Supporting information

Construction of strains and plasmids

To create pTC67, a PCR product obtained with primers *cc2570-162F* (gcatgggcaagcttgaggacggcgtcatgt) and *cc2570+512F* (gaggccgtggtaccatagaggcgggcg), that includes 512 bp of *cc2570* coding sequence and 162 bp upstream of its ATG codon, was cut with HindIII-KpnI and inserted between the same sites of pBGent (Gent^R). Conjugation of strain PC7167 (CB15 *divA305*) with strain CJW2130 (*E. coli* S17-1 carrying pTC67) resulted in the insertion of pTC67 at the *cc2570* locus of PC7167, which is about 10-kb downstream the *ftsI* gene (previously called *divA*), maintaining a full copy of the coding sequence of *cc2570* and yielding strain CJW2142. A similar strain construction was obtained by insertion of pTC67 into wild-type CB15N by conjugation, creating CJW2138. CJW2138 cells were indistinguishable from wild-type cells with respect to cell morphology and growth rate, verifying that insertion of pTC67 does not interfere with cell division. To move *divA305* (*ftsI*(Ts)) mutation into a synchronizable cell background, CB15N was transduced with a ΦCR30 phage lysate prepared from strain CJW2142, and transductants were selected for gentamycin resistance, creating strain CJW2141. The presence of *divA305 (ftsI*(Ts)*)* mutation in CJW2141 was confirmed by DNA sequencing. Strain CJW2577 expressing *ftsZ-yfp* under the control of *Pvan* was obtained by transduction of CJW2141 with a ΦCR30 phage lysate prepared from strain CJW1438.

To construct pTC34-3, the *ftsI* coding region (1770-bp) was PCR amplified with primers *egfp-pbp3D* (ccgctcaagcttcgatgagcctctcgaacctgggtccc) and *egfp-pbp3R* (gttgaacaggtaccacagtcttttggtcatagg), cleaved with HindIII and KpnI, and cloned into pXGFP4C1 (Kan^R) using the same sites. pTC34-3 was then inserted into the *xylX* locus of CB15N by

transformation to create strain CJW1822 carrying a *gfp-ftsI* fusion under the control of the xylose-inducible promoter (*Pxyl*) as a second *ftsI* copy on the chromosome.

To obtain pTC49, a DNA fragment containing the entire *ftsI* coding region was first obtained by PCR with primers *egfp-pbp3D* and *egfp-pbp3R* (see above) and cloned into the TOPO pCR2.1 vector (Invitrogen). This plasmid was used as template to create a S296A mutation by site-directed mutagenesis with primers *pbp3*-S296Af (gtctacgagatgggcgcgaccttcaaggccttc) and *pbp3*-S296Ar (gaaggccttgaaggtcgcgcccatctcgtagac), digested with HindIII and KpnI, and the $ftsI_{S296A}$ fragment cloned between the same sites of pXGFP4C1. pTC49 was then inserted into the *xylX* locus of CB15N by transformation to generate strain CJW2136 carrying a $gfp\text{-}ftsI_{S296A}$ fusion under the control of *Pxyl*. Strains CJW1823 and CJW2137 were obtained by transduction of strains CJW1822 and CJW2136, respectively, with a ΦCR30 phage lysate prepared from strain CJW1550 to insert the *ftsZ-mcherry* fusion under the control of *Pvan* at the *vanA* chromosomal locus. Strains CJW2235 and CJW2236 were obtained by mating CB15N carrying the *ftsI*(Ts) mutation (CJW2141) with *E. coli* strains CJW1821 (*Pxyl-gfp-ftsI*) and CJW2127 ($Pxyl\text{-}gfp\text{-}ftsI_{S296A}$), respectively.

To create pTC71, the *cc*2561 coding region, corresponding to the region upstream the *ftsI* gene locus, was PCR amplified with primers *cc*2561-200F (ctggactagtgggccggcaaggcacccg) and *cc*2561+449R (gaggctcatatgaacgccccctggac), digested with SpeI and NdeI, and a *gfp-ftsI* DNA fragment was released from pTC34-3 by digestion with NdeI and PstI. A triple ligation of the two fragments was then performed into pNPTS138 digested with SpeI and PstI. pTC71 was inserted into CB15N by transformation and selection for Kan^R. Transformants were then incubated in PYE medium without antibiotics and streaked on PYE plates containing 3% sucrose in order to select for those in which the pNPTS138 vector has been excised, maintaining a *gfp-*

ftsI fusion under control of the native *ftsI* promoter, yielding strain CJW2144. Strain CJW2144 was confirmed by PCR and DNA sequencing. To create strain CJW2143, a ΦCR30 phage lysate prepared from strain YB1585 was used to replace native *ftsZ* with *ftsZ* under the control of *Pxyl* by transduction.

A *gfp-ftsI*$_{1\text{-}90}$ fragment was PCR amplified from plasmid pTC34-3 using primers *gfp*+1F (ggcatatggtgagcaagggcgaggagc) and *cc*2560+270R (ccgggatcctcaatcgccccgcgcgccttcgac) and cloned into pXGFP4C1 using Nde1 and Kpn1 restriction sites. The plasmid pRP3 was then inserted into the *xylX* locus of CB15N by transformation to create strain CJW2851 (CB15N *Pxyl*::*Pxyl-gfp-ftsI*$_{1\text{-}90}$) carrying the *gfp-ftsI*$_{1\text{-}90}$ fusion under the control of *Pxyl*. A ΦCR30 phage lysate was prepared from strain CJW2851 and used to introduce *gfp-ftsI*$_{1\text{-}90}$ under the control of *Pxyl* by transduction into CJW2141 (CB15N *ftsI*(Ts)) to obtain strain CJW2852 (CB15N *ftsI*(Ts) *Pxyl*::*Pxyl-gfp-ftsI*$_{1\text{-}90}$).

Legends of supporting figures

Fig. S1. Immunoblot analysis of GFP-PBP3 or GFP-PBP3$_{S296A}$ in different backgrounds and conditions. Various strains (as indicated) were grown to log phase at 30˚C. When indicated, the cultures contained xylose or glucose and were incubated at 37˚C for 1.5 h. Whole cell extracts of log-phase cultures were prepared by sonicating cells producing GFP-PBP3 or GFP-PBP3$_{S296A}$ under various conditions. Thirty micrograms of these extracts were analyzed by immunoblotting using anti-GFP antibody. The asterisk shows a degradation product that may result from the known C-terminal proteolytic processing of PBP3 (Hara *et al.*, 1989; Nagasawa *et al.*, 1989; Nakamura *et al.*, 1983). The arrowheads mark the position of GFP-PBP3 and free GFP

(produced from the CJW2144 control strain) as indicated. Molecular weight markers (MW) are also shown.

Fig. S2. The dynamic behavior of PBP3 also occurs under conditions of endogenous production and in the absence of *de novo* protein synthesis. The FRAP experiments were performed as described as in Fig. 5 except that only the fluorescence intensities of a bleached region (closed diamonds) is shown over time. The solid red line is the predicted recovery curve determined by least-square fitting of the data as described in the Experimental Procedures ($r^2 \geq 0.95$).

A. Polar GFP-PBP3 signals of CJW1822 cells (CB15N *Pxyl*::P*xyl*::*gfp-ftsI*) grown overnight in the presence of xylose to accumulate GFP-PBP3 were photobleached in time-lapse sequences.

B. Same as (A) except that CJW1822 cells (CB15N *Pxyl*::*Pxyl*::*gfp-ftsI*) grown in the presence of xylose were washed in medium containing glucose and incubated in the same glucose medium for 30 min to stop synthesis of GFP-PBP3 from *Pxyl*. Cells were then incubated with 20 μg/ml chloramphenicol (CAM) for 20 min to block protein synthesis before being placed on an agarose-padded slide that contained glucose and CAM. Whole cell intensity did not increase and remained constant for as long as 60 min under these conditions (data not shown), verifying that GFP-PBP3 synthesis was effectively blocked.

C. Same as (A) except that GFP-PBP3 was produced at the native level as the only source of PBP3 from CJW2144 cells (CB15N *ftsI*::*gfp-ftsI*).

D. Same as (B) except that the medial GFP-PBP3 signals from predivisional CJW1822 cells were photobleached.

E. Same as (C) except that the medial GFP-PBP3 signals from predivisional CJW2144 cells, which produce GFP-PBP3 at endogenous levels, were photobleached.

Fig. S3. Cephalexin binds to several PBPs and fosfomycin affects PBP3 localization.

A. The specificity of cephalexin binding to PBP3 was tested using a BOCILLIN-FL competition binding assay. The membrane-enriched fractions of CB15N Δ*bla* cells (lacking the β-lactamase-encoding gene) were incubated in a buffer containing increasing amounts of cephalexin or an excess of Penicillin G (+). They were then incubated with 10μM of the fluorescent penicillin derivative, BOCILLIN-FL. Lane 1 and 2, samples incubated with 0 (-) or 10 mM (+) Penicillin G, respectively. Lane 3 to 7, samples incubated with 0, 0.001, 0.01, 0.1 and 1 mM cephalexin, respectively. Fluorescent bands that disappeared in the presence of a large excess of penicillin identify PBPs whereas bands that remain present correspond to unspecific binding of the BOCILLIN-FL (indicated by the bracket on the right). Incubation with 0.01 mM or higher of cephalexin (lanes 5-7) results in the concomitant disappearance of several specific PBP fluorescent bands, suggesting that cephalexin is not specific for a particular PBP (such as PBP3). Molecular weight markers (MW) are shown.

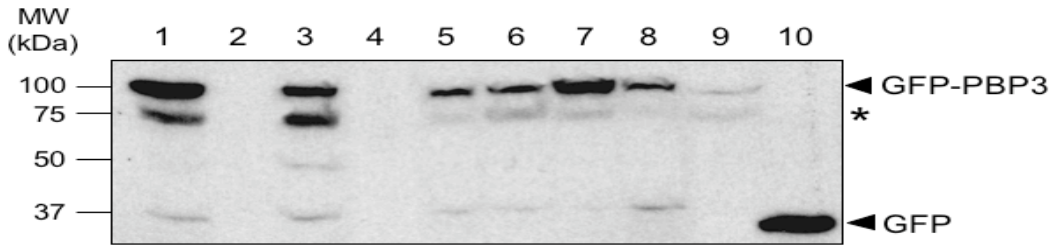
B. Quantitative analysis of GFP-PBP3 and FtsZ-mCherry localization upon treatment with fosfomycin. At least 114 cells from 3 separate microscopy experiments were analyzed. Polar GFP-PBP3 localization pattern was analyzed in cells showing no visible constriction by DIC microscopy, while medial GFP-PBP3 localization was considered in terms of colocalization with FtsZ-mCherry in cells showing clear constriction.

Movie legend

Movie S1. Time-lapse microscopy of *E. coli* cells producing a $PBP3_{N361S}$ mutant. *E. coli* K-12 cells (LMC502 *leu*$^{+}$ *pbpB*^{r1}) producing $PBP3_{N361S}$ (Taschner *et al.*, 1988) were placed on LB-

containing agarose-padded slide and imaged every 5 min during the course of several cell cycles at 37ºC. Representative images of phase contrast are shown. Division produces pointed poles. Scale bar, 1μm.

Fig. S1



Lane 1: CJW2235 (*ftsI*(Ts) *Pxyl*::*Pxyl-gfp-ftsI*$_{wt}$), xylose, 30°C
Lane 2: CJW2235 (*ftsI*(Ts) *Pxyl*::*Pxyl-gfp-ftsI*$_{wt}$), glucose, 30°C
Lane 3: CJW2235 (*ftsI*(Ts) *Pxyl*::*Pxyl-gfp-ftsI*$_{wt}$), xylose, 37°C
Lane 4: CJW2235 (*ftsI*(Ts) *Pxyl*::*Pxyl-gfp-ftsI*$_{wt}$), glucose, 37°C
Lane 5: CJW2236 (*ftsI*(Ts) *Pxyl*::*Pxyl-gfp-ftsI*$_{S296A}$), xylose, 30°C
Lane 6: CJW2236 (*ftsI*(Ts) *Pxyl*::*Pxyl-gfp-ftsI*$_{S296A}$), xylose, 37°C
Lane 7: CJW1822 (*Pxyl*::*Pxyl-gfp-ftsI*$_{wt}$), xylose
Lane 8: CJW2136 (*Pxyl*::*Pxyl-gfp-ftsI*$_{S296A}$), xylose
Lane 9: CJW2144 (*ftsI*::*gfp-ftsI*)
Lane10: CJW1472 (*Pxyl*::*Pxyl-gfp*), xylose (free GFP)

Fig. S2

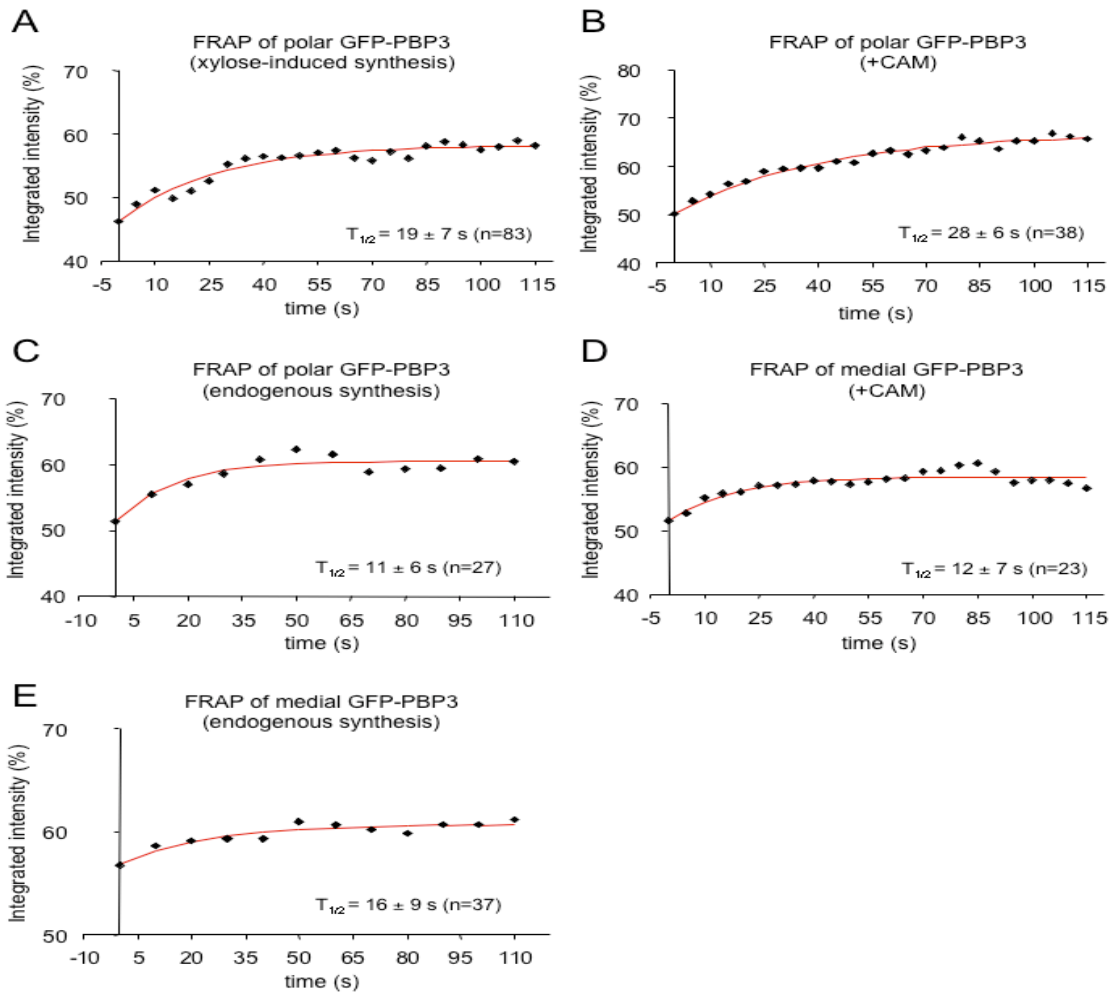
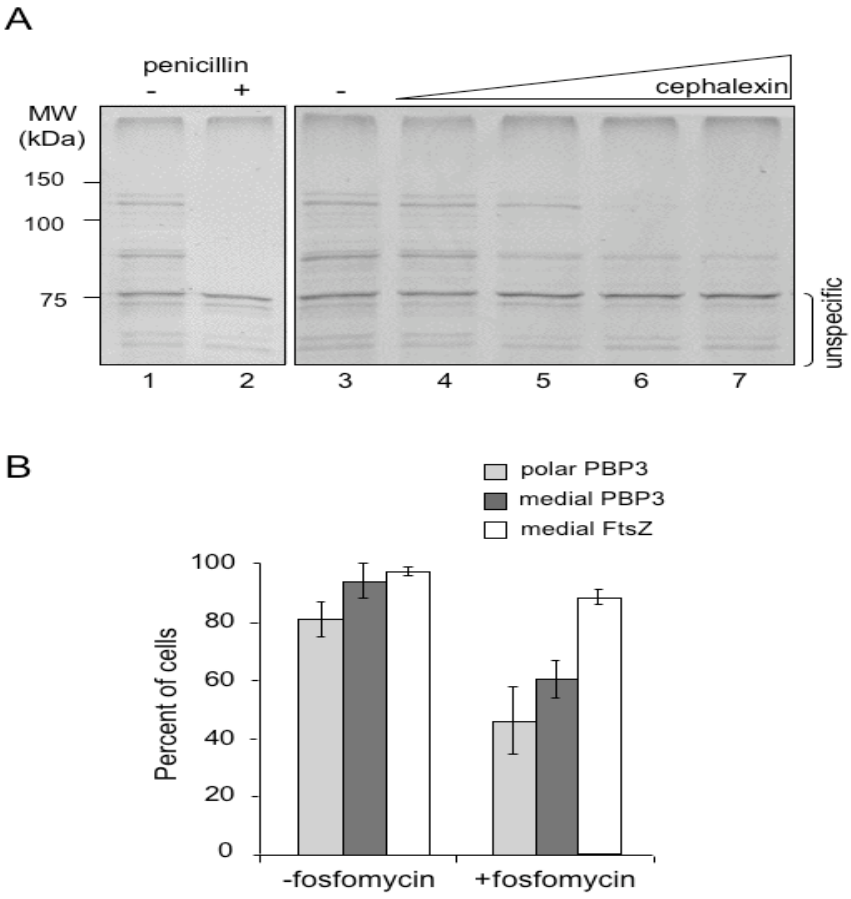

A
FRAP of polar GFP-PBP3
(xylose-induced synthesis)
Integrated intensity (%)
time (s)
T1/2 = 19 ± 7 s (n=83)
B
FRAP of polar GFP-PBP3
(+CAM)
Integrated intensity (%)
time (s)
T1/2 = 28 ± 6 s (n=38)
C
FRAP of polar GFP-PBP3
(endogenous synthesis)
Integrated intensity (%)
time (s)
T1/2 = 11 ± 6 s (n=27)
D
FRAP of medial GFP-PBP3
(+CAM)
Integrated intensity (%)
time (s)
T1/2 = 12 ± 7 s (n=23)
E
FRAP of medial GFP-PBP3
(endogenous synthesis)
Integrated intensity (%)
time (s)
T1/2 = 16 ± 9 s (n=37)

Fig. S3


A
penicillin
-
+
-
cephalexin
MW
(kDa)
150
100
75
unspecific
1
2
3
4
5
6
7
B
polar PBP3
medial PBP3
medial FtsZ
Percent of cells
100
80
60
40
20
0
-fosfomycin
+fosfomycin