SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmid construction

pJK005 [*amyE::Phy-refZ-gfp (spec)(amp)*] was generated in a two-way ligation with a PCR product containing *refZ-gfp* (oligonucleotide primers oJW020 and oJW043 and pLM029 DNA as template) cut with HindIII and NheI and pDR111 cut with the same enzymes. pLM029 [*refZ-gfp (spec)(amp)*] was generated in a two-way ligation with a PCR product containing *refZ* and its promoter (oligonucleotide primers oLM039 and oLM038 and PY79 genomic DNA as template) cut with EcoRI and XhoI and pKL147 (3) cut with the same enzymes. pDR111 [*amyE::Phyperspank (spec)*] is an ectopic integration vector with a strong IPTG-inducible promoter (D.Z.R. unpublished).

pJK013 [*amyE-Phy-refZ (spec)(amp)*] was generated in a two-way ligation with a PCR product containing *refZ* (oligonucleotide primers oJW014 and oJW015 and PY79 genomic DNA as template) cut with HindIII and NheI and pDR111 cut with HindIII and NheI.

pJW014 [*yhdG::Phy-refZ (phleo)*] was generated in a two-way ligation with a PCR product containing *refZ* (oligonucleotide primers oJW014 and oJW015 and PY79 genomic DNA as template) cut with HindIII and NheI and pJW004 [*yhdG*::*Phyperspank (phleo)*] cut with the same enzymes. pJW004 [*ycgO*::*Phy* (*phleo*)] was generated in a two-way ligation with an EcoRI-BamHI fragment containing the P*hyperspank* promoter and *lacI* from pDR111 and pBB280 cut with the same enzymes. pBB280 [*yhdG*::*phleo*] is an ectopic integration vector for insertions into the nonessential *yhdG* locus (B. Burton and D.Z.R., unpublished).

pJW105 [*ycgO::Phy-refZ (tet)(amp)*] was generated in a two-way ligation with a HindIII-NheI fragment containing *refZ* from pJK013 and pJW033 cut with the same enzymes. pJW033 [*ycgO*::*Phy* (*tet*)] was generated in a two-way ligation with an EcoRI-BamHI fragment containing the P*hyperspan*k promoter and *lacI* from pDR111 and pKM086 cut with the same enzymes. pKM086 [*ycgO*::*tet*] is an ectopic integration vector for insertions into the non-essential *ycgO* locus (K. Marquis and D.Z.R., unpublished).

pJW110 [*yhdG::RBMwt (erm)(amp)*] was generated by linker cloning using oligonucleotides oDR984 and oDR985 and plasmid pBB279 cut with XhoI and HindIII. pBB279 [*yhdG::erm*] is an ectopic integration vector made for double crossover insertions into the nonessential *yhdG* locus (B. Burton and D.Z.R., unpublished).

pJW111 [*yhdG::RBMmut (erm)(amp)*] was generated by linker mutagenesis using oDR986 and oDR987 and plasmid pBB279 cut with XhoI and HindIII.

pJW113 [*RBM from ywzF (erm)(cat)(RC-replicon)*] was generated in a two-way ligation with a PCR product containing the *ywzF* RBM (oligonucleotide primers oDR982 and oDR983 with PY79 genomic DNA as template) cut with XhoI and HindIII and pKM006 cut with the same enzymes.

pKM006 [*B. subtilis* RC plasmid (cat)(erm)] was generated by linker cloning using oligonucleotides oDR268 and oDR269 and plasmid pHB201 (2) cut with KpnI and HindIII. The linker contains a large multi-cloning site.

pLM028 [*refZ-gfp (spec)(amp)*] was generated in a two-way ligation with the 3' end of the *refZ* gene (oligonucleotide primers oLM037 and oLM038 and PY79 genomic DNA as template) cut with XhoI and EcoRI and pKL147(3) cut with the same enzymes.

pLM029 [*refZ-gfp (spec)(amp)*] was generated in a two-way ligation with the *refZ* gene including its promoter (oligonucleotide primers oLM039 and oLM038 and PY79 genomic DNA as template) cut with XhoI and EcoRI and pKL147 cut with the same enzymes.

pRB083 [*amyE::refZ-gfp (spec)(amp)*] was generated in a two-way ligation with an EcoRI-HindIII *refZ-gfp* fusion from pLM029 and pDR190 cut with EcoRI and HindIII. pDR190 [*amyE::spec*] is an ectopic for double crossover insertions into the nonessential *amyE* locus (D.Z.R., unpublished).

pRD002 [*amyE::Phy-refZ(E107A) (spec)(amp)*] was generated by site-directed mutagenesis using oligonucleotide oRB100 and plasmid pJK013.

pRD010 [*amyE::Phy-refZ(Y43A) (spec)(amp)*] was generated by site-directed mutagenesis using oligonucleotide oRB108 and plasmid pJK013.

pRD017 [*amyE::Phy-refZ(E107A)-gfp (spec)(amp)*] was generated by site-directed mutagenesis using oligonucleotide oRB100 and plasmid pJK005.

pRD018 [*amyE::Phy-refZ(Y43A)-gfp (spec)(amp)*] was generated by site-directed mutagenesis using oligonucleotide oRB108 and plasmid pJK005.

pRD021 [*amyE::refZ(E107A)-gfp (spec)(amp)*] was generated by site-directed mutagenesis using oligonucleotide oRB100 and plasmid pRB083.

pRD022 [*amyE::refZ(Y43A)-gfp (spec)(amp)*] was generated by site-directed mutagenesis using oligonucleotide oRB108 and plasmid pRB083.

SUPPLEMENTAL REFERENCES

1. **Bernard, R., K. A. Marquis, and D. Z. Rudner.** 2010. Nucleoid occlusion prevents cell division during replication fork arrest in Bacillus subtilis. Mol Microbiol **78:**866-882.
2. **Bron, S., A. Bolhuis, H. Tjalsma, S. Holsappel, G. Venema, and J. M. van Dijl.** 1998. Protein secretion and possible roles for multiple signal peptidases for precursor processing in bacilli. J Biotechnol **64:**3-13.
3. **Lemon, K. P., and A. D. Grossman.** 1998. Localization of bacterial DNA polymerase: evidence for a factory model of replication. Science **282:**1516-1519.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 Expression of RefZ and RefZ-GFP during sporulation and vegetative growth. (**A**) Immunoblot analysis of RefZ-GFP during sporulation and vegetative growth. Extracts from cells (BJW190) harboring *refZ-gfp* under the control of its native promoter and rbs were harvested at 0, 30, 60, and 75 min following the initiation of sporulation by resuspension at 37˚C (left) and from exponentially growing cells (BJK001) harboring P*hy-refZ-gfp* induced with either 0 or 10 μM IPTG for 60 min at 37˚C (right). RefZ-GFP levels were analyzed by immunoblot using anti-GFP antibodies. The images are from the same autoradiogram and were not adjusted independently (**B**) Induction of P*hy*-refZ with 7.5 μM IPTG leads to partial inhibition of cell division. Representative images of cells (BJW538) with and without 7.5 μM IPTG stained with the fluorescent membrane dye TMA-DPH. The cell extent of cell filamentation is quantitated in Fig. S9.

Figure S2 Expression of RefZ during vegetative growth does not induce the SOS response. Visualization of an SOS reporter (*PyneA-cfp*) (1) in wild (strain BRB24), in response to inhibition of DNA replication after addition of HPUra for 60 min (strain BRB24) or following induction of RefZ (Phy-*refZ*, strain BJW139) for 60 min. Images show membranes visualized with FM4-64 (left) and CFP (right). Two exposures of the CFP images are shown.

Figure S3 Expression of RefZ during vegetative growth disrupts FtsZ rings. Localization of FtsZ-GFP before induction of *refZ* (BJW429) and at indicated times (min) after induction. Top panel shows FtsZ-

GFP images. Bottom panel shows an overlay of membranes stained with FM4-64 (red) and FtsZ-GFP (green).

Figure S4 RefZ facilitates the switch of the FtsZ ring from a medial to polar position during sporulation. Histograms show a quantification of FtsZ-GFP localizations (medial, shifting, or polar) from wild type (purple, strain RL3056), a *refZ* mutant (dark blue, strain BRB455), a *spoIIE* mutant (light blue, strain BRB457) and a *refZ*, *spoIIE* double mutant (green, strain BRB459) 90 min, 120 min, and 150 min after induction of sporulation. Representative images of the three stages are shown below the histogram. Values are an average ± SD from 2 independent experiments. >300 cells were scored for each strain in each experiment.

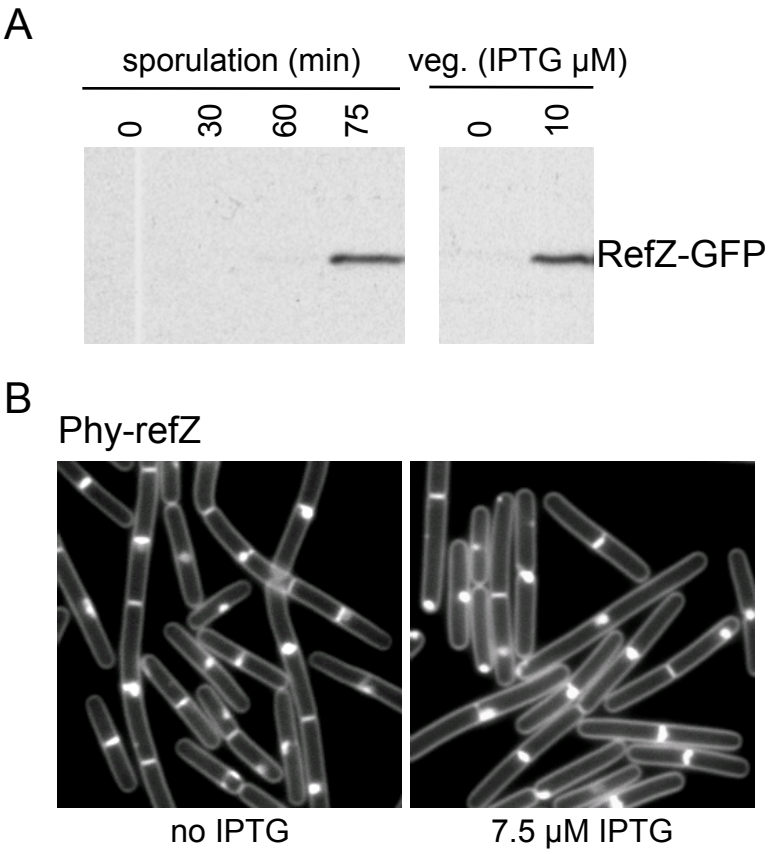
Figure S5 RefZ-GFP localizes in discrete foci that require an intact HTH motif when expressed during vegetative growth. Cells harboring *refZ-gfp* (strain BJK001) or *refZ(Y43A)-gfp* (strain BRB677) under the control of the IPTG-inducible promoter P*hy* were grown in CH medium. 30 min after addition of IPTG (10µM final), RefZ-GFP was visualized by fluorescence microscopy. Images show representative fields of RefZ-GFP (left), or overlaid (green) with DAPI-stained DNA (blue) and FM4-64-stained membranes (red).

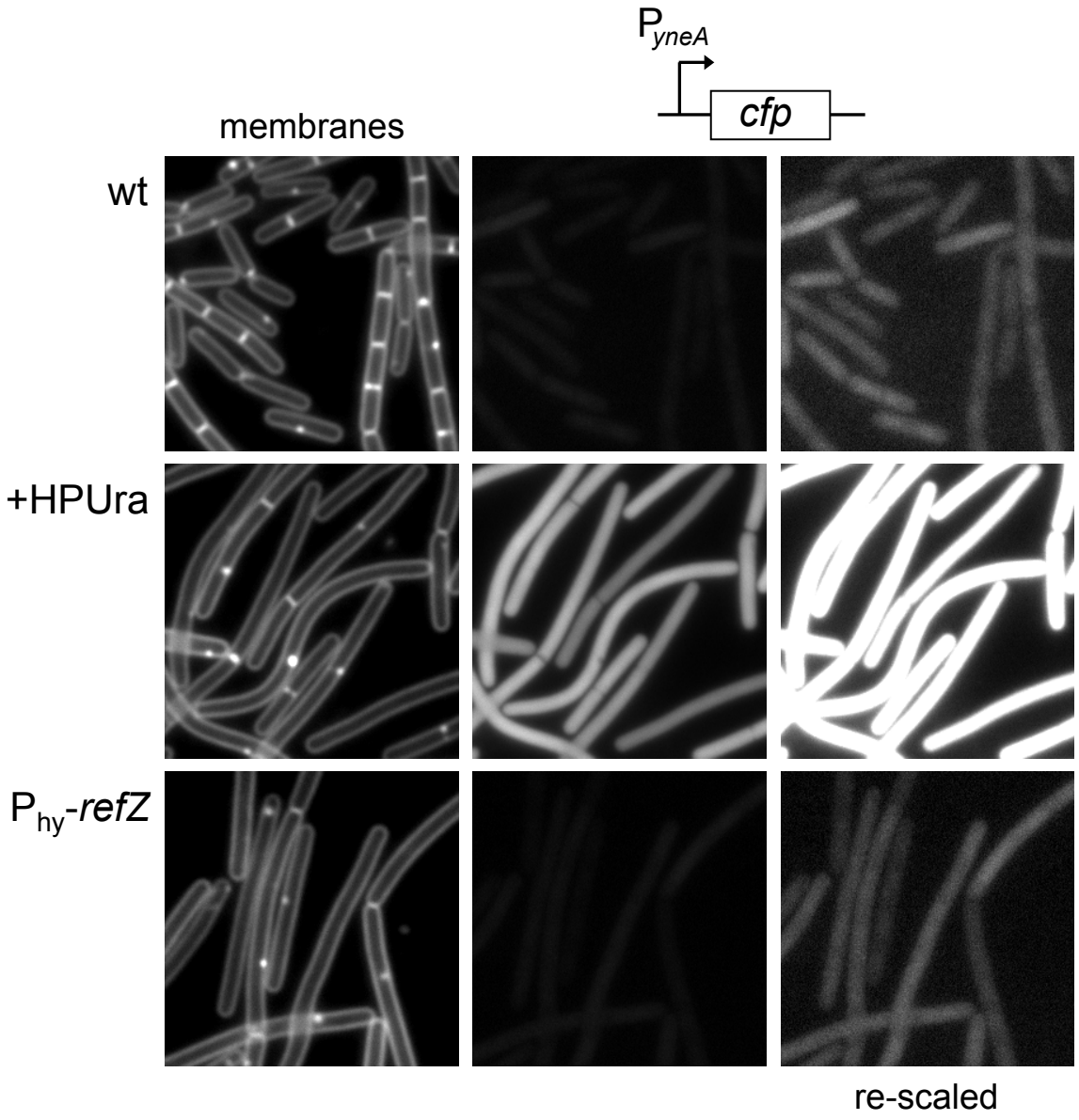
Figure S6 Broad RefZ binding sites identified by ChIP-seq. Plots show relative enrichment values (normalized over the input control DNA) for regions of interest (4-8 kb surrounding the peaks). These broad peaks were identified in two independent biological replicates. Only the RefZ binding site in the *yydB* locus was confirmed by ChIP-qPCR. The *ycgK* locus (blue) was identified as strong peak, and is shown here only for comparison. The relative enrichment scale (Y-axis) for *spo0J* was adjusted in the bottom graph to more clearly show the overall peak structure.

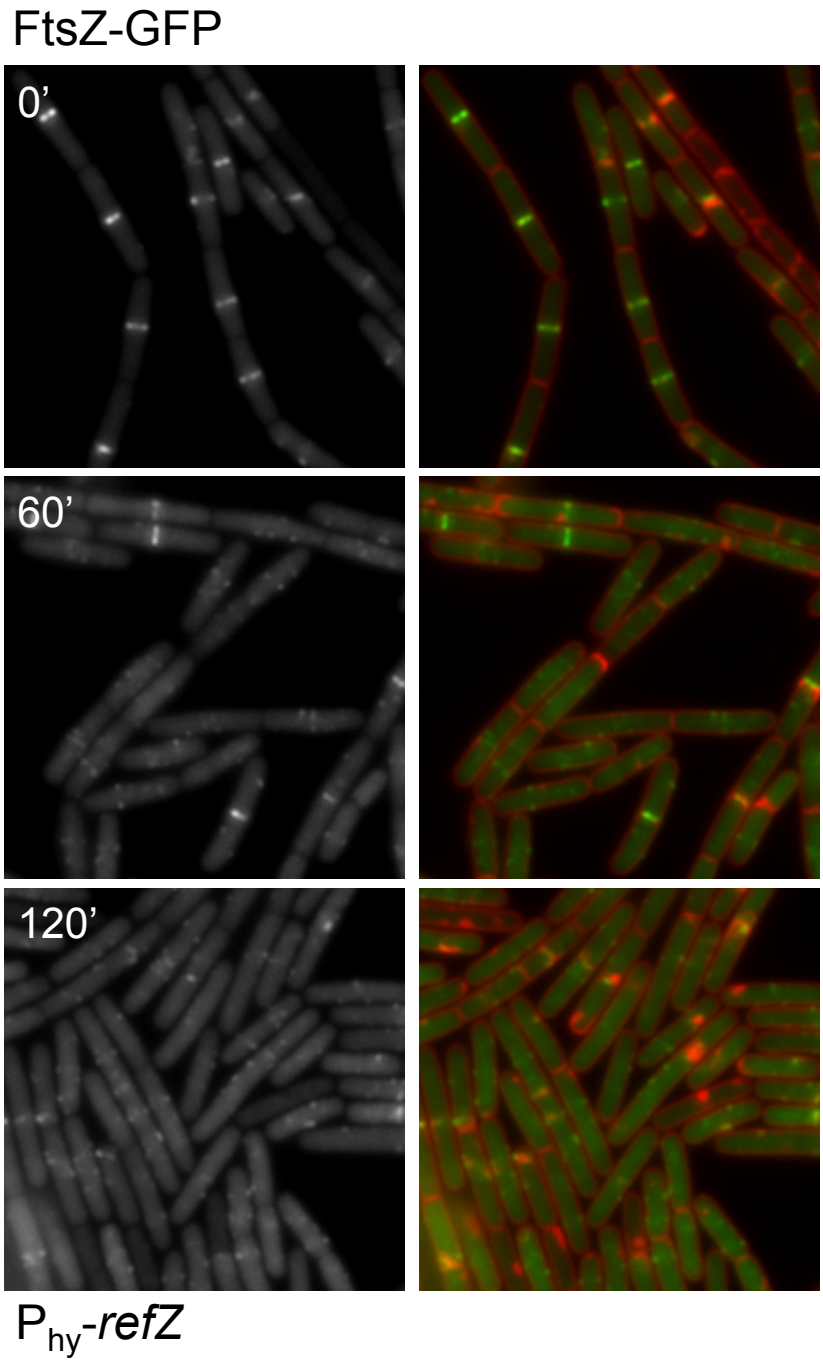
Figure S7 Confirmation of RefZ-enrichment by ChIP-qPCR. Plots show ChIP fold-enrichment values calculated relative to an amplicon internal to the *citZ* gene (a non-binding control). Mock reactions were from an identically treated strain lacking the carboxyl-terminal RefZ-GFP fusion. All ChIP fold-enrichment values represent the average of three biological replicates.

Figure S8 RefZ-GFP forms weak midcell foci in sporulating cells lacking the terminus-proximal RBM located in the *hrcA* locus. Cells harboring an intact RBM at *hrcA* (strain BRB672) and a mutant RBM (strain BJW555) were visualized by fluorescence microscopy 75 min after the induction of sporulation. Images show RefZ-GFP (left panel) and an overlay (right panel) of RefZ-GFP (green) and membranes stained with TMA-DPH (red). Mid-cell foci (yellow carets) are indicated.

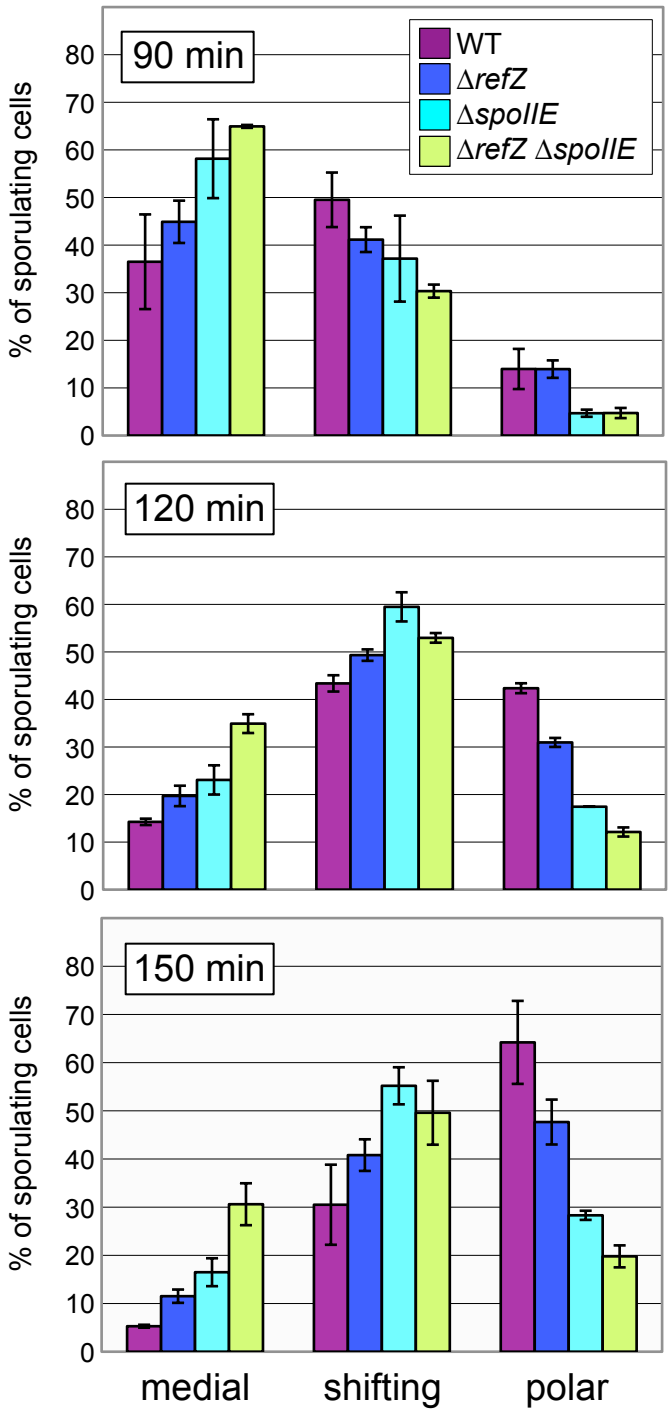
Figure S9 RefZ is a more potent regulator of FtsZ when bound to DNA. Cells expressing low levels of RefZ harboring an empty plasmid (strain BJW538) or a plasmid harboring the RBM from the *ywzF* locus (strain BJW537). Cells were grown to mid-log and induced with 7.5 μM IPTG. Time (in min) after addition of IPTG is indicated. Histograms show the cell lengths of all cells counted before (grey) and after (black) IPTG induction (n=300 for each sample), ordered from smallest to largest along the X-axis.


A
sporulation (min)
veg. (IPTG μM)
0
30
60
75
0
10
RefZ-GFP
B
Phy-refZ
no IPTG
7.5 μM IPTG

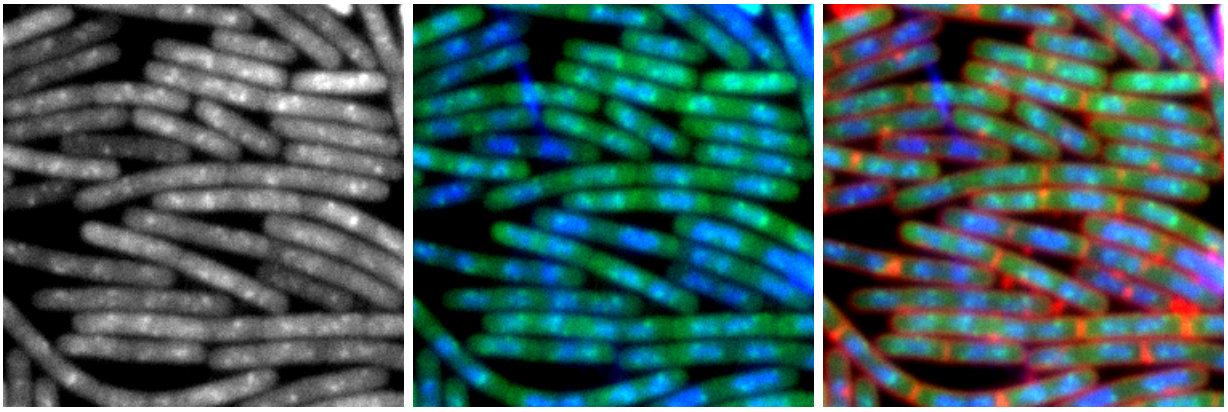

P yneA
cfp
membranes
wt
+HPUra
P hy-refZ
re-scaled


FtsZ-GFP
0’
60’
120’
P hy -refZ

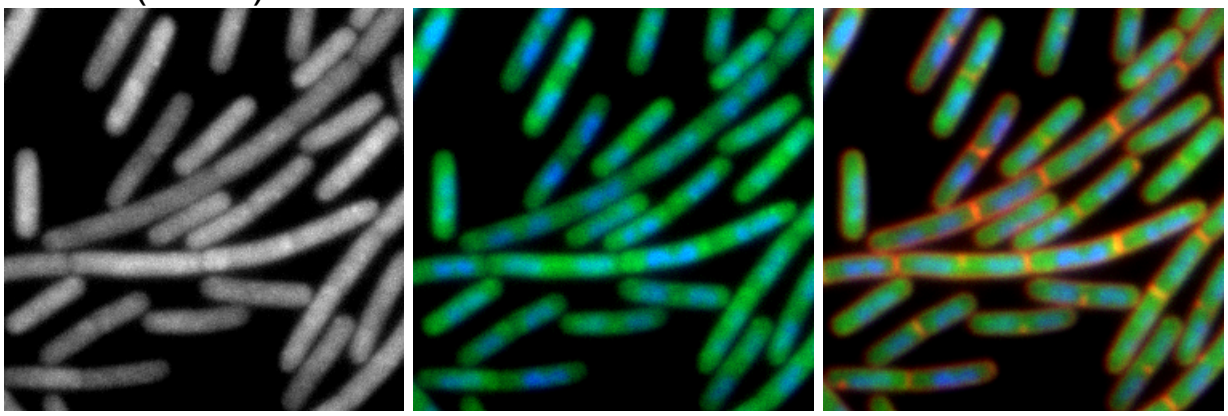
Herman_Fig S4



RefZ-GFP

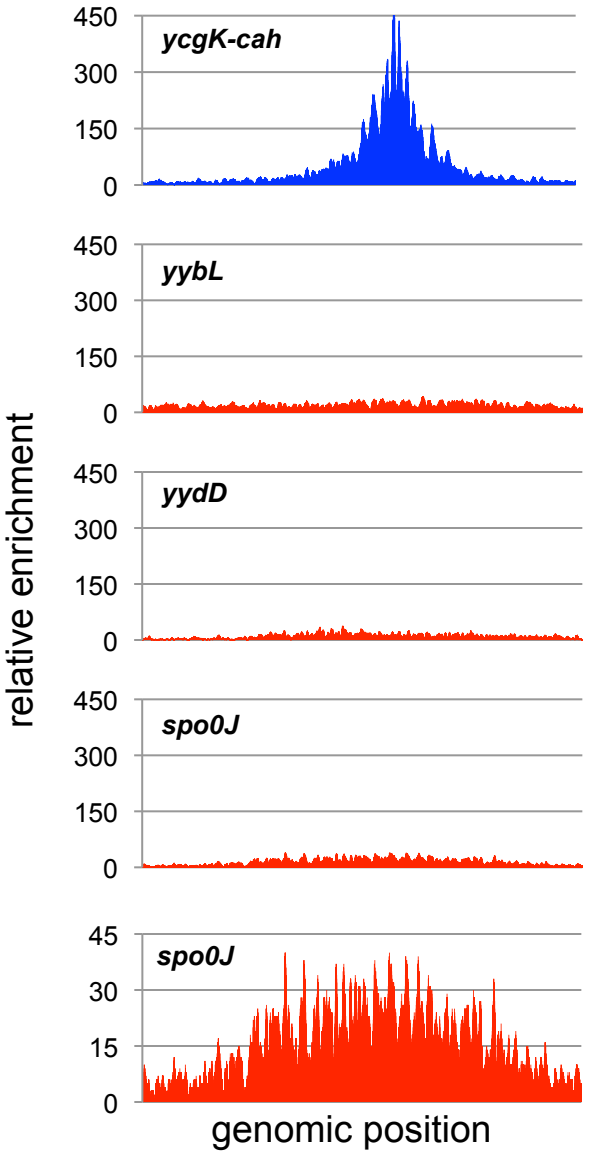


RefZ(Y43A)-GFP

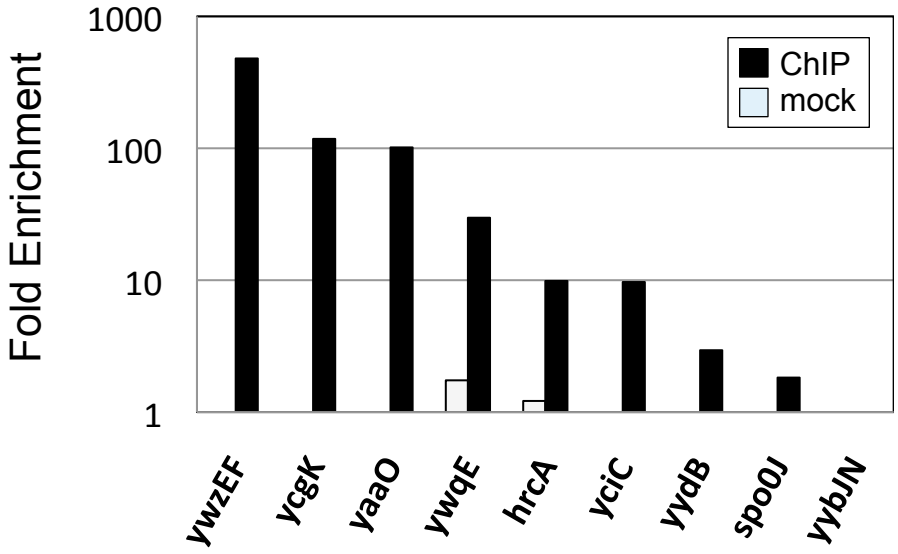


GFP GFP/DNA GFP/DNA/membranes

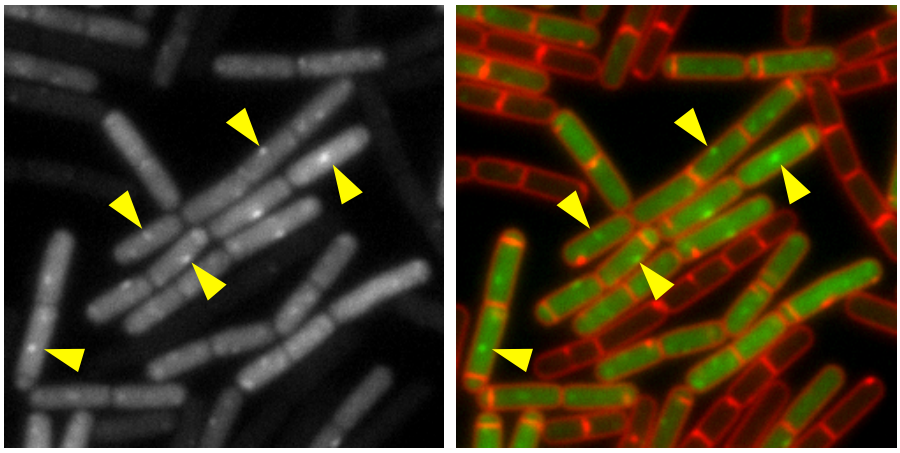
Herman_Fig S6



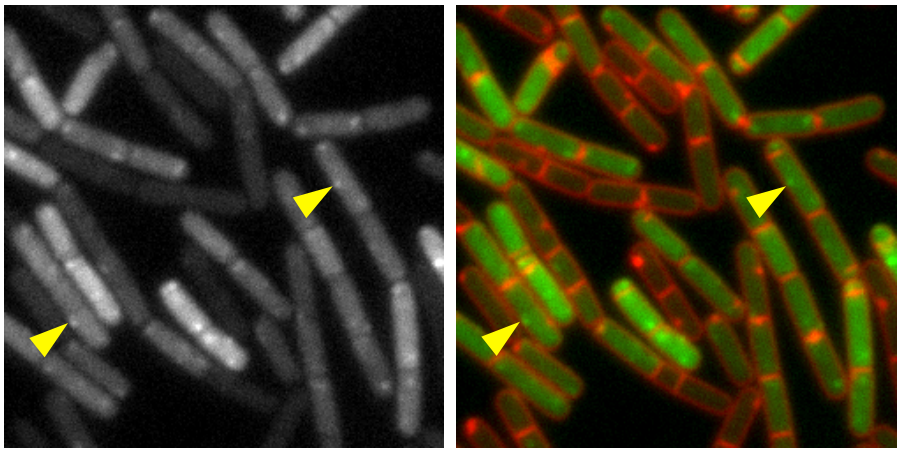
Herman_Fig S7

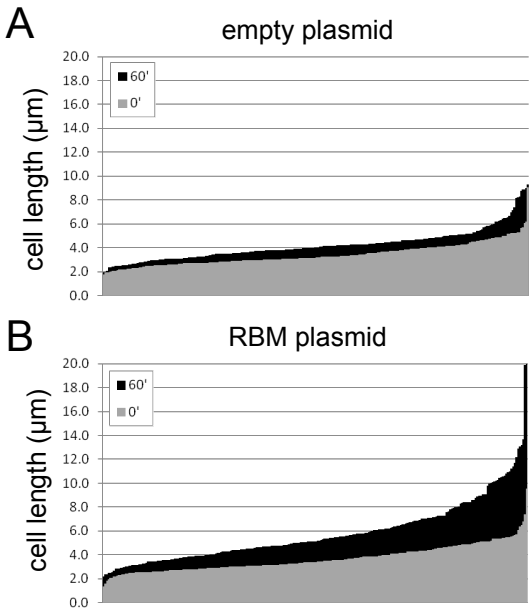


wt



ΔDRM in *hrcA*




A
empty plasmid
cell length (µm)
60'
0'
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
B
RBM plasmid
cell length (µm)
60'
0'
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0

Sensitivity to Inhibitor
sensitive
weakly resistant
strongly resistant

over-expressed protein

FtsZ mutants

	DimZ	MinCD	ZapA-MTS	MciZ
N24D				
N24I				
G62A				
G68R				
A71V				
P75Q				
E84D				
S85G				
G104S				
G104D				
M105L				
T111A				
A113V				
T232I				
K243R				
S247I				
A285V				
I293T				

Confidence Intervals for 120 min timepoint

MEDIAL	Low 95% CI	High 95% CI
WT	0.09	0.19
refZ	0.15	0.25
spoIIE	0.17	0.29
refZ/spoIIE	0.29	0.40
SHIFTING	Low 95% CI	High 95% CI
WT	0.39	0.47
refZ	0.45	0.53
spoIIE	0.55	0.64
refZ/spoIIE	0.48	0.58
POLAR	Low 95% CI	High 95% CI
WT	0.38	0.46
refZ	0.26	0.36
spoIIE	0.12	0.23
refZ/spoIIE	0.06	0.18

Table S2 Genomic regions with RefZ ChIP-Seq peaks (*B. subtilis* 168 coordinates)

Peak Region	**Start**	**End**
yaaO-tmk	38,000	39,500
yciC-yckA	366,000	369,000
ycgK-cah	340,000	344,200
hrcA-grpE	2,627,500	2,630,500
ywqE-ptkA	3,730,000	3,733,000
ywzEF	3,767,500	3,771,500
yydB	4,128,500	4,135,000
yybJN	4,172,500	4,176,500
spo0J	4,203,000	4,209,000

Table S4

Strains used in this study

Strain	Genotype	Reference
PY79	wild-type	Youngman et al. 1983
RL3056	*amyE::Pspac-ftsZ-gfp (cat)*	Ben-Yehuda & Losick 2002
RL3063	*amyE::ftsAZ (cat)*	Ben-Yehuda & Losick 2002
BJK001	*amyE::Phy-refZ-gfp (spec)*	This work
BJW123	*amyE::Phy-refZ (spec)*	This work
BJW139	*amyE::Phy-refZ (spec), sacA::PyneA-cfp (erm)*	This work
BJW144	*yhdG::Phy-refZ (phleo)*	This work
BJW147	*yhdG::Phy-refZ (phleo), amyE::ftsAZ (cat)*	This work
BJW190	*refZΩrefZ-gfp (spec)*	This work
BJW304	*amyE::phyperspank (spec)*	This work
BJW429	*amyE::ftsAZ-gfp (cat, kan), yhdG::Phy-refZ (phleo)*	This work
BJW469	*yhdG::Phy-refZ (phleo), ftsZ(K243R)ΩftsZ (tet)*	This work
BJW470	*yhdG::Phy-refZ (phleo), ftsZ(T232I)ΩftsZ (tet)*	This work
BJW471	*yhdG::Phy-refZ (phleo), ftsZ(A285V)ΩftsZ (tet)*	This work
BJW472	*yhdG::Phy-refZ (phleo), ftsZ(WT)ΩftsZ (tet)*	This work
BJW479	*yhdG::Phy-refZ (phleo), ftsZ(S85G)ΩftsZ (tet)*	This work
BJW480	*yhdG::Phy-refZ (phleo), ftsZ(E84D)ΩftsZ (tet)*	This work
BJW481	*amyE:: ftsAZ-gfp (cat, kan), yvbJ::PxylA-mciZ (erm)*	This work
BJW537	*amyE::Phy-refZ (spec) + plasmid (with ywzF RBM)(cat)(erm)*	This work
BJW538	*amyE::Phy-refZ (spec) + plasmid (empty)(cat)(erm)*	This work
BLM43	*SpoIIIE36, yycR::PspoIIQ-yfp (phleo), lacA::PspoIIQ-cfp (erm)*	This work
BLM51	*SpoIIIE36, yycR::PspoIIQ-yfp (phleo), lacA::PspoIIQ-cfp (erm), refZ::tet*	This work
BRB24	*sacA::PyneA-cfp (erm)*	Bernard et al. 2010
BRB455	*refZ::tet, amyE::Pspac-ftsZ-gfp (cat)*	This work
BRB457	*spoIIEΔ::kan, amyE::Pspac-ftsZ-gfp (cat)*	Ben-Yehuda & Losick 2002
BRB459	*spoIIEΔ::kan, refZ::tet, amyE::Pspac-ftsZ-gfp (cat)*	This work
BRB642	*amyE::Phy-refZ(E107A) (spec)*	This work
BRB658	*amyE::Phy-refZ(Y43A) (spec)*	This work
BRB672	*amyE::refZ-gfp (spec)*	This work
BRB677	*amyE::Phy-refZ(Y43A)-gfp (spec)*	This work
BRB697	*amyE::refZ(E107A)-gfp (spec)*	This work
BRB698	*amyE::refZ(Y43A)-gfp (spec)*	This work
BRB789	*yhdG::RBMwt (erm), refZΩrefZ-gfp (spec)*	This work
BRB790	*yhdG::RBMmut (erm), refZΩrefZ-gfp (spec)*	This work
AB88	*zapA::tet, yhdG::Phy-refZ (phleo)*	This work

Table S5
Plasmids used in this study

plasmid	description	reference
pHB201	*B. subtilis* plasmid *(RC-replicon)* (*erm*)(*cat*)	Bron et al. 1998
pJK005	*amyE::Phy-refZ-gfp (spec)(amp)*	This work
pJK013	*amyE-Phy-refZ (spec)(amp)*	This work
pJW014	*yhdG::Phy-refZ (phleo)(amp)*	This work
pJW105	*ycgO::Phy-refZ (tet)(amp)*	This work
pJW110	*yhdG::RBMwt (erm)(amp)*	This work
pJW111	*yhdG::RBMmut (erm)(amp)*	This work
pJW113	*RBM (*from *ywzF) (RC-replicon) (erm)(cat)*	This work
pKM006	pHB201 with improved MCS	This work
pRD002	*amyE::Phy-refZ(E107A) (spec)(amp)*	This work
pRD010	*amyE::Phy-refZ(Y43A) (spec)(amp)*	This work
pRD017	*amyE::Phy-refZ(E107A)-gfo (spec)(amp)*	This work
pRD018	*amyE::Phy-refZ(Y43A)-gfp (spec)(amp)*	This work
pRD021	*amyE::refZ(E107A)-gfp (spec)(amp)*	This work
pRD022	*amyE::refZ(Y43A)-gfp (spec)(amp)*	This work
pLM028	*3' end of refZ-gfp (spec)(amp)*	This work
pLM029	*refZ-gfp (spec)(amp)*	This work

Table S6

Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')
oDR268	CATGTCGACTAGCATGCTTGCTAGCTAGGATCCATCTCGAGTCAAGCTTGTGAATTCAGTCTAGAC
oDR269	AGCTGTCTAGACTGAATTCACAAGCTTGACTCGAGATGGATCCTAGCTAGCAAGCATGCTAGTCGACATGGTAC
oDR982	CGGAAGCTTGCGATCAGCGAAGTGACCG
oDR983	CGGCTCGAGCCGTTCCCCTGTCGATCAC
oDR984	AGCTTGTTTTAATCAAACGTTTGTTCAAAACGC
oDR985	TCGAGCGTTTTGAACAAACGTTTGATTAAAACA
oDR986	AGCTTGTTTTACTCAGAAGTGTGTTCCAAACGC
oDR987	TCGAGCGTTTGGAACACACTTCTGAGTAAAACA
oJW014	CGCAAGCTTACATAAGGAGGAACTACTATGAAAGTAAGCACCAAAGACAAA
oJW015	TTTGCTAGCGGATCCCGGTGCTAGTTGGTGAGCGCCAC
oJW020	CGCCGCTAGCGGATCCTGCATGCCTGCAGGTCTGGACATTTA
oJW043	GCGAAGCTTACATAAGGAGGAACTACTATGAAAGTAAGC
oLM037	GCCGAATTCATCTCCTACTATTTTAAAGGC
oLM038	CGGCTCGAGGTTGGTGAGCGCCACGTCTCC
oLM039	GCCGAATTCCCCTCTGCCGAAAGGAATCC
oRB100	AACTCGTTTTGTCTACCGGGCAGTCACAATTGACTCCACAT
oRB108	GTAAATGTTGCGCACATCTCCGCCTATTTTAAAGGCAAAGGAGGC