Supporting Information

SI Materials and Methods

Media, bacterial strains, plasmids and growth conditions

Cells were grown in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 0.05 g/L thymine), LB without NaCl (LB0N) or LB with 0.2 M sucrose media at indicated temperatures. When needed, antibiotics were used at the following concentrations: ampicillin = 100 μg/ml; spectinomycin = 25 μg/ml; kanamycin = 25 μg/ml; tetracycline = 25 μg/ml; and chloramphenicol = 20 μg/ml. When cells carried the *bla* or *cat* marker integrated in the chromosome, ampicillin or chloramphenicol was used at 25 μg/ml and 10 μg/ml, respectively. FtsE^{D162N}X suppressor mutations, strains, plasmids and primers used in this study are listed in Table S1-S4, respectively. Construction of the plasmids and strains was described in detail in the section for construction of plasmids and construction of strains respectively.

Screen for FtsE^{D162N}X resistant mutants by EMS mutagenesis

The procedure for EMS mutagenesis (1) was used with slight modifications. Cells of SD221 [W3110 *leu::Tn10 ftsA*^{R286W} *ftsEX::cat*] were grown overnight in LB + 0.2 M sucrose medium at 37 °C. The next morning, the culture was sub-cultured 1:100 in LB + 0.2 M sucrose medium and grown to OD_{540}=0.4. Cells were spun down and washed twice in equal volume of minimal A buffer (1 L buffer: 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 1 g of $[NH_4]_2SO_4$, and 0.5 g of sodium citrate-$2H_2O$). After the wash, the cells were resuspended in half the original volume of minimal A buffer. 2 ml of the culture was transferred to a 10 ml tube and 30 μL of EMS was added to the culture and incubated at 37°C with shaking for 45 min. After the EMS treatment, the cells were spun down, washed twice in 5 ml of minimal A buffer and resuspended in 2 ml of minimal A buffer. The mutagenized culture was then diluted 1:10 in fresh LB + 0.2 M sucrose medium and grown to OD_{540}=0.8. Cells were collected and made eletrocompetent. Plasmid pSD221-D162N was transformed into these mutagenenized cells by electroporation and transformants were

selected on LB + 0.2 M sucrose plates containing chloramphenicol, kanamycin, and 0.2% glucose or 250 µM IPTG at 37°C. A control culture without EMS treatment was subjected to the same selection and FtsE^{D162N}X resistant colonies arose on plates with 250 µM IPTG at a frequency of 10^{-5}. EMS treatment increased the number of FtsE^{D162N}X resistant colonies on IPTG plates by 50 to 100 fold. The larger colonies on IPTG plates were further purified on the same selection plates with 250 µM IPTG. In total, 41 FtsE^{D162N}X resistant mutants were isolated following one round of EMS mutagenesis.

To rule out the suppressors that contained a mutated plasmid, the plasmid (pSD221-D162N) from suppressor strains was isolated and retransformed into SD221 to test its ability to kill the cells in the presence of IPTG. Plasmids from 21 of the 41 FtsE^{D162N}X resistant mutants no longer killed the cells in the presence of IPTG and these were discarded. Plasmids from the remaining 20 FtsE^{D162N}X resistant mutants killed naïve cells as well as the original pSD221-D162N plasmid in the presence of IPTG, suggesting that the resistance was due to mutations in the bacterial chromosome. To determine the location of the mutations that confer resistance to FtsE^{D162N}X, we first tested whether the resistance was linked with the *leu::Tn10* marker, which is located close to the 2 min region of the chromosome that harbors most of the essential cell division genes. Resistance to FtsE^{D162N}X was co-transducible for 8 of the suppressors at a frequency of 60-90%. Therefore, the division genes within the 2 min region of these 8 FtsE^{D162N}X resistant mutants were sequenced. Sequencing results showed that 5 of them contained missense mutations in *ftsL*, 2 contained missense mutations in *ftsA* and 1 had a missense mutation in *ftsW*. In all 8 cases, the missense mutations resulted from a single nucleotide change (Table S1). For the 12 FtsE^{D162N}X resistant mutants that displayed no co-transducible resistance with *leu::Tn10*, *ftsB* was sequenced. Strikingly, 6 of them contained a single missense mutation in *ftsB* while one contained two missense mutations in *ftsB* (Table S1). The other 5 did not have a mutation in *ftsB* and were not further studied here.

BTH assay

To detect the interaction among the division proteins, appropriate plasmid pairs were co-transformed into BTH101. The next day, single colonies were resuspended in 1 ml LB medium,

and 3 µL of each aliquot was spotted on LB plates containing 100 µg/ml ampicillin, 25 µg/ml kanamycin, 40 µg/ml X-gal and 25 µM IPTG. Plates were incubated at 30°C overnight before imaging.

Immunofluorescence microscopy

Overnight cultures of CH2/pDB280 (*ftsA*^{0} *recA56-Tn10*/pSC101^{ts}, P_{ftsA}::*ftsA*) harboring the plasmid pSD266 (pACYC184, P_{ftsA}::*ftsA*) or its variants were diluted 1:100 in fresh LB medium with appropriate antibiotics and grown at 30 °C for about 3 hours. The cultures were then diluted 1:10 in LB medium and shifted to 42°C. To keep the cells in exponential phase, the cultures were diluted once after they were shifted to 42°C for 1.5 hours. Samples were taken after the cultures were shifted to 42°C for 3 hours. The cells were fixed with 2% paraformaldehyde and 0.4% glutaraldehyde, and prepared for immunostaining and photography as previously described (2). Antisera were used as the following concentration: FtsZ (1/5000), FtsA (1/1000) and FtsK (1/250).

Western blot

To measure the expression level of FtsA mutants, strains were grown as described for immunofluorescence microscopy. Overnight cultures of CH2/pDB280 (*ftsA*^{0} *recA56-srlD::Tn10*/pSC101^{ts}, P_{ftsA}::*ftsA*), or CH2/pDB280 harboring the plasmid pSD266 (pACYC184, P_{ftsA}::*ftsA*) or its variants were diluted 1:100 in fresh LB medium with appropriate antibiotics and grown at 30 °C for about 3 hours. The cultures were then diluted 1:10 in LB medium and split in half. One was kept at 30 °C, while the other half was shifted to 42°C. Cultures were kept in exponential phase by dilution and samples were taken after they were grown for 3 hours. To make sure that same amount of cell mass was used for each culture, the volumes taken from each culture were adjusted according to their OD_{540}. Cells were collected, resuspended in SDS-PAGE sample buffer and boiled for 10 min before they were loaded on the SDS-PAGE gel for analysis. Western blotting and detection of FtsA were performed as previously described (3). Anti-FtsA serum was used at a final concentration of 1/1000.

To measure the expression level of FtsN and its mutants, overnight cultures of S3 (W3110, leu::Tn10) harboring the plasmid pBR322 or pKD140 (pBR322-FtsN) and its derivatives were diluted 1:100 in fresh LB medium with ampicillin and grown at 30°C until OD_{540} reached about 0.4. Samples were then taken according to their OD_{540} to make sure equal amount of cell mass was used for each strain. Cells were spun down, resuspended in SDS sample buffers, boiled and 15 µL of each sample was loaded into the SDS-PAGE gel for comparison. Anti-FtsN serum was used at a final concentration of 1/1000.

Localization of GFP-fusion proteins

To visualize ZapA-GFP localization in cells expressing $FtsE^{D162N}X$, overnight cultures of HC261/pSD221 (TB28, *zapA-gfp*, *cat*/pEXT22, P_{tac}::*ftsEX*) or HC261/pSD221-D162N were diluted 1:100 in fresh LB + sucrose medium with appropriate antibiotics at 30°C. At OD540 ≈ 0.4, the cultures were diluted 1:10 in LB + sucrose medium with 125 µM IPTG and grown for another 2 hours. Cells were then immobilized on LB + 2% agarose pad for photography.

To visualize GFP-FtsI and GFP-FtsN localization in cells expressing $FtsE^{D162N}X$, overnight cultures of EC436/pDSW610-D162N (P_{lac}::*gfp-ftsI*/pBAD33, P_{BAD}::$ftsE^{D162N}X$), and EC440/pDSW610-D162N (P_{lac}::*gfp-ftsN*/pBAD33, P_{BAD}::$ftsE^{D162N}X$) were diluted 1:100 in fresh LB medium with appropriate antibiotics. At OD540 ≈ 0.4, the cultures were diluted 1:10 in LB medium with 2.5 µM IPTG and 0.2% arabinose and grown for 5 hours. During this long period of induction, the cultures were diluted 1:10 twice to keep the cells in exponential phase. Cells were then immobilized on LB + 2% agarose pad for photography.

To visualize GFP-FtsW localization in cells expressing $FtsA^{R63H}$ or $FtsA^{G366D}$ as the only source of FtsA, overnight cultures of CH2/pDB280 carrying plasmid pDSW311 (pDSW209, P_{lac}::*gfp-ftsW*) and pSD273 or its variants were diluted 1:100 in fresh LB medium with appropriate antibiotics. At OD540 ≈ 0.4, the cultures were diluted 1:10 in LB medium with 10 µM IPTG and shifted to 42°C. The cultures were then grown for 3 hours at 42°C to deplete the wild type FtsA. As a control, an exponentially growing culture of CH2/pDB280 carrying plasmid pDSW311 and pSD273 at 30°C was treated with 20 µg/ml of cephalexin to inhibit cell division. Cells were immobilized on LB + 2% agarose pad for photography.

To visualize FtsX-GFP and FtsEX-GFP localization in cells expressing wild type FtsA, the FtsA^{R286W}, FtsA$^{R286W, R63H}$ or FtsA$^{R286W, G366D}$ mutant, overnight cultures of SD220 (S3, *ftsEX::cat*), SD221 (S3, *ftsA^{R286W} ftsEX::cat*), SD261 (S3, *ftsA$^{R286W, R63H}$ ftsEX::cat*) or SD262 (S3, *ftsA$^{R286W, G366D}$ ftsEX::cat*) carrying plasmid pSD226 (pDSW210, P_{lac}::*ftsX-gfp*) or pSD242 (pDSW210, P_{lac}::*ftsEX-gfp*) were diluted 1:100 in fresh LB + 0.2 M sucrose medium with appropriate antibiotics at 30 °C. At OD540 ≈ 0.4, the cultures were diluted 1:10 in LB + 0.2 M sucrose medium with 20 µM IPTG. The cultures were then grown for 2 hours before samples were taken for photography. Localization of FtsX-GFP and FtsEX-GFP in wild type cells S3 was performed similarly but in LB medium.

Construction of strains

Construction of strains involved P1-mediated transduction (4), λ-Red mediated recombineering (5) and allelic replacement using plasmids that are temperature sensitive for replication (6).

Strains carrying the *ftsEX::cat* marker were obtained by P1-mediated transduction of *ftsEX::cat* from EC1779 (BW25113, *ftsEX::cat*) into respective strains (Table S2). Transductants were selected on LB + 0.2M sucrose plates containing chloramphenicol.

Strains carrying the *ftsN::kan* marker were constructed by P1-mediated transduction of *ftsN::kan* from strain CH34/pMG20 (TB28, *ftsN<>aph*/P_{BAD}::*ftsN*) or SD303 (TB28, *ftsN<>aph*/pSEB413) into respective strains (Table S3). Transductants were selected on plates with spectinomycin and kanamycin if the strains were complemented with pBL154 (pSC101^{ts}, P_{syn}::*ftsN*). If the strain s complemented with pMG20 (pBAD33, P_{BAD}::^{ss}*tor-bfp-ftsN$^{71-105}$-le*), transductants would be selected on plates with chloramphenicol, kanamycin and 0.4% arabinose. If the strains were complemented with pSD414-D5N (pDSW210, P_{lac}::*ftsN$^{1-140-D5N}$*), transductants were selected on plates with ampicillin, kanamycin and 30 µM IPTG.

Strains SD238 to SD244 were obtained by replacing the chromosomal *ftsB^{WT}* allele of TB28 with respective *ftsB* alleles from plasmid pSD255 (pSC101^{ts}, P_{syn}::*ftsB*) by the methods of Hamilton et

al. (6) Presence of the desired allele in each strain was verified by amplification and sequencing of the chromosomal DNA using primers 5-ftsB-seq and 3-ftsB-seq.

SD246 and SD247 were obtained by replacing the chromosomal *ftsL*^{WT} allele or *ftsW*^{WT} allele of S3 with the *ftsL*^{G92D} allele or *ftsW*^{M269I} allele from plasmid pSD256-G92D (pSC101^{ts}, P_{syn}::*ftsL*^{G92D}) or pSD257-M269I (pSC101^{ts}, P_{syn}::*ftsW*^{M269I}) by the methods of Hamilton et al. (6). The presence of the mutation in each strain was verified by amplification and sequencing of the chromosomal DNA using primers 5-ftsL-HindIII and 3-ftsL-EcoRI for SD246 and 5-HindIII-ftsW and 3-ftsW-EcoRI for SD247.

Strains SD248 and SD249 were constructed by λ-Red mediated recombineering using strain PS236 (W3110, *leu::Tn10 ftsA12*) as the host. DNA fragments containing the *ftsA*$^{R286W,\ R63H}$ and *ftsA*$^{R286W,\ G366D}$ were amplified from the chromosomal DNA of the FtsE^{D162N}X resistant isolates DNEXRM59 (dominant negative FtsEX resistant mutant 59) and DNEXRM42 using primers m-ftsQ-seq and 3-ftsA-EcoRI. Gel purified DNA fragments were electroporated into PS236/pKD46 competent cells and recombinants selected at 42°C overnight. 4-10 recombinants of each strain were sub-cloned and the presence of the mutation was confirmed by sequencing of *ftsA* using primers m-ftsQ-seq, m-ftsA-seq and 3-ftsA-EcoRI.

Strain SD290 (S3, ftsA^0/pSD263 [pSC101^{ts}, P_{ftsA}::*ftsA*]) was constructed by P1-mediated transduction of ftsA^0 from CH2/pDB280 into strain S3 (W3110 *leu::Tn10*). Transductants were selected on M9 minimum medium with 0.2% glucose. Transductants obtained on M9 medium were then checked for sensitivity to tetracycline and for temperature sensitive growth.

Strain SD296 (S3, ftsA^0 *leu::Tn10*/pSD263 [pSC101^{ts}, P_{ftsA}::*ftsA*]) was constructed by P1-mediated transduction of *leu::Tn10* from S3 into strain SD290. Transductants were selected on LB medium with tetracycline and then checked for temperature sensitive growth.

Strain SD318 (TB28, ftsA^0 *leu::Tn10/*pDB280), SD319 (TB28, ftsA^0 *leu::Tn10 ftsB*^{E56A}*/*pDB280) and SD320 (TB28, ftsA^0 *leu::Tn10 ftsB*^{D59H}*/*pDB280) were constructed by P1-mediated transduction of *leu::Tn10-ftsA*^{0} from SD296 into strains TB28, BL140 and BL154, respectively.

Strain SD321 (TB28, ftsA^0/pDB280), SD322 (TB28, ftsA^0 *ftsB*^{E56A}/pDB280) and SD323 (TB28, ftsA^0 *ftsB*^{D59H}/pDB280) were constructed by P1-mediated transduction of *leu*$^+$ from strain SD303 [TB28, *ftsN::kan*/pSEB413] into strain SD318, SD319 and SD320 respectively. Transductants were selected on M9 minimum medium with 0.2% glucose and then checked for sensitivity to tetracycline and for temperature sensitive growth.

Strain SD324 (TB28, ftsA^0 *recA56-srlD::Tn10/*pDB280), SD325 (TB28, ftsA^0 *ftsB*^{E56A} *recA56-srlD::Tn10/*pDB280) and SD326 (TB28, ftsA^0 *ftsB*^{D59H} *recA56-srlD::Tn10/*pDB280) were constructed by P1-mediated transduction of *recA56-srlD::Tn10* from strain W3110 *recA56-srlD::Tn10* into strain SD321, SD322and SD323 respectively. Transductants were selected on LB plates with tetracycline and then checked for UV sensitivity and temperature sensitive growth.

Construction of plasmids

Plasmid pDSW610-D162N was obtained by site-directed mutagenesis of plasmid pDSW610 with primers ftsE-D162N-F and ftsE-D162N-R.

Plasmid pEX-T18 was constructed by ligation of a BamHI/EcoRI digested DNA fragment containing *ftsEX* into BamHI/EcoRI digested pUT18. The *ftsEX* containing DNA fragment was amplified from W3110 chromosomal DNA using primers 5-BamHI-ftsE and 3-ftsX-EcoRI.

Plasmids pKD140-D5N and pKD140-WYAA were obtained by site-directed mutagenesis of plasmid pKD140 using primer pairs ftsN-D5N-F/ftsN-D5N-R and ftsN-WYAA-F/ftsN-WYAA-R.

Plasmid pKT25-E was constructed by ligation of an XbaI/KpnI digested DNA fragment containing *ftsE* into XbaI/KpnI digested pKT25. The *ftsE* containing DNA fragment was amplified from W3110 chromosomal DNA using primers 5-XbaI-ftsE and 3-ftsE-KpnI.

Plasmid pKT25-EX was constructed by ligation of a XbaI/KpnI digested DNA fragment containing *ftsEX* into XbaI/KpnI digested pKT25. The *ftsEX* containing DNA fragment was amplified from W3110 chromosomal DNA using primers 5-XbaI-ftsE and 3-ftsX-KpnI.

Plasmid pSD221 was obtained by ligation of a SacI/HindIII digested DNA fragment containing *ftsEX* into SacI/HindIII digested pEXT22. The *ftsEX* containing DNA fragment was amplified from W3110 chromosomal DNA using primers 5-SacI-ftsE and ftsX-R-HindIII.

Plasmid pSD221-D162N was obtained by site-directed mutagenesis of plasmid pSD221 using primers ftsE-D162N-F and ftsE-D162N-R.

Plasmid pSD226 was obtained by ligation of an EcoRI/PstI digested DNA fragment containing *ftsX* into EcoRI/PstI digested pDSW210. The DNA fragment was amplified from W3110 chromosomal DNA using primers 5-ftsX-EcoRI/3-ftsX-PstI.

Plasmid pSD242 was obtained by ligation of an EcoRI/SalI digested DNA fragment containing *ftsEX* into EcoRI/SalI digested pDSW210. The DNA fragment was amplified from W3110 chromosomal DNA using primers 5-ftsE-EcoRI/3-ftsX-SalI.

Plasmid pSD255 was obtained by ligation of a HindIII/EcoRI digested *ftsB* containing DNA fragment into HindIII/EcoRI digested pHGB2. The DNA fragment was amplified from W3110 chromosomal DNA using primers 5-HindIII-ftsB/3-ftsB-EcoRI. Derivatives of pSD255 carrying different alleles of *ftsB* were obtained by site-directed mutagenesis using primer pairs listed in Table S4.

Plasmid pSD256 was obtained by ligation of a HindIII/EcoRI digested *ftsL* containing DNA fragment into HindIII/EcoRI digested pHGB2. The DNA fragment was amplified from W3110 chromosomal DNA using primers 5-HindIII-ftsL/3-ftsL-EcoRI. Derivative of pSD256 carrying the $ftsL^{D92G}$ allele was obtained by site-directed mutagenesis using primer pairs listed in Table S4.

Plasmid pSD257 was obtained by ligation of a HindIII/EcoRI digested *ftsW* containing DNA fragment into HindIII/EcoRI digested pHGB2. The DNA fragment was amplified from W3110 chromosomal DNA using primers 5-HindIII-ftsW/3-ftsW-EcoRI. Derivative of pSD255 carrying the $ftsW^{M269I}$ allele was obtained by site-directed mutagenesis using primer ftsW-M269I-F and ftsW-M269I-R.

Plasmid pSD263 was obtained by ligation of a PstI/EcoRI digested DNA fragment containing *ftsA* into PstI/EcoRI digested pHGB2. The *ftsA* containing DNA fragment was cut from pSEB24 using PstI/EcoRI

Plasmid pSD265 was obtained by ligation of a PstI/EcoRI digested DNA fragment containing *ftsA* into PstI/EcoRI digested pBANG112. The *ftsA* containing DNA fragment was cut from pSEB24 using PstI/EcoRI.

Plasmid pSD266 was obtained by self-ligation of AvaI digested pSD265. AvaI digestion removes a 1221bp fragment from pSD265 without changing the expression level of FtsA. Derivatives of pSD266 containing different alleles of *ftsA* were obtained by site-directed mutagenesis using primer pairs listed in Table S4.

Plasmid pSD273 was obtained by ligation of PstI/AvaI digested pSD266 with a PstI/AvaI digested fragment from pBANG100. pSD273 differs from pSD266 in that it carries the kanamycin resistant *aph* gene instead of the ampicillin resistant *bla* gene. Derivatives of pSD273 containing different alleles of *ftsA* were obtained by site-directed mutagenesis using primer pairs listed in Table S4.

Derivatives of pSEB428 carrying different *ftsE* mutations were obtained by site-directed mutagenesis using primer pairs listed in Table S4.

Plasmid pSEB428-EX$^{\Delta 152\text{-}161}$ was obtained by ligation of an NcoI/HindIII digested DNA fragment containing *ftsEX*$^{\Delta 152\text{-}161}$ into NcoI/HindIII digested pSEB428. The *ftsEX*$^{\Delta 152\text{-}169}$ DNA fragment was obtained by overlap extension PCR. Two DNA fragments containing *ftsEX*$^{1\text{-}152}$ and *ftsX*$^{161\text{-}352}$ were amplified from pSEB428 using primer pairs 5-NcoI-ftsE/ftsX152-R and ftsX161-F/3-ftsX-HindIII, respectively. The *ftsEX*$^{1\text{-}152}$ and *ftsX*$^{161\text{-}352}$ fragments were used together as template to obtain the *ftsEX*$^{\Delta 152\text{-}161}$ fragment by PCR using primers 5-NcoI-ftsE/3-ftsX-HindIII.

Plasmid pSEB428-E^{D162N}X$^{\Delta 152\text{-}161}$ was obtained by site-directed mutagenesis using primer pair ftsE-D162N-F/ftsE-D162N-R.

Plasmid pT18-E was constructed by ligation of an XbaI/KpnI digested DNA fragment containing *ftsE* into XbaI/KpnI digested pUT18C. The *ftsE* containing DNA fragment was amplified from W3110 chromosomal DNA using primers 5-XbaI-ftsE and 3-ftsX-KpnI.

Plasmid pT18-EX was constructed by ligation of an XbaI/KpnI digested DNA fragment containing *ftsEX* into XbaI/KpnI digested pUT18C. The *ftsEX* containing DNA fragment was amplified from W3110 chromosomal DNA using primers 5-XbaI-ftsE and 3-ftsX-KpnI.

Derivatives of pWM3014 were obtained by site-directed mutagenesis using primer pairs listed in Table S4.

Plasmid pX-T18 was obtained by ligation of a BamHI/EcoRI digested DNA fragment containing *ftsX* into BamHI/EcoRI digested pUT18. The *ftsX* DNA fragment was amplified from plasmid pSD221 using primer pair 5-BamHI-ftsX and 3-ftsX-EcoRI.

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Supplemental Figure legends

Figure S1. The ATPase activity of FtsEX but not its role in amidase activation is essential for cell division. Cells of SD205 (*W3110 ftsEX::cat*) harboring plasmid pDSW208, pSEB428 (pDSW208-*ftsEX*) or its variants were spotted on LB0N or LB + 0.2 M sucrose plates to determine the ability of the FtsEX variants to restore growth on LB0N medium. Plasmid pDSW208, pSEB428 and its variants were transformed into strain SD205, and a single transformant of each strain from LB + 0.2M sucrose plates was resuspended in 1 ml LB medium and serially diluted. 3 µL of each aliquot was then spotted on the LB0N plates or LB + 0.2 M sucrose plates with appropriate antibiotics and increasing concentrations of IPTG. The plates were incubated at 30°C overnight before photography. Note that at higher concentrations of inducer expression of FtsEX and the variants are toxic.

Figure S2. Toxicity of FtsEX or its mutants in wild type cells growing in LB or LB + 0.2 M sucrose. Plasmid pSEB428 (pDSW208, P_{lac}::*ftsEX*) or its variants was transformed into strain S3 [*W3110 leu::Tn10*] and a single transformant of each strain from LB + 0.2M sucrose plates was then resuspended in 1 ml LB medium and serially diluted. 3 µL of each aliquot was then spotted on LB plates or LB + 0.2M sucrose plates with appropriate antibiotics and increasing concentration of IPTG. The plates were incubated at 30°C overnight before photography.

Figure S3. Morphology of *ΔftsEX* cells overexpressing $FtsE^{D162N}X$. Cells of SD205 (*W3110 ftsEX::cat*) containing the plasmid pSEB428 or variants containing FtsE mutations were grown to exponential phase in LB + 0.2 M sucrose at 30°C. The cultures were then diluted 1:10 in fresh LB +0.2 M sucrose medium with antibiotics and IPTG was added to the culture to a final concentration as indicated. Samples were taken for photography after 2 hours of induction. The scale bar is 3 µm.

Figure S4. Plasmid pSD221-D162N provides enough $FtsE^{D162N}X$ to kill cells and ZapA-GFP does not confer resistance to the toxicity of $FtsE^{D162N}X$. Plasmid pSD221 (pEXT22, P_{tac}::*ftsEX*)or its

variant pSD221-D162N (pEXT22, P_{tac}::*ftsE*^{D162N}*X*) was transformed into strain TB28 and HC261 (TB28, *zapA-gfp*), and a single transformant of each strain from LB + 0.2M sucrose plates was resuspended in 1 ml LB medium and serially diluted. 3 µL of each aliquot was then spotted on the LB + 0.2 M sucrose plates with appropriate antibiotics and increasing concentrations of IPTG. The plates were incubated at 30°C overnight before photography. Note that growth of both strains was inhibited by FtsE^{D162N}X at 125 µM IPTG.

Figure S5. Western blot to determine the level of FtsN mutant proteins. Overnight cultures of S3 (W3110, *leu*::Tn*10*) harboring the plasmid pBR322 or pKD140 (pBR322-FtsN) and its derivatives were diluted 1:100 in fresh LB medium with ampicillin and grown at 30°C until OD_{540} reached about 0.4. Samples were then taken and adjusted to make sure equal amount of OD_{540} equivalents was used for each strain. Cells were spun down, resuspended in SDS sample buffers, boiled and 15 µL of each sample was loaded into the SDS-PAGE gel. Anti-FtsN serum was used at a final concentration of 1/1000.

Figure S6. Hyperactive *ftsB* and *ftsL*, but not hyperactive *ftsA* mutations provide resistance to overexpressed FtsE^{D162N}X in the absence of wild type FtsEX. Plasmid pSD221 (pEXT22, P_{tac}::*ftsEX*) or its derivative pSD221-D162N was transformed into TB28 and W3110 derivatives carrying the *ftsEX::cat* allele and different mutations in *ftsA*, *ftsB* or *ftsL*. Single transformants obtained on LB + 0.2 M sucrose plates were then spot tested to access sensitivity to FtsE^{D162N}X as Figure. 4A. Strain names were listed next to the spots on the right.

Figure S7. Locations of the *ftsA* mutations and evidence they require less FtsN. (A) Shown is a modeled structure of *E. coli* FtsA by I-TASSER using the *Thermotoga maritima* FtsA structure as a template (PDB#4A2A). Mutations that bypass ZipA and require less FtsN$^{1\text{-}140\text{-}D5N}$ for division are indicated in cyan and mutations that impair interaction with FtsX are indicated in red. The 1C sub-domain of FtsA is indicated. ((B) FtsA mutants that bypass ZipA require less FtsN for division. A single colony of strains SD327 (*S3, ftsN::kan/pSEB414-D5N*), SD328 (*S3, ftsA*^{E124A} *ftsN::kan/pSEB414-D5N*), SD329 (*S3, ftsA*^{I143L} *ftsN::kan/pSEB414-D5N*), SD330 (*S3, ftsA*^{M167I} *ftsN::kan/pSEB414-D5N*), SD331 (*S3, ftsA*^{R177C} *ftsN::kan/pSEB414-D5N*), SD332 (*S3, ftsA*^{L204A} *ftsN::kan/pSEB414-D5N*) and SD333 (*S3, ftsA*^{R286W} *ftsN::kan/pSEB414-D5N*) was resuspended in

1 ml LB medium and serially diluted. 3 µL of each aliquot was then spotted on LB plates with or without 15 µM IPTG. The plates were then incubated at 37°C overnight before photography.

Figure S8. Hyperactive *ftsA*, *ftsB* and *ftsL* mutations enable the cells to grow without FtsEX. *W3110 ftsEX::cat* or *TB28 ftsEX::cat* cells harboring different alleles of *ftsA*, *ftsB* or *ftsL* were grown overnight on LB + 0.2M sucrose plates. The next day, single colonies of each strain were resuspended in 1 ml LB medium and serially diluted. 3 µL of each aliquot was then spotted on LB0N, LB or the LB + 0.2 M sucrose plates with appropriate antibiotics. The plates were incubated at 30°C overnight before photography. Strain names are listed to the right of the panel.

Figure S9. Newly isolated FtsB, FtsL and FtsW mutants increase survival of cells depleted of FtsN. *W3110 ftsN::kan* or *TB28 ftsN::kan* cells harboring different alleles of *ftsB*, *ftsL* or *ftsW* and the plasmid pBL154 (pSC101^{ts}, P_{syn}::*ftsN*) were grown on LB plates with appropriate antibiotics at 30°C overnight. The next day, single colonies of each strain were resuspended in 1 ml LB medium and serially diluted. 3 µL of each aliquot was then spotted on LB plates with appropriate antibiotics. The plates were incubated at 30°C, 37°C or 42°C overnight before photography. Strain names are listed to the right of the panel.

Figure S10. Newly isolated FtsB, FtsL or FtsW mutants allow the cells to grow without FtsEX. Test was done as described in Figure S5. Strain names are listed to the right of the panel.

Figure S11. BTH assay to assess the interaction between division proteins. (A) FtsE does not interact with FtsA, FtsZ or itself unless it is coexpressed with FtsX. Pairs of plasmids were co-transformed into BTH101. A single transformant of each combination of plasmids was resuspended in LB medium and 3 µL of each aliquot was spotted on LB plates containing appropriate antibiotics, 25 µM IPTG and 40 µg/ml X-gal. The plates were then incubated at 30°C overnight before photography. Note that the R300E mutation does not affect FtsA self-interaction or its interaction with other division proteins in this test. (B) FtsA interaction with FtsX and FtsEX. Test was performed as in (A). Note that introduction of R300E and R286W into FtsA does not reduce the interaction with FtsX and FtsEX.

Figure S12. Western blot to determine the level of FtsA mutant proteins. Overnight cultures of CH2/pDB280 (*ftsA*^{0} *recA56-Tn10*/pSC101^{ts}, P_{ftsA}::*ftsA*) or CH2/pDB280 harboring the plasmid pSD266 (pACYC184, P_{ftsA}::*ftsA*) or its derivatives were diluted 1:100 in fresh LB medium with appropriate antibiotics and grown at 30°C for 3 hours. The cultures were then diluted 1:10 in fresh LB medium with appropriate antibiotics, split in half, with one growing at 30°C and one shifted to 42°C for 3 hours. Samples were then taken according to their OD_{540} to make sure equal amount of cell mass was used for each strain. Cells were spun down, resuspended in SDS sample buffers, boiled and 15 µL of each sample was loaded into the SDS-PAGE gel for comparison. Anti-FtsA serum was used at a final concentration of 1/1000. Lanes 1-6 contain samples obtained at 30°C, while lanes 7-12 contain samples from 42°C. The identity of each sample was provided below the image.

Figure S13. *ftsA*^{R286W}-like mutations display different ability to suppress the FtsA mutants impaired for interaction with FtsX. Test was done as in Figure 7B.

Figure S14. Hyperactive FtsB mutants can suppress the growth defects of cells expressing the FtsA mutants impaired for interaction with FtsX as the only copy of FtsA. Plasmid pSD266 or its derivatives were transformed into FtsA depletion strains SD324 (TB28, *ftsA*^{0} *recA56-srlD::Tn10*/pDB280), SD325 (TB28, *ftsA*^{0} *ftsB*^{E56A} *recA56-srlD::Tn10*/pDB280)and SD326 (TB28, *ftsA*^{0} *ftsB*^{D59H} *recA56-srlD::Tn10*/pDB280) harboring wild type FtsB or FtsB mutants. A single transformant of each strain was then resuspended in 1 ml LB medium and serially diluted. 3 µL of each aliquot was spotted on the LB plates with appropriate antibiotics. The plates were incubated at 30°C, 37°C and 42°C overnight before photography.

Figure S15. Localization of FtsX-GFP and FtsEX-GFP in wild type cells (S3), *ftsEX::cat* cells (SD220) or in *ftsEX::cat* cells harboring mutations to suppress the growth defect of mutations impaired for the FtsA-FtsX interaction (strains SD221, SD261 and SD262). Overnight cultures of each strain carrying plasmid pSD226 (pDSW210, P_{lac}::*ftsX-gfp*) or pSD242 (pDSW210, P_{lac}::*ftsEX-gfp*) was diluted 1:100 in fresh LB (for strain S3) or LB + 0.2 M sucrose with appropriate antibiotics and grown at 30°C for 2 hours. The cultures were then diluted 1:10 and IPTG was added to a final concentration of 20 µM to induce FtsX-GFP or FtsEX-GFP. 2 hours later, samples were

taken for photography. Note that FtsX-GFP localizes to midcell poorly without FtsE and *ftsEX::cat* cells expressing FtsEX-GFP are shorter and do not form chains compared to cells expressing FtsX-GFP, indicating complementation of *ftsEX::cat by* FtsEX-GFP. Arrows indicate localization of FtsX-GFP to potential division sites.

Figure S16. GFP-FtsW does not localize in cells expressing the FtsA mutants impaired for interaction with FtsX as the only source of FtsA. Overnight cultures of CH2/pDB280 carrying plasmid pDSW311 (pDSW209, P_{lac}::*gfp-ftsW*) and pSD273 (pACYC184, P_{ftsA}::*ftsA, kan*^{r})or its variants were diluted 1:100 in fresh LB medium with appropriate antibiotics. At $OD_{540} \approx 0.4$, the cultures were diluted 1:10 in LB medium with 10 μM IPTG and shifted to 42°C. The cultures were then grown for 3 hours at 42°C to deplete the wild type FtsA. As a control, an exponentially growing culture of CH2/pDB280 carrying plasmid pDSW311 and pSD273 at 30 °C was treated with 20 μg/ml of cephalexin to inhibit cell division. Cells were immobilized on LB + 2% agarose pad for photography.

Figure S17. Localization of FtsZ, FtsA and FtsK in cells expressing $FtsA^{L367E}$ or $FtsA^{D369R}$ as the only source of FtsA. Detailed information about the experiment was provided in the supplemental information.

Figure S18. Both ^{N}FtsN and ^{E}FtsN are required to bypass FtsEX. Plasmid pDSW208, pSEB417 and its variants were transformed into strain EC1779 (BW25113, *ftsEX::cat*), and a single transformant of each strain from LB + 0.2M sucrose plates was resuspended in 1 ml LB medium and serially diluted. 3 μL of each aliquot was then spotted on the LB plates or LB + 0.2 M sucrose plates with appropriate antibiotics and with or without IPTG. The plates were incubated at 37°C overnight before photography.

Fig. S1

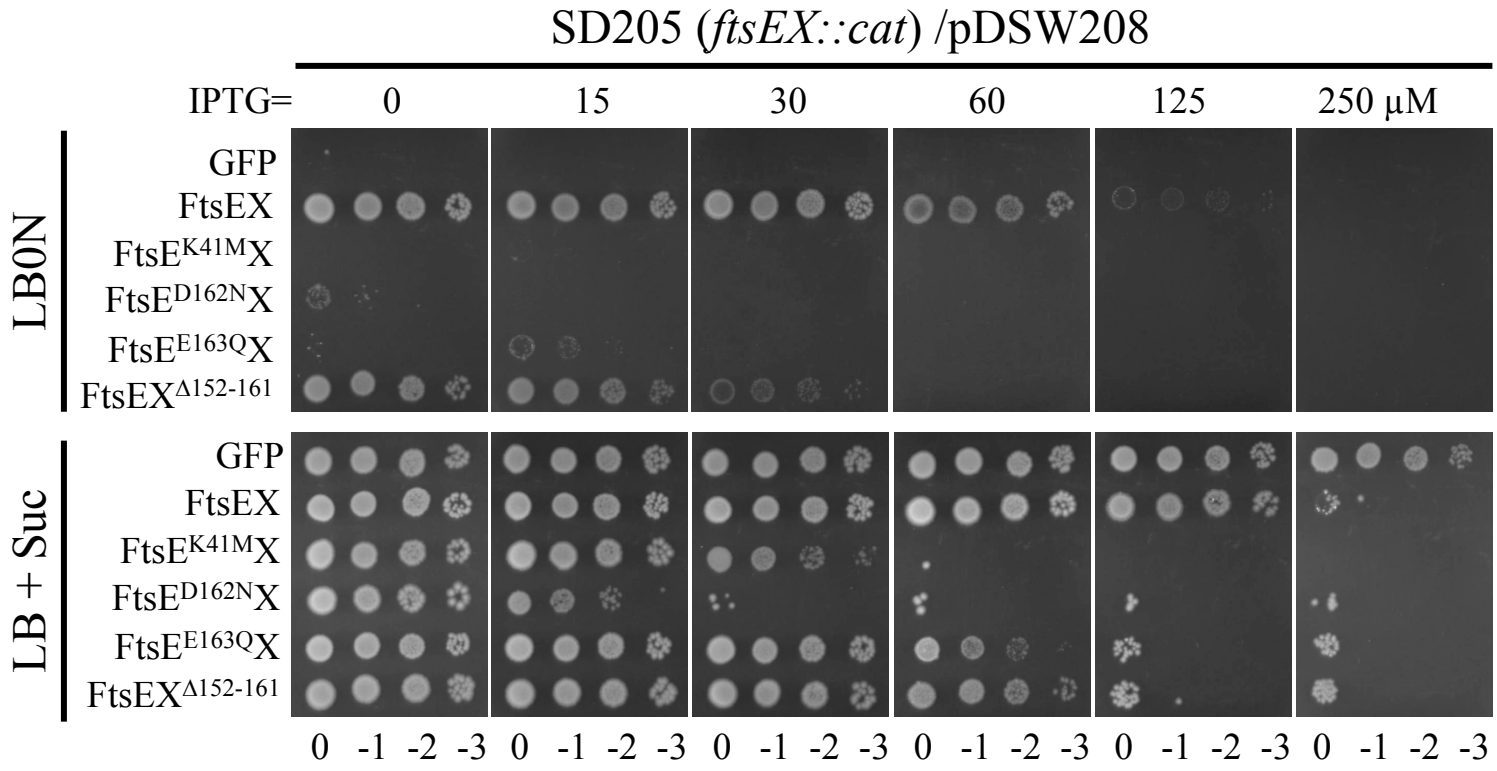

SD205 (*ftsEX::cat*) /pDSW208
IPTG= 0 15 30 60 125 250 μM
LB0N
GFP
FtsEX
FtsE^{K41M}X
FtsE^{D162N}X
FtsE^{E163Q}X
FtsEX$^{\Delta 152\text{-}161}$
LB + Suc
GFP
FtsEX
FtsE^{K41M}X
FtsE^{D162N}X
FtsE^{E163Q}X
FtsEX$^{\Delta 152\text{-}161}$
0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3

Fig. S2

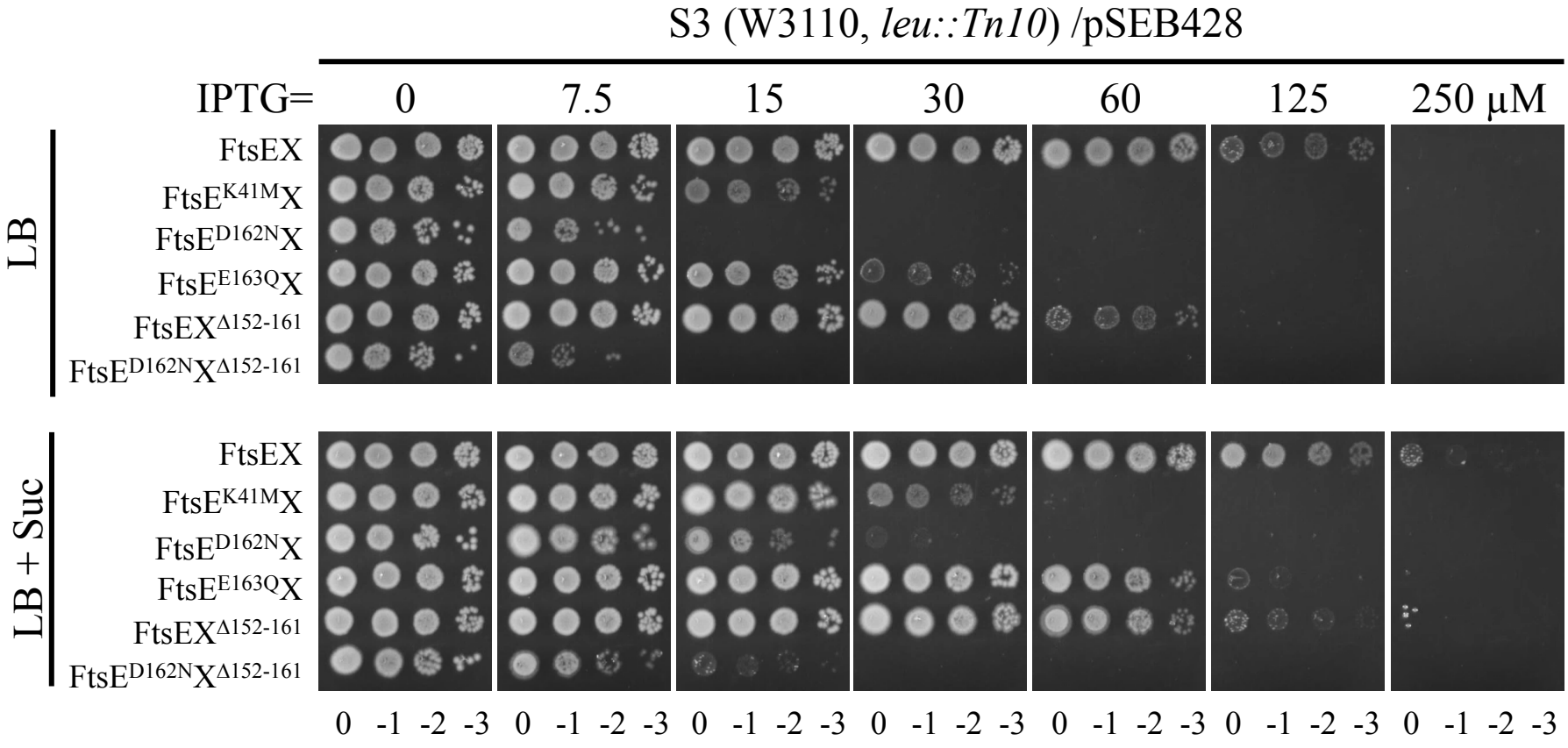

S3 (W3110, leu::Tn10) /pSEB428
IPTG= 0 7.5 15 30 60 125 250 μM
LB
LB + Suc
FtsEX
FtsE^K41M X
FtsE^D162N X
FtsE^E163Q X
FtsEX^Δ152-161
FtsE^D162N X^Δ152-161
0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3

Fig. S3

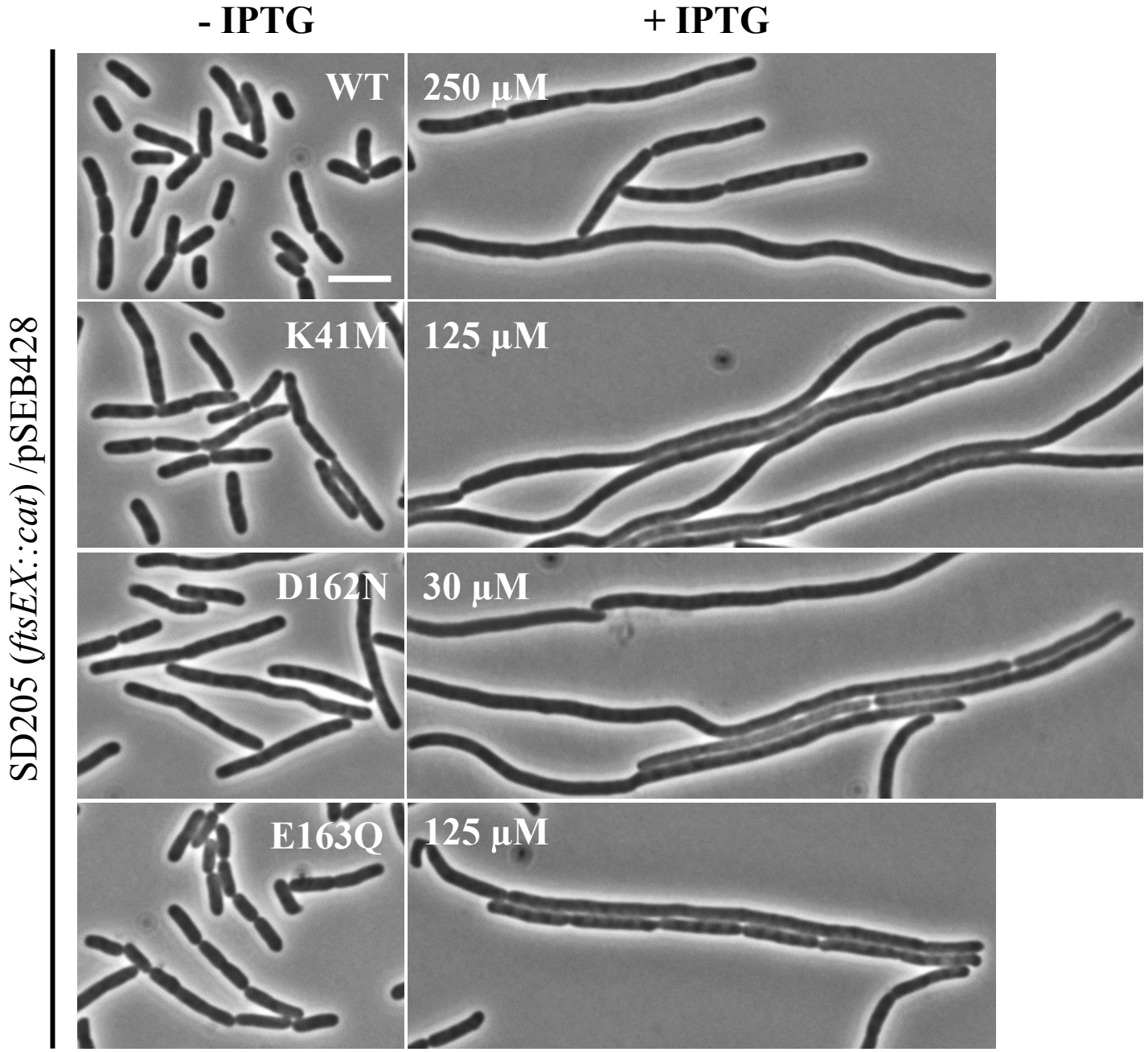

- IPTG
+ IPTG
SD205 (*ftsEX::cat*) /pSEB428
WT
250 µM
K41M
125 µM
D162N
30 µM
E163Q
125 µM

Fig. S4

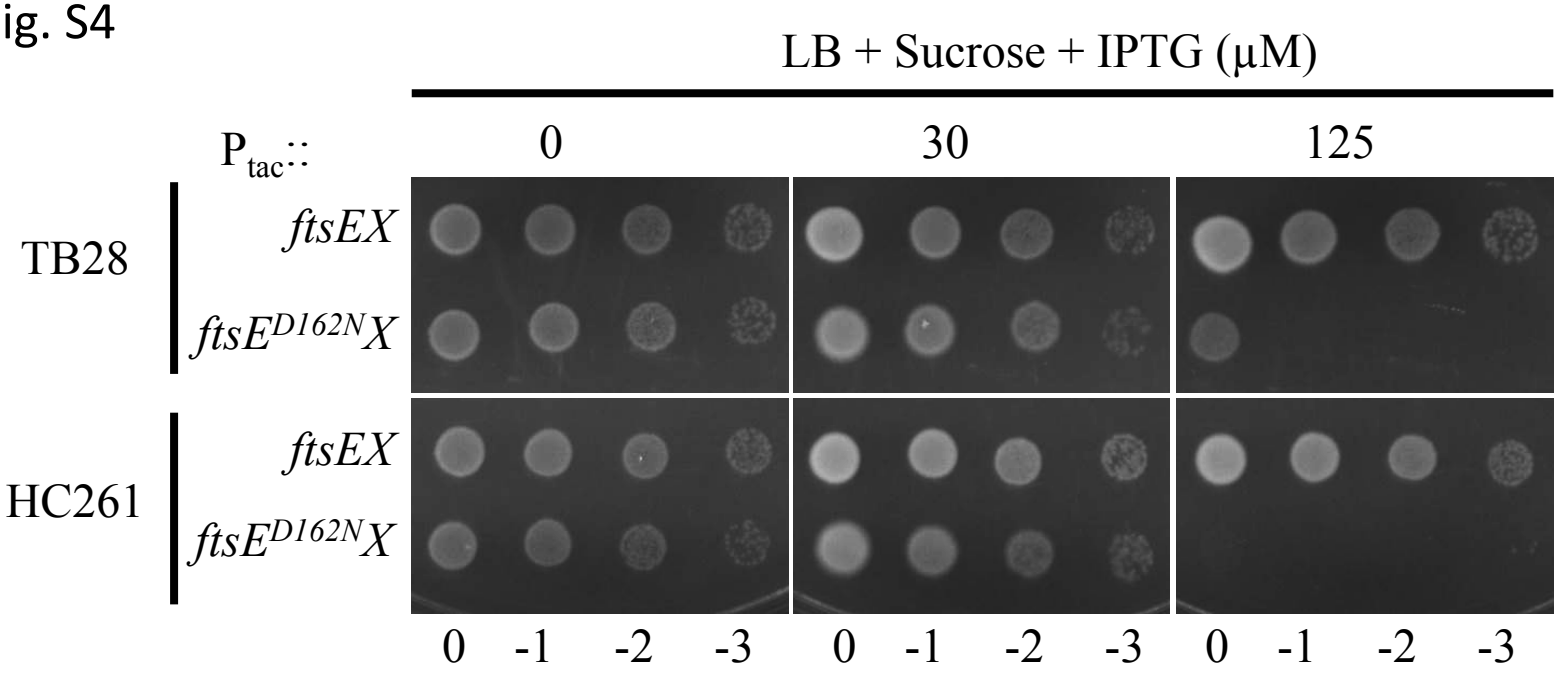

LB + Sucrose + IPTG (μM)
P tac::
0
30
125
TB28
ftsEX
ftsE D162N X
HC261
ftsEX
ftsE D162N X
0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3

Fig. S5

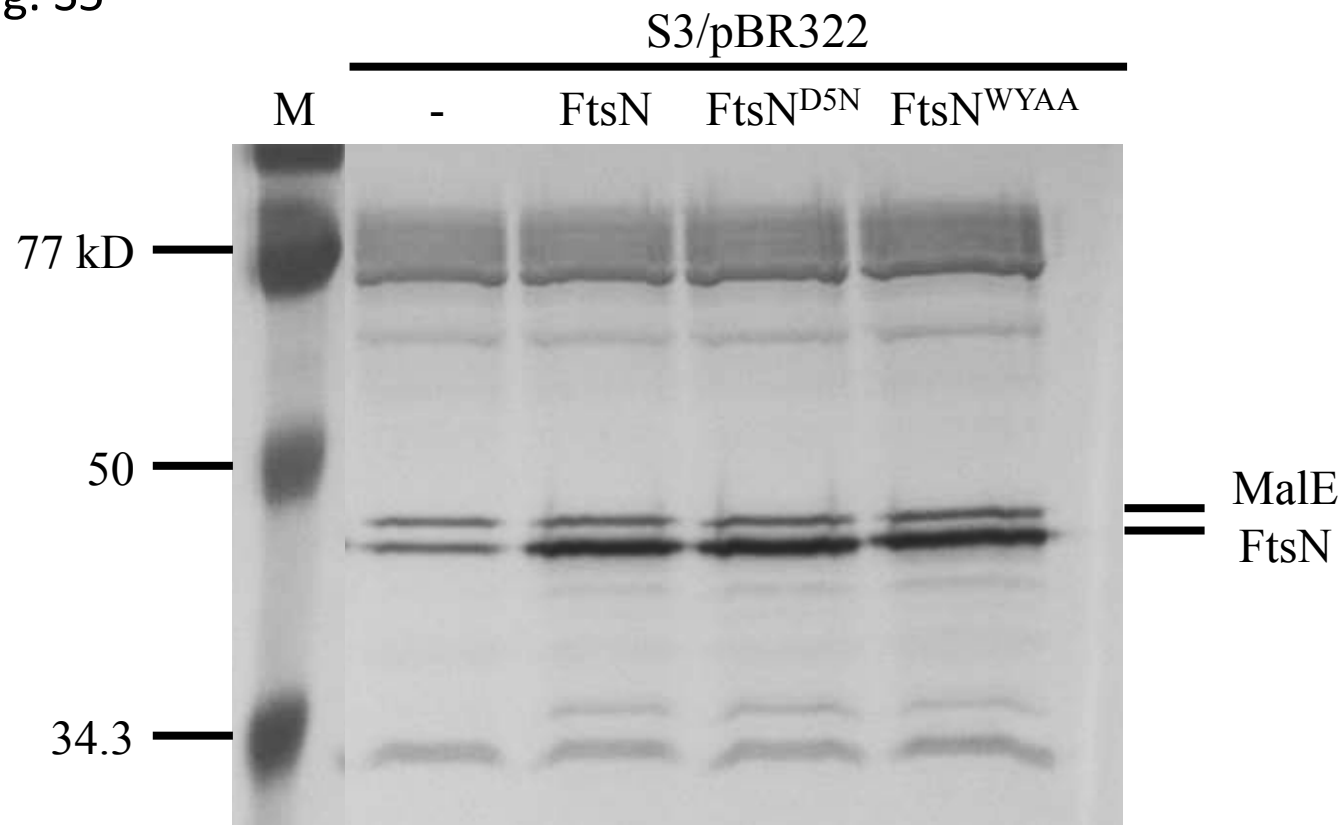
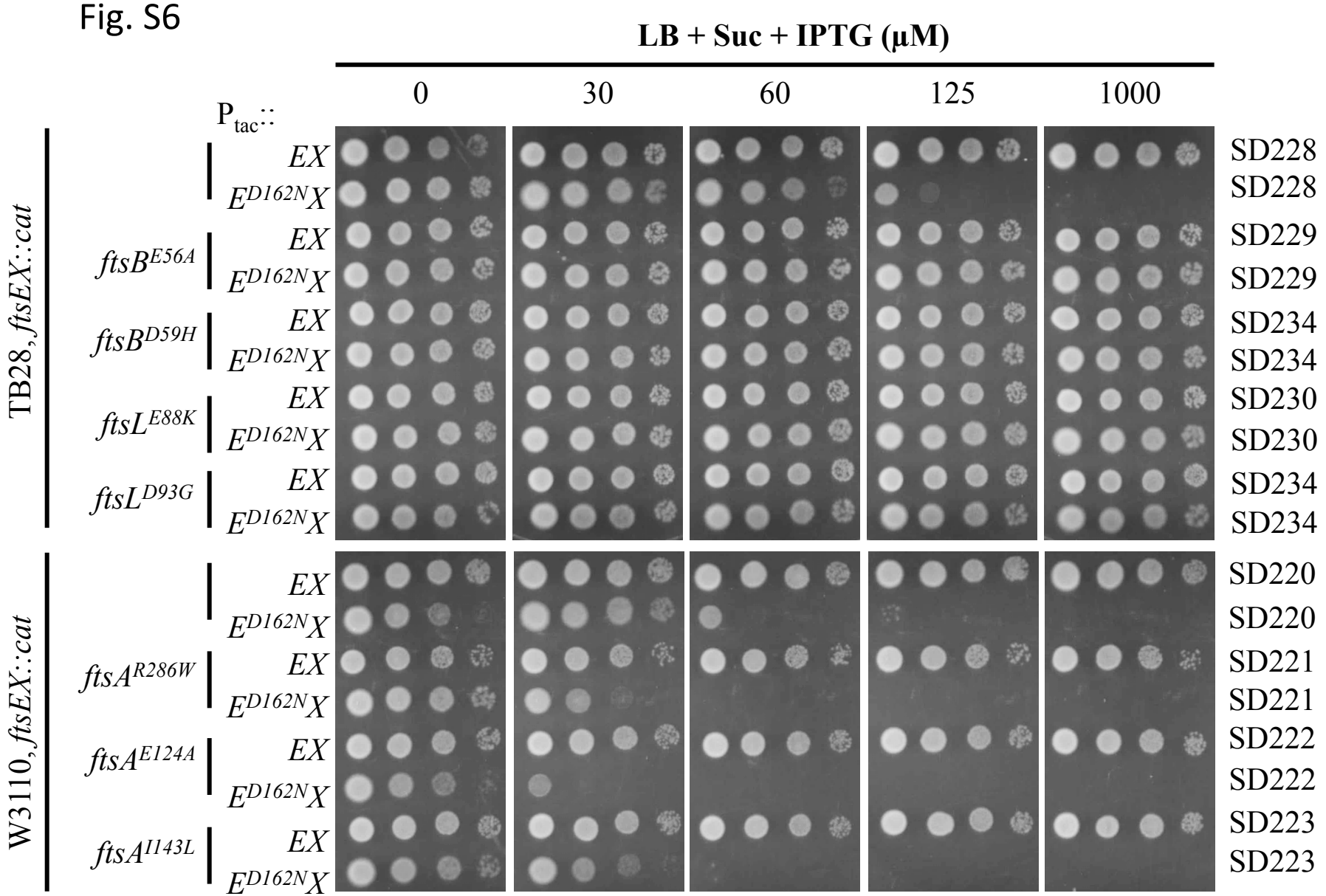


Fig. S6


LB + Suc + IPTG (μM)
0
30
60
125
1000
P tac ::
TB28, ftsEX::cat
W3110, ftsEX::cat
EX
E D162N X
ftsB E56A
ftsB D59H
ftsL E88K
ftsL D93G
ftsA R286W
ftsA E124A
ftsA I143L
SD228
SD228
SD229
SD229
SD234
SD234
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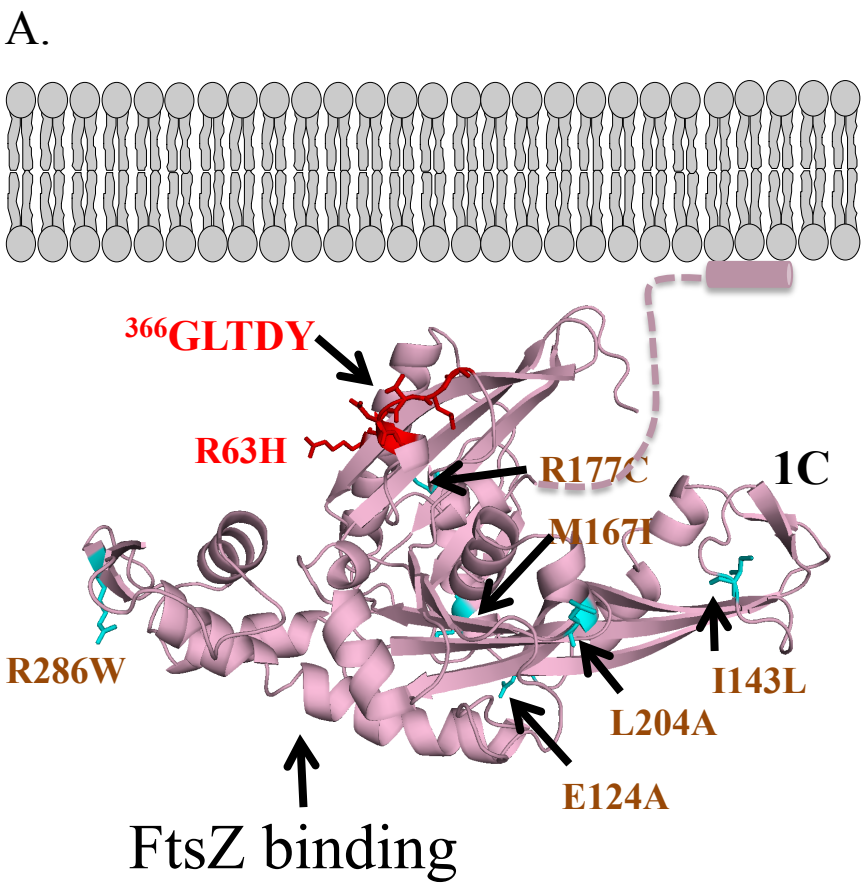
SD234
SD234
SD220
SD220
SD221
SD221
SD222
SD222
SD223
SD223

Fig. S7


A.
366GLTDY
R63H
R177C
1C
M167I
R286W
I143L
L204A
E124A
FtsZ binding

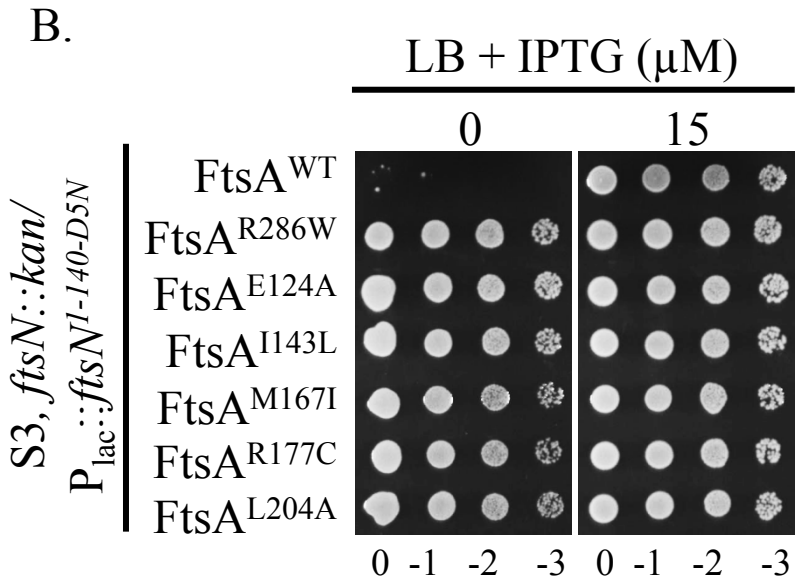

B.
LB + IPTG (μM)
0
15
S3, ftsN::kan/
P lac::ftsN 1-140-D5N
FtsA WT
FtsA R286W
FtsA E124A
FtsA I143L
FtsA M167I
FtsA R177C
FtsA L204A
0 -1 -2 -3 0 -1 -2 -3

Fig. S8

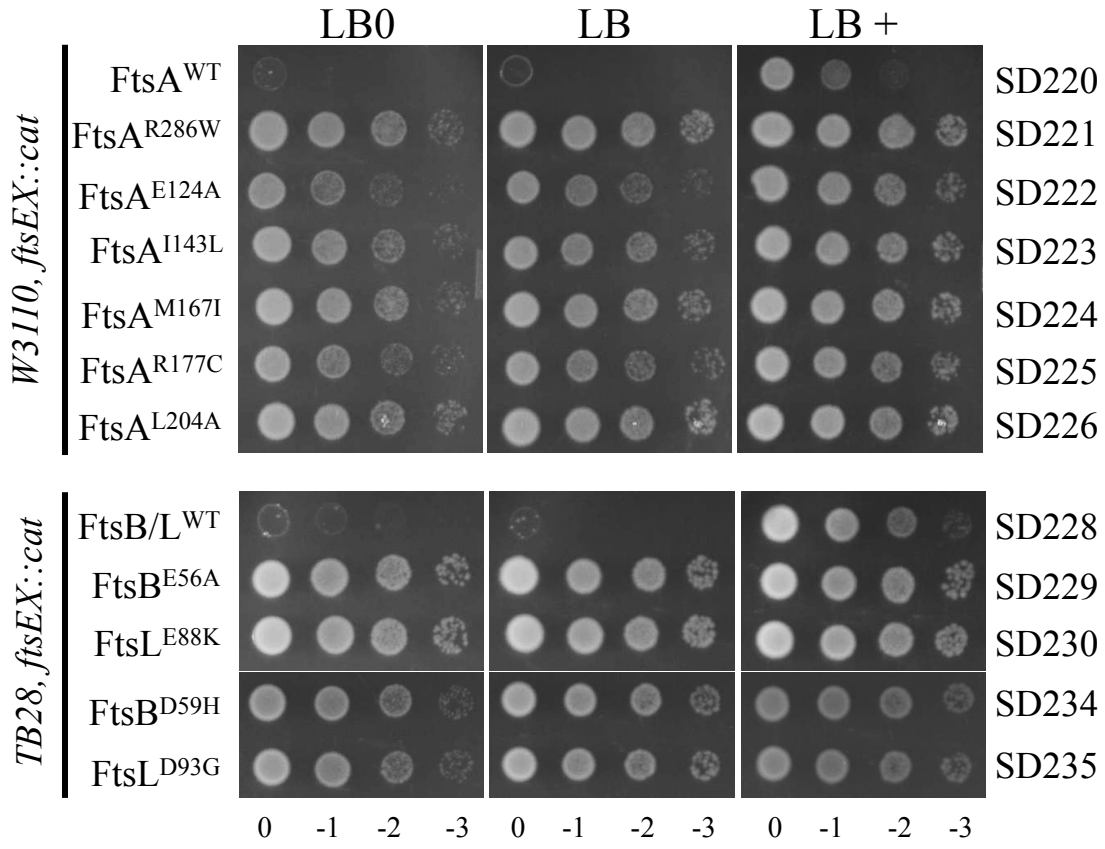

LB0
LB
LB +
W3110, ftsEX::cat
FtsA^WT
FtsA^R286W
FtsA^E124A
FtsA^I143L
FtsA^M167I
FtsA^R177C
FtsA^L204A
SD220
SD221
SD222
SD223
SD224
SD225
SD226
TB28, ftsEX::cat
FtsB/L^WT
FtsB^E56A
FtsL^E88K
FtsB^D59H
FtsL^D93G
SD228
SD229
SD230
SD234
SD235
0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3

Fig. S9

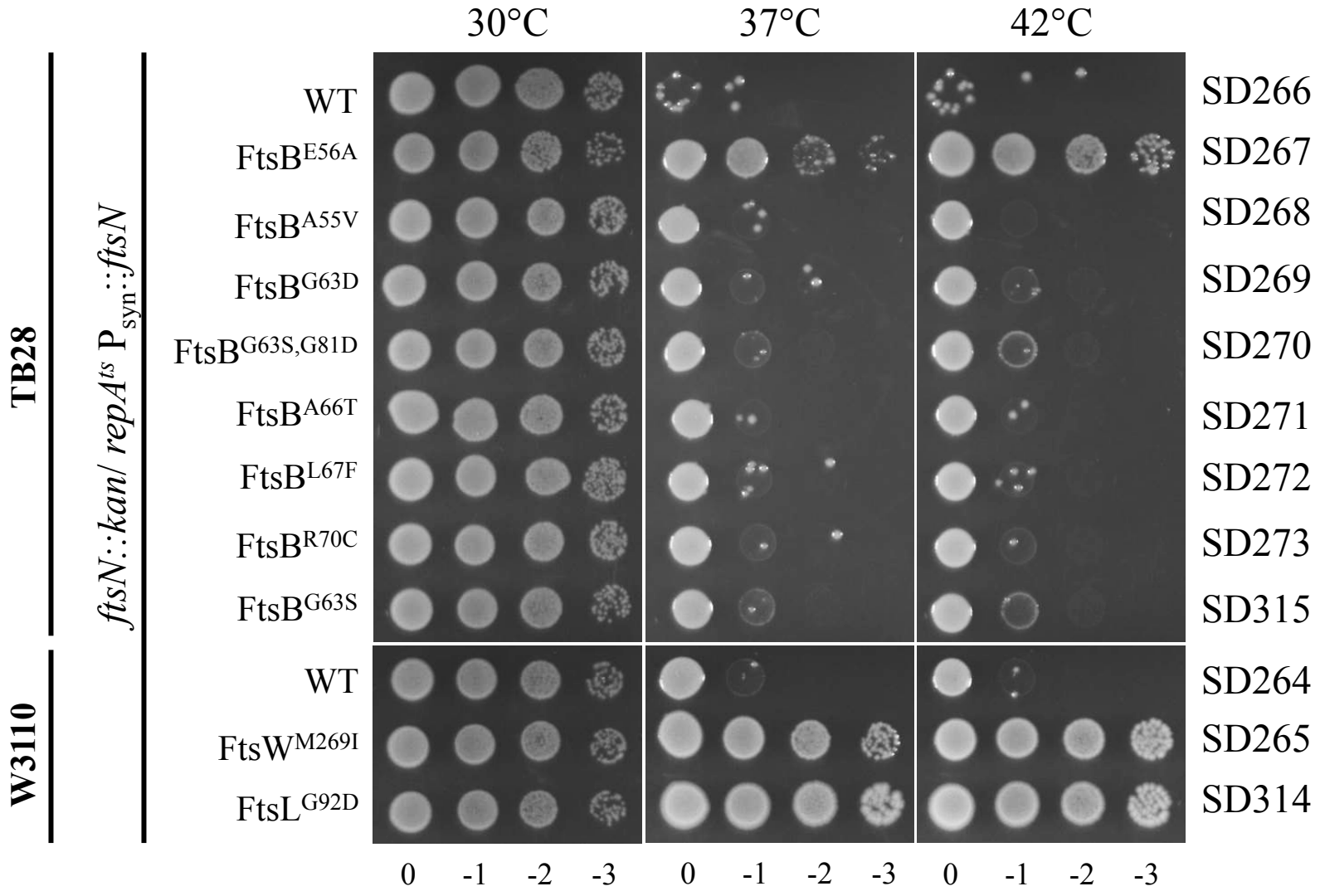

30°C
37°C
42°C
TB28
W3110
ftsN::kan/ repA^ts P_syn::ftsN
WT
FtsB^E56A
FtsB^A55V
FtsB^G63D
FtsB^G63S,G81D
FtsB^A66T
FtsB^L67F
FtsB^R70C
FtsB^G63S
WT
FtsW^M269I
FtsL^G92D
SD266
SD267
SD268
SD269
SD270
SD271
SD272
SD273
SD315
SD264
SD265
SD314
0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3

Fig. S10

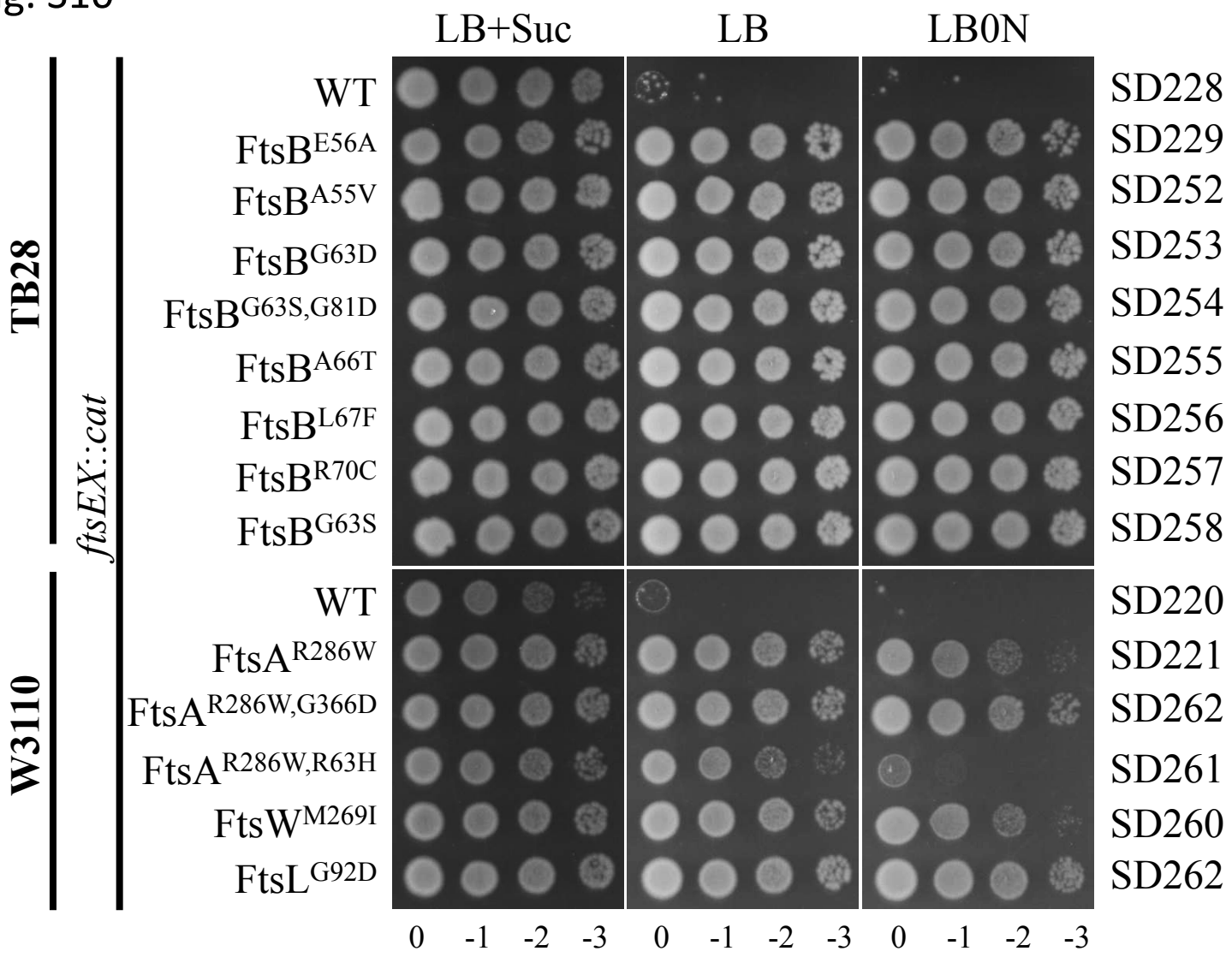

LB+Suc
LB
LB0N
TB28
W3110
ftsEX::cat
WT
FtsB^E56A
FtsB^A55V
FtsB^G63D
FtsB^G63S,G81D
FtsB^A66T
FtsB^L67F
FtsB^R70C
FtsB^G63S
WT
FtsA^R286W
FtsA^R286W,G366D
FtsA^R286W,R63H
FtsW^M269I
FtsL^G92D
SD228
SD229
SD252
SD253
SD254
SD255
SD256
SD257
SD258
SD220
SD221
SD262
SD261
SD260
SD262
0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3

Fig. S11

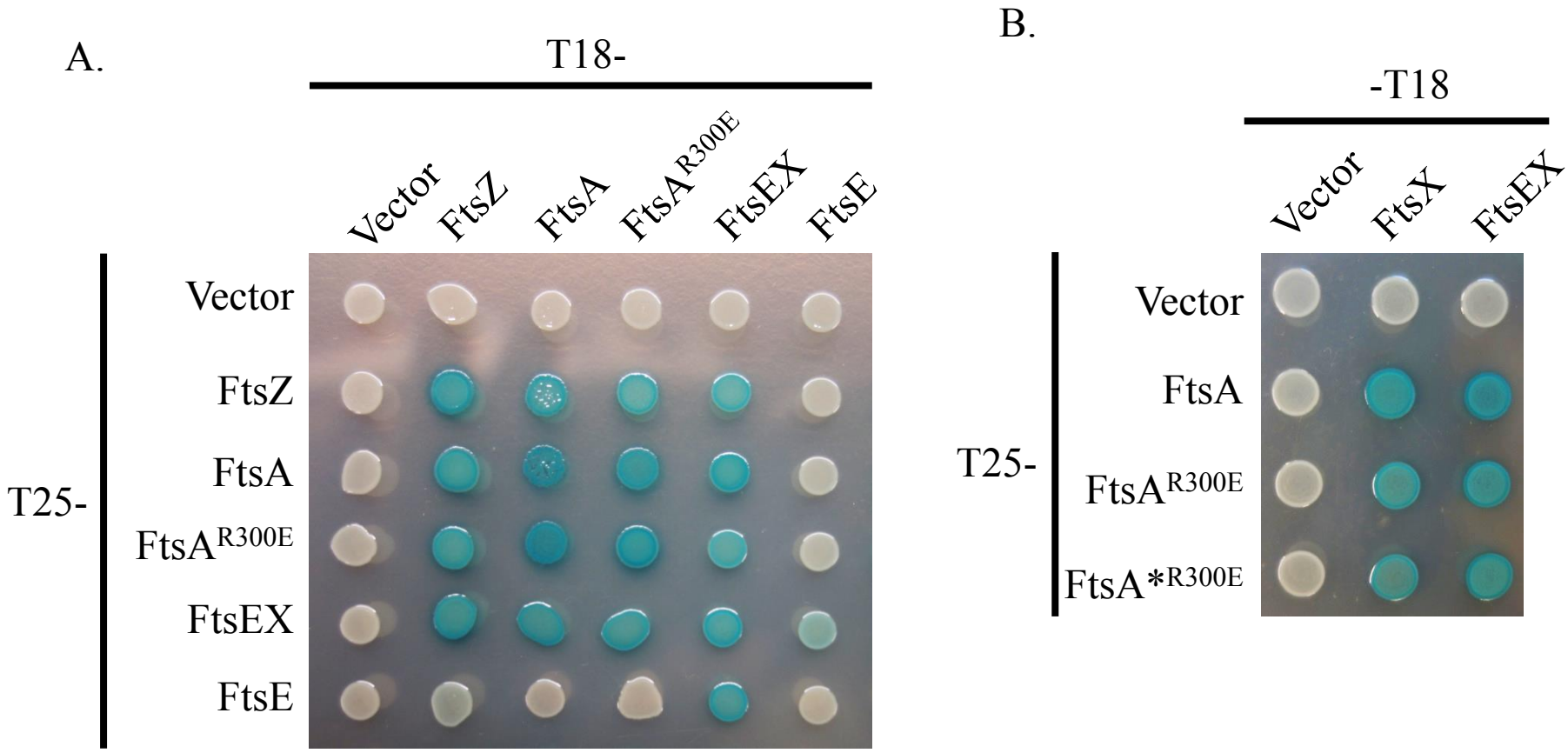
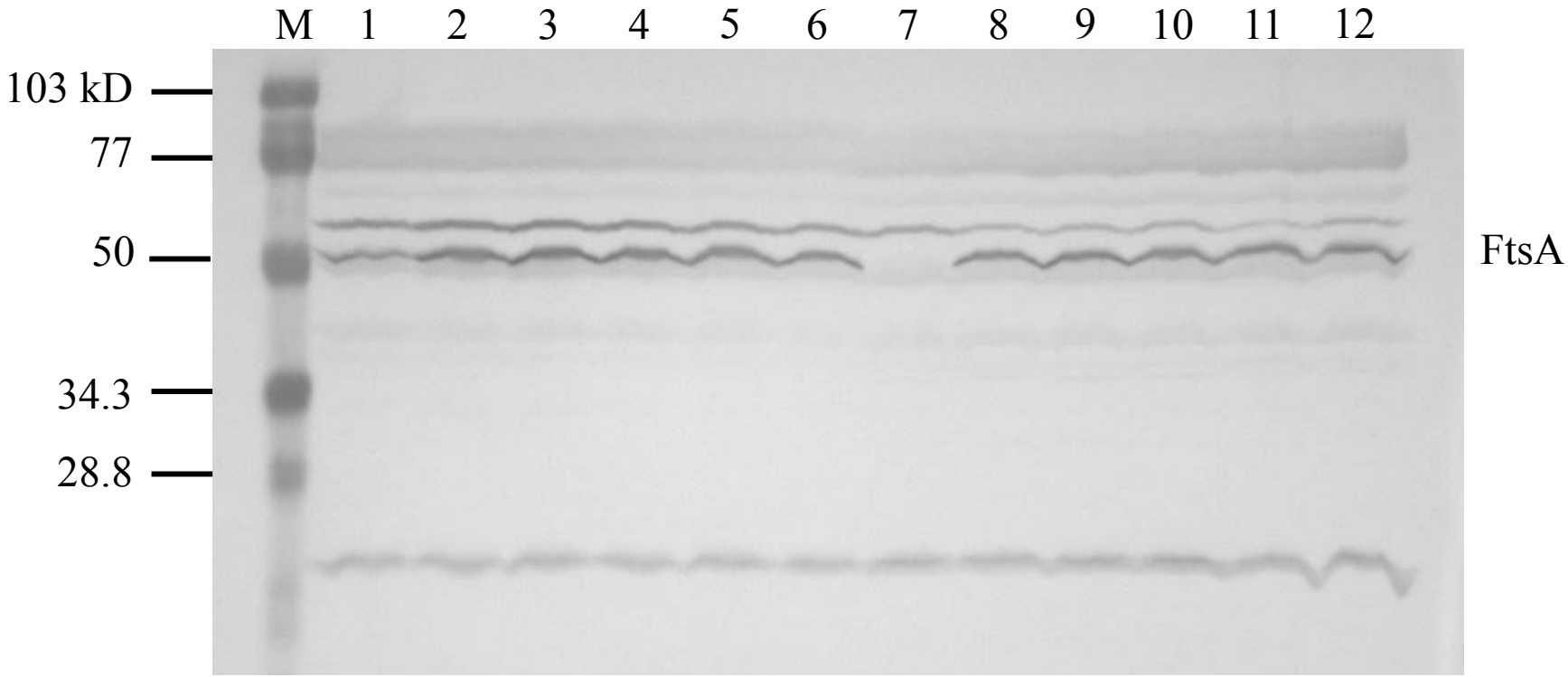

A.
T18-
Vector
FtsZ
FtsA
FtsA R300E
FtsEX
FtsE
T25-
Vector
FtsZ
FtsA
FtsA R300E
FtsEX
FtsE
B.
-T18
Vector
FtsX
FtsEX
T25-
Vector
FtsA
FtsA R300E
FtsA*R300E

Fig. S12



M: Marker
1&7: CH2/pDB280 (FtsA)
2&8: CH2/pDB280 & pSD266 (FtsA)
3&9: CH2/pDB280 & pSD266-R63H ($FtsA^{R63H}$)
4&10: CH2/pDB280 & pSD266-G366D ($FtsA^{G366D}$)
5&11: CH2/pDB280 & pSD266-L367E ($FtsA^{L367E}$)
6&12: CH2/pDB280 & pSD266-D369R ($FtsA^{D369R}$)

Fig. S13

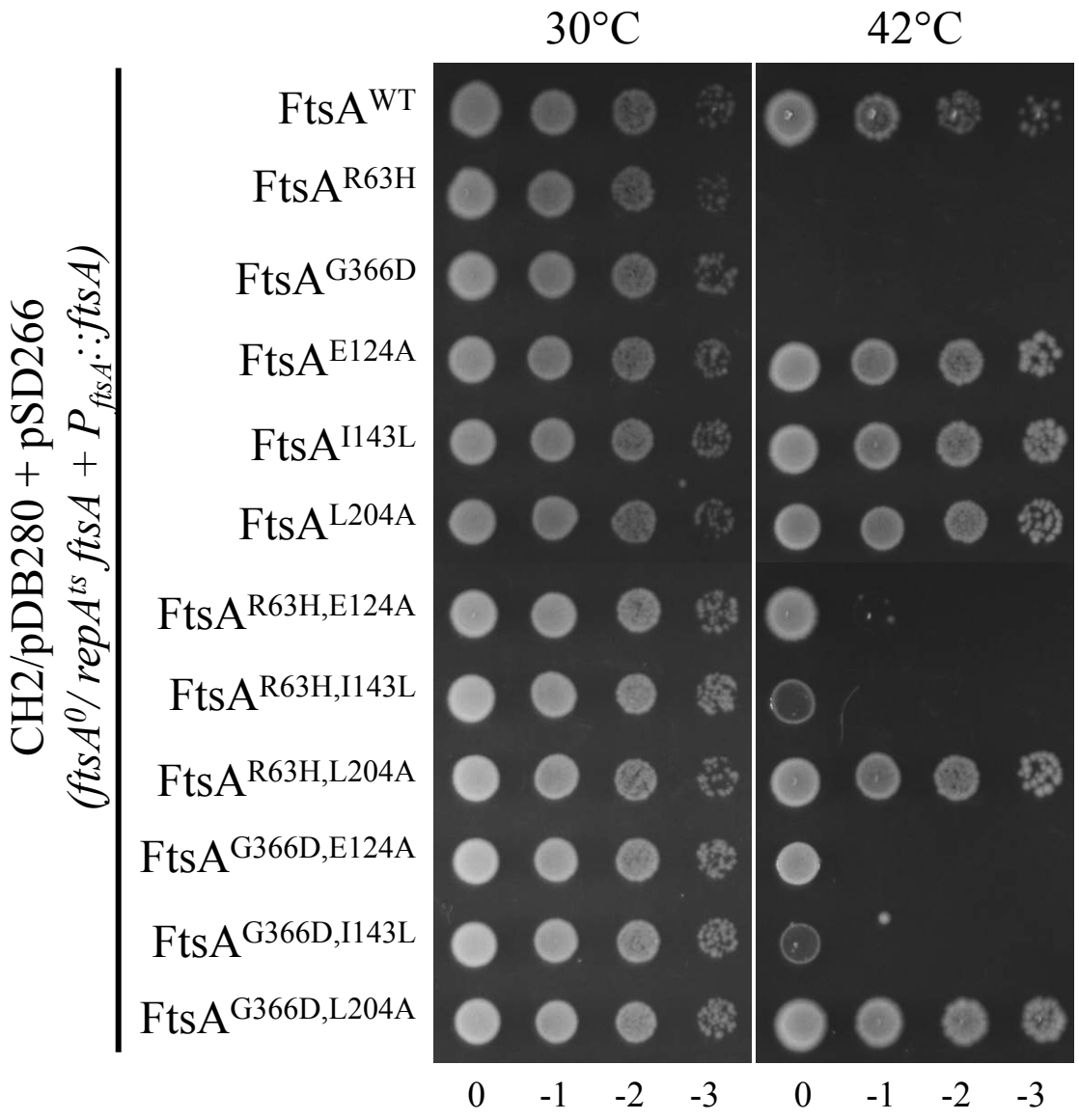

30°C
42°C
CH2/pDB280 + pSD266
(ftsA0/ repAts ftsA + PftsA::ftsA)
FtsAWT
FtsAR63H
FtsAG366D
FtsAE124A
FtsAI143L
FtsAL204A
FtsAR63H,E124A
FtsAR63H,I143L
FtsAR63H,L204A
FtsAG366D,E124A
FtsAG366D,I143L
FtsAG366D,L204A
0 -1 -2 -3 0 -1 -2 -3

Fig. S14

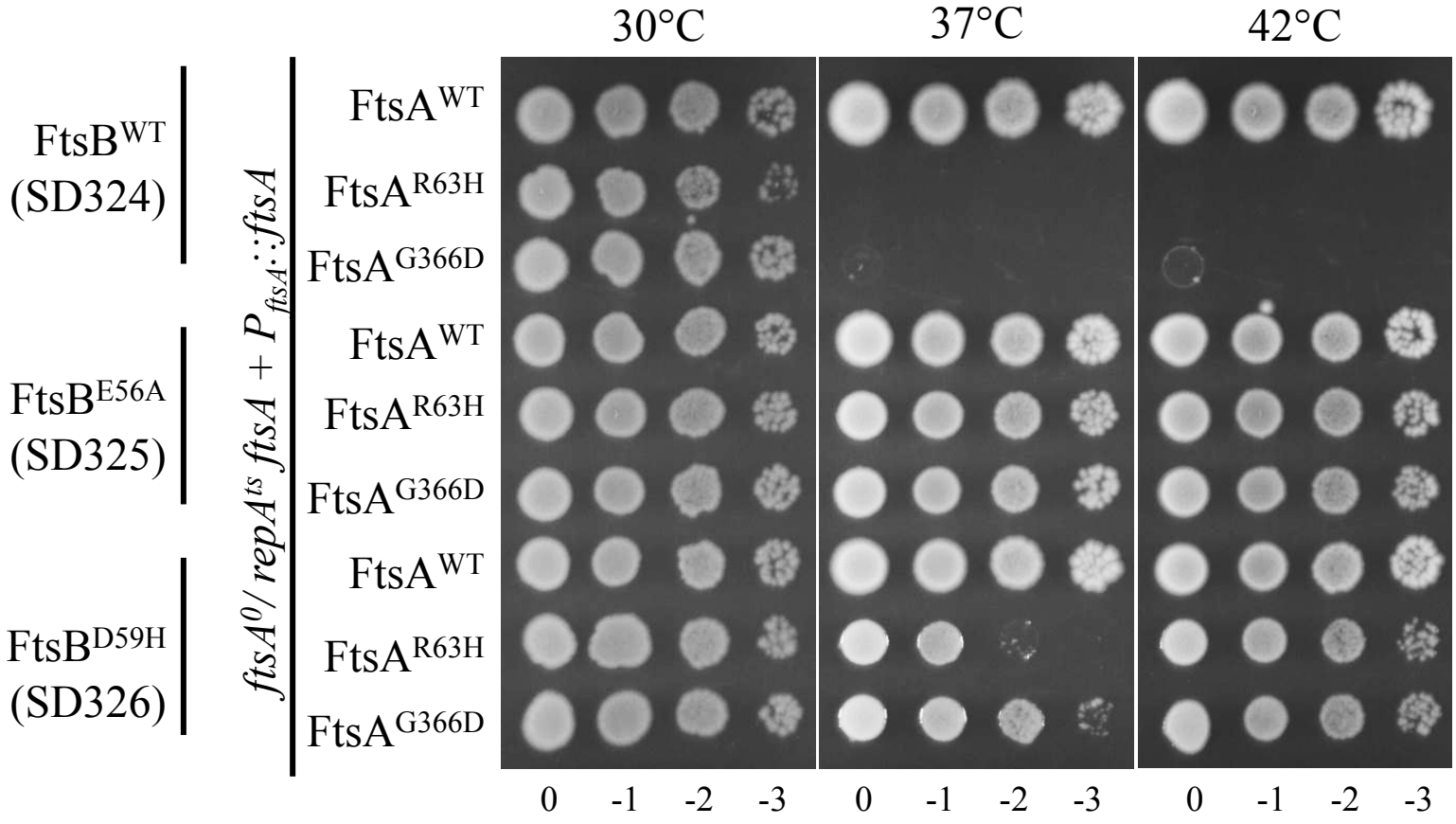

30°C
37°C
42°C
FtsB^WT (SD324)
FtsB^E56A (SD325)
FtsB^D59H (SD326)
ftsA^0/ repA^ts ftsA + P_ftsA::ftsA
FtsA^WT
FtsA^R63H
FtsA^G366D
FtsA^WT
FtsA^R63H
FtsA^G366D
FtsA^WT
FtsA^R63H
FtsA^G366D
0 -1 -2 -3 0 -1 -2 -3 0 -1 -2 -3

Fig. S15

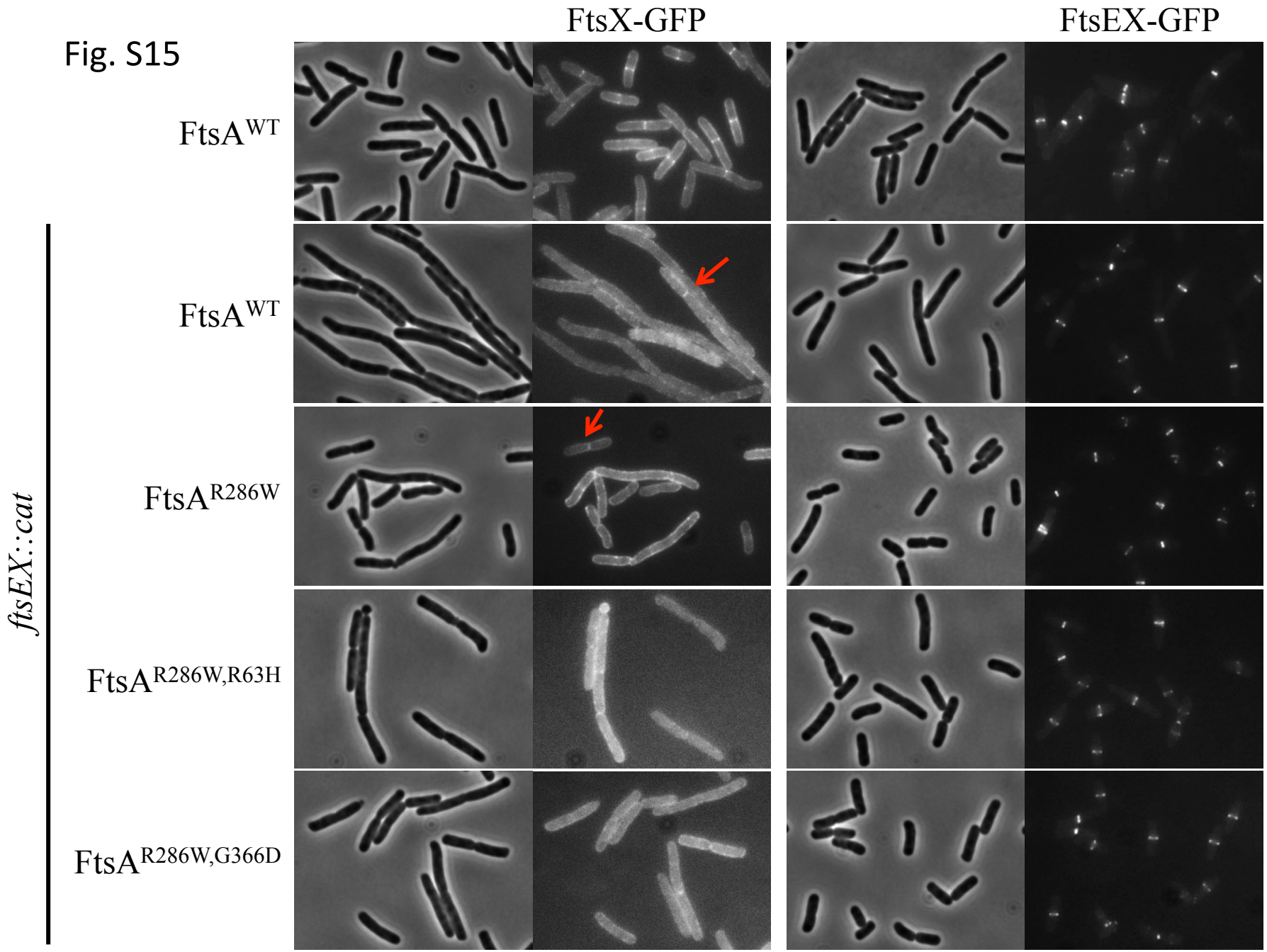

FtsX-GFP
FtsEX-GFP
FtsA^WT
FtsA^WT
FtsA^R286W
FtsA^R286W,R63H
FtsA^R286W,G366D
ftsEX::cat

Fig. S16

CH2/pDB280 + pSD272 + pDSW311

(ftsA^0/ repA^{ts} ftsA + P_{ftsA}::ftsA + P_{lac}::gfp-ftsW)

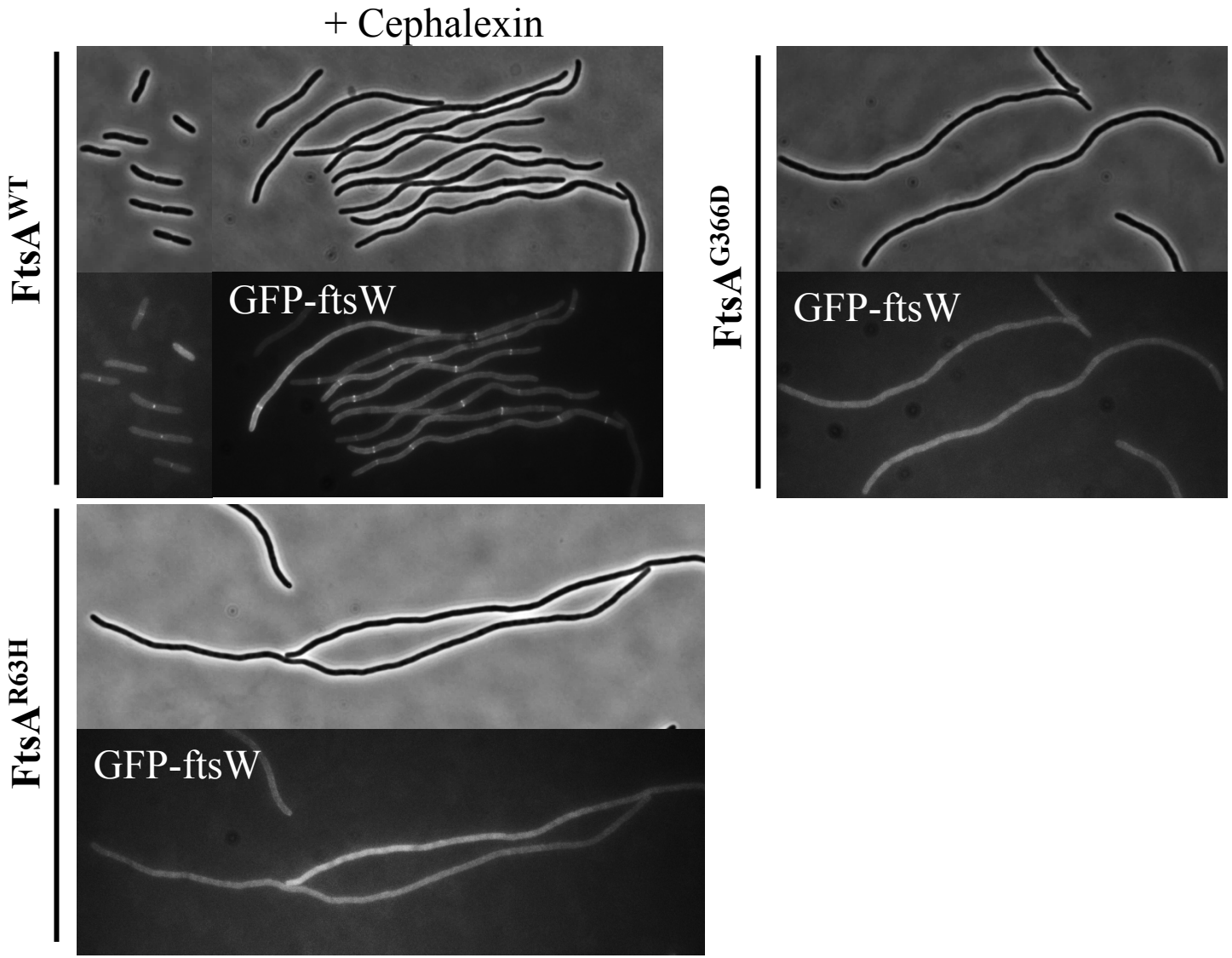


Fig. S17

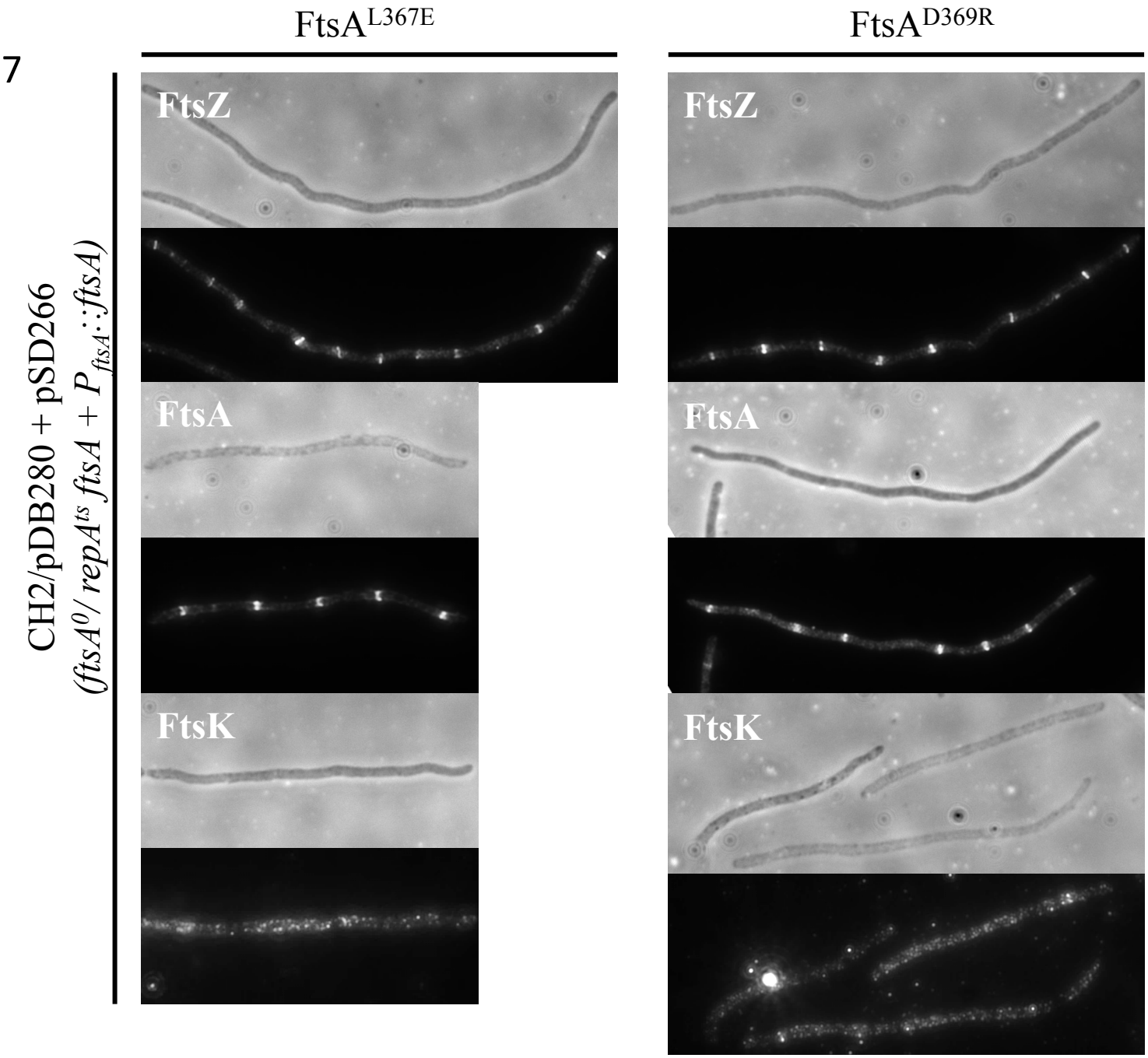

FtsA^L367E
FtsA^D369R
CH2/pDB280 + pSD266
(ftsA0/ repAts ftsA + PftsA::ftsA)
FtsZ
FtsA
FtsK
FtsZ
FtsA
FtsK

Fig. S18

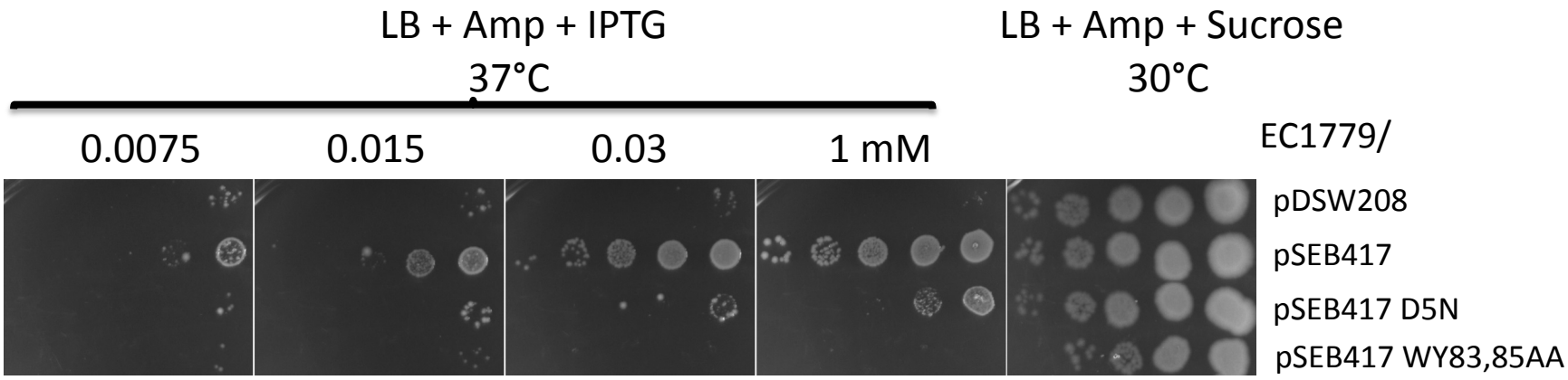

LB + Amp + IPTG
37°C
LB + Amp + Sucrose
30°C
0.0075
0.015
0.03
1 mM
EC1779/
pDSW208
pSEB417
pSEB417 D5N
pSEB417 WY83,85AA

Table S1. List of mutations identified in division genes of the FtsE^{D162N}X suppressors

Gene	AA substitution	Base change	Frequency	Confirmation of resistance
ftsA	R63H	CGC/CAC	1	Yes
	G366D	GGT/GAT	1	Yes
ftsB	A55V	GCC/GTC	1	Yes
	G63D	GGC/GAC	2	Yes
	G63S, G81D	GGC/AGC; GGC/GAC	1	Yes
	A66T	GCG/ACG	1	Yes
	L67F	CTC/TTC	1	Yes
	R70C	CGT/TGT	1	Yes
ftsL	E88K	GAG/AAG	2	Yes
	G92D	GGC/GAC	2	Yes
	H94Y	CAT/TAT	1	NO
ftsW	M269I	ATG/ATA	1	Yes

Table S2. Bacterial strains used in this study.

Strain	Genotype	Source/Reference
BL140	TB28, *ftsB*^{D59H}	(7)
BL154	TB28, *leu::Tn10 ftsL*^{D93G}	(7)
BL167	TB28, *ftsB*^{E56A}	(7)
BTH101	*cya-99 araD139 galE15 galK16 rpsL1 hsdR2 mcrA1 mcrB1*	(8)
CH2/pDB280	PB103, *ftsA*^{0} *recA56-srlD::Tn*10	(9)
EC436	MC4100 Δ(λ*attL-lom*)::*bla lacI* ^{q} P_{207}::*gfp-ftsI*	(10)
EC440	MC4100 *Δ* (λ*att*L-*lom*)::*bla lacI*^{q}P_{207}*::gfp-ftsN*	(11)
EC1779	BW25113, *ftsEX::cat*	Gift from Weiss DS
HC261	TB28, *zapA-gfp Cam*^{r}	(12)
MT10	TB28, *ftsL*^{E88K}	(13)
PS2272	S3, *ftsA*^{E124A}	(14)
PS2273	S3, *ftsA*^{I143L}	(14)
PS2279	S3, *ftsA*^{M167I}	(14)
PS2280	S3, *ftsA*^{R177C}	(14)
PS2281	S3, *ftsA*^{L204A}	(14)
PS2343	S3, *ftsA*^{R286W}	(14)
S3	W3110, *leu::Tn10*	(15)
SD205	W3110, *ftsEX::cat*	P1 (EC1779) × W3110
SD220	S3, *ftsEX::cat*	P1 (EC1779) × S3

SD221	S3, *ftsA*^{R286W} *ftsEX::cat*	P1 (EC1779) × PS2343
SD222	S3, *ftsA*^{E124A} *ftsEX::cat*	P1 (EC1779) × PS2272
SD223	S3, *ftsA*^{I143L} *ftsEX::cat*	P1 (EC1779) × PS2273
SD224	S3, *ftsA*^{M167I} *ftsEX::cat*	P1 (EC1779) × PS2279
SD225	S3, *ftsA*^{R177C} *ftsEX::cat*	P1 (EC1779) × PS2280
SD226	S3, *ftsA*^{L204A} *ftsEX::cat*	P1 (EC1779) × PS2281
SD228	TB28, *ftsEX::cat*	P1 (EC1779) × TB28
SD229	TB28, *ftsB*^{E56A} *ftsEX::cat*	P1 (EC1779) × BL167
SD230	TB28, *ftsL*^{E88K} *ftsEX::cat*	P1 (EC1779) × MT10
SD234	TB28, *ftsB*^{D59H} *ftsEX::cat*	P1 (EC1779) × W3110
SD235	TB28, *leu::Tn10 ftsL*^{D93G} *ftsEX::cat*	P1 (EC1779) × BL140
SD238	TB28, *ftsB*^{A55V}	Allelic replacement
SD239	TB28, *ftsB*^{G63D}	Allelic replacement
SD240	TB28, *ftsB*^{G63S,G81D}	Allelic replacement
SD241	TB28, *ftsB*^{A66T}	Allelic replacement
SD242	TB28, *ftsB*^{L67F}	Allelic replacement
SD243	TB28, *ftsB*^{R70C}	Allelic replacement
SD244	TB28, *ftsB*^{G63S}	Allelic replacement
SD246	S3, *ftsL*^{G92D}	Allelic replacement
SD247	S3, *ftsW*^{M269I}	Allelic replacement
SD248	S3, *ftsA*^{R63H,R286W}	Allelic replacement
SD249	S3, *ftsA*^{G366D,R286W}	Allelic replacement
SD252	TB28, *ftsB*^{A55V} *ftsEX::cat*	P1 (EC1779) × SD238

SD253	TB28, *ftsB*^{G63D} *ftsEX::cat*	P1 (EC1779) × SD239
SD254	TB28, *ftsB*^{G63S,G81D} *ftsEX::cat*	P1 (EC1779) × SD240
SD255	TB28, *ftsB*^{A66T} *ftsEX::cat*	P1 (EC1779) × SD241
SD256	TB28, *ftsB*^{L67F} *ftsEX::cat*	P1 (EC1779) × SD242
SD257	TB28, *ftsB*^{R70C} *ftsEX::cat*	P1 (EC1779) × SD243
SD258	TB28, *ftsB*^{G63S} *ftsEX::cat*	P1 (EC1779) × SD244
SD260	S3, *ftsW*^{M269I} *ftsEX::cat*	P1 (EC1779) × SD247
SD261	S3, *ftsA*^{R63H,R286W} *ftsEX::cat*	P1 (EC1779) × SD248
SD262	S3, *ftsA*^{G366D,R286W} *ftsEX::cat*	P1 (EC1779) × SD249
SD263	S3, *ftsL*^{G92D} *ftsEX::cat*	P1 (EC1779) × SD246
SD264	S3, *ftsN::kan*/pBL154	P1 (CH34) × S3/pBL154
SD265	S3, *ftsW*^{M269I} *ftsN::kan*/pBL154	P1 (CH34) × SD247/pBL154
SD266	TB28, *ftsN::kan*/pBL154	P1 (CH34) × TB28/pBL154
SD267	TB28, *ftsB*^{E56A} *ftsN::kan*/pBL154	P1 (CH34) × BL167/pBL154
SD268	TB28, *ftsB*^{A55V} *ftsN::kan*/pBL154	P1 (CH34) × SD238/pBL154
SD269	TB28, *ftsB*^{G63D} *ftsN::kan*/pBL154	P1 (CH34) × SD239/pBL154
SD270	TB28, *ftsB*^{G63S,G81D} *ftsN::kan*/pBL154	P1 (CH34) × SD240/pBL154
SD271	TB28, *ftsB*^{A66T} *ftsN::kan*/pBL154	P1 (CH34) × SD241/pBL154
SD272	TB28, *ftsB*^{L67F} *ftsN::kan*/pBL154	P1 (CH34) × SD242/pBL154
SD273	TB28, *ftsB*^{R70C} *ftsN::kan*/pBL154	P1 (CH34) × SD243/pBL154
SD274	S3, *ftsN::kan*/pMG20	P1 (CH34) × S3/pMG20
SD275	S3, *ftsW*^{M269I} *ftsN::kan*/pMG20	P1 (CH34) × SD247/pMG20
SD276	TB28, *ftsN::kan*/pMG20	P1 (CH34) × TB28/pMG20

SD277	TB28, *ftsB*^{E56A} *ftsN::kan*/pMG20	P1 (CH34) × BL167/pMG20
SD278	TB28, *ftsB*^{A55V} *ftsN::kan*/pMG20	P1 (CH34) × SD238/pMG20
SD279	TB28, *ftsB*^{G63D} *ftsN::kan*/pMG20	P1 (CH34) × SD239/pMG20
SD280	TB28, *ftsB*^{G63S,G81D} *ftsN::kan*/pMG20	P1 (CH34) × SD240/pMG20
SD281	TB28, *ftsB*^{A66T} *ftsN::kan*/pMG20	P1 (CH34) × SD241/pMG20
SD282	TB28, *ftsB*^{L67F} *ftsN::kan*/pMG20	P1 (CH34) × SD242/pMG20
SD283	TB28, *ftsB*^{R70C} *ftsN::kan*/pMG20	P1 (CH34) × SD243/pMG20
SD290	W3110, *ftsA*^{0}/pSD263	P1 (CH2) × S3/pSD263
SD296	S3, *ftsA*^{0}/pSD263	P1 (S3) × SD290
SD303	TB28, *ftsN::kan*/pSEB413	P1 (CH34) × TB28/pSEB413
SD314	S3, *ftsL*^{G92D} *ftsN::kan*/pBL154	P1 (CH34) × SD246/pBL154
SD315	TB28, *ftsB*^{G63S} *ftsN::kan*/pBL154	P1 (CH34) × SD244/pBL154
SD316	S3, *ftsL*^{G92D} *ftsN::kan*/pMG20	P1 (CH34) × SD246/pMG20
SD317	TB28, *ftsB*^{G63S} *ftsN::kan*/pMG20	P1 (CH34) × SD244/pMG20
SD318	TB28, *leu::Tn10 ftsA*^{0} /pDB280	P1 (SD296) × TB28/pDB280
SD319	TB28, *leu::Tn10 ftsA*^{0} *ftsB*^{E56A}/pDB280	P1 (SD296) × BL167/pDB280
SD320	TB28, *leu::Tn10 ftsA*^{0} *ftsB*^{D59H}/pDB280	P1 (SD296) × BL140/pDB280
SD321	TB28, *ftsA*^{0}/pDB280	P1 (SD303) × SD318
SD322	TB28, *ftsA*^{0} *ftsB*^{E56A}/pDB280	P1 (SD303) × SD319
SD323	TB28, *ftsA*^{0} *ftsB*^{D59H}/pDB280	P1 (SD303) × SD320
SD324	TB28, *ftsA*^{0} *recA56-srlD::Tn10*/pDB280	P1 (CH2) × SD321
SD325	TB28, *ftsA*^{0} *ftsB*^{E56A} *recA56-srlD::Tn10*/pDB280	P1 (CH2) × SD322
SD326	TB28, *ftsA*^{0} *ftsB*^{D59H} *recA56-srlD::Tn10*/pDB280	P1 (CH2) × SD323

SD327	S3, *ftsN::kan*/pSEB414-D5N	P1 (SD303) × S3/pSEB414-D5N
SD328	S3, *ftsA*^{E124A} *ftsN::kan*/pSEB414-D5N	P1 (SD303) × PS2272/pSEB414-D5N
SD329	S3, *ftsA*^{I143L} *ftsN::kan*/pSEB414-D5N	P1 (SD303) × PS2273/pSEB414-D5N
SD330	S3, *ftsA*^{M167I} *ftsN::kan*/pSEB414-D5N	P1 (SD303) × PS2279/pSEB414-D5N
SD331	S3, *ftsA*^{R177C} *ftsN::kan*/pSEB414-D5N	P1 (SD303) × PS2280/pSEB414-D5N
SD332	S3, *ftsA*^{L204A} *ftsN::kan*/pSEB414-D5N	P1 (SD303) × PS2281/pSEB414-D5N
SD333	S3, *ftsA*^{R286W} *ftsN::kan*/pSEB414-D5N	P1 (SD303) × PS2343/pSEB414-D5N
TB28	MG1655, *lacIZYA<>frt*	(16)

Table S3. Plasmid used in this study.

Plasmid	Genotype	Source/ Reference
pBANG100	pACYC184, *ftsZ*$^{+}$, *Kan*^{r}	Bang Shen
pBANG112	pACYC184, *ftsZ*$^{+}$, *Amp*^{r}	(17)
pBL154	pSC101^{ts}, P_{syn}::*ftsN*, *Spc*^{r}	(7)
pBR322	pMB1, *Amp*^{r}	Lab collection
pDB280	pSC101^{ts}, *ftsA*$^{+}$, *Cam*^{r}	(9)
pDSW208	pDSW204-MCS-*gfp*, *Amp*^{r}	(10)
pDSW209	pDSW206-*gfp*-MCS, *Amp*^{r}	(10)
pDSW210	pDSW206-MCS-*gfp*, *Amp*^{r}	(10)
pDSW311	pDSW209, P_{206}::*gfp-ftsW*, *Amp*^{r}	(18)
pDSW610	pBAD33, P_{BAD}::*ftsEX*, *Cam*^{r}	(19)
pDSW610-D162N	pBAD33, P_{BAD}::*ftsE*^{D162N}*X*, *Cam*^{r}	This study
pEXT22	*ori* (R100) P_{tac}::, *Kan*^{r}	(20)
pEX-T18	pUT18, P_{lac}::*ftsEX-T18*, *Amp*^{r}	This study
pKD46	pSC101^{ts}, P_{BAD}:: *exo*, *bet*, *gam*, *Kan*^{r}	(5)
pKD140	pBR322, *ftsN*$^{+}$, *Amp*^{r}	(21)
pKD140-D5N	pBR322, *ftsN*^{D5N}, *Amp*^{r}	(22)
pKD140-WYAA	pBR322, *ftsN*^{W83A,Y85A}, *Amp*^{r}	This study
pKT25	pACYC184, P_{lac}::*T25-*, *Kan*^{r}	(8)
pKT25-E	pACYC184, P_{lac}::*T25-ftsE*, *Kan*^{r}	This study
pKT25-EX	pACYC184, P_{lac}::*T25-ftsEX*, *Kan*^{r}	This study

pMG20	pBAD33, P_{BAD}::^{ss}*torA-bfp-ftsN*$^{71-105}$*-le*	(23)
pSD221	pEXT22, P_{tac}::*ftsEX*, *Kan*^{r}	This study
pSD221-D162N	pEXT22, P_{tac}::*ftsE*^{D162N}*X*, *Kan*^{r}	This study
pSD226	pDSW210, P_{206}:: *ftsX*-gfp, *Amp*^{r}	This study
pSD242	pDSW210, P_{206}:: *ftsEX-gfp*, *Amp*^{r}	This study
pSD255	pSC101^{ts}, P_{syn}::*ftsB*, *Spc*^{r}	This study
pSD255-A55V	pSC101^{ts}, P_{syn}::*ftsB*^{A55V}, *Spc*^{r}	This study
pSD255-G63D	pSC101^{ts}, P_{syn}::*ftsB*^{G63D}, *Spc*^{r}	This study
pSD255-G&D	pSC101^{ts}, P_{syn}::*ftsB*^{G63S,G81D}, *Spc*^{r}	This study
pSD255-G63S	pSC101^{ts}, P_{syn}::*ftsB*^{G63S}, *Spc*^{r}	This study
pSD255-A66T	pSC101^{ts}, P_{syn}::*ftsB*^{A66T}, *Spc*^{r}	This study
pSD255-L67F	pSC101^{ts}, P_{syn}::*ftsB*^{L67F}, *Spc*^{r}	This study
pSD255-R70C	pSC101^{ts}, P_{syn}::*ftsB*^{R70C}, *Spc*^{r}	This study
pSD256	pSC101^{ts}, P_{syn}::*ftsL*, *Spc*^{r}	This study
pSD256-G92D	pSC101^{ts}, P_{syn}::*ftsL*^{G92D}, *Spc*^{r}	This study
pSD257	pSC101^{ts}, P_{syn}::*ftsW*, *Spc*^{r}	This study
pSD257-M269I	pSC101^{ts}, P_{syn}::*ftsW*^{M269I}, *Spc*^{r}	This study
pSD265	pACYC184, P_{ftsA}::*ftsA*, *Amp*^{r}	This study
pSD266	pACYC184, P_{ftsA}::*ftsA*, *Amp*^{r}	This study
pSD266*	pACYC184, P_{ftsA}::*ftsA*^{R286W}, *Amp*^{r}	This study
pSD266-R63H	pACYC184, P_{ftsA}::*ftsA*^{R63H}, *Amp*^{r}	This study
pSD266-G366D	pACYC184, P_{ftsA}::ftsA^{G366D}, Amp^{r}	This study
pSD266-L367E	pACYC184, P_{ftsA}::*ftsA*^{L367E}, *Amp*^{r}	This study

pSD266-D369R	pACYC184, P_{ftsA}::*ftsA*^{D369R}, *Amp*^{r}	This study
pSD266*-R63H	pACYC184, P_{ftsA}::*ftsA*^{R286W,R63H}, *Amp*^{r}	This study
pSD266*-G366D	pACYC184, P_{ftsA}::*ftsA*^{R286W,G366D}, *Amp*^{r}	This study
pSD266*-L367E	pACYC184, P_{ftsA}::*ftsA*^{R286W,L367E}, *Amp*^{r}	This study
pSD266*-D369R	pACYC184, P_{ftsA}::*ftsA*^{R286W,D369R}, *Amp*^{r}	This study
pSD266-R63H&E124A	pACYC184, P_{ftsA}::*ftsA*^{R63H,E124A}, *Amp*^{r}	This study
pSD266-R63H&I143L	pACYC184, P_{ftsQ}::*ftsA*^{R63H,I143L}, *Amp*^{r}	This study
pSD266-R63H&L204A	pACYC184, P_{ftsA}::*ftsA*^{R63H,L204A}, *Amp*^{r}	This study
pSD266-G366D&E124A	pACYC184, P_{ftsA}::*ftsA*^{G366D,E124A}, *Amp*^{r}	This study
pSD266-G366D&I143L	pACYC184, P_{ftsA}::*ftsA*^{G366D,I143L}, *Amp*^{r}	This study
pSD266-G366D&L204A	pACYC184, P_{ftsA}::*ftsA*^{G366D,L204A}, *Amp*^{r}	This study
pSD273	pACYC184, P_{ftsA}::*ftsA*, *Kan*^{r}	This study
pSD273-R63H	pACYC184, P_{ftsA}::*ftsA*^{R63H}, *Kan*^{r}	This study
pSD273-G366D	pACYC184, P_{ftsA}::*ftsA*^{G366D}, *Kan*^{r}	This study
pSEB428	pDSW208, P_{204}::*ftsEX*, *Amp*^{r}	(22)
pSEB413	pDSW210, P_{206}::*ftsN*, *Amp*^{r}	(22)
pSEB414-D5N	pDSW210, P_{206}::*ftsN*$^{1\text{-}140\text{-}D5N}$, *Amp*^{r}	(22)
pSEB428-K41M	pDSW208, P_{204}::*ftsE*^{K41M}*X*, *Amp*^{r}	This study
pSEB428-D162N	pDSW208, P_{204}::*ftsE*^{D162N}*X*, *Amp*^{r}	This study
pSEB428-E163Q	pDSW208, P_{204}::*ftsE*^{E163Q}*X*, *Amp*^{r}	This study
pSEB428-EX$^{\Delta 152\text{-}161}$	pDSW208, P_{204}::*ftsEX*$^{\Delta 152\text{-}169}$, *Amp*^{r}	This study
pSEB428-E^{D162N}X$^{\Delta 152\text{-}161}$	pDSW208, P_{204}::*ftsE*^{D162N}*X*$^{\Delta 152\text{-}169}$, *Amp*^{r}	This study
pT18-E	pMB1, P_{lac}::*T18-ftsE, Amp*^{r}	This tudy

pT18-EX	pMB1, P_{lac}::*T18-ftsEX, Amp^r*	This study
pWM3014	pKT25, P_{lac}::*T25-ftsA*, *Kan^r*	(24)
pWM3014-A^{R300E}	pKT25, P_{lac}::*T25-ftsA^{R300E}*, *Kan^r*	(22)
pWM3014-A*^{R300E}	pKT25, P_{lac}::*T25-ftsA^{R286W,R300E}*, *Kan^r*	This study
pWM3014-A*^{R300E,R63H}	pKT25, P_{lac}::*T25-ftsA^{R286W,R300E,R63H}*, *Kan^r*	This study
pWM3014-A*^{R300E,G366D}	pKT25, P_{lac}::*T25-ftsA^{R286W,R300E,G366D}*, *Kan^r*	This study
pWM3014-A* ^{R300E,L367E}	pKT25, P_{lac}::*T25-ftsA^{R286W,R300E,L367E}*, *Kan^r*	This study
pWM3014-A*^{R300E,D369R}	pKT25, P_{lac}::*T25-ftsA^{R286W,R300E,D369R}*, *Kan^r*	This study
pWM3021	pUT18C, P_{lac}::*ftsA*, *Amp^r*	(24)
pWM3021-A^{R300E}	pUT18C, P_{lac}::*ftsA^{R300E}*, *Amp^r*	(22)
pUT18	pMB1, P_{lac}::-*T18, Amp^r*	(8)
pUT18C	pMB1, P_{lac}::*T18-, Amp^r*	(8)
pX-T18	pUT18, P_{lac}::*ftsX-T18*, *Amp^r*	This study
pZT18	pUT18, P_{lac}::*FtsZ-T18*, *Amp^r*	(15)
pZT25	pKNT, P_{lac}::FtsZ-*T25*, *Kan^r*	(15)

Table S4. Oligonucleotide primers used in this study.

Primer name	Sequence (5’-3’)
ftsA-R63H-F	GTCAAGTGCGTACAACACGCCATTGACCAGGCAG
ftsA-R63H-R	CTGCCTGGTCAATGGCGTGTTGTACGCACTTGAC
ftsA-G366D-F	CCGCTGAACATTACCGATTTAACGGATTATGCTC
ftsA-G366D-R	GAGCATAATCCGTTAAATCGGTAATGTTCAGCGG
ftsA-L367E-F	CTGAACATTACCGGTGAAACGGATTATGCTCAG
ftsA-L367E-R	CTGAGCATAATCCGTTTCACCGGTAATGTTAG
ftsA-D369R-F	CATTACCGGTTTAACGCGTTATGCTCAGGAGCCG
ftsA-D369R-R	CGGCTCCTGAGCATAACGCGTTAAACCGGTAATG
ftsB-A55V-F	GAAACGATCAACTTTTTGTCGAAATTGACGATCTC
ftsB-A55V-R	GAGATCGTCAATTTCGACAAAAAGTTGATCGTTTC
ftsB-G63D-F	GACGATCTCAATGGCGACCAGGAGGCGCTCGAAG
ftsB-G63D-R	CTTCGAGCGCCTCCTGGTCGCCATTGAGATCGTC
ftsB-G63S-F	GACGATCTCAATGGCTCCCAGGAGGCGCTCGAAG
ftsB-G63S-R	CTTCGAGCGCCTCCTGGGAGCCATTGAGATCGTC
ftsB-A66T-F	CAATGGCGGCCAGGAGACGCTCGAAGAGCGTGC
ftsB-A66T-R	GCACGCTCTTCGAGCGTCTCCTGGCCGCCATTG
ftsB-L67F-F	GGCGGCCAGGAGGCGTTCGAAGAGCGTGCGC
ftsB-L67F-R	GCGCACGCTCTTCGAACGCCTCCTGGCCGCC
ftsB-R70C-F	GGAGGCGCTCGAAGAGTGTGCGCGTAATGAAC
ftsB-R70C-R	GTTCATTACGCGCACACTCTTCGAGCGCCTCC
ftsE-K41M-F	CATTCCGGCGCAGGGATGAGTACCCTCCTGAAG

ftsE-K41M-R	CTTCAGGAGGGTACTCATCCCTGCGCCGGAATG
ftsE-D162N-F	GCGGTACTGCTGGCGAACGAACCGACTGGTAAC
ftsE-D162N-R	GTTACCAGTCGGTTCGTTCGCCAGCAGTACCGC
ftsE-E163Q-F	GTACTGCTGGCGGACCAACCGACTGGTAACCTG
ftsE-E163Q-R	CAGGTTACCAGTCGGTTGGTCCGCCAGCAGTAC
ftsN-D5N-FW	TGGCACAACGAAATTATGTACGCCGCAGCCAACC
ftsN-D5N-BW	CCGGTTGGCTGCGGCGTACATAATTTCGTTGTGC
ftsN-WYAA-F	CCAGAAGAACGCGCTCGCGCCATTAAAGAGCTGG
ftsN-WYAA-R	CCAGCTCTTTAATGGCGCGAGCGCGTTCTTCTGG
ftsL-G92D-F	GAAGAGAATGCGCTCGACGACCATAGCCGGGTG
ftsL-G92D-R	CACCCGGCTATGGTCGTCGAGCGCATTCTCTTC
ftsW-M269I-F	GTTAACGCAATCGCTGATAGCGTTTGGTCGCGGCG
ftsW-M269I-R	CGCCGCGACCAAACGCTATCAGCGATTGCGTTAAC
5-NcoI-ftsE	CACCCATGGCCCGAGAGGATTAACAATGATTCG
3-ftsX-HindIII	CACAAGCTTATTATTCAGGCGTAAAGTGG
ftsX161-F	GAAGACGCACTGGGTGAGCTGGATATGCTGGAAGAA
ftsX152-R	CTTCCAGCATATCCAGCTCACCCAGTGCGTCTTCAC
5-BamHI-ftsE	GACTGGATCCCAGACCATGGCCCGAGAG
3-ftsX-EcoRI	GACTGAATTCGCTTCAGGCGTAAAGTGGCG
5-XbaI-ftsE	GACTCTAGACCAGGTTCAGCAGCCAGTGATGGATCGCTACATTCGCTTTGAACATG
3-ftsE-KpnI	GACTGGTACCGCGCTTATTCATGGCCCAC
3-ftsX-KpnI	GACTGGTACCTTATTCAGGCGTAAAGTGGC
5-SacI-ftsE	GACTGAGCTCGACCATGGCCCGAGAGGATT
ftsX-R-HindIII	ATGCAAGCTTGTGAATAAATTAC

5-ftsX-EcoRI	CATGGAATTCAATAAGCGCGATGCAATC
3-ftsX-PstI	CATGCTGCAGTTCAGGCGTAAAGTGGCGTA
5-ftsE-EcoRI	GACGAATTCAGGCCCGAGAGGATTAACAATG
3-SalI-ftsX	GACTGTCGACTTCAGGCGTAAAGTGGCGTA
5-HindIII-ftsB	GACTAAGCTTGGGCTAATTTGTACTTTCCC
3-ftsB-EcoRI	GATCGAATTCCCAGGACTTATGGCAATGAC
5-HindIII-ftsL	GCTAAGCTTCGTATTGTGAAACGTTTTATG
3-ftsL-EcoRI	GACTGAATTCAGAGAACGCATGTCGCCCTC
5-HindIII-ftsW	GACTAAGCTTCGGTCGTGACGGCGCGCAGC
3-ftsW-EcoRI	GACTGAATTCCACGCAGACCAGAGA
5-BamHI-ftsX	GACTGGATCCTGGTCACTTGCATGGAGGC
5-ftsL-seq	GGCAGGGTTACCGATGACTG
5-ftsI-seq	GCGACATGCGTTCTCTTCGC
m-ftsI-seq	GAAAGAGGCGATGCGTAACC
3-ftsI-seq	GATCGCCCGCCGCAGCCACA
5-ftsW-seq	GAAACTATGGAACAGGCGATGC
3-ftsW-seq	GCGAACTTGCCAACCCTGAG
5-ftsQ-seq	GCCAATACCTCACCGGGTATG
m-ftsQ-seq	CGTTCCTGGCAGTTGACGCTGA
m-ftsA-seq	GCAGGCAAAAGTGCACCTGATC
3-ftsA-seq	GAGTCACATCTTAACGGTGAAG
m-ftsZ-seq	GATGCGTTTGGCGCAGCGAAC
3-ftsZ-seq	CGTATTATCTCGCCAAATTACC
5-ftsB-seq	GGGCTAATTTGTACTTTCCC

3-ftsB-seq	CCAGGACTTATGGCAATGAC