SUPPLEMENTARY INFORMATION

SepF supports the recruitment of the DNA translocase SftA to the Z-ring

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Content:

General bacterial growth conditions

E. coli strain TOP10 was used for the construction and propagation of all plasmids. All *B. subtilis* strains were derived from strain 168ca *trpC2* (Zeigler *et al.*, 2008). Strains were grown at 37 °C in Lysogeny broth (LB) medium supplemented with antibiotics. For transformation, *B. subtilis* was grown in minimal medium as described before (Hamoen *et al.*, 2006). Antibiotic concentrations used were 100 µg/ml ampicillin, 5 µg/ml chloramphenicol, 5 µg/ml kanamycin, 10 µg/ml tetracycline, 2 µg/ml phleomycin and 2 µg/ml erythromycin.

The PSIPRED secondary structure prediction method was used to predict secondary structures in the SftA sequences (Jones, 1999). Two helices were found in the first 21 amino acids sequence and Amphipaseek predicts the first one likely to be an amphipathic helix.

Plasmid and strain construction

Strains and plasmids used are listed in table S1 and S2, respectively. Relevant primers used for cloning are shown in table S3. For cloning, either classical restriction or Gibson Assembly was used (Gibson *et al.*, 2009). The deletion mutants of *sftA* (BKE29805, BKK29805) were acquired from the Bacillus genomic stock centre (Koo *et al.*, 2017). Chromosomal DNA of these strains was transformed into wild type *B. subtilis* 168 to achieve strains TNVS083 (*sftA*::*erm*) and TNVS825 (*sftA*::*kan*), respectively.

To localize SftA, YdeL and PsdS, we constructed strains TNVS455, TNVS366 and TNVS810, respectively. To this end, the *sftA* gene was amplified with primers TerS279 and TerS280 from *B. subtilis* 168 genomic DNA and Gibson assembled into plasmid pMW1 (Müller *et al.*, 2016), linearized with primer pairs TerS153/TerS274, resulting in plasmid pTNV144 (P*xyl*-*sftA*-*msfGFP*). This placed the *sftA*-*msfGFP* reporter under control of the xylose-inducible P*xyl* promoter. The primers added an affinity tag of 6 histidines to allow future pulldown experiments, but this was not used in this manuscript. Next, plasmid pTNV144 was

transformed into TNVS83, resulting in strain TNVS455 (*sftA*::*erm* P*xyl*-*sftA*-*msfGFP*). The *ydeL* gene was amplified from *B. subtilis* 168 genomic DNA using primers TerS127 and TerS128. The product was inserted into the *amyE* locus integration plasmid pSG1729 (Lewis and Marston, 1999), using *XhoI* and *NotI* sites, resulting in plasmid pTNV022. Integration of plasmid pTNV022 into wild type *B. subtilis* resulted in strain TNVS042. To construct a PsdS-GFP fusion, first plasmid pTNV143 was created. The monomeric super-folder GFP variant from pTNV064 (Müller *et al.*, 2016) was amplified using primers TerS283/TerS413 and Gibson assembled into pSG1729, which was linearized with primers TerS274 and TerS414, resulting in pTNV100. This plasmid contains no unwanted additional amino acids in the multiple cloning site and can be used to tag proteins of interest at their C-termini with monomeric super folder GFP. Subsequently, pTNV100 was linearized with primers TerS484 and TerS283 and circularized using Gibson assembly, resulting in plasmid pTNV143 (P*xyl*-*msfGFP*). The *psdS* gene was amplified with primers TerS621 and TerS622 from *B. subtilis* 168 genomic DNA and Gibson assembled into pTNV143 linearized with primers TerS274 and TerS368. The Gibson assembly product was transformed directly into competent *B. subtilis* 168 cells, resulting in strain TNVS810 (P*xyl*-*psdS*-*msfgfp*). The fusion proteins were expressed from the xylose inducible P*xyl* promoter.

To test whether SftA is recruited to cell division site by the early or late cell division proteins, two strains were constructed each harbouring the mCherry-ZapA and SftA-GFP reporter fusions, and one containing IPTG-inducible *ftsZ* and the other IPTG-inducible *pbpB*. *sftA* was amplified from *B. subtilis* genomic DNA with primers TerS001 and TerS002, which adds a unique *ApaI* and *EcoRI* restriction site at each flank. Subsequently, the *ApaI* and *EcoRI* digested product was inserted into linearized pSG1154 (Lewis and Marston, 1999), resulting in plasmid pTNV001. This plasmid was transformed into competent *B. subtilis* 168 cells, resulting in strain TNVS001 (*Pxyl*-*sftA*-*gfp*). To simultaneously express mCherry-ZapA and

SftA-GFP, competent cells of strain EKB36 (*zapA*::*Pxyl-mCherry-zapA*, kind gift from Edward de Koning) was transformed with chromosomal DNA from strain TNVS001 (*Pxyl-sftA-gfp*), resulting in strain TNVS353. To tightly control the expression of FtsZ and PbpB from the IPTG-inducible P*spac* promoter, an extra copy of the LacI repressor from strain DS7996 (*lacA*::*lacI*) (Pozsgai *et al.*, 2011) was introduced into *B. subtilis* strain 168, resulting in strain TNVS209. Chromosomal DNA of TNVS209 was used to transform competent cells of strain TNVS353 resulting in strain TNVS758. Inducible FtsZ was acquired by transforming chromosomal DNA of strain 1801 (*ftsZ*::*Pspac-ftsZ*) (Marston *et al.*, 1998) into competent TNVS758, resulting in strain TNVS786. To obtain the IPTG inducible PbpB strain, chromosomal DNA of strain 3294 (*chr*::*PdivIVA-gfp-divIVA Pspac-pbpB*) (Hamoen and Errington, 2003) was first transformed to *B. subtilis* 168 cells to isolate the *Pspac-pbpB* locus, resulting in strain TNVS087 (*Pspac-pbpB*). Finally, chromosomal DNA of strain TNVS87 was transformed into competent TNVS758, resulting in strain TNVS783 (*Pxyl-mCherry-zapA Pxyl-sftA-gfp lacA::lacI Pspac-pbpB*).

To test whether amino acid residues 30 to 75 were required for recruitment of SftA, SftA was first fused to GFP by amplifying the *sftA* gene from genomic DNA with primers TerS279 and TerS280, and linearizing plasmid pMW1 (Müller *et al.*, 2016) with primers TerS153 and TerS274, followed by Gibson assembly, resulting in plasmid pTNV063 (*Pxyl-sftA-msfgfp*). A truncated variant of SftA comprising the first 149 amino acids was then constructed. For this plasmid pTNV063 was PCR amplified with primer pairs TerS153 and TerS145, and TerS203 and TerS146 to yield two separate products. These two products were Gibson assembled to create plasmid pTNV069 (*Pxyl-sftA*(M1-P149)-*msfgfp*). Two different PCR products with plasmid pTNV063 with primer pairs TerS153 and TerS145, and TerS203 and TerS146, which were Gibson assembled, resulting in plasmid pTNV124 (*Pxyl-sftA*(M1-P149)-*msfgfp*). Plasmid pTNV124 was transformed to competent cells of TNVS083 (*sftA*::*erm*) resulting in strain TNVS456. Next, pTNV069 was also PCR amplified with primer pairs TerS180

and TerS342, and TerS179 and TerS341 to give two different products. Gibson assembly of the two products removed the amino acids Q30 to R75 to yield plasmid pTNV093 (*Pxyl-sftA*(M1-P149(Δ30-75))-*msfgfp*). Plasmid pTNV093 was transformed to competent wild type cells to result in strain TNVS224, and subsequent transformation of this strain with genomic DNA from strain TNVS083 (*sftA*::*erm*) resulted in strain TNVS234.

To test whether the amphipathic helix of SftA is sufficient to target the protein to the membrane, this helix was fused to monomeric GFP. To this end, plasmid pTNV124 (P*xyl-sftA*(M1-P149)-*msfgfp*) was amplified with primer pairs TerS153 and TerS274, and TerS287 and TerS573, and Gibson assembled to obtain pTNV187 (P*xyl-sftA*(M1-E14)-*msfgfp*). This plasmid was transformed into competent wild type cells, resulting in strain TNVS586.

To test which early cell division protein is important for SftA recruitment of a SftA variant lacking the N-terminal amphipathic membrane targeting helix, plasmid pTNV144 (*Pxyl-sftA-msfgfp*) was linearized with primer pair TerS274 and TerS282 followed by subsequent recirculation using Gibson assembly, resulting in pTNV202 (*Pxyl-sftA*Δ(S2-E14)-*msfgfp*). This plasmid was then transformed into competent wild type cells, resulting in strain TNVS729. To remove wild type *sftA*, genomic DNA of strain BKK29805 (Koo *et al.*, 2017) was transformed into TNVS729. To evaluate the effect of different cell division proteins, chromosomal DNA from either strain BFA2863 (*sepF*::*erm*) (Hamoen *et al.*, 2006), YK206 (*ftsA*::*erm*) (Ishikawa *et al.*, 2006), LH28 (*ezrA::cat*) (Gamba *et al.*, 2015), or 1356 (*zapA::tet*) (Feucht and Errington, 2005) was transformed into competent TNVS947 cells, resulting in strains TNVS949, TNVS950, TNVS951, and TNVS952, respectively.

To test whether FtsA is important for SftA recruitment, we constructed a minimal 61 amino acid long N-terminal domain that still showed some cell division localization activity by PCR amplifying from plasmid pTNV063 (*Pxyl-sftA-msfgfp*) using primer pair ZT189 and EKP22 followed by recircularization using Gibson assembly, resulting in plasmid pZH014 (*Pxyl-*

sftA(M1-P61)-msfgfp). pZH014 was transformed into competent *B. subtilis* 168 cells, resulting in strain TZH029. To remove wild type *sftA*, genomic DNA of strain BKK29805 (Koo *et al.*, 2017) was transformed into TZH029 resulting in strain TZH035. To label the location of the Z-ring, we amplified a fragment of *zapA::*P_{xy}*-mcherry-zapA-cat* from strain TNVS758 using primer pair TerS314 and TerS315, then transformed this into TZH035, resulting in strain TZH119. To evaluate the effect of a *sepF* or *ftsA* deletions, chromosomal DNA from either strain BFA2863 (*sepF*::*erm*) (Hamoen *et al.*, 2006) or TB07 (*ftsA*::*erm*), were transformed into competent TZH119 cells, resulting in strains TZH120 and TZH121, respectively.

To determine the effect of an SftA null mutant on cell division in different cell division mutants, we first introduced these mutations into the same *B. subtilis* 168 wild type background. To this end genomic DNA from either a *zapA*::*tet* (strain 1356 (Feucht and Errington, 2005)), *sepF*::*spc* (strain YK204 (Ishikawa *et al.*, 2006)), *ftsA*::*erm* (strain YK206) (Ishikawa *et al.*, 2006) or *ezrA*::*spc* (strain PG049 (Gamba *et al.*, 2015)) strain was used to first transform *B. subtilis* 168, resulting in strains TNVS193, TNVS159, TNVS281 and TNVS158, respectively. Subsequently, *sftA* was deleted by introducing *sftA*::*erm* from TNVS083, resulting in TNVS197, TNVS152 and TNVS171, respectively. Strain TNVS825 (*sftA*::*kan*) was combined with YK206 (ftsA::erm) (Ishikawa *et al.*, 2006) to result in strain TNVS869 (*sftA*::*kan ftsA*::*erm*).

To test whether overexpression of SftA affected cell division in the different cell division mutants, the *amyE* locus integration plasmid pSG1729 (Lewis and Marston, 1999), containing a xylose-inducible (Pxyl) *gfp* gene, was PCR amplified to remove the *gfp* gene using primer pairs TerS261 and TerS146, and TerS262 and TerS145. Ligation with Gibson assembly resulted in plasmid pTNV061. We encountered some problems with the cloning in *E. coli* of certain *sftA* containing plasmids, which was bypassed by utilizing a low copy number plasmid. To achieve this, the low copy number origin pSC101 from plasmid pSEN29 (Genevaux *et al.*,

2004) was amplified with primer pair TerS478 and TerS479, to replace the ORI of plasmid pTNV061 by PCR using primer pair TerS226 and TerS382. The two PCR products were Gibson assembled to form the low copy number plasmid pTNV122. To introduce the *sftA* gene, the plasmid was PCR linearized using primer pair TerS263 and TerS274, and the *sftA* gene was amplified with primer pair TerS279 and TerS357. Gibson assembly of both PCR products resulted in plasmid pTNV125 (*Pxyl-sftA*), which was subsequently integrated into the *amyE* locus of wild type *B. subtilis* 168, resulting in strain TNVS390. TNVS390 was transformed with chromosomal DNA from strains LH028 (*ezrA*::*cat*), BFA2863 (*sepF*::*erm*), 1356 (*zapA*::*tet*) and YK206 (*ftsA*::*erm*), resulting in TNVS591, TNVS605, TNVS606 and TNVS622, respectively.

To test the effect of overexpression of the amino acid 1-149 N-terminal domain of SftA on viability, this fragment was amplified with primer pair TerS279 and AH002 from genomic DNA of *B. subtilis* 168 and cloned into the *amyE* integration plasmid pTNV143 linearized with primer pair TerS274 and AH001, using Gibson assembly. The resulting plasmid pTNV166 was transformed to *B. subtilis* 168, resulting in strain TNVS541 (*Pxyl-sftA*(M1-P149)). Strain TNVS541 was transformed with chromosomal DNA of LH028 (Gamba *et al.*, 2015) containing the *ezrA*::*cat* deletion resulting in strain TNVS602. The N-terminal domain of SftA was overproduced by the addition of xylose.

To test whether SftA overexpression in Δ*ezrA* cells influenced Z-ring formation or the recruitment of late cell division proteins, firstly, the spectinomycin marker of strain TNVS390 was replaced with the kanamycin marker of plasmid pYQ29 (unpublished plasmid from Y. Gao), resulting in strain TNVS608 (*Pxyl-sftA*). Subsequently, TNVS608 was transformed with chromosomal DNA from either strain PG62 (*Pspac-yfp-ftsA*) (Gamba *et al.*, 2009), PG227 (*Pspac-yfp-pbpB*) (Gamba *et al.*, 2009) or YK203 (*Pspac-yfp-sepF*) (Ishikawa *et al.*, 2006), resulting in strains TNVS650, TNVS652 and TNVS708, respectively. These strains were subsequently transformed with chromosomal DNA from strain LH028 (*ezrA*::*cat*) (Gamba *et*

175 *al.*, 2015), resulting in the final test strains TNVS650, TNVS652 and TNVS708, respectively.

176 To test the effect of *sftA* and *sepF* deletions on chromosome bisection, strains
177 TNVS371 (*yneABC*::*tet*), TNVS089 (*sftA*::*erm yneABC*::*tet*), TNVS373 (*sepF*::*spc yneABC*::*tet*)
178 and TNVS431 (*sftA*::*erm sepF*::*spc yneABC*::*tet*) were created by transforming genomic DNA
179 of strain YK138 (Bohorquez *et al.*, 2018) into strains *B. subtilis* 168, TNVS083 (*sftA*::*erm*),
180 TNVS159 (*sepF*::*spc*) and TNVS152 (*sftA*::*erm sepF*::*spc*), respectively.

181 To quantify FtsZ-rings formation over nucleoids strain TNVS354 (*chr*::*Pxyl-mCherry-*
182 *zapA sftA::erm*) was constructed by transforming strain EKB036 (kind gift from Edward de
183 Koning) with chromosomal DNA from TNVS083.

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185 **Table S1. *B. subtilis* trains used in this study.**

strain	genotype	reference
168	*trpC2*	(Zeigler *et al.*, 2008)
DS7996	*lacA::lacI-tet amyE::lacZ-cat*	(Pozsgai *et al.*, 2011)
BKE29805	*sftA::erm*	(Koo *et al.*, 2017)
BKK29805	*sftA::kan*	(Koo *et al.*, 2017)
BFA2863	*sepF::erm*	(Hamoen *et al.*, 2006)
3294	*chr::P_{divIVA}-gfp-divIVA - cat chr::(P_{spac}-pbpB neo)*	(Hamoen and Errington, 2003)
1801	*chr::(P_{spac}-ftsZ ble)*	(Marston *et al.*, 1998)
1356	*zapA-yshB::tet*	(Feucht and Errington, 2005)
EKB036	*chr::(P_{xyl}-mCherry-zapA-cat*)	Gift from E. de Koning unpublished
TB07	*ftsA::erm*	This work
LH028	*ezrA::cat*	(Gamba *et al.*, 2015)
PG049	*ezrA::spc*	(Gamba *et al.*, 2015)
PG062	*aprE3'-spc-P_{spac}-yfp-ftsA-aprE5'*	(Gamba *et al.*, 2009)
PG227	*aprE3'-spc-P_{spac}-yfp-pbpB-aprE5'*	(Gamba *et al.*, 2009)
TNVS001	*amyE3'-spc-P_{xyl}-sftA-gfp-amyE5'*	This work
TNVS042	*amyE3'-P_{xyl}-mgfp-ydeL-amyE5'*	This work
TNVS083	*sftA::erm* (BKE29805 transformed to 168)	This work
TNVS087	*chr::(P_{spac}-pbpB neo)* (3294 transformed to 168)	This work
TNVS089	*sftA::erm yneABC::tet*	This work
TNVS152	*sftA::erm sepF::spc*	This work
TNVS158	*ezrA::spc* (PG049 transformed to 168)	This work
TNVS159	*sepF::spc* (YK204 transformed to 168)	This work
TNVS171	*sftA::erm ezrA::spc*	This work
TNVS193	*zapA::tet* (1356 transformed to 168)	This work
TNVS197	*sftA::erm zapA::tet*	This work
TNVS209	*lacA::lacI-tet* (DS7996 transformed to 168)	This work
TNVS224	*amyE3'-spc-P_{xyl}-sftA(M1-P149)Δ(Q30-R75)-mgfp-amyE5'*	This work
TNVS234	*sftA::erm amyE3'-spc-P_{xyl}-sftA(M1-P149)Δ(Q30-R75)-mgfp-amyE5'*	This work
TNVS248	*ydeL::erm* (BKE05240 transformed to 168)	This work
TNVS254	*amyE3'-spc-P_{xyl}-sftA-mgfp-amyE5' sftA::erm ylmFGH::kan*	This work
TNVS281	*ftsA::erm P_{spac}-ftsZ* (YK206 transformed to 168)	This work
TNVS330	*amyE3'-spc-P_{xyl}-sftA-mgfp-amyE5' ftsA::erm*	This work
TNVS342	*amyE3'-spc-P_{xyl}-sftA-mgfp-amyE5' zapA::tet*	This work
TNVS343	*amyE3'-spc-P_{xyl}-sftA-mgfp-amyE5' ezrA::tet*	This work
TNVS353	*chr::(P_{xyl}-mCherry-zapA-cat*) *amyE3'-spc-P_{xyl}-sftA-mgfp-amyE5'*	This work
TNVS366	*amyE3'-P_{xyl}-mgfp-ydeL-amyE5' ydeL::erm*	This work
TNVS371	*yneABC::tet* (YK138 transformed to 168)	This work
TNVS373	*yneABC::tet sepF::spc*	This work
TNVS390	*amyE3'-spc-P_{xyl}-sftA-amyE5'*	This work
TNVS431	*yneABC::tet sepF::spc sftA::erm*	This work
TNVS456	*sftA::erm amyE3'-spc-P_{xyl}-sftA(M1-P149)-mgfp-amyE5*	This work
TNVS541	*amyE3'-spc-P_{xyl}-sftA(M1-P149)-His6-amyE5'*	This work
TNVS586	*amyE3'-spc-P_{xyl}-sftA (M1-E14)-mgfp-amyE5'*	This work
TNVS591	*amyE3'-spc-P_{xyl}-sftA-amyE5' ezrA::cat*	This work
TNVS602	*amyE3'-spc-P_{xyl}-sftA(M1-P149)-2GS-His6-amyE5' ezrA::cat*	This work
TNVS605	*amyE3'-spc-P_{xyl}-sftA-amyE5' sepF::erm*	This work
TNVS606	*amyE3'-spc-P_{xyl}-sftA-amyE5' zapA::tet*	This work
TNVS608	*amyE3'-spc::kan-P_{xyl}-sftA-amyE5'*	This work
TNVS622	*amyE3'-spc-P_{xyl}-sftA-amyE5' ftsA::erm*	This work
TNVS650	*amyE3'-kan-P_{xyl}-sftA-amyE5'- aprE3'-spc-P_{spac}-yfp-ftsA-aprE5'*	This work
TNVS652	*amyE3'-kan-P_{xyl}-sftA-amyE5'- aprE3'-spc-P_{spac}-yfp-sepF-aprE5'*	This work
TNVS708	*amyE3'-kan-P_{xyl}-sftA-amyE5'- aprE3'-spc-P_{spac}-yfp-pbpB-aprE5'*	This work

TNVS710	*amyE3'-kan-P_{xyl}-sftA-amyE5'- aprE3'-spc-P_{spac}-yfp-ftsA-aprE5' ezrA::cat*	This work
TNVS712	*amyE3'-kan-P_{xyl}-sftA-amyE5'- aprE3'-spc-P_{spac}-yfp-sepF-aprE5' ezrA::cat*	This work
TNVS713	*amyE3'-kan-P_{xyl}-sftA-amyE5'- aprE3'-spc-P_{spac}-yfp-pbpB-aprE5' ezrA::cat*	This work
TNVS729	*amyE3'-spc-P_{xyl}-sftAΔ(S2-L12)-4GS-msfGFP-His6-amyE5'*	This work
TNVS758	*chr::(P_{xyl}-mCherry-zapA-cat) cat amyE3'-spc-P_{xyl}-sftA-mgfp-amyE5' lacA::lacI-tet*	This work
TNVS776	*psdS::erm* (BKE34710 transform to 168)	This work
TNVS783	*chr::(P_{xyl}-mCherry-zapA-cat) amyE3'-spc-P_{xyl}-sftA-mgfp-amyE5' lacA::lacI-tet chr::(Pspac-pbpB-neo)*	This work
TNVS786	*chr::(P_{xyl}-mCherry-zapA-cat) amyE3'-spc-P_{xyl}-sftA-mgfp-amyE5' lacA::lacI-tet chr::(P_{spac}-ftsZ ble)*	This work
TNVS810	*amyE3'-spc-P_{xyl}-psdS-mgfp-amyE5'*	This work
TNVS825	*sftA::kan* (BKK29805 transformed to 168)	This work
TNVS947	*amyE3'-spc-P_{xyl}-sftAΔ(S2-L12)-amyE5' sftA::kan*	This work
TNVS949	*amyE3'-spc-P_{xyl}-sftAΔ(S2-L12)-amyE5' sftA::kan ΔsepF::erm*	This work
TNVS950	*amyE3'-spc-P_{xyl}-sftAΔ(S2-L12)-amyE5' sftA::kan ΔftsA::erm*	This work
TNVS951	*amyE3'-spc-P_{xyl}-sftAΔ(S2-L12)-amyE5' sftA::kan ΔezrA::cat*	This work
TNVS952	*amyE3'-spc-P_{xyl}-sftAΔ(S2-L12)-amyE5' sftA::kan ΔzapA::tet*	This work
TZH010	*amyE3'-spc-P_{xyl}-msfgfp-sftAΔ(M1-E250)-amyE5'*	This work
TZH018	*amyE3'-spc-P_{xyl}-msfgfp-sftAΔ(M1-E250)-amyE5' sftA::kan sepF::erm*	This work
TZH021	*amyE3'-spc-P_{xyl}-msfgfp-sftAΔ(M1-E250)-amyE5' sftA::kan*	This work
TZH022	*amyE3'-spc-P_{xyl}-msfgfp-sftAΔ(M1-E250)-amyE5' sftA::kan ftsA::cat*	This work
TZH029	*amyE3'-spc-P_{xyl}-sftA(M1-P61)-msfgfp-amyE5'*	This work
TZH035	*amyE3'-spc-P_{xyl}-sftA(M1-P61)-msfgfp-amyE5' sftA::kan*	This work
TZH051	*amyE3'-spc-P_{xyl}-sftA(M1-P61)-msfgfp-amyE5' sftA::kan ftsA::cat*	This work
TZH053	*amyE3'-spc-Pxyl-sftA(M1-P61)-msfgfp-amyE5' sftA::kan sepF::erm*	This work
TZH119	*amyE3'-spc-P_{xyl}-sftA(M1-P61)-msfgfp-amyE5' chr::(P_{xyl}-mCherry-zapA-cat) sftA::kan*	This work
TZH120	*amyE3'-spc-P_{xyl}-sftA(M1-P61)-msfgfp-amyE5' chr::(P_{xyl}-mCherry-zapA-cat) sftA::kan ftsA::erm*	This work
TZH121	*amyE3'-spc-P_{xyl}-sftA(M1-P61)-msfgfp-amyE5' chr::(P_{xyl}-mCherry-zapA-cat) sftA::kan sepF::erm*	This work
YK138	*yneABC::tet*	(Bohorquez *et al.*, 2018)
YK203	*aprE3'-spc-P_{spac}-yfp-ylmF-aprE5'*	(Ishikawa *et al.*, 2006)
YK204	*sepF::spc*	(Ishikawa *et al.*, 2006)
YK206	*ftsA::erm P_{spac}-ftsZ*	(Ishikawa *et al.*, 2006)
GYQ300	*ftsA::cat*	Kind gift of Y. Gao

g*fp, mgfp* and *msfgfp* indicate green fluorescent protein (GFP), the monomeric and monomeric super folder variants, respectively. *tet, cat, spec* and *erm* indicate tetracycline, chloramphenicol, spectinomycin and erythromycin resistance cassettes, respectively.

Table S2. Plasmids used in this study.

plasmid	relevant genotype	reference
pET24A	*lacI-T_7-lacO-kan*	Novagen
pMW1*	*amyE3'-spc-P_{xyl}-MCS-msfgfp-amyE5' bla*	(Müller *et al.*, 2016)
pNC12	*P_{tac}-malE-sepF*	(Duman *et al.*, 2013)
pSEN29	*bla Rep101 pSC101 ori*	(Genevaux *et al.*, 2004)
pSG1154	*amyE3'-spc-P_{xyl}-mcs-gfp-amyE5' bla*	(Lewis and Marston, 1999)
pSG1729	*amyE3'-spc-P_{xyl}-gfp-mcs-amyE5' bla*	(Lewis and Marston, 1999)
pTNV001	*amyE3'-spc-P_{xyl}-sftA-gfp-amyE5' bla*	This work
pTNV022	*amyE3'-spec-P_{xyl}-mgfp-ydeL-amyE5' bla*	This work
pTNV061	*amyE3'-spec-P_{xyl}-MCS-amyE5' bla*	This work
pTNV063	*amyE3'-spc-P_{xyl}-sftA-msfgfp-amyE5' bla*	This work
pTNV064*	*amyE3': spec-P_{xyl}-MCS-msfgfp-amyE5' bla*	This work
pTNV069	*amyE3': spec-P_{xyl}-sftA(M1-P149)-msfgfp-amyE5' bla*	This work
pTNV082	*lacI-T_7-lacO-sftA-His_6-kan*	This work
pTNV093	*amyE3': spec-P_{xyl}-sftA(M1-P149)Δ(Q30-R75)-mgfp-amyE5' bla*	This work
pTNV100	*amyE3'-spec-P_{xyl}-msfgfp-MCS-amyE5' bla*	This work
pTNV122	*amyE3'-spec-P_{xyl}-MCS-amyE5' bla Rep101 pSC101 ori*	This work
pTNV124	*amyE3': spec-P_{xyl}-sftA(M1-P149)-msfgfp-His_6-amyE5' bla*	This work
pTNV125	*amyE3'-spec-P_{xyl}-sftA-amyE5' bla Rep101 pSC101 ori*	This work
pTNV143	*amyE3': spec-P_{xyl}-msfgfp-His_6-amyE5' bla*	This work
pTNV144	*amyE3'-spc-P_{xyl}-sftA-msfgfp-His_6-amyE5' bla*	This work
pTNV166	*amyE3': spec-P_{xyl}-sftA(M1-P149)-His_6-amyE5' bla*	This work
pTNV187	*amyE3': spec-P_{xyl}-sftA(M1-E14)-His_6-amyE5' bla*	This work
pTNV202	*amyE3': spec-P_{xyl}-sftAΔ(S2-L12)-msfgfp-His_6-amyE5' bla*	This work
pZH008	*amyE3'-spc-P_{xyl}-msfgfp-sftAΔ(M1-E250)-amyE5' bla*	This work
pZH014	*amyE3'-spc-P_{xyl}-sftA(M1-P61)-msfgfp-amyE5' bla*	This work
pYQ29	*spc::kan bla*	Kind gift of Y. Gao

***** the MCS of plasmid pMW1 adds two amino acids to the fused protein.

g*fp, mgfp* and *msfgfp* indicate green fluorescent protein (GFP), the monomeric and monomeric super folder variants, respectively. *tet, cat, spec* and *erm* indicate tetracycline, chloramphenicol, spectinomycin and erythromycin resistance cassettes, respectively. *mcs* indicates multiple cloning site.

Table S3. Primers used in this study.

primer	sequence 5'→3'
AH001	GGCTCAGGAAGCCATCATCACCATCACCACTAG
AH002	TGATGATGGCTTCCTGAGCCCGGTTCGTTCAATGTTTCAGAAGGCT
TerS001	GGGGGGGGGCCCATGAGTTGGCTTCATAAAT
TerS002	CCCCGAATTCGGATCCTGAGCCGCTTCCTGAGCCTTCGTTTATTAAATCAC
TerS127	GGGCTCGAGGGCTCAGGAAGCGGCTCAGGATCCGATATCACGCCTTTTTTGAATAGA
TerS128	CCCGCGGCCGCCTCATATCTTCCAGGCAGCCTTCA
TerS145	ATAAACAAATAGGGGTTCCGCGCA
TerS146	TGCGCGGAACCCCTATTTGTTTAT
TerS153	GGCTCAGGAAGCGGCTCAGGATCCAGTAAAGGAGAAGAACT
TerS179	CGCACACCATCGGACGAACCGA
TerS180	GGTTCGTCCGATGGTGTGCGCGGAATTTGAGCGGGTTTTGTCT
TerS203	CCTGAGCCGCTTCCTGAGCCCGGTTCGTTCAATGTTTCA
TerS226	GGGGAAGGCCATCCAGCCTCGCGT
TerS261	TGGATCCGAAGTCTGGACATCTGCAGGGTACCCATCCTAGGA
TerS262	ATGTCCAGACTTCGGATCCA
TerS263	CAATTCTAGTTCTAGAGCGGCCGCGA
TerS274	CATCCTAGGAATCTCCTTTCTAGA
TerS279	GAAAGGAGATTCCTAGGATGAGTTGGCTTCATAAATTTTTTGA
TerS280	CCTGAGCCGCTTCCTGAGCCTTCGTTTATTAAATCACTTGCT
TerS282	GAAAGGAGATTCCTAGGATGGGCGAAAGCGAAGAGGATGCTGA
TerS283	TTTGTAGAGCTCATCCATGCCATGTGT
TerS287	CTGTAGACAAATTGTGAAAGGAT
TerS341	CGCGGTCATCAATCATACCACCA
TerS342	TGGTATGATTGATGACCGCGTCA
TerS357	CCGCTCTAGAACTAGAATTGTTATTCGTTTATTAAATCACTTGCTGTGA
TerS368	GGCTCAGGAAGCGGCTCAGGATCCAAAGGAGAAGAACTTTTCACTGGAGT
TerS382	CAAAAAGGATCTTCACCTAGATCCT
TerS413	GAAAGGAGATTCCTAGGATGAGCAAAGGAGAAGAACTTTTCACT
TerS414	GCATGGATGAGCTCTACAAAGGCTCAGGAAGCGGCTCAGGATCCTCCAGACTTCGGATCCACGGGCCCCCCCT
TerS478	AGGATCTAGGTGAAGATCCTTTTTGCACCGTTTTCATCTGTGCATATGGA
TerS479	CGCGACGCGAGGCTGGATGGCCTTCCCCAT
TerS484	GGCATGGATGAGCTCTACAAAGGCTCAGGAAGCCATCATCACCATCACCACTAGATGTCCAGACTTCAGATCCA
TerS573	CCTGAGCCGCTTCCTGAGCCTTCGCCTAAAAACAAATCAAAAAATTTATGAAGCCA
TerS621	GAAAGGAGATTCCTAGGATGTTAAAGACTTATCTCATCGACCGCT
TerS622	CCTGAGCCGCTTCCTGAGCCCAATGCTGTCACATTCACGAGGGTGT
ZT059	AAGGCTCAGGAAGCGGCTCAATGCTCACAGATACGGCGGC
ZT060	AGCTTATCGATACCGTCGACTTATTCGTTTATTAAATCACTTGCTGTG
ZT088	TCTAGAAAGGAGATTCCTAGGATGAGTTGGCTTCATAAATTTTTTGATTTG
ZT089	ATTGAGCCGCTTCCTGAGCCTGGAAAACGGAATTTGCCTTTTG
ZT189	CCTAGGAATCTCCTTTCTAGATGC
ZT200	TGAGCCGCTTCCTGAGCCTTTGTAGAGCTCATCCATGCC
EKP22	GTCGACGGTATCGATAAGCTTGAT

Fig. S1

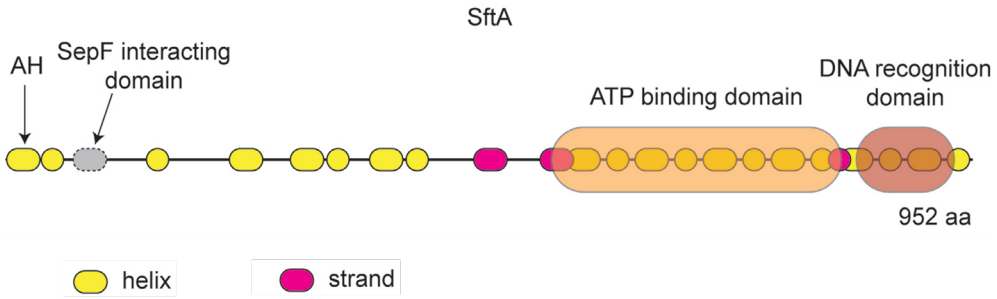

Fig. S1 Secondary structure prediction of SftA using PSIPred.

Secondary structure analysis was performed using PSIPred (Jones et al. 1999). The cartoon depicts the main predicted alpha helices (helix), beta strands (strand) and the putative ATP binding domain and DNA binding domain of SftA. The putative SepF interacting domain of SftA (amino acids P61-R75) found in the yeast two-hybrid analysis is indicated in grey, and the membrane-binding amphipathic helix is indicated as AH.

Fig. S2

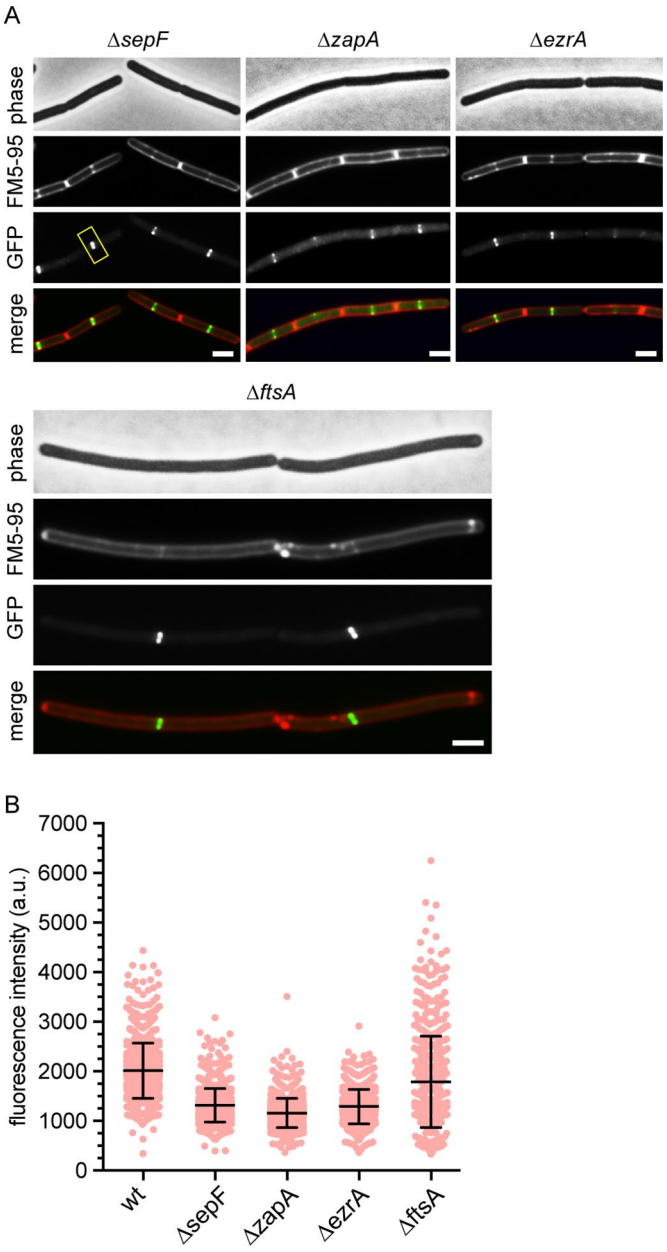

Fig. S2. Localization of SftA in different cell division mutants.

(A) Fluorescence microscopy images of SftA-GFP in different *B. subtilis* cell division mutants. SftA-GFP expression was induced with 0.1 % xylose. Membranes were stained with FM5-95. Scale bars are 2 µm. (B) Scatter plot indicating the SftA-GFP fluorescence intensities at cell division sites (yellow box in A) of the different mutants. On average 500 cells were measured. Strains used: TNVS001, TNVS254, TNVS342, TNVS343 and TNVS330, respectively.

Fig. S3

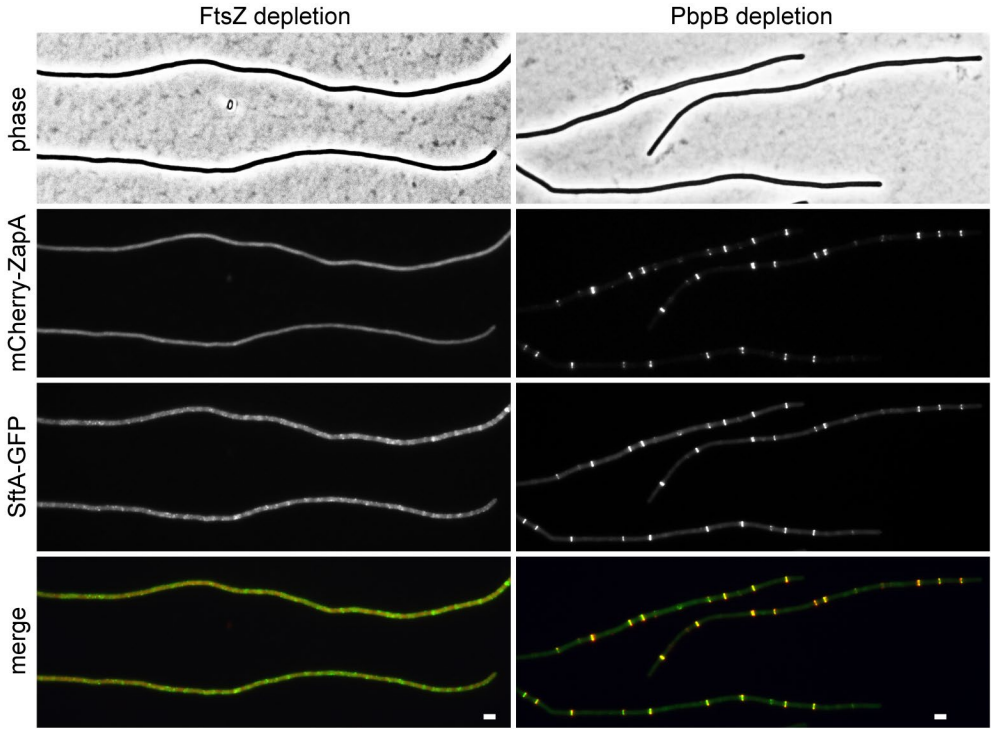

Fig. S3. SftA recruitment does not depend on late cell division proteins.

Localization of SftA-GFP and mCherry-ZapA in cells containing either an IPTG-inducible *ftsZ* (left panels) or *pbp2B* gene (right panels). Cells were grown for 3 h in the absence of IPTG resulting in filamentation. In the absence of Pbp2B, Z-rings are still being formed. SftA-GFP and mCherry-ZapA were induced with 0.1 % xylose. Scale bars are 2 µm. Strains used: TNVS783 and TNVS786, respectively.

Fig. S4

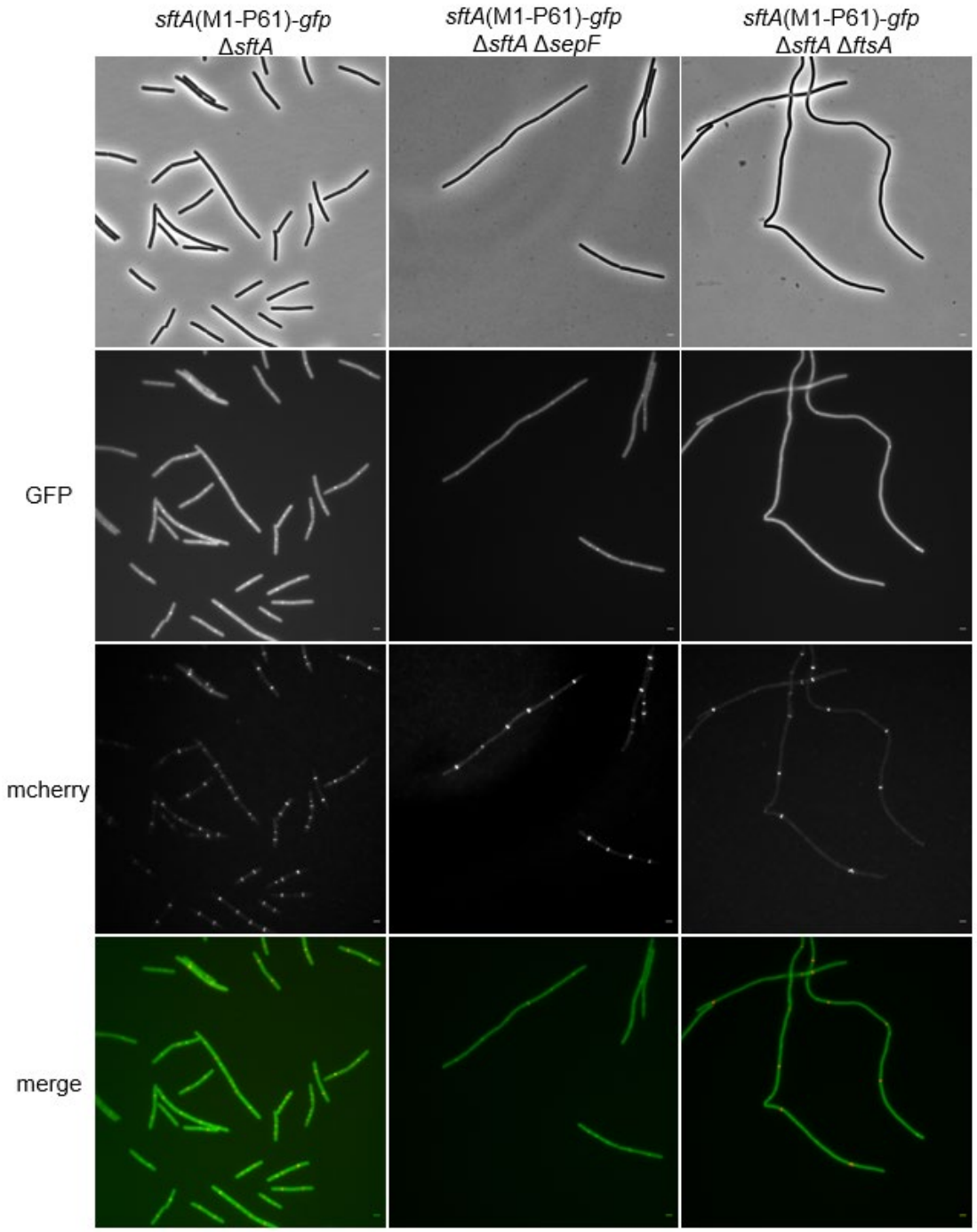

Fig. S4. Large field microscopy images of mutants shown in Fig. 5.

Large field images related to main text Fig. 5. Localization of GFP fused to the first 61 amino acids of SftA (green) in different *B. subtilis* mutants. All strains lacked wild type *sftA*, and all strains contained an mCherry-ZapA fusion (red) to indicate the localization of Z-rings. Expression of the fusion proteins was induced with 0.1 % xylose. Scale bars are 2 µm. Strains used, TZH029, TZH119, TZH120, TZH121, respectively.

Fig. S5

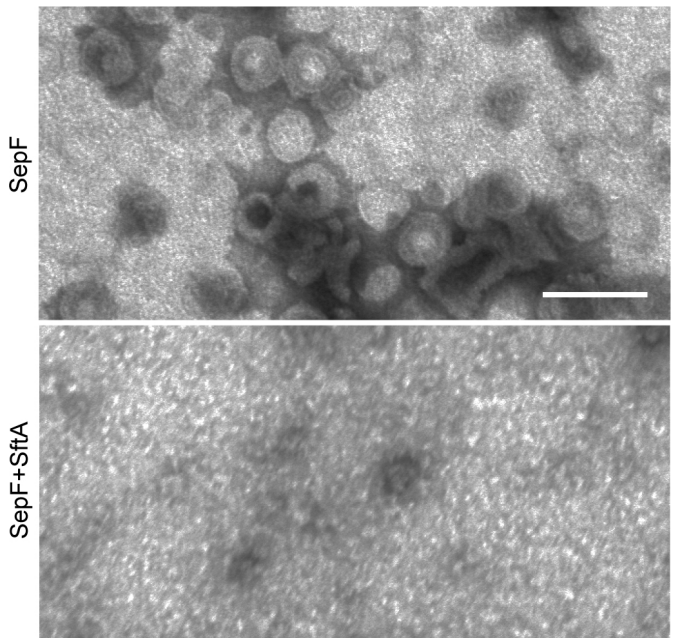

Fig. S5. SftA inhibits SepF ring formation.

SepF forms large protein rings with a diameter of approximately 50 nm (top EM image), after cleavage of the MBP (Maltose binding protein) moiety from the purified MBP-SepF fusion by factor Xa. The formation of these rings is inhibited when the cleavage reaction is carried out in the presence of SftA at a molar ratio of MBP-SepF:SftA of 3:1. Scale bar is 100 nm.

Fig. S6

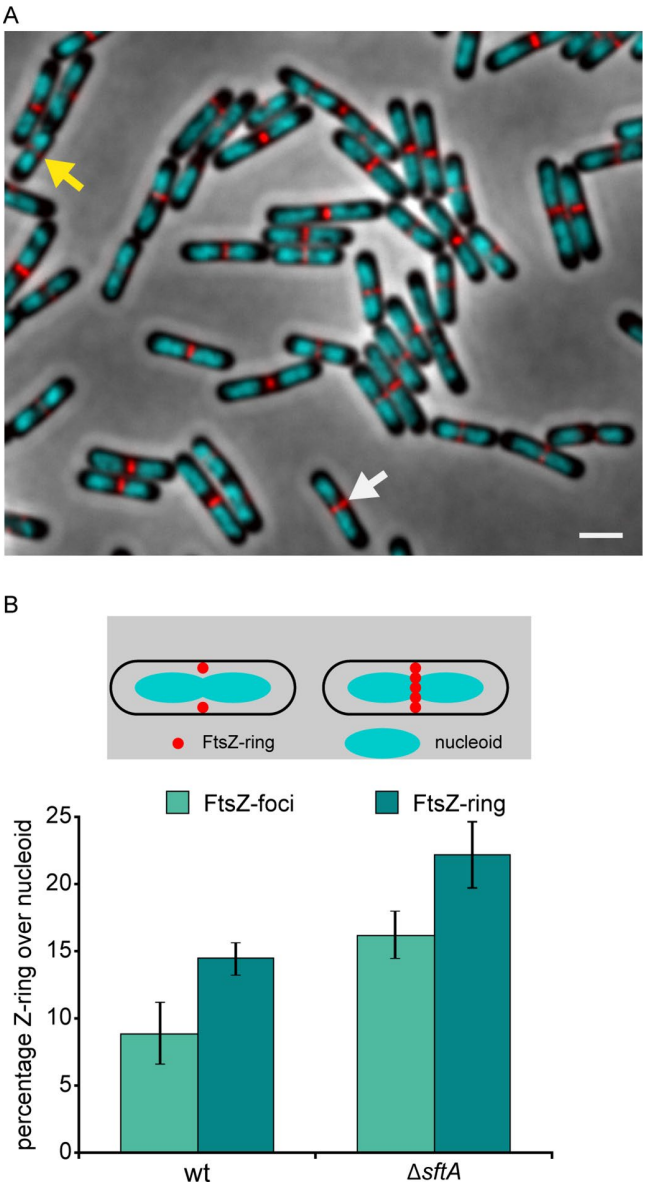

Fig. S6. Assembly of FtsZ-rings over nucleoids.

(A) Δ*sftA* cells expressing mCherry-ZapA (red) as FtsZ-ring marker with nucleoids (turquoise) stained with DAPI. The start of a Z-ring can be observed in microscopic images by the formation of two opposite red fluorescence foci at midcell (yellow arrow). Over time the Z-ring becomes more intense and septum synthesis begins resulting in a clear fluorescent band (white arrow) that eventually becomes smaller, since the Z-ring forms the leading edge of the closing septal wall. Scale bar is 2 µm. (B) Bar diagram indicating the average localization of early FtsZ foci and Z-rings over nucleoids, based on 3 independent biological experiments (approximately 100 cells counted). Cartoon above the bar diagram illustrates how FtsZ-rings over nucleoids were scored. Standard errors are indicated. Strains used: TNVS354 and EKB036.

References

Bohorquez, L.C., Surdova, K., Jonker, M.J., and Hamoen, L.W. (2018) The Conserved DNA Binding Protein WhiA Influences Chromosome Segregation in Bacillus subtilis. *J Bacteriol* 200.

Duman, R., Ishikawa, S., Celik, I., Strahl, H., Ogasawara, N., Troc, P., *et al.* (2013) Structural and genetic analyses reveal the protein SepF as a new membrane anchor for the Z ring. *Proc National Acad Sci* 110: E4601–E4610.

Feucht, A., and Errington, J. (2005) ftsZ mutations affecting cell division frequency, placement and morphology in Bacillus subtilis. *Microbiology+* 151: 2053–2064.

Gamba, P., Rietkötter, E., Daniel, R.A., and Hamoen, L.W. (2015) Tetracycline hypersensitivity of an ezrA mutant links GalE and TseB (YpmB) to cell division. *Front Microbiol* 6: 346.

Gamba, P., Veening, J.-W., Saunders, N.J., Hamoen, L.W., and Daniel, R.A. (2009) Two-Step Assembly Dynamics of the Bacillus subtilis Divisome. *J Bacteriol* 191: 4186–4194.

Genevaux, P., Keppel, F., Schwager, F., Langendijk-Genevaux, P.S., Hartl, F.U., and Georgopoulos, C. (2004) In vivo analysis of the overlapping functions of DnaK and trigger factor. *Embo Rep* 5: 195–200.

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6: 343–345.

Hamoen, L.W., and Errington, J. (2003) Polar Targeting of DivIVA in Bacillus subtilis Is Not Directly Dependent on FtsZ or PBP 2B. *J Bacteriol* 185: 693–697.

Hamoen, L.W., Meile, J., Jong, W.D., Noirot, P., and Errington, J. (2006) SepF, a novel FtsZ-interacting protein required for a late step in cell division. *Mol Microbiol* 59: 989–999.

Ishikawa, S., Kawai, Y., Hiramatsu, K., Kuwano, M., and Ogasawara, N. (2006) A new FtsZ-interacting protein, YlmF, complements the activity of FtsA during progression of cell division in Bacillus subtilis. *Mol Microbiol* 60: 1364–1380.

Jones, D.T. (1999) Protein secondary structure prediction based on position-specific scoring matrices1 1Edited by G. Von Heijne. *J Mol Biol* 292: 195–202.

Koo, B.-M., Kritikos, G., Farelli, J.D., Todor, H., Tong, K., Kimsey, H., *et al.* (2017) Construction and Analysis of Two Genome-Scale Deletion Libraries for Bacillus subtilis. *Cell Syst* 4: 291-305.e7.

Lewis, P.J., and Marston, A.L. (1999) GFP vectors for controlled expression and dual labelling of protein fusions in Bacillus subtilis. *Gene* 227: 101–109.

345 Marston, A.L., Thomaides, H.B., Edwards, D.H., Sharpe, M.E., and Errington, J. (1998) Polar
346 localization of the MinD protein of Bacillus subtilis and its role in selection of the mid-cell
347 division site. *Gene Dev* 12: 3419–3430.

348 Müller, A., Wenzel, M., Strahl, H., Grein, F., Saaki, T.N.V., Kohl, B., *et al.* (2016) Daptomycin
349 inhibits cell envelope synthesis by interfering with fluid membrane microdomains. *Proc*
350 *National Acad Sci* 113: E7077–E7086.

351 Pozsgai, E.R., Blair, K.M., and Kearns, D.B. (2011) Modified mariner Transposons for Random
352 Inducible-Expression Insertions and Transcriptional Reporter Fusion Insertions in Bacillus
353 subtilis. *Appl Environ Microb* 78: 778–785.

354 Zeigler, D.R., Prágai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., *et al.* (2008) The
355 Origins of 168, W23, and Other Bacillus subtilis Legacy Strains. *J Bacteriol* 190: 6983–6995.

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