonization and invasion. *Nat Rev Microbiol* **16**, 355-367 (2018).

24

3. A. Zapun, T. Vernet, M. G. Pinho, The different shapes of cocci. *FEMS Microbiol Rev* **32**, 345-360 (2008).

4. W. Vollmer, O. Massidda, A. Tomasz, The cell wall of *Streptococcus pneumoniae*. *Microbiol Spectr* **7,** 10.1128/microbiolspec.GPP3-0018-2018 (2019).

5. A. J. F. Egan, J. Errington, W. Vollmer, Regulation of peptidoglycan synthesis and remodelling. *Nat Rev Microbiol* **18**, 446-460 (2020).

6. A. J. Egan, J. Biboy, I. van't Veer, E. Breukink, W. Vollmer, Activities and regulation of peptidoglycan synthases. *Phil Trans Royal Soc London. Series B, Biolog Sci* **370**, 10.1098/rstb.2015.0031 (2015).

7. S. J. Siegel, J. N. Weiser, Mechanisms of Bacterial Colonization of the respiratory tract. *Ann Rev Microbiol* **69**, 425-444 (2015).

8. J. L. Rodriguez, A. B. Dalia, J. N. Weiser, Increased chain length promotes pneumococcal adherence and colonization. *Infect Immun* **80**, 3454-3459 (2012).

9. K. H. Berg, D. Straume, L. S. Havarstein, The function of the transmembrane and cytoplasmic domains of pneumococcal penicillin-binding proteins 2x and 2b extends beyond that of simple anchoring devices. *Microbiol* 10.1099/mic.0.078535-0 (2014).

10. B. E. Rued *et al.*, Suppression and synthetic-lethal genetic relationships of Δ*gpsB* mutations indicate that GpsB mediates protein phosphorylation and penicillin-binding protein interactions in *Streptococcus pneumoniae* D39. *Molec Microbiol* **103**, 931-957 (2017).

11. H. C. Tsui *et al.*, Pbp2x localizes separately from Pbp2b and other peptidoglycan synthesis proteins during later stages of cell division of *Streptococcus pneumoniae* D39. *Molec Microbiol* **94**, 21-40 (2014).

12. A. D. Land *et al.*, Requirement of essential Pbp2x and GpsB for septal ring closure in *Streptococcus pneumoniae* D39. *Molec Microbiol* **90**, 939-955 (2013).

13. A. J. Perez *et al.*, Movement dynamics of divisome proteins and PBP2x:FtsW in cells of *Streptococcus pneumoniae*. *Proc Nat Acad Sci USA* **116**, 3211-3220 (2019).

14. D. Straume *et al.*, Class A PBPs have a distinct and unique role in the construction of the pneumococcal cell wall. *Proc Nat Acad Sci USA* **117**, 6129-6138 (2020).

15. M. Aaron *et al.*, The tubulin homologue FtsZ contributes to cell elongation by guiding cell wall precursor synthesis in *Caulobacter crescentus*. *Molec Microbiol* **64**, 938-952 (2007).

25

16. L. P. Potluri, S. Kannan, K. D. Young, ZipA is required for FtsZ-dependent preseptal peptidoglycan synthesis prior to invagination during cell division. *J Bacteriol* **194**, 5334-5342 (2012).

17. R. Wheeler, S. Mesnage, I. G. Boneca, J. K. Hobbs, S. J. Foster, Super-resolution microscopy reveals cell wall dynamics and peptidoglycan architecture in ovococcal bacteria. *Molec Microbiol* **82**, 1096-1109 (2011).

18. A. Taguchi *et al.*, FtsW is a peptidoglycan polymerase that is functional only in complex with its cognate penicillin-binding protein. *Nat Microbiol* **4**, 587-594 (2019).

19. A. J. Meeske *et al.*, SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature* **537**, 634-638 (2016).

20. M. Sjodt *et al.*, Structural coordination of polymerization and crosslinking by a SEDS-bPBP peptidoglycan synthase complex. *Nat Microbiol* **5**, 813-820 (2020).

21. H. C. Tsui *et al.*, Suppression of a deletion mutation in the gene encoding essential PBP2b reveals a new lytic transglycosylase involved in peripheral peptidoglycan synthesis in *Streptococcus pneumoniae* D39. *Molec Microbiol* **100**, 1039-1065 (2016).

22. R. A. Daniel, J. Errington, Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. *Cell* **113**, 767-776 (2003).

23. Y. P. Hsu *et al.*, Full color palette of fluorescent d-amino acids for in situ labeling of bacterial cell walls. *Chem Sci* **8**, 6313-6321 (2017).

24. E. Kuru *et al.*, In Situ probing of newly synthesized peptidoglycan in live bacteria with fluorescent D-amino acids. *Angew Chem Int Ed Engl* **51**, 12519-12523 (2012).

25. S. Sharifzadeh *et al.*, Novel electrophilic scaffold for imaging of essential penicillin-binding proteins in *Streptococcus pneumoniae*. *ACS Chem Biol* **12**, 2849-2857 (2017).

26. S. Sharifzadeh *et al.*, Chemical tools for selective activity profiling of bacterial penicillin-binding proteins. *Meth Enzymol* **638**, 27-55 (2020).

27. S. Holden, Probing the mechanistic principles of bacterial cell division with super-resolution microscopy. *Curr Opin Microbiol* **43**, 84-91 (2018).

28. M. Alcorlo, D. Straume, J. Lutkenhaus, L. S. Havarstein, J. A. Hermoso, Structural characterization of the essential cell division protein FtsE and its interaction with FtsX in *Streptococcus pneumoniae*. *mBio*, **11**, 10.1128/mBio.01488-20 (2020).

29. B. E. Rued *et al.*, Structure of the large extracellular loop of Ftsx and its interaction with the essential peptidoglycan hydrolase Pcsb in *Streptococcus pneumoniae*. *mBio*, **10** 10.1128/mBio.02622-18 (2019).

30. L. T. Sham, S. M. Barendt, K. E. Kopecky, M. E. Winkler, Essential PcsB putative peptidoglycan hydrolase interacts with the essential FtsX^{Spn} cell division protein in *Streptococcus pneumoniae* D39. *Proc Nat Acad Sci USA*, **108**, E1061-1069 (2011).

31. L. T. Sham, K. R. Jensen, K. E. Bruce, M. E. Winkler, Involvement of FtsE ATPase and FtsX extracellular loops 1 and 2 in FtsEX-PcsB complex function in cell division of *Streptococcus pneumoniae* D39. *mBio* **4**, 10.1128/mBio.00431-13 (2013).

32. S. G. Bartual *et al.*, Structural basis of PcsB-mediated cell separation in *Streptococcus pneumoniae*. *Nature Comm* **5**, 3842 (2014).

33. W. L. Ng, K. M. Kazmierczak, M. E. Winkler, Defective cell wall synthesis in *Streptococcus pneumoniae* R6 depleted for the essential PcsB putative murein hydrolase or the VicR (YycF) response regulator. *Molec Microbiol* **53**, 1161-1175 (2004).

34. G. Komis *et al.*, Superresolution live imaging of plant cells using structured illumination microscopy. *Nature Prot* **10**, 1248-1263 (2015).

35. A. D. Land, Q. Luo, P. A. Levin, Functional domain analysis of the cell division inhibitor EzrA. *PloS One* **9**, 10.1371/journal.pone.0102616 (2014).

36. R. M. Cleverley *et al.*, The cell cycle regulator GpsB functions as cytosolic adaptor for multiple cell wall enzymes. *Nature Comm* **10**, 10.1038/s41467-018-08056-2 (2019).

37. A. D. Land, M. E. Winkler, The requirement for pneumococcal MreC and MreD is relieved by inactivation of the gene encoding PBP1a. *J Bacteriol* **193**, 4166-4179 (2011).

38. J. J. Zheng, A. J. Perez, H. T. Tsui, O. Massidda, M. E. Winkler, Absence of the KhpA and KhpB (JAG/EloR) RNA-binding proteins suppresses the requirement for PBP2b by overproduction of FtsA in *Streptococcus pneumoniae* D39. *Molec Microbiol* **106**, 793-814 (2017).

39. O. Kocaoglu *et al.*, Selective penicillin-binding protein imaging probes reveal substructure in bacterial cell division. *ACS Chem Biol* **7**, 1746-1753 (2012).

40. X. Y. Gao, X. Y. Zhi, H. W. Li, H. P. Klenk, W. J. Li, Comparative genomics of the bacterial genus *Streptococcus* illuminates evolutionary implications of species groups. *PloS One* **9**, 10.1371/journal.pone.0101229 (2014).

41. W. Ludwig *et al.*, The phylogenetic position of *Streptococcus* and *Enterococcus*. *J Gen Microbiol* **131**, 543-551 (1985).

42. O. Massidda, L. Novakova, W. Vollmer, From models to pathogens: how much have we learned about *Streptococcus pneumoniae* cell division? *Environ Microbiol* **15**, 3133-3157 (2013).

43. A. Fleurie *et al.*, Interplay of the serine/threonine-kinase StkP and the paralogs DivIVA and GpsB in pneumococcal cell elongation and division. *PLoS Genet* **10**, 10.1371/journal.pgen.1004275 (2014).

44. D. Straume, G. A. Stamsas, K. H. Berg, Z. Salehian, L. S. Havarstein, Identification of pneumococcal proteins that are functionally linked to penicillin-binding protein 2b (PBP2b). *Molec Microbiol* 10.1111/mmi.13543 (2016).

45. B. David *et al.*, PBP2b plays a key role in both peripheral growth and septum positioning in Lactococcus lactis. *PloS One* **13**, 10.1371/journal.pone.0198014 (2018).

46. K. H. Berg, G. A. Stamsas, D. Straume, L. S. Havarstein, Effects of low PBP2b levels on cell morphology and peptidoglycan composition in *Streptococcus pneumoniae* R6. *J Bacteriol* **195**, 4342-4354 (2013).

47. Y. R. Brunet, X. Wang, D. Z. Rudner, SweC and SweD are essential co-factors of the FtsEX-CwlO cell wall hydrolase complex in *Bacillus subtilis*. *PLoS Genet* **15**, 10.1371/journal.pgen.1008296 (2019).

48. J. Meisner *et al.*, FtsEX is required for CwlO peptidoglycan hydrolase activity during cell wall elongation in *Bacillus subtilis*. *Molec Microbiol* **89**, 1069-1083 (2013).

49. S. Pichoff, S. Du, J. Lutkenhaus, Roles of FtsEX in cell division. *Res Microbiol* **170**, 374-380 (2019).

50. X. Yang *et al.*, GTPase activity-coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell wall synthesis. *Science* **355**, 744-747 (2017).

51. B. Söderström, H. Chan, D. O. Daley, Super-resolution images of peptidoglycan remodelling enzymes at the division site of *Escherichia coli*. *Curr Genet* **65**, 99-101 (2019).

52. V. A. Lund *et al.*, Molecular coordination of *Staphylococcus aureus* cell division. *eLife* **7**, 10.7554/eLife.32057 (2018).

53. J. M. Monteiro *et al.*, Peptidoglycan synthesis drives an FtsZ-treadmilling-independent step of cytokinesis. *Nature* **554**, 528-532 (2018).

28

54. M. P. Strauss *et al.*, 3D-SIM super resolution microscopy reveals a bead-like arrangement for FtsZ and the division machinery: implications for triggering cytokinesis. *PLoS Biol* **10**, 10.1371/journal.pbio.1001389 (2012).

55. A. W. Bisson-Filho *et al.*, Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. *Science* **355**, 739-743 (2017).

56. J. Garcia-Lara *et al.*, Supramolecular structure in the membrane of *Staphylococcus aureus*. *Proc Nat Acad Sci USA* **112**, 15725-15730 (2015).

57. F. Weihs *et al.*, Heterogeneous localisation of membrane proteins in *Staphylococcus aureus*. *Sci Reports* **8**, 10.1038/s41598-018-21750-x (2018).

58. J. W. McCausland *et al.*, Treadmilling FtsZ polymers drive the directional movement of sPG-synthesis enzymes via a Brownian ratchet mechanism. *BioRxiv* 10.1101/857813 (2020).

FIGURE LEGENDS

Fig. 1. 3D-SIM of vertically oriented *Spn* cells labeled with FDAAs reveals spatially distinct, concentric midcell ring intermediates of TP activity at different stages of division. (*A*) Schematic of labeling procedure (*top*). WT cells (IU1945) were labeled with 125 µM HADA for several generations (old cell wall; cyan), washed, and labeled with 125 µM TADA for 2.5 min (new cell wall from ≈7% of a generation; red). Cells were fixed and prepared for vertical cell imaging by 3D-SIM (see *SI Appendix, Experimental Procedures*). Representative images are shown of rings from pre-, early, and late-division stages estimated by the diameters of outer TADA rings. Left images, HADA and TADA channels overlaid; right images TADA channel only. (*B*) Representative horizontally oriented cells from the same field as (*A)* locating the concentric rings at constricting midcell regions. *(C)* Montage of manually sorted *Spn* cells in different stages of cell division. Only the TADA is shown, and images are representative of >50 cells from more than three independent biological replicates. *(D)* Diagrammatic summary based on data in *Results* of the

29

716 organization of sPG and pPG synthesis in *Spn*, including concentric rings of new PG

717 synthesis, membrane invagination, and proteins located in this study. Curved arrows

718 indicate the image rotations indicated.

719 **Fig. 2.** bPBP2x or bPBP2b locate to the leading edge of the septal ring annulus or to

720 the outer peripheral ring, respectively, at midcell in vertically oriented *Spn* cells. Cells

721 expressing isfGFP-bPBP2x (IU11157) or sfGFP-bPBP2b (IU9965) were labeled with 125

722 µM TADA for 2.5 min, fixed, and prepared for vertical cell imaging by 3D-SIM (see *SI*

723 *Appendix, Experimental Procedures*). (*A*) Representative images of cells (6 to 29) at

724 different stages of division. isfGFP-bPBP2x and sfGFP-bPBP2b, pseudo-colored cyan;

725 TADA labeling, pseudo-colored red. Brightness and contrast were manually adjusted to

726 show signals associated with division rings and to reduce background lacking TADA

727 labeling. Progression of cell division was indicated by the separation of the inner (septal)

728 and outer (peripheral) rings. Cellular amounts of fluorescent-proteins produced relative to

729 untagged WT protein in a control strain (see Fig. S3C) are in parentheses. (*B*)

730 Quantitation of nodal distributions of isfGFP-bPBP2x and sfGFP-bPBP2b (but not TADA)

731 in predivisional *Spn* cells with single, overlapping rings of TADA and GFP labeling.

732 Distributions of ring diameters (left), nodes per ring (middle), and arc distances (right)

733 were determined as described in *SI Appendix, Experimental Procedures.* Graphs show

734 median (interquartile range; whiskers, 5th-95th percentile; +, mean). Means ± SD from two

735 independent biological replicates are shown below the graphs and compiled in Table S3.

736 Differences between means were compared by a two-tailed *t* test (GraphPad Prism). *,

737 *P*<0.05 and ***, *P*<0.001.

738   **Fig. 3.** PBP TP activity is organized into regular nodes at the midcell of predivisional

739   *Spn* cells. WT cells (IU1945) were incubated for 17 s with a low concentration of TADA

740   (45.5 µM), fixed, and prepared for vertical cell imaging by 3D-SIM (see *SI Appendix,*

741   *Experimental Procedures*). (*A*) Representative images of 12 predivisional *Spn* cells

742   pulsed-labeled with TADA. Mean ring diameter ± SD of >60 cells from >3 independent

743   biological replicates was determined as described in *SI Appendix, Experimental*

744   *Procedures*. (*B*) Distributions of arc distances (*top*) and number of nodes per ring (*bottom*)

745   determined for the data set of cells in (*A*) and compiled in Table S3.

746   **Fig. 4.** TADA-labeled nodes at the midcell of vertically oriented predivisional *Spn* cells

747   are observed by widefield microscopy and at different 3D-SIM filter settings. WT cells

748   (IU1945) were labeled with 45.5 µM TADA for 17 s, fixed, and prepared for vertical cell

749   imaging as described in *SI Appendix, Experimental Procedures*. (*A*) Representative

750   widefield-microscopy images of four separate cells, before and after deconvolution.

751   Arrows point to nodes of TADA labeling. (*B*) 3D-SIM images processed with different

752   Wiener filter settings of the midcell of a single TADA-labeled vertical cell (top row) or a

753   background region lacking a cell (bottom row). Red box, 0.001 Wiener filter setting used

754   throughout this paper for 3D-SIM imaging. Scale bar = 1 µm.

755   **Fig. 5.** FtsZ-depleted cells have enlarged midcell diameters, and a majority of cells

756   have irregularly spaced nodes of TP activity. IU8124 (Δ*ftsZ*//P$_{Zn}$-*ftsZ*$^+$) cells were grown

757   in BHI broth containing 0.2 mM ZnCl$_2$ to ectopically express FtsZ for 12 h prior to

758   depletion. IU8124 cells were diluted into BHI broth without added ZnCl$_2$ and incubated to

759   deplete FtsZ (see *SI Appendix, Experimental Procedures*). After 2.5 h, WT (IU1945) and

760   FtsZ-depleted cells were labeled with 45.5 µM TADA for 17 s, fixed, and prepared for

31

761 vertical cell imaging by 3D-SIM (*SI Appendix, Experimental Procedures*). (*A*)

762 Representative 3D-SIM images of nodes of TP activity in midcell rings of predivisional

763 cells. Labeling was classified as regular (Reg) or irregular (Irreg) with gaps of varying

764 sizes between nodes (white arrow) as described in *Results.* Percentages refer to analysis

765 of 76 WT and 47 FtsZ-depleted cells*.* (*B*) Distributions of midcell ring diameters (left),

766 nodes per ring (middle), and arc distances (right) of WT and FtsZ-depleted cells were

767 determined as described in *SI Appendix, Experimental Procedures*. Ring diameters and

768 nodes per ring were determined for 76 WT cells and for 19 or 28 Reg or Irreg FtsZ-

769 depleted cells, respectively. Arc distance were determined for 746 nodes in WT cells and

770 for 230 or 293 nodes in Reg or Irreg FtsZ-depleted cells, respectively. Graphs show

771 median (interquartile range; whiskers, 5th-95th percentile; +, mean). Means ± SD are

772 compiled in Table S3. Differences in means relative to WT were determined by one-way

773 ANOVA with Bonferonni's multiple comparison posttest (GraphPad Prism). ***, *P*<0.001.

774 **Fig. 6.** Arc distance between regularly spaced midcell TP nodes is constant in

775 predivisional *Spn* mutants with decreased or increased diameters compared to WT.

776 Mutant strains of *S. pneumoniae* with altered midcell diameters were labeled with 45.5

777 μM TADA for 17 s, fixed, and prepared for vertical cell imaging by 3D-SIM as described

778 in *SI Appendix*, *Experimental Procedures*. Strains used were: Δ*pbp1a* (K164), Δ*khpA*

779 (E751), WT (IU1945), *ftsZ*(G107S) (IU10612), *ezrA*(T506I) (IU11034), and *gpsB*(K96N)

780 (IU11956) (Table S1). (*A*) Representative 3D-SIM images of TP activity in midcell rings

781 of predivisional cells. Percentages indicate the frequency of regularly spaced nodes (Reg)

782 or irregularly spaced nodes (Irreg) with gaps (see white arrows) for the number of cells

783 analyzed of each strain. Irregularly spaced nodes were observed in the majority of

32

784   *ftsZ*(G107S), *ezrA*(T506I) and *gpsB*(K96N) cells, whose diameters were greater than that

785   of WT (see Fig. S4 and Table S3). (*B*) Distributions of arc distances between TP nodes

786   of WT and mutant strains were determined as described in *SI Appendix, Experimental*

787   *Procedures*. Graphs show median (interquartile range; whiskers, 5th-95th percentile; +,

788   mean). Means ± SD are compiled in Table S3. Cells with irregular midcell nodal patterns

789   exhibit large SDs. Differences in means relative to WT were determined by one-way

790   ANOVA with Bonferonni's multiple comparison posttest (GraphPad Prism). ***, *P*<0.001.

791   (*C*) Linear relationship between ring diameter (X-axis) versus nodes per ring (Y-axis) for

792   WT and mutant strains with regular nodal patterns. Colored crosses represent

793   measurements from single rings determined as described in *SI Appendix, Experimental*

794   *Procedures* and shown in Fig. S4. Line of best fit and r^2 values for the combined data set

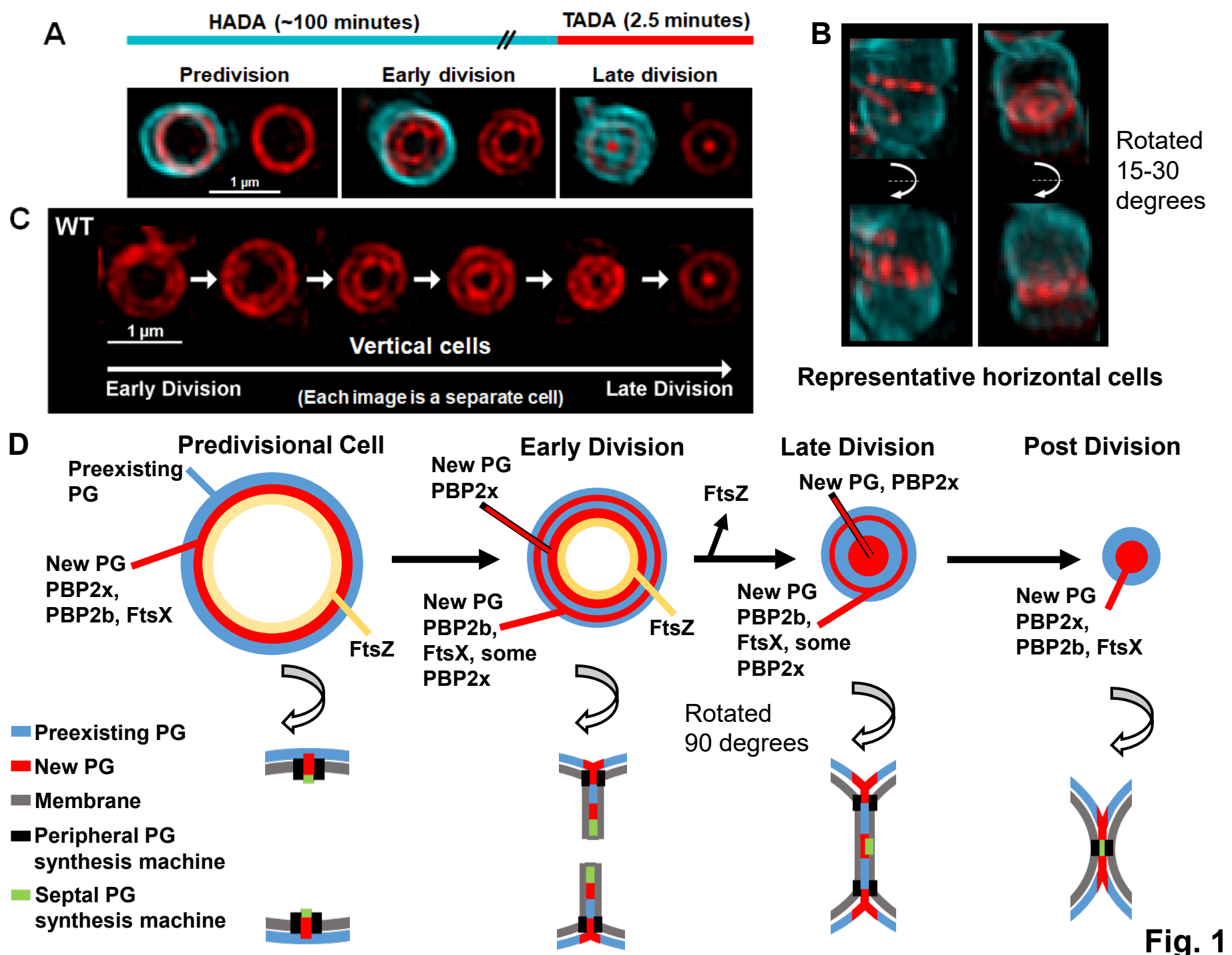795   were determined using GraphPad Prism.

796   **Fig. 7.** sfGFP-bPBP2b and Bocillin-FL (Boc-FL) labeling are organized in nodal

797   patterns at the midcell of predivisional *Spn* cells. Cells expressing sfGFP-bPBP2b

798   (IU9965) were labeled with 45.5 µM of TADA for 17 s, fixed, and prepared for vertical cell

799   imaging by 3D-SIM as described in *SI Appendix*, *Experimental Procedures*. WT (IU1945)

800   cells were pulse-labeled with TADA for 17 s, followed by labeling with 2 µg/mL of Boc-FL

801   for 21 s, fixed, and prepared for imaging (see *SI, Appendix, Experimental Procedures*).

802   (*A*) Representative images of IU9965 localizing sfGFP-bPBP2b and TADA as nodes.

803   Each row is a separate cell. (*B*) Representative images of WT cells localizing Boc-FL and

804   TADA as nodes. Each row is a separate cell. (*C*) Distributions of ring diameters (left),

805   nodes per ring (middle), and arc distances (right) for WT cells labeled with TADA alone,

806   IU9965 (sfGFP-bPBP2b) labeled with TADA, IU9965 (sfGFP-bPBP2b) not labeled with
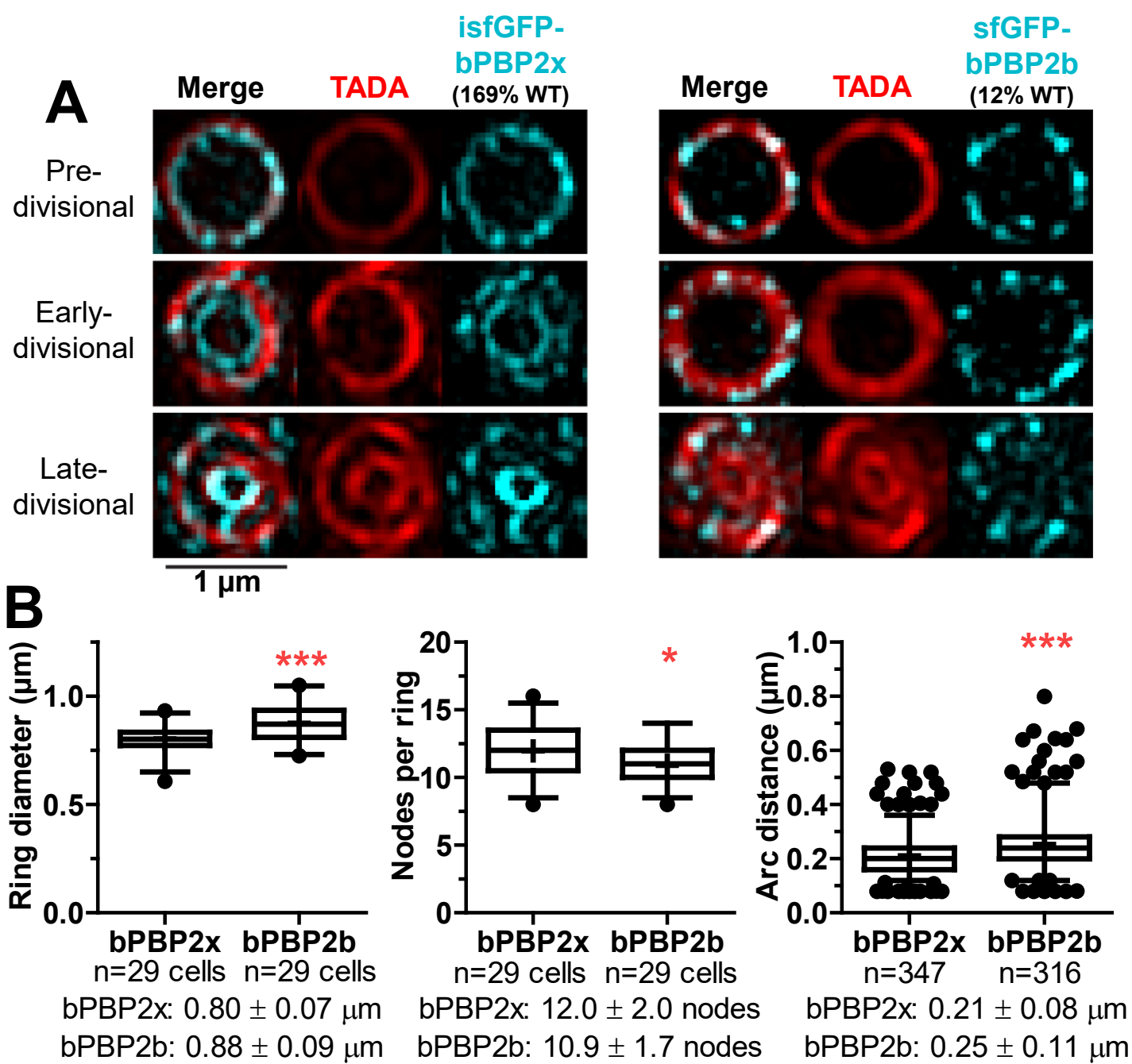
807 TADA, WT labeled with TADA followed by Boc-FL, and WT labeled with Boc-FL alone.

808 Measurements were performed as described in *SI Appendix*, *Experimental Procedures*.

809 Graphs show median (interquartile range; whiskers, 5th-95th percentile; +, mean). Means

810 ± SD are compiled in Table S3. Differences in means relative to WT labeled with TADA

811 were determined by one-way ANOVA with Bonferonni's multiple comparison posttest

812 (GraphPad Prism). *, $P<0.05$; ***, $P<0.001$. Correlation coefficient analyses are presented

813 in Fig. S5 and *Results*.

814 **Fig. 8.** The nodal pattern of regularly spaced TP activity at the midcell of predivisional

815 cells is conserved in other ovoid-shaped bacterial species. *S. pneumoniae* (*Spn*, IU1945),

816 *S. mitis* (*Smi*, ATCC 49456) and *E. faecalis* (*Efa*, ATCC 51299) cells were labeled with

817 45.5 µM TADA for 17 s, fixed, and prepared for vertical cell imaging by 3D-SIM (see *SI*

818 *Appendix*, *Experimental Procedures*). (A) Representative images of two predivisional

819 cells pulse-labeled with TADA are shown for *Spn* (left), *S. mitis* (middle), and *E. faecalis*

820 (right) from the total number of cells analyzed (bottom). (B) Distributions of ring diameters

821 (left), nodes per ring (middle), and arc distances of ovoid-shaped cells. Measurements

822 were performed as described in *SI Appendix*, *Experimental Procedures*. Graphs show

823 median (interquartile range; whiskers, 5th-95th percentile; +, mean). Means ± SD are

824 compiled in Table S3. Differences in means relative to *Spn* were determined by one-way

825 ANOVA with Bonferonni's multiple comparison posttest (GraphPad Prism).**, $P<0.01$ and
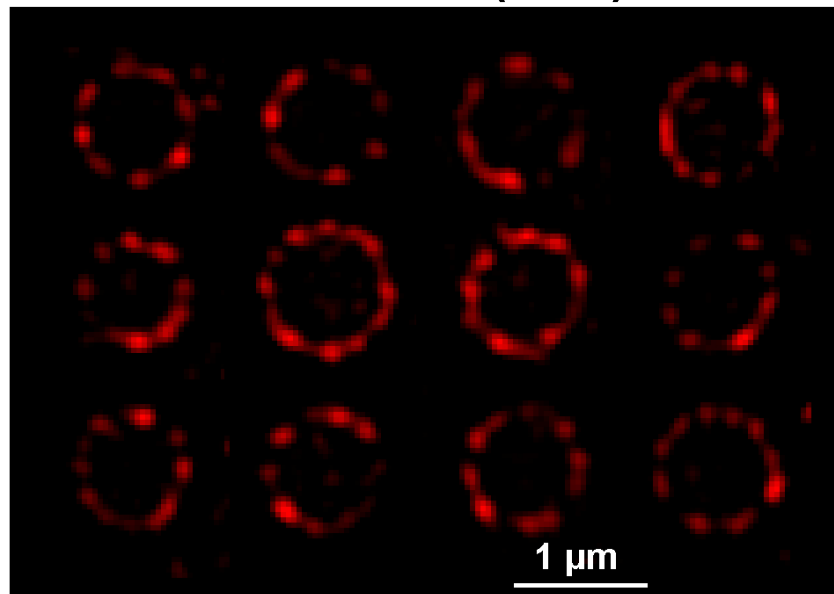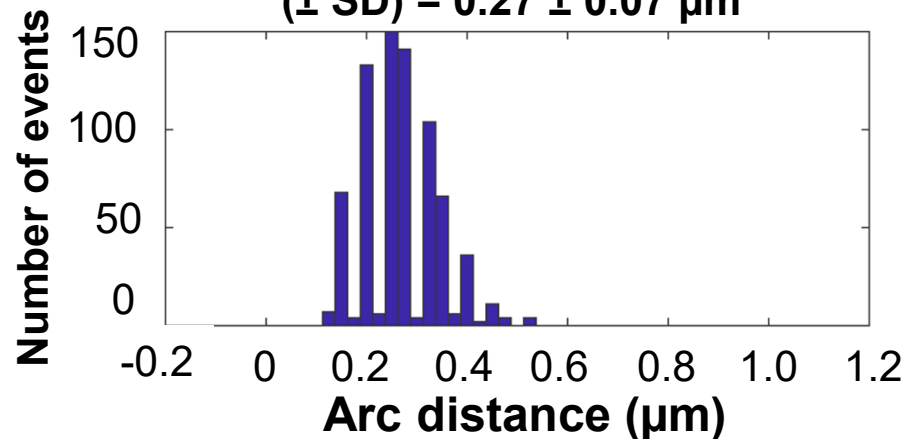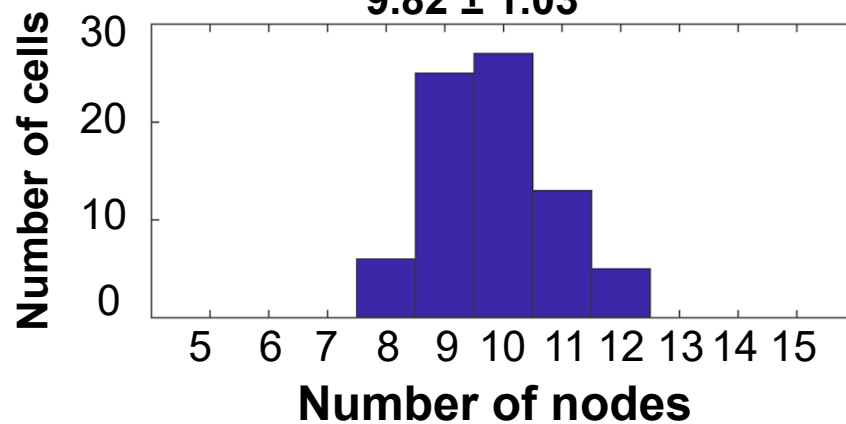
826 ***, $P<0.001$.

827 **Fig. 9.** Summary diagram of the localization PG synthesis in *Spn* based on results in

828 this paper. (*A*) Separation of sPG and pPG (elongasome) machines at the midcell of

829 dividing *Spn* cells. Cell division begins at the midcell equator of newly divided
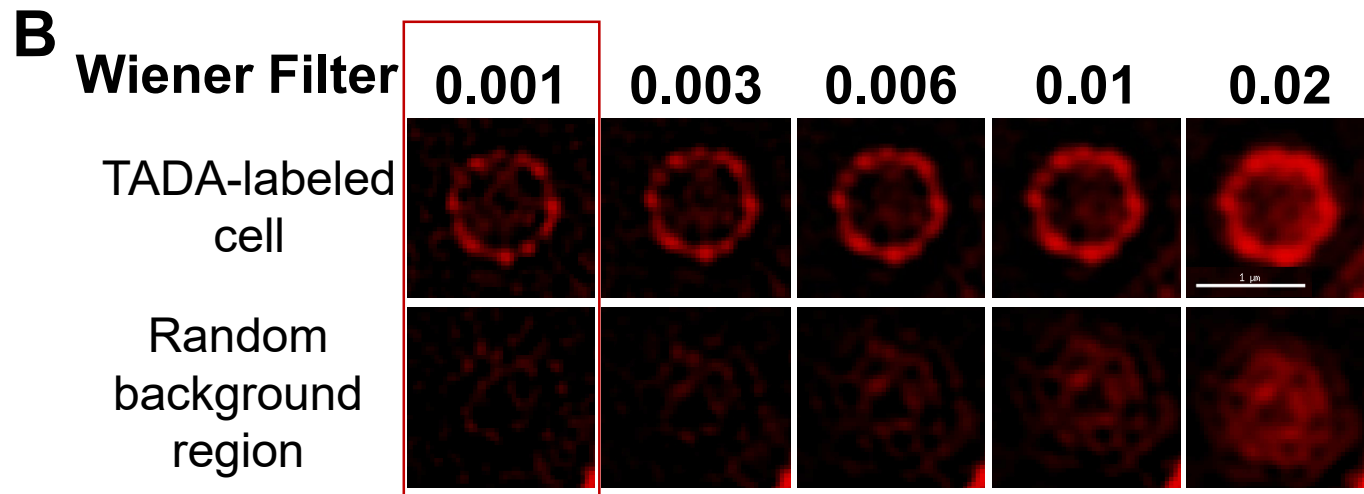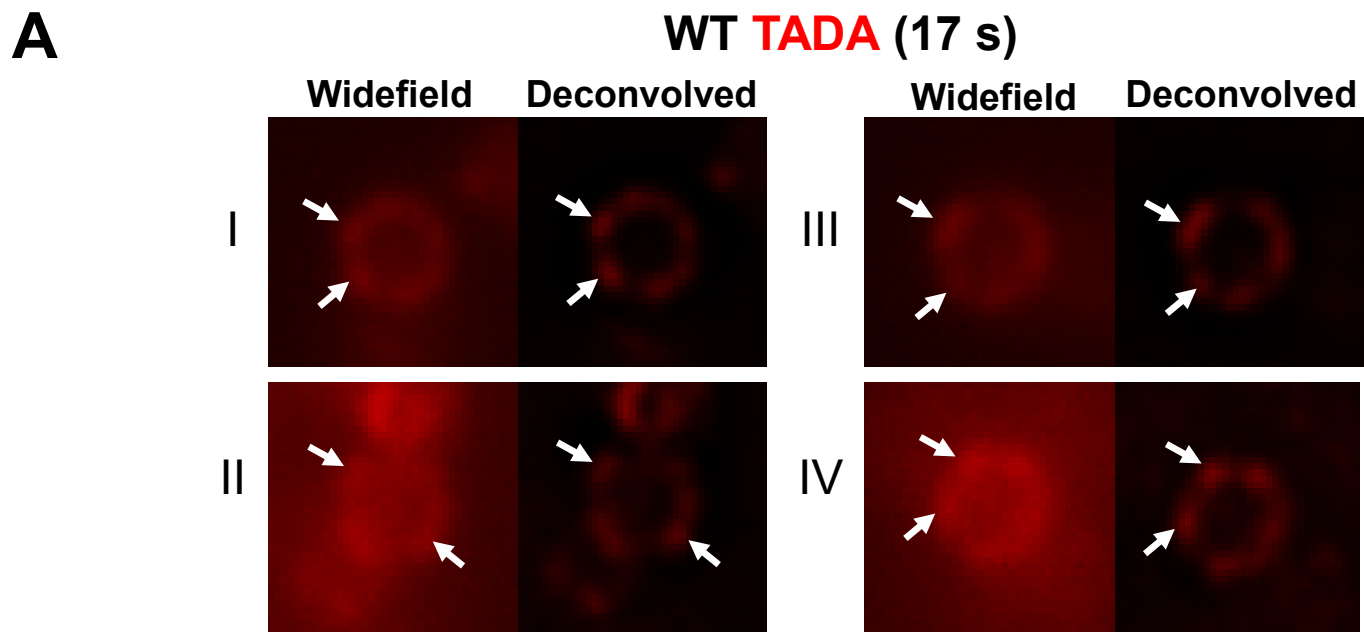
830 predivisional cells in FtsZ-organized divisome rings containing the components of the

831 sPG and pPG synthesis machines. Early in cell division the septal annulus forms and

832 begins to close. The leading edge of the closing septal annulus separates the sPG

833 machine from the pPG synthesis machine that remains at the outer edge of the annulus

834 and elongates the PG outward from the midcell. The sPG machine contains bPBP2x TP,

835 its partner FtsW GT, and other components, while the pPG synthesis machine is made

836 up of bPBP2b TP and its partner RodA GT, other "Rod" complex proteins, and the

837 FtsEX:PcsB remodeling PG hydrolase. The constricting FtsZ ring tracks with the leading

838 edge of the septal annulus, such that the outer peripheral ring is not organized by FtsZ

839 beyond the predivisional stage. Later in division, FtsZ remaining at the septum migrates

840 to the developing equatorial rings in daughter cells. The inner sPG synthesis machine

841 constricts into a dot surrounded by the closing outer pPG synthesis ring, which eventually

842 constricts and merges with the sPG dot as the new cell pole is completed and the PG

843 synthesis enzymes migrate to the new equatorial rings in daughter cells (see Fig. 1D

844 also). (*B*) In predivisional cells, TP activity, sPG synthesis complexes, and pPG

845 complexes are organized into a pattern of regularly spaced nodes (red circles). The

846 placement of PBPs and TP activity is not fixed at single positions on equators of early

847 divisional cells, but rather, is dynamic and distributive, likely driven by PG synthesis itself

848 (13). Aggregate movements of these heterogenous nodal complexes with time would

849 account for the distributive, non-correlated nodal patterns observed in two-color labeling

850 experiments. A constant distance (≈0.27 μm) is maintained between nodes of PBP TP

851 activity and between the PBPs themselves. Midcell rings of predivisional cells with smaller

852 or larger diameters contain fewer or more nodes, respectively, than WT (≈ 10 nodes).

35

A HADA (~100 minutes) // TADA (2.5 minutes)

Predivision | Early division | Late division

1 µm

B Rotated 15-30 degrees

Representative horizontal cells

C WT

1 µm

Vertical cells

Early Division → Late Division

(Each image is a separate cell)

D Predivisional Cell | Early Division | Late Division | Post Division

Preexisting PG

New PG PBP2x, PBP2b, FtsX

FtsZ

New PG PBP2x

New PG PBP2b, FtsX, some PBP2x

FtsZ

FtsZ

New PG, PBP2x

New PG PBP2b, FtsX, some PBP2x

New PG PBP2x, PBP2b, FtsX

Rotated 90 degrees

Preexisting PG
New PG
Membrane
Peripheral PG synthesis machine
Septal PG synthesis machine

Fig. 1

Fig. 2

A

WT TADA (17 s)

1 µm

Mean ring diameter (±SD) = 0.84 ±0.05 µm

B

Mean arc distance between nodes
(± SD) = 0.27 ± 0.07 µm

Mean nodes per ring (± SD) =
9.82 ± 1.03

Fig. 3

A

WT TADA (17 s)

	Widefield	Deconvolved		Widefield	Deconvolved
I			III		
II			IV		

B

Wiener Filter 0.001 0.003 0.006 0.01 0.02

TADA-labeled cell

Random background region

1 µm

Fig. 4

A

WT

FtsZ-depleted
(2.5 h depletion)

Regular (94%)

Regular (40%)   Irregular (60%)

1 µm

B

Ring diameter (µm)

*** ***

WT Reg Irreg

FtsZ-depleted

Nodes per ring

***

WT Reg Irreg

FtsZ-depleted

Arc distance (µm)

*** ***

WT Reg Irreg

FtsZ-depleted

Fig. 5

Fig. 6

A

TADA (17 s)   sfGFP-bPBP2b   Overlay

1 μm

B

TADA (17 s)   Boc-FL (21 s)   Overlay

1 μm

C

Ring diameter (μm)

WT (TADA)
sfGFP-bPBP2b (TADA)
sfGFP-bPBP2b (sfGFP)
WT + Boc-FL (TADA)
WT + Boc-FL (Boc-FL)

Nodes per ring

*

WT (TADA)
sfGFP-bPBP2b (TADA)
sfGFP-bPBP2b (sfGFP)
WT + Boc-FL (TADA)
WT + Boc-FL (Boc-FL)

Arc distance (μm)

***   *

WT (TADA)
sfGFP-bPBP2b (TADA)
sfGFP-bPBP2b (sfGFP)
WT + Boc-FL (TADA)
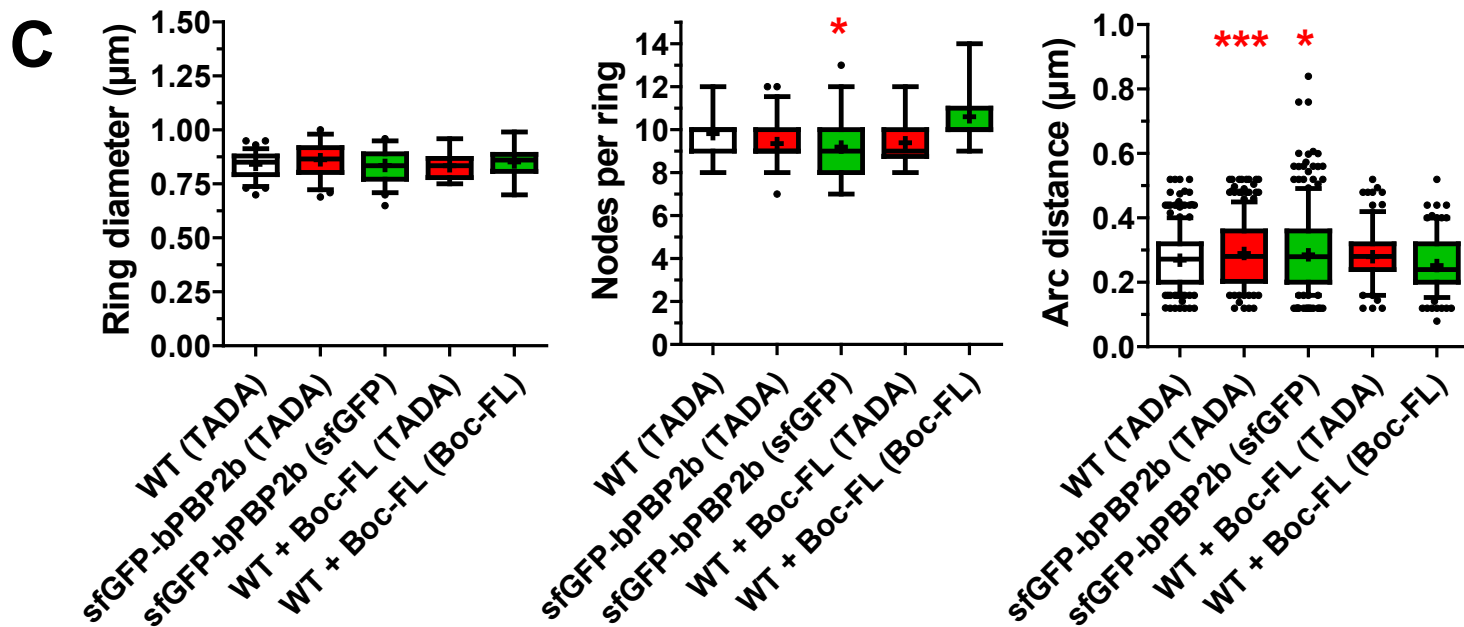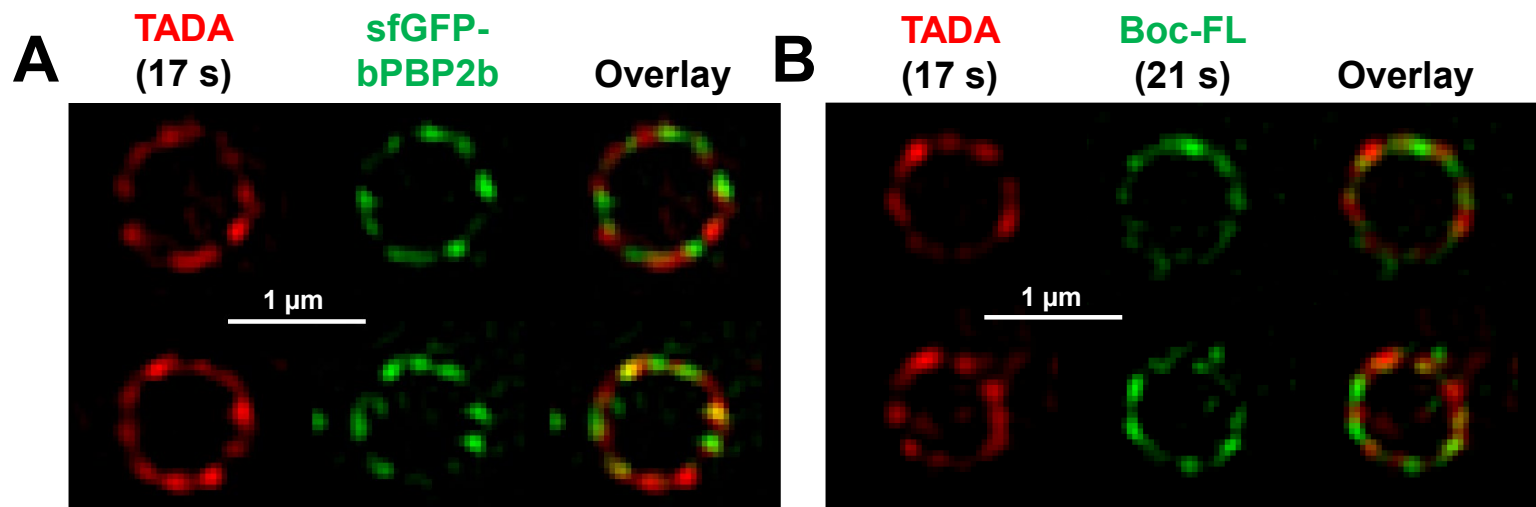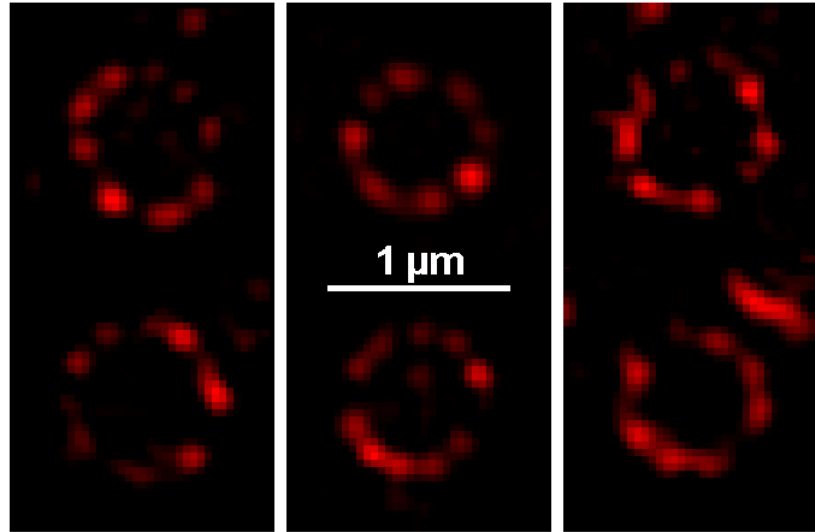WT + Boc-FL (Boc-FL)

Fig. 7

A *S. pneumoniae*   *S. mitis*   *E. faecalis*

1 µm

n (cells) =   76   19   20

B

Ring diameter (µm)

*** ***

Spn Smi Efa

Nodes per ring

** ***

Spn Smi Efa

Arc distance (µm)

Spn Smi Efa

Fig. 8

A

Predivisional **Early Division** **Late Division**

- - - Tracks of newly synthesized PG
- Certain PG hydrolases (FtsEX:PcsB) ⎫ Peripheral PG
- Elongation synthases (bPBP2b:RodA) ⎬ synthesis
- Septal synthases (bPBP2x:FtsW) and FtsZ ⎭ machine parts
- Ring of closing septal annulus

B

Average arc distance between TADA nodes
0.27 (±0.07) μm

Predivisional cells

- Pre-existing PG
- Membrane
- TADA-labeled TP activity

Larger Diameter (More Nodes) ⟷ Smaller Diameter (Fewer Nodes)

Fig. 9