SUPPLEMENTAL INFORMATION

A two-step transport pathway allows the mother cell to nurture the developing spore in *Bacillus subtilis*

Fernando H. Ramírez-Guadiana, Alexander J. Meeske, Christopher D. A. Rodrigues, Rocío del Carmen Barajas-Ornelas, Andrew C. Kruse, and David Z. Rudner

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SUPPLEMENTAL METHODS

Strain constructions

Deletion mutants from the *Bacillus subtilis* knock-out collection were all confirmed by PCR using an oligonucleotide primer (oKO0) within the erythromycin resistance gene and a gene-specific primer.

BDR3414 [Δ*spoVV* Δ*gerA::spec*] was generated by transforming *B. subtilis* BDR3154 (Δ*spoVV*) with a PCR product containing the *gerA::spec* mutation (amplified with oligonucleotide primers oFR5 and oFR8 and template DNA from BDR3371).

BDR3416 [*ycgO::cat*] was generated by transforming *B. subtilis* 168 with pKM77. pKM77 (*ycgO::cat*) is a double-crossover vector for ectopic integration into the nonessential *ycgO* locus (Rudner Lab stock).

BDR3430 [*ycgO::*$P_{hyperspank}$*-spoVFAB* (*erm*)] was generated by transforming *B. subtilis* BDR3416 with pFR001.

BDR3432 [*ycgO::*$P_{hyperspank}$*-spoVFAB* (*erm*) *amyE*::P_{xylA}*-spoVV* (*spec*)] was generated by transforming *B. subtilis* BDR342 with pFR002.

BDR3449 [*spoVV-gfp* (*spec*)] was generated by direct transformation of *B. subtilis* 168 with an isothermal assembly product derived from 3 PCR products: 1) a PCR product containing the *spoVV* gene lacking its stop codon amplified with oligonucleotide primers oDR1262 and oDR1263 and *B. subtilis* 168 genomic DNA as template; 2) a PCR product containing *gfp* (*mgfpmut3a*) and the *spec* cassette amplified with oligonucleotide primers oDR1264 and oDR1265 and DNA from pWX429a as template; 3) a PCR product containing the region downstream of *spoVV* amplified with oligonucleotide primers oDR1266 and oDR1267 and *B. subtilis* 168 genomic DNA as template.

BDR3458 [Δ*spoIIQ::kan*] was generated by two back-crosses into *B. subtilis* 168 with genomic DNA from BCR267 (Rodrigues *et al.,* 2016)

BDR3465 [*ycgO::*P_{yeeK}*-optRBS-spoVV-gfp* (*erm*)] was generated by transforming *B. subtilis* BDR3416 with pFR008.

BDR3466 [*ycgO::*P_{spoVV}*-optRBS-spoVV-gfp* (*erm*)] was generated by transforming *B. subtilis* BDR3416 with pFR009.

BDR3468 [Δ*spoVV ycgO::*P_{yeeK}*-optRBS-spoVV-gfp* (*erm*)] was generated by transforming *B. subtilis* BDR3154 with gDNA from BDR3465.

BDR3469 [Δ*spoVV ycgO::*P_{spoVV}*-optRBS-spoVV-gfp* (*erm*)] was generated by transforming *B. subtilis* BDR3154 with gDNA from BDR3466.

BDR3471 [Δ*gerA::spec* Δ*spoVV ycgO::*P_{yeeK}*-optRBS-spoVV-gfp* (*erm*)] was generated by transforming *B. subtilis* BDR3414 with gDNA from BDR3465.

BDR3472 [Δ*spoVV* Δ*spoIIQ::kan ycgO::*P_{spoVV}*-optRBS-spoVV-gfp* (*erm*)] was generated by transforming *B. subtilis* BDR3469 with gDNA from BDR3458.

BDR3474 [Δ*spoIIIAH* Δ*spoVV::spec ycgO::*P_{spoVV}*-optRBS-spoVV-gfp* (*erm*)] was generated in two steps: first, *B. subtilis* BCR1117 was transformed with gDNA from BDR3466, and this intermediate strain was transformed with a PCR product containing Δ*spoVV::spec* (amplified with oligonucleotide primers oKO260 and oFR3 and gDNA from BDR3312).

BDR3507 [*ycgO::spoVV-gfp* (*spec*)] was generated by transforming *B. subtilis* BDR3416 with pFR11.

BDR3527 [Δ*spoVV ycgO::spoVV-gfp* (*spec)*] was generated by transforming *B. subtilis* BDR3154 with gDNA from *B. subtilis* BDR3507.

BDR3558 [Δ*spoVV ycgO::cat*] was generated by transforming *B. subtilis* BDR3154 with pKM77.

BDR3562 [Δ*spoVV ycgO::spoVV*(*N97A*)*-gfp* (*spec*)] was generated by transforming *B. subtilis* BDR3558 with pFR017.

BDR3563 [Δ*spoVV ycgO::spoVV*(*F302A*)*-gfp* (*spec*)] was generated by transforming *B. subtilis* BDR3558 with pFR018.

BDR3564 [Δ*spoVV ycgO::spoVV-gfp* (*Q310A*) (*spec*)] was generated by transforming *B. subtilis* BDR3558 with pFR019.

BDR3615 [Δ*spoVV ycgO::spoVV*(*F141A*)*-gfp* (*spec*)] was generated by transforming *B. subtilis* BDR3558 with pFR027.

BDR3632 [Δ*spoVV ycgO::spoVV*(*G96A*)*-gfp* (*spec*)] was generated by transforming *B. subtilis* BDR3558 with pFR024.

BDR3646 [Δ*gerAB*::*erm* Δ*spoVV ycgO::spoVV-gfp* (*wt*) (*spec*)], **BDR3647** [Δ*gerAB*::*erm* Δ*spoVV ycgO::spoVV*(*G96A*)*-gfp* (*spec*)], **BDR3648** [Δ*gerAB*::*erm* Δ*spoVV ycgO::spoVV*(*N97A*)*-gfp* (*spec*)], **BDR3649** [Δ*gerAB*::*erm* Δ*spoVV ycgO::spoVV*(*F141A*)*-gfp* (*spec*)], **BDR3650** [Δ*gerAB*::*erm* Δ*spoVV ycgO::spoVV*(*F302A*)*-gfp* (*spec*)] and **BDR3651** [Δ*gerAB*::*erm* Δ*spoVV ycgO::spoVV*(*Q310A*)*-gfp* (*spec*)] were generated by transforming *B. subtilis* BDR3527, BDR3632, BDR3562, BDR3615, BDR3563 and BDR3564, respectively, with a PCR product containing Δ*gerAB::erm* (amplified with the oligonucleotide primers oFR1 and oFR2 and gDNA from the strain BAM786 as template).

BDR3699 (Δ*spoVFB::erm*) and **BDR3700** (Δ*gerAB* Δ*spoVFB::erm*) were generated by direct transformation of *B. subtilis* 168 and BDR3158, respectively, with a PCR product containing the mutation Δ*spoVFB::erm* [amplified with the oligonucleotide primers oFR60 + oFR61 and gDNA of the strain Δ*spoVFB::erm* (BKE collection) as template].

Plasmid construction

pFR001 [*ycgO*::$P_{hyperspank}$-*spoVFAB* (*erm*)] was constructed in a two-way ligation with a *Spe*I-*Sph*I PCR product containing the *spoVFAB* operon (amplified with oligonucleotide primers oDR1247 and oDR1257 and gDNA from *B. subtilis* 168 as template) and pER67 cut with *Spe*I and *Sph*I. pER67 [*ycgO::*$P_{hyperspank}$ (*lacI*) (*erm*)] is a double crossover vector with an IPTG-inducible promoter for ectopic integration at the *ycgO* locus (Rudner Lab stock).

pFR002 [*amyE*::P_{xylA}-*spoVV* (*spec*)] was constructed in a two-way ligation with a *Sal*I-*BamH*I PCR product containing *spoVV* (amplified with oligonucleotide primers oDR1250 and oDR1251 and gDNA from *B. subtilis* 168 as template) and pDR150 cut with *Sal*I and *BamH*I. pDR150 [*amyE::*P_{xylA} (*xylR*) (*spec*)] is a double crossover vector with a xylose inducible promoter for ectopic integration at the *amyE* locus (Rudner Lab stock).

pFR008 [*ycgO::*P_{yeeK}-*optRBS-spoVV-gfp* (*erm*)] was constructed in a three-way ligation with an *EcoR*I-*BamH*I PCR product containing the SigK-responsive *yeeK* promoter (amplified with oligonucleotide primers oFR11 and oFR12 and gDNA from *B. subtilis* 168 as template) and a *Nhe*I-*BamH*I PCR product containing *spoVV* with an optimized RBS fused to the *mGFPmut3a* (amplified with oligonucleotide primers oFR13 and oFR15 and gDNA from BDR3449 as template) into pER61 cut with *EcoR*I and *BamH*I. pER61 (*ycgO*::*erm*) is a double-crossover vector for ectopic integration at the *ycgO* locus (Rudner Lab stock).

pFR009 [*ycgO::*P_{spoVV}-*optRBS-spoVV-gfp* (*erm*)] was constructed in a three-way ligation with a *Hind*III-*Nhe*I PCR product containing the promoter of *spoVV* (amplified with oligonucleotide primers oFR16 and oFR17 and gDNA from *B. subtilis* 168 as template) and a *Nhe*I-*BamH*I PCR product containing *spoVV* with an optimized RBS fused to the *mGFPmut3a* reporter (amplified with oligonucleotide primers oFR13 and oFR15 and gDNA from BDR3449 as template) into pER61 between *Hind*III and *BamH*I sites.

pFR011 [*ycgO*::*spoVV-gfp* (*spec*)] was constructed in a two-way ligation with a *Spe*I-*BamH*I PCR product containing *spoVV* fused to the *mGFPmut3a* reporter (amplified with oligonucleotide primers oFR23 + oFR24 and gDNA from BDR3449 as template) and pKM83 cut with *Spe*I and *BamH*I. pKM83 (*ycgO*::*spec*) is a double-crossover vector for ectopic integration at the *ycgO* locus (Rudner Lab stock).

pFR017 [*ycgO*::*spoVV*(*N97A*)-*gfp* (*spec*)] was constructed by site-directed mutagenesis using oligonucleotide primers oFR32 + oFR33 and plasmid pFR011.

pFR018 [*ycgO*::*spoVV*(*F302A*)-*gfp* (*spec*)] was constructed by site-directed mutagenesis using oligonucleotide primer oFR34 and plasmid pFR011.

pFR019 [*ycgO*::*spoVV*(*Q310A*)-*gfp* (*spec*)] was constructed by site-directed mutagenesis using oligonucleotide primer oFR36 and plasmid pFR011.

pFR024 [*ycgO*::*spoVV*(*G96A*)-*gfp* (*spec*)] was constructed by site-directed mutagenesis using oligonucleotide primers oFR40 + oFR41 and plasmid pFR011.

pFR027 [*ycgO*::*spoVV*(*F141A*)-*gfp* (*spec*)] was constructed by site-directed mutagenesis using oligonucleotide primers oFR46 + oFR47 and plasmid pFR011.

SUPPLEMENTAL FIGURE LEGENDS

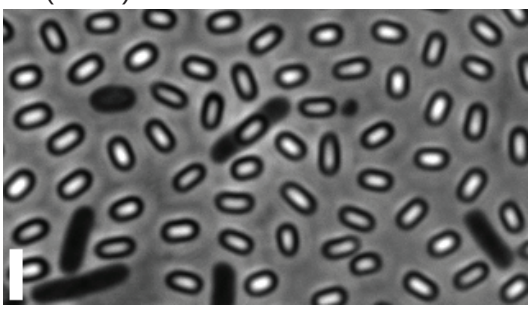
Figure S1. Suppression of Δ*spoVFA* and Δ*spoVFB* in the absence of a functional GerA receptor.
Representative phase-contrast images of the indicated strains sporulated for 30 h at 37°C in liquid DSM are shown. Sporulation efficiencies are indicated above each image. Strains lacking the B subunit of the GerA receptor (GerAB) are designated Δ*gerA* for clarity. Scale bar indicates 2 µm.

Figure S2. Sporulation by resuspension: Suppression of Δ*spoVV* and Δ*spoVFA* in the absence of a functional GerA receptor.
Representative phase-contrast images of the indicated strains sporulated by resuspensin for 30 h at 37°C. Sporulation efficiencies are indicated above each image. Strains lacking the B subunit of the GerA receptor (GerAB) are designated Δ*gerA* for clarity. We note that the homogenous spore populations shown in Figure 1B were achieved after spore purification (see Materials and Methods). Scale bar indicates 2 µm.

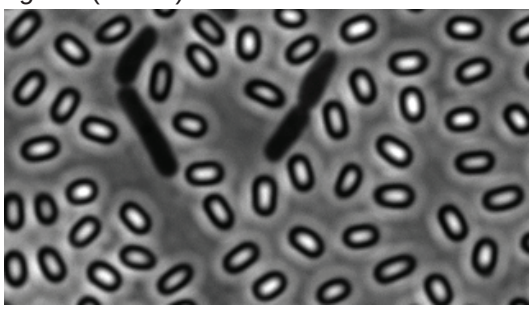
Figure S3. Expression of SpoVV and the SpoVFAB synthase during vegetative results in secretion of DPA into the medium.
A. Growth curve of wild-type and the strains engineered to over-express the DPA synthase in the presence and absence of SpoVV. Indicated strains were grown in minimal medium supplemented with 33 mM xylose. When the cultures reached an OD_{600} of 0.3, IPTG was added (0.5 mM, final concentration) to induce expression of SpoVFA and SpoVFB. Samples were collected before and after IPTG addition at the indicated times to assay DPA levels in the medium. **B.** Representative phase-contrast and fluorescence microscopy images of the indicated strains collected 3 h after the addition of IPTG. Membranes were stained with TMA-DPH (false-colored red) and membrane permeability was assessed with propidium iodide (PI) (false-colored blue). The number of PI-positive cells was quantified for each strain. Scale bar indicates 2 µm.

Figure S4. Conservation of the DPA transport pathway in endospore-forming bacteria. Phylogenetic tree showing the co-occurrence of the DPA synthases (SpoVFA and SpoVFB) and transporters (SpoVV and SpoVAA-AF) in a diverse set of 1,773 bacterial taxa. The amino acid sequences of *B. subtilis* SpoVFA, SpoVFB, SpoVV, and the SpoVA proteins served as queries in a BLASTp search against the NCBI 'nr' database with an *e*-value cutoff of $1x10^{-4}$. This analysis was performed through the Harvard Medical School Research Computing Orchestra cluster. The phylogenetic tree was constructed in PhyloT (http://phylot.biobyte.de/) and the BLASTp search results were plotted against the tree. The tree was visualized and annotated using the Interactive Tree Of Life web-based tool (iTOL, v3; http://itol.embl.de).

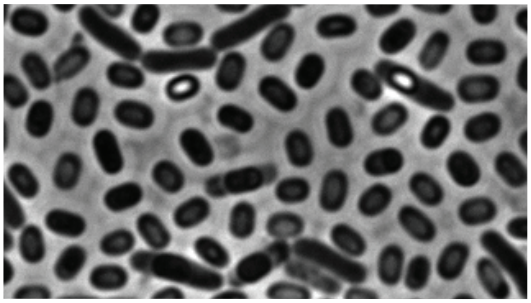
wt (100%)



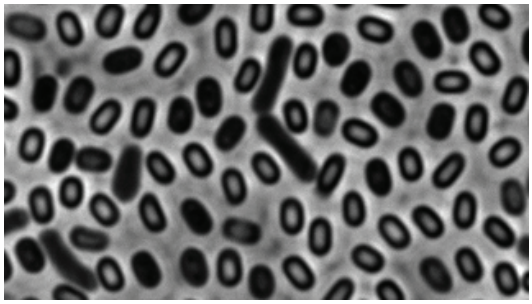
Δ*gerA* (86.1%)



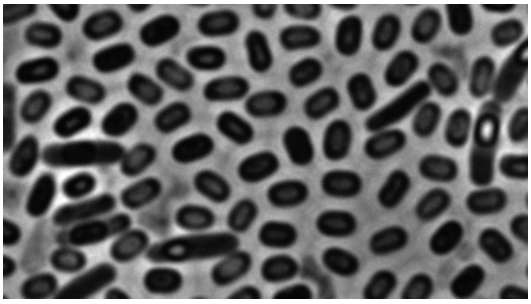
Δ*spoVFA* (0.0017%)



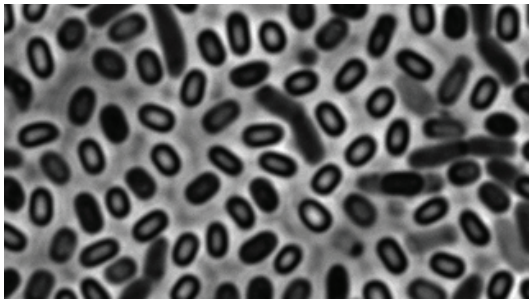
Δ*spoVFA* Δ*gerA* (0.207%)



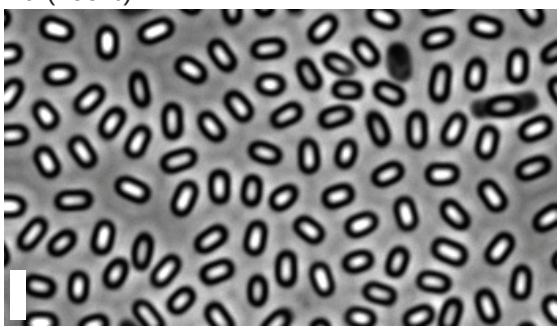
Δ*spoVFB* (0.0012%)



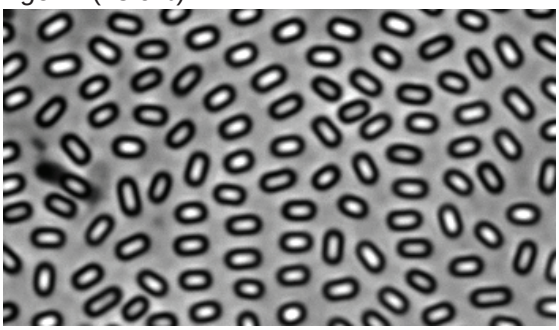
Δ*spoVFB* Δ*gerA* (0.196%)



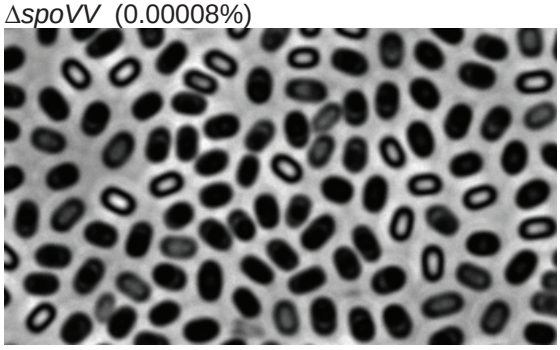
wt (100%)



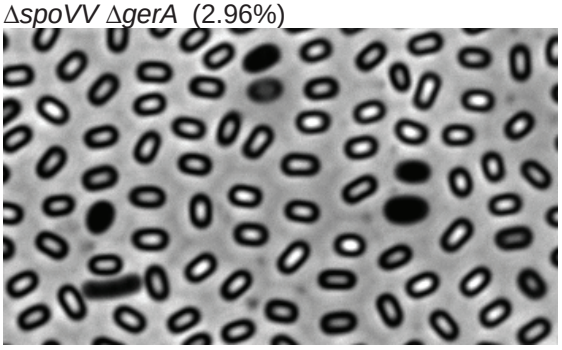
Δ*gerA* (13.9%)



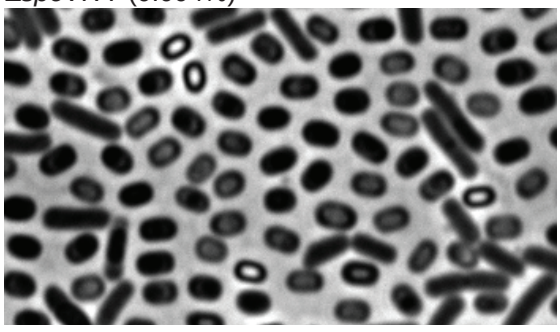
Δ*spoVV* (0.00008%)



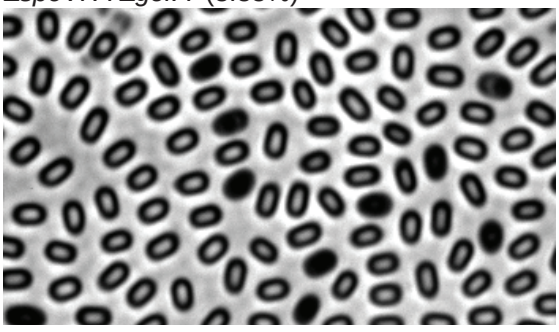
Δ*spoVV* Δ*gerA* (2.96%)



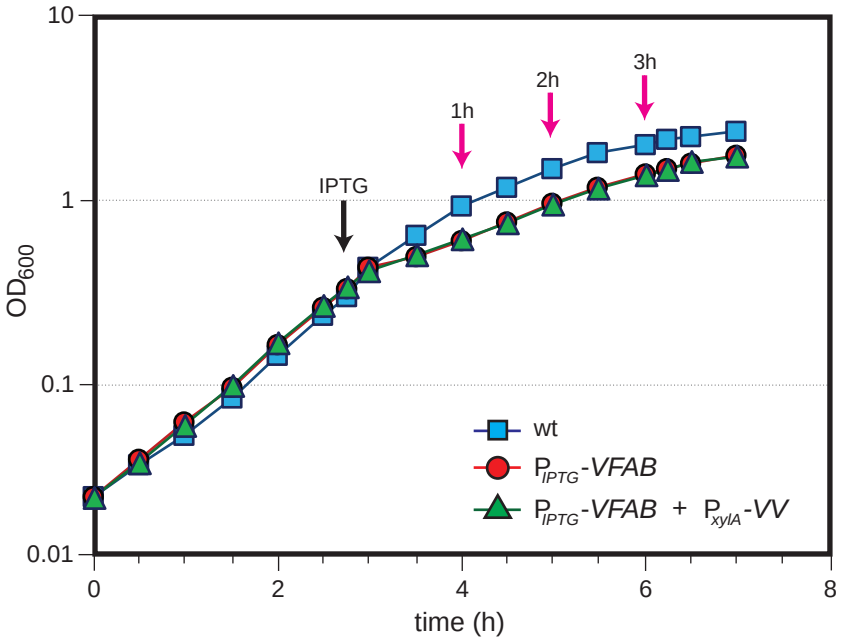
Δ*spoVFA* (0.004%)



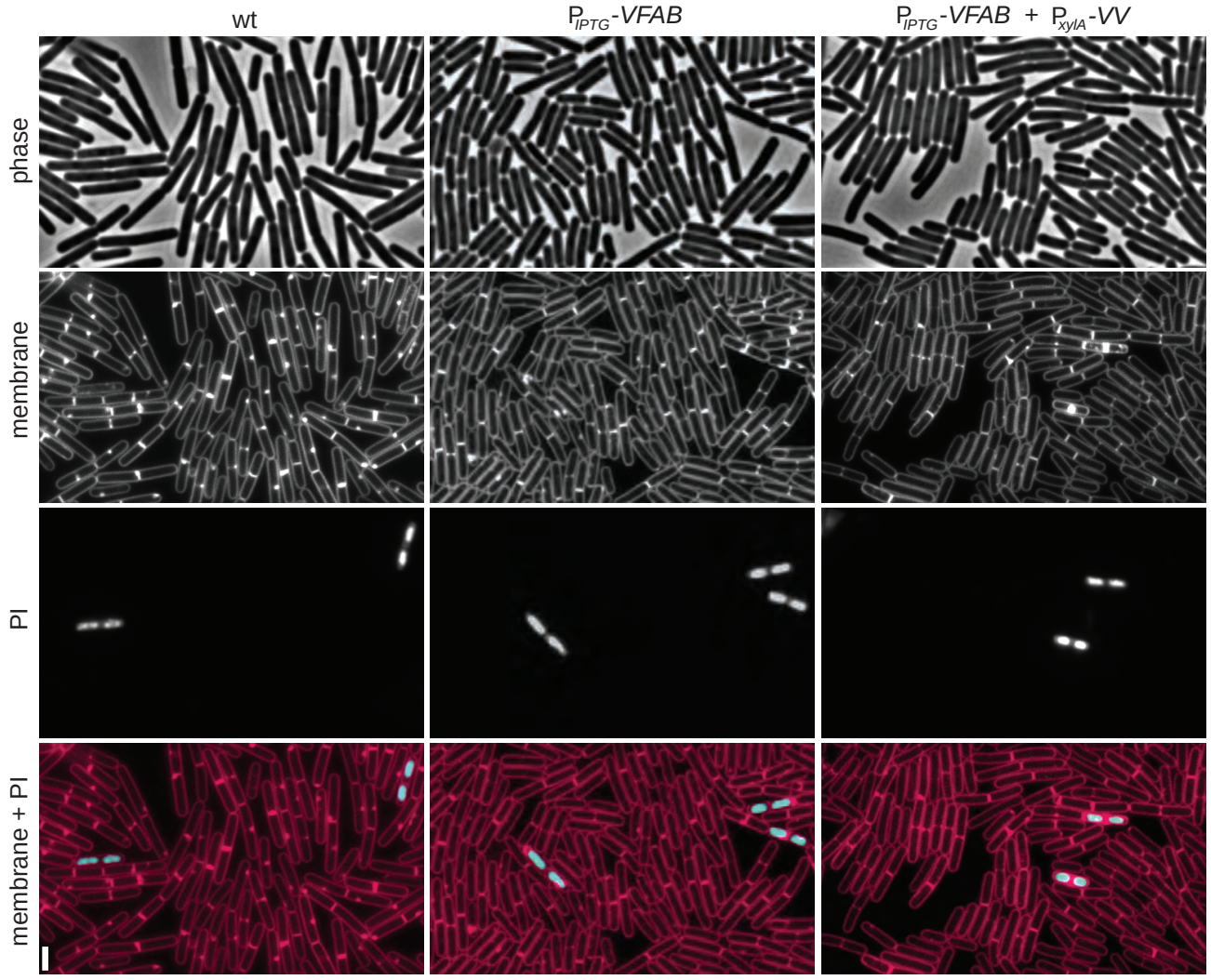
Δ*spoVFA* Δ*gerA* (3.83%)



A



B



PI-positive cells:	0.9% *n* > 2,100	0.9% *n* > 1,500	0.8% *n* > 1,700

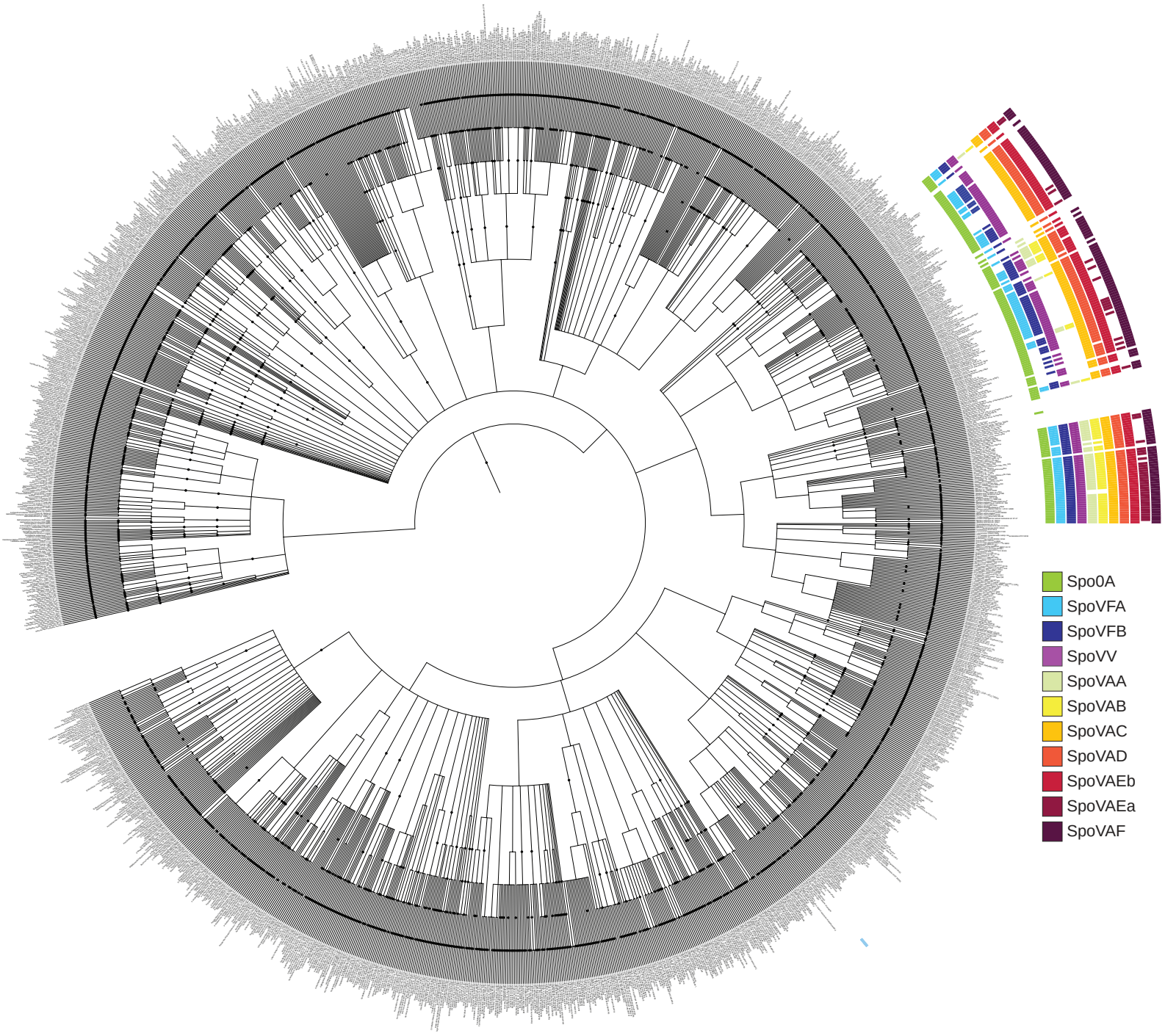

Spo0A
SpoVFA
SpoVFB
SpoVV
SpoVAA
SpoVAB
SpoVAC
SpoVAD
SpoVAEb
SpoVAEa
SpoVAF

Table S1. *Bacillus subtilis* strains used in this study.

Strain	Genotype	Source	Figure(s)
168	Wild-type (*trpC2*)	Zeigler *et al.* 2008	1, 3, 5, S1, S2 and S3
BAM786	Δ*gerAB::erm*	Koo *et al.*, 2017	
BDR3158	Δ*gerAB*	Ramírez-Guadiana *et al., 2017*	1, S1 and S2
BDR3151	Δ*spoVFA*	Ramírez-Guadiana *et al., 2017*	1, S1 and S2
BDR3154	Δ*spoVV*	Ramírez-Guadiana *et al., 2017*	1, 5 and S2
BDR3205	Δ*gerAB* Δ*spoVV*	Ramírez-Guadiana *et al., 2017*	1, 3, 5 and S2
BDR3205	Δ*gerAB* Δ*spoVT*	Ramírez-Guadiana *et al., 2017*	1
BDR3206	Δ*gerAB* Δ*spoVFA*	Ramírez-Guadiana *et al., 2017*	1, S1 and S2
BDR3205	Δ*gerAB* Δ*ytaF*	Ramírez-Guadiana *et al., 2017*	1
BDR3312	Δ*spoVV::spec*	Ramírez-Guadiana *et al., 2017*	
BDR3699	Δ*spoVFB*::*erm*	This work	S1
BDR3700	Δ*gerAB* Δ*spoVFB*::*erm*	This work	S1
BDR3414	Δ*spoVV* Δ*gerA::spec*	This work	3
BDR3416	*ycgO::cat*	This work	
BDR3430	*ycgO::* $P_{hyperspank}$-*spoVFAB* (*erm*)	This work	4 and S3
BDR3432	*ycgO::* $P_{hyperspank}$-*spoVFAB* (*erm*) *amyE::*P_{xylA}-*spoVV* (*spec*)	This work	4 and S3
BDR3449	*spoVV-gfp* (*spec*)	This work	
BDR3465	*ycgO::*P_{yeeK}-*optRBS-spoVV-gfp* (*erm*)	This work	
BDR3466	*ycgO::*P_{spoVV}-*optRBS-spoVV-gfp* (*erm*)	This work	
BDR3468	Δ*spoVV ycgO::*P_{yeeK}-*optRBS-spoVV-gfp* (*erm*)	This work	
BDR3469	Δ*spoVV ycgO::*P_{spoVV}-*optRBS-spoVV-gfp* (*erm*)	This work	2
BDR3471	Δ*gerA::spec* Δ*spoVV ycgO::*P_{yeeK}-*optRBS-spoVV-gfp* (*erm*)	This work	3
BDR3458	Δ*spoIIQ::kan*	This work	
BDR3472	Δ*spoIIQ::kan* Δ*spoVV ycgO::*P_{spoVV}-*optRBS-spoVV-gfp* (*erm*)	This work	2
BCR1117	Δ*spoIIIAH*	Laboratory stock	
BDR3474	Δ*spoIIIAH* Δ*spoVV::spec ycgO::*P_{spoVV}-*optRBS-spoVV-gfp* (*erm*)	This work	2
BDR3507	*ycgO::spoVV-gfp* (*spec*)	This work	
BDR3527	Δ*spoVV ycgO::spoVV-gfp* (*spec*)	This work	5
BDR3558	Δ*spoVV ycgO::cat*	This work	
BDR3632	Δ*spoVV ycgO::spoVV-gfp* (*G96A*) (*spec*)	This work	5
BDR3562	Δ*spoVV ycgO::spoVV-gfp* (*N97A*) (*spec*)	This work	5
BDR3615	Δ*spoVV ycgO::spoVV-gfp* (*F141A*) (*spec*)	This work	5
BDR3563	Δ*spoVV ycgO::spoVV-gfp* (*F302A*) (*spec*)	This work	5
BDR3564	Δ*spoVV ycgO::spoVV-gfp* (*Q310A*) (*spec*)	This work	5
BDR3646	Δ*gerAB::erm* Δ*spoVV ycgO*::*spoVV-gfp* (wt) (*spec*)	This work	5
BDR3647	Δ*gerAB::erm* Δ*spoVV ycgO*::*spoVV-gfp* (G96A) (*spec*)	This work	5
BDR3648	Δ*gerAB::erm* Δ*spoVV ycgO*::*spoVV-gfp* (N97A) (*spec*)	This work	5
BDR3649	Δ*gerAB::erm* Δ*spoVV ycgO*::*spoVV-gfp* (F141A) (*spec*)	This work	5
BDR3650	Δ*gerAB::erm* Δ*spoVV ycgO*::*spoVV-gfp* (F302A) (*spec*)	This work	5
BDR3651	Δ*gerAB::erm* Δ*spoVV ycgO*::*spoVV-gfp* (Q310A) (*spec*)	This work	5

All unmarked mutants are in-frame deletions generated by Cre-mediated recombination and contain a *lox72* scar.

Table S2. Plasmids used in this study.

Plasmid	Description	Source
pFR001	*ycgO*::$P_{hyperspank}$-*spoVFAB* (*erm*) (*amp*)	This work
pFR002	*amyE*::P_{xylA}-*spoVV* (*spec*) (*amp*)	This work
pFR008	*ycgO*::P_{yeeK}-*optRBS-spoVV-gfp* (*erm*) (*amp*)	This work
pFR009	*ycgO*::P_{spoVV}-*optRBS-spoVV-gfp* (*erm*) (*amp*)	This work
pFR011	*ycgO*::*spoVV-gfp* (*spec*) (*amp*)	This work
pFR017	*ycgO*::*spoVV*(N97A)-*gfp* (*spec*) (*amp*)	This work
pFR018	*ycgO*::*spoVV*(F302A)-*gfp* (*spec*) (*amp*)	This work
pFR019	*ycgO*::*spoVV*(Q310A)-*gfp* (*spec*) (*amp*)	This work
pFR024	*ycgO*::*spoVV*(QG96A)-*gfp* (*spec*) (*amp*)	This work
pFR027	*ycgO*::*spoVV*(F141A)-*gfp* (*spec*) (*amp*)	This work

Table S3. List of oligonucleotide primers used in this study.

Primer	Sequence
oDR1247	ggACTAGTcaaagaaggtgaacgtttagaatg
oDR1257	gccGCATGCtagaatatgcgggtgatgaccg
oDR1250	gccGTCGACagcatcggggaggtaagaatg
oDR1251	cgcGGATCCgccggaagtagagttggcgc
oDR1262	ggcggcttcgttcttgctatg
oDR1263	tttacttccggaaccctcgagtccttttttgttccgtttgtcag
oDR1264	ctcgagggttccggaagtaaa
oDR1265	gttccatccgattatcacggg
oDR1266	cccgtgataatcggatggaacggatgagtcaaaaccctcatcc
oDR1267	agtggtgtggaacatgagagt
oFR1	cttgtttccttccatcaggg
oFR2	ttctgacctcgtttcccagc
oFR3	agggcatggttgtgtatctgc
oFR5	tgaatggtttctttattaggc
oFR8	gtttcgcctcagggtatatg
oFR11	gccGAATTCctgtaatcggtgtggaaggc
oFR12	gccGCTAGCcataggttacagtccagagt
oFR13	gccGCTAGCacataaggaggaactactatgaacttgtcgaagattaataca
oFR15	gccGGATCCttatttgtatagttcatccatgccatgtgt
oFR16	gccAAGCTTagcatgcacaagctgtctgac
oFR17	gccGCTAGCtgattctttgtactaatatacgagcatagg
oFR23	ggcACTAGTacaccgagatgagcaagccct
oFR24	gccGGATCCttatttgtatagttcatccatgccatgtgt
oFR32	atgggaatggcatcaggagcccagcgggcgcaaaactcac
oFR33	gtgagttttgcgcccgctgggggctcctgatgccattcccat
oFR34	gtcagctttattcttggggcaagcggcttttccgtacaagc
oFR36	ggcttttccgtacaagctgcagtggcaggtattttatcgga
oFR40	gctatgggaatggcatcagcgaacccagcgggcgcaaaact
oFR41	agttttgcgcccgctgggttcgctgatgccattcccatagc
oFR46	ggcgctgttgccgtcggtgcgtttcaaaacgcatcactagg
oFR47	cctagtgatgcgttttgaaacgcaccgacggcaacagcgcc
oFR60	ggtcatgacattcgtgtccc
oFR61	gctgctggcttggatcatcc
oKO260	cgcgtacgctgcatatgtcta
oKO0	ctcgttcatagtagttcctcc

Capital letters indicate the recognition sequence for restriction enzymes and underlines indicate mutated bases.