SUPPLEMENTAL MATERIAL

Perturbations to engulfment do not impair B processing activity.

We have shown that when engulfment is impaired, σ^K activation is blocked due to the failure to accumulate the IVB signaling protein. Recently, Pogliano and co-workers have proposed that perturbations to engulfment *directly* inhibit the B processing enzyme (Jiang *et al*., 2005). These researchers analyzed the same engulfment mutants described here, however, in their analysis they used a *bofAΔ* strain to bypass the requirement for σ^G. In the absence of BofA, pro-σ^K processing is constitutive and thus completely bypasses the forespore signal (Cutting *et al*., 1990). In this bypass strain, mutations that impair engulfment blocked σ^K activation. Since these experiments were done in a strain that bypasses the IVB signaling protein, these researchers hypothesized the existence of a morphological checkpoint that couples B-dependent pro-σ^K processing activity to proper engulfment (Jiang *et al*., 2005).

One complication of these experiments is that the level of the B processing enzyme is significantly reduced in the absence of BofA (Rudner and Losick, 2002). Thus, in a *bofAΔ* background, pro-σ^K processing is delicately balanced: the B processing enzyme is constitutively active but the levels of B are barely high enough to support σ^K activation. We therefore wondered whether the block to σ^K activation when engulfment is impaired was due to a reduction in the level of B. To address this, we monitored B by immunoblot in a *bofAΔ* bypass strain that also lacked IIQ or IID. As reported previously, in the absence of BofA, the level of the B processing enzyme was significantly reduced compared to wild type (Fig. S3A) (Rudner and Losick, 2002). Importantly, there was a further reduction in the level of B in the absence of IID or IIQ and this correlated with the block in pro-σ^K processing (Fig. S3A). We note that the reduction in B protein levels when engulfment is impaired was only observed in the *bofAΔ* bypass strain but not when BofA was present in the signaling complex (compare Fig. 1B and Fig. S3A). These results are consistent with the idea that the absence of σ^K activity in this bypass strain, when engulfment is perturbed, is due to the instability of the B protein.

If this is true, a more stable version of B should support constitutive pro-σ^K processing even when engulfment is impaired. To test this, we used a functional B-GFP fusion (Fig. S3C) that has been shown previously to be more stable than the untagged protein (Resnekov and Losick, 1998). We inserted this fusion into the *bofAΔ* bypass strain and monitored pro-σ^K processing in the presence or absence of IIQ or IID. In all backgrounds tested, B-GFP remained stable and supported efficient pro-σ^K processing (Fig. S3B). These experiments indicate that the principal defect in the *bofAΔ* bypass strain is a reduction in B protein levels. We conclude that perturbations to engulfment impair σ^K activation primarily at the level of expression and accumulation of IVB.

SUPPLEMENTAL FIGURES

Figure S1. Full-length IVB is membrane-associated. Biochemical fractionation of IVB and A (an integral membrane control). Lysates were prepared from sporulating cells (PY79 and BDR94) collected at hour 3. Soluble (S100) and membrane fractions (P100) were separated by centrifugation at 100,000X g and subjected to immunoblot analysis. A nonspecific (soluble) protein recognized by the anti-IVB antibody is indicated by an asterisk.

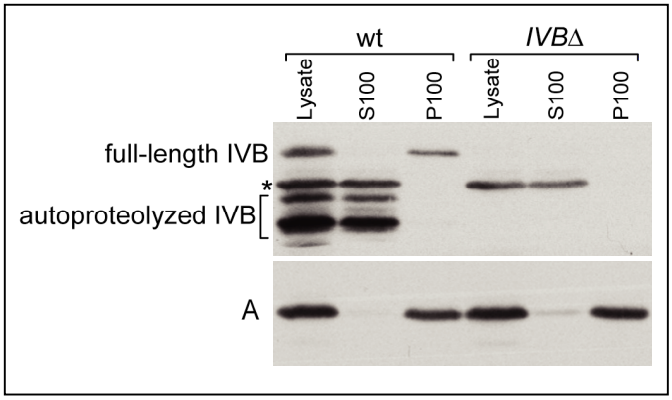


Figure S2. Protoplast engulfment protects membrane-anchored proteins that localize to the intermembrane space from protease digestion. Immunoblots from protease susceptibility assays. Cells were induced to sporulate by resuspension. At hour 2.2 of sporulation, prior to the completion of engulfment, the sporangia were protoplasted and incubated for indicated times (in minutes) with Trypsin or Trypsin and Triton X-100 (+Triton). Full-length IVB^{S378A}, YFP-A (an integral membrane protein) and σ^F (a cytoplasmic protein) were analyzed in the *sigG*Δ bypass strain (wt) (BTD2073), in a *IIIAHΔ* mutant (BTD2081), and in a *IIIAΔ*, *IIDΔ* double mutant (BTD2089). Broder and Pogliano have recently reported that zipper-like interactions between IIQ and IIIAH allow engulfment to proceed even upon protoplast treatment (Broder and Pogliano, 2006). Thus, in the *IIIAH*+ strain (wt) (BTD2073), membrane-anchored proteins that localize to the intermembrane space are protected from trypsin degradation.

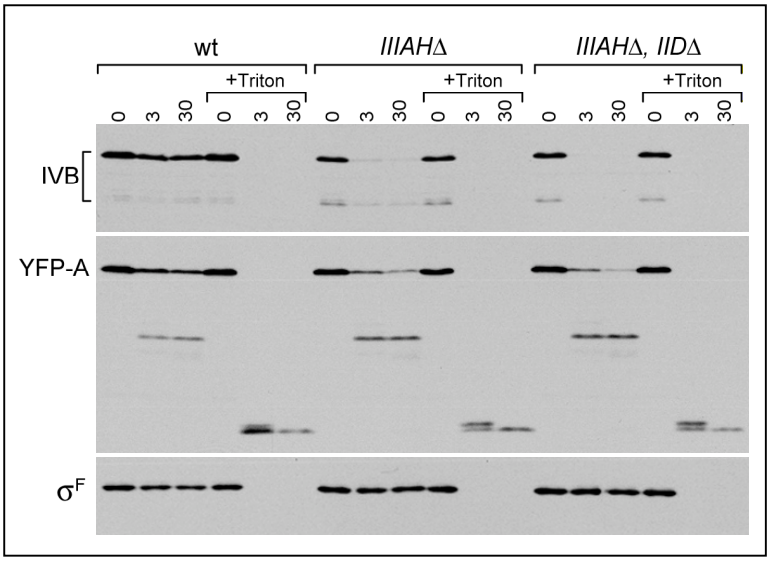


Figure S3. Perturbations to engulfment do not affect B-dependent pro-σ^K processing activity.
Immunoblot analysis of whole cell lysates from sporulating cells. Cells were induced to sporulate by resuspension and samples were collected at indicated times (in hours). **(A)** Perturbations to engulfment reduce B levels in a *bofAΔ* bypass background. B levels and pro-σ^K processing were compared in *bofAΔ* (BDR103), *bofAΔ,IIQΔ* (BTD2021) and *bofAΔ, IIDΔ* (BTD2023) mutants. Levels of B and pro-σ^K processing in a *bofA+* strain (wt) (PY79) at hour 3.25 are shown for comparison. **(B)** A stable version of B (B-GFP) supports efficient pro-σ^K processing in the *bofAΔ* bypass strain even when engulfment is impaired. Pro-σ^K processing and B-GFP levels were compared in *bofAΔ* (BTD2025), *bofAΔ,IIQΔ* (BTD2029) and *bofAΔ,IIDΔ* (BTD2031) mutants. Levels of B-GFP and pro-σ^K processing in a *bofA+* strain (BDR347) at hour 3.25 are shown for comparison. **(C)** B-GFP is a functional fusion. B-GFP, like wild-type B, is held inactive by A and BofA in the absence of the IVB signal. Pro-σ^K processing and the levels of B and B-GFP were compared in wild-type (PY79), *B-GFP* (BDR347), *IVBΔ* (BDR94) and *IVBΔ, B-GFP* (BTD2027) strains.

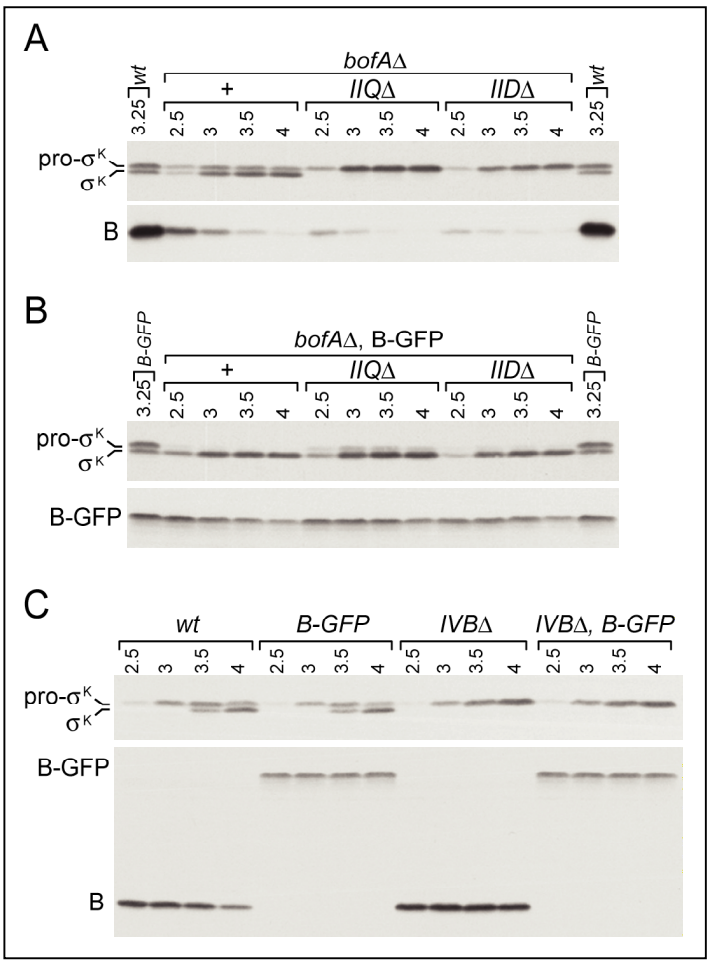
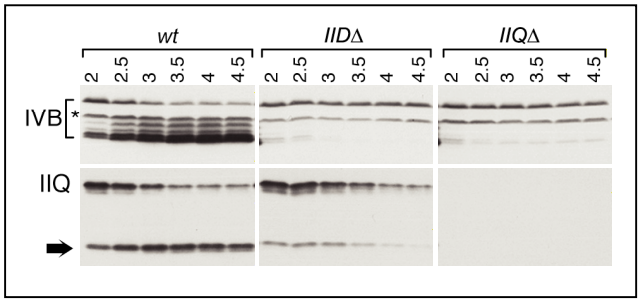


Figure S4. IVB activity is not coupled to the completion of engulfment.
Immunoblot analysis of whole cell lysates from sporulating cells. Cells were induced to sporulate by resuspension and samples were collected at indicated times (in hours). IVB levels and IVB-dependent cleavage of IIQ were compared in the *sigGΔ* bypass strain in which the *IVB* gene was fused to the σ^F-responsive promoter P_{IIQ} (BTD409) and this same strain containing the engulfment mutants *IIDΔ* (BTD1057) or *IIQΔ* (BTD1053). The IIQ cleavage product is indicated by an arrow. A nonspecific band recognized by the anti-IVB antibody is indicated by an asterisk. The immunoblots were obtained from samples collected in the same experiment and analyzed on the same day but on separate gels. Exposure times were the same for all three strains.



Plasmid construction

pDT47 [*amyE::P*$_{spoIIQ}$*-spoIVB (cat)*] was generated by inserting a *Hind*III-*Bam*HI PCR fragment containing the *spoIVB* gene (oligonucleotide primers oTD32 and oTD33 and PY79 genomic DNA template) into pDT28 (Doan *et al*., 2005) between *Hind*III and *Bam*HI.

pDT75 [*lacA::P*$_{spoIIQ}$*-spoIVB (erm)*] was created by inserting P_{spoIIQ}-*spoIVB* from pDT47 into pDR183 (Doan *et al*., 2005) between *Eco*RI and *Bam*HI.

pNS58 [*yycR::P*$_{spoIIQ}$*-cfp(Bs) (spec)*] was generated by inserting P_{spoIIQ}-*cfp* from pKM8 (Doan *et al*., 2005) into pNS44 between *Eco*RI and *Bam*HI. pNS44 [*yycR::spec*] is an ectopic integration vector for double crossover insertions into the nonessential *yycR* locus (N.L. Sullivan and DZR unpublished).

pDR203 [*amyE::P*$_{spoIIQ}$*-bofC (cat)*] was created by inserting a *Hind*III-*Bam*HI PCR fragment containing the *bofC* gene (oligonucleotide primers oDR394 and oDR395 and PY79 genomic DNA template) into pDT28 between *Hind*III and *Bam*HI.

pDT83 [*ycgO::P*$_{spoIIQ}$*-SS(amyE)-malE (erm)*] was generated by inserting a *Hind*III-*Xho*I PCR fragment encoding the signal sequence from AmyE and an optimized RBS (oligonucleotide primers oTD69 and oTD70 and PY79 genomic DNA template) into pDT80 between *Hind*III and *Xho*I. pDT80 [*ycgO::*P_{spoIIQ}*-malE (erm)*] was created by inserting an *Xho*I-*Bam*HI PCR fragment encoding mature MBP (oligonucleotide primers oTD71 and oTD72 and *E. coli* MG1655 genomic DNA template) into pDT79 between *Xho*I and *Bam*HI. pDT79 [*ycgO::*P_{spoIIQ} *(erm)*] was generated by inserting P_{spoIIQ} from pDT28 into pKM84 between *Eco*RI and *Hind*III. pKM84 [*ycgO::erm*] is an ectopic integration vector for double crossover insertions into the nonessential *ycgO* locus (K. Marquis and DZR unpublished).

pDT104 [*ycgO::P*$_{spoIIQ}$*-SS(amyE)-dsbA (erm)*] was generated by inserting an *Xho*I-*Bam*HI PCR fragment encoding mature DsbA (oligonucleotide primers oTD87 and oTD88 and *E. coli* MG1655 genomic DNA template) into pDT83 between *Xho*I and *Bam*HI.

pDT74 [*amyE::P*$_{spoIIQ}$*-spoIVB*^{S378A} *(cat)*] was constructed by inserting a *Hind*III-*Bam*HI PCR fragment encoding SpoIVB^{S378A} (oligonucleotide primers oTD32 and oTD33 and plasmid pQP50-1 template (Oke *et al*., 1997)) into pDT28 between *Hind*III and *Bam*HI.

pKM48 [*pelB::P$_{spoIVF}$-yfp(JF)-spoIVFA (tet)*] was generated by inserting an *Eco*RI-*Bam*HI fragment containing *PspoIVF-yfp(JF)-spoIVFA* from pKM26 (K. Marquis and DZR, unpublished) into pKM33 between *Eco*RI and *Bam*HI. pKM33 [*pelB::tet*] is an ectopic integration vector for double crossover insertions into the nonessential *pelB* locus (K. Marquis and DZR unpublished).

pNS50 [*yycR::P$_{spoIIQ}$-cfp(Bs) (phleo)*] was created by inserting *P$_{spoIIQ}$-cfp* from pKM8 (Doan *et al.*, 2005) into pNS42 between *Eco*RI and *Bam*HI. pNS42 [*yycR::phleo*] is an ectopic integration vector for double crossover insertions into the nonessential *yycR* locus (N.L. Sullivan and D.Z.R. unpublished).

pDT117 [*yvbJ::P$_{spoIVCB}$-spoIID (spec)*] was generated in a three-way ligation with an *Eco*RI-*Nhe*I PCR fragment containing the *spoIVCB* promoter including the first 129 base pairs of *spoICVB* coding sequence, a stop codon and an optimized RBS (oligonucleotide primers oTD102 and oTD103 and PY79 genomic DNA template) and an *Nhe*I-*Bam*HI PCR fragment encoding SpoIID (oligonucleotide primers oTD97 and oDR385 and PY79 genomic DNA template) and pNS28 cut with *Eco*RI and *Bam*HI. pNS28 [*yvbJ::spec*] is an ectopic integration vector for double crossover insertions into the nonessential *yvbJ* locus (N.L. Sullivan and DZR unpublished).

pDT124 [*lacA::P$_{spoIVCB}$-spoIID (tet)*] was created by inserting the *P$_{spoICVB}$-spoIID* fragment from pDT117 into pNC18 between *Eco*RI and *Bam*HI. pNC18 [*lacA::tet*] is an ectopic integration vector for double crossover insertions into the nonessential *lacA* locus (N.J. Campo and D.Z.R. unpublished).

pDR199 [*his$_6$-IID*] was generated by inserting an *Nhe*I-*Xho*I PCR fragment encoding the extracellular domain of SpoIID (oligonucleotide primers oDR379 and oDR380) into pRsetA (Invitrogen) between *Nhe*I and *Xho*I.

pKM67 [*his$_6$-BofC*] was created by inserting an *Nhe*I-*Xho*I PCR fragment encoding the last 140 amino acids of the BofC protein (oligonucleotide primers oKM2 and oKM4) into pRsetA (Invitrogen) between *Nhe*I and *Xho*I.

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Oke, V., Shchepetov, M., and Cutting, S. (1997) SpoIVB has two distinct functions during spore formation in Bacillus subtilis. *Mol Microbiol* **23**: 223-230.

Resnekov, O., and Losick, R. (1998) Negative regulation of the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **95**: 3162-3167.

Rudner, D.Z., and Losick, R. (2002) A sporulation membrane protein tethers the pro-sigmaK processing enzyme to its inhibitor and dictates its subcellular localization. *Genes Dev* **16**: 1007-1018.

Supplemental Table 1. *Strains used in this study*

Strain	Genotype	Source
PY79	Prototrophic wild-type strain	Youngman *et al.*, 1983
BDR104	*spoIIIG ::neo*	Margolis *et al.*, 1991.
BTD409	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat)*	This work
BTD1017	*spoIIIG::neo, lacA::P_{spoIIQ}-spoIVB (erm), amyE::P_{spoIIQ}-cfp(Bs) (cat)*	This work
BTD1053	*spoIIIG::neo, lacA::P_{spoIIQ}-spoIVB (erm), amyE::P_{spoIIQ}-cfp(Bs) (cat), spoIIQ::spec*	This work
BTD1057	*spoIIIG::neo, lacA::P_{spoIIQ}-spoIVB (erm), amyE::P_{spoIIQ}-cfp(Bs) (cat), spoIID::cat::spec*	This work
BTD2019	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), yycR::P_{spoIIQ}-cfp(Bs) (spec), spoVG::Tn917 (erm), spoIIB::erm*	This work
BTD823	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), spoIIQ::spec*	This work
BTD937	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), spoIID::Tn917 (erm)*	This work
BTD1549	*spoIIIG::neo, lacA::P_{spoIIQ}-spoIVB (erm), amyE::P_{spoIIQ}-bofC (cat)*	This work
BTD1551	*spoIIIG::neo, lacA::P_{spoIIQ}-spoIVB (erm), amyE::P_{spoIIQ}-bofC (cat), spoIIQ::spec*	This work
BTD1569	*spoIIIG::neo, lacA::P_{spoIIQ}-spoIVB (erm), amyE::P_{spoIIQ}-bofC (cat), spoIID::cat::spec*	This work
BTD1209	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), ycgO::P_{spoIIQ}-SS(AmyE)-malE (erm)*	This work
BTD1221	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), ycgO::P_{spoIIQ}-SS(AmyE)-malE (erm), spoIIQ::spec*	This work
BTD1567	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), ycgO::P_{spoIIQ}-SS(AmyE)-malE (erm), spoIID::cat::spec*	This work
BTD1423	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), ycgO::P_{spoIIQ}-SS(AmyE)-dsbA (erm)*	This work
BTD1425	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), ycgO::P_{spoIIQ}-SS(AmyE)-dsbA (erm), spoIIQ::spec*	This work
BTD1571	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), ycgO::P_{spoIIQ}-SS(spoIVB)-dsbA (erm), spoIID::cat::spec*	This work
BTD489	*spoIIIG::neo, spoIVB::spec, amyE::P_{spoIIQ}-spoIVB (cat)*	This work
BTD1099	*spoIIIG::neo, spoIVB::spec, amyE::P_{spoIIQ}-spoIVB S378A (cat)*	This work
BTD1101	*spoIIIG::neo, spoIVB::spec, amyE::P_{spoIIQ}-spoIVB S378A (cat), spoIID::Tn917 (erm)*	This work
BTD1245	*spoIIIG::neo, spoIVB::erm, amyE::P_{spoIIQ}-spoIVB S378A (cat), spoIIQ::spec*	This work
BTD2073	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB S378A (cat), spoIVB::spec, pelB::P_{spoIVF}-yfp(JF)-spoIVFA (tet)*	This work
BTD2081	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB S378A (cat), spoIVB::spec, pelB::P_{spoIVF}-yfp(JF)-spoIVFA (tet), spoIIIAH (phleo)*	This work
BTD2089	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB S378A (cat), spoIVB::spec, pelB::P_{spoIVF}-yfp(JF)-spoIVFA (tet), spoIIIAH (phleo), spoIID::Tn917 (erm)*	This work
BTD1575	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), yycR::P_{spoIIQ}-cfp(Bs) (phleo)*	This work
BTD1595	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), yycR::P_{spoIIQ}-cfp(Bs) (phleo), spoIID::Tn917 (erm)*	This work
BTD1597	*spoIIIG::neo, amyE::P_{spoIIQ}-spoIVB (cat), yycR::P_{spoIIQ}-cfp(Bs) (phleo), spoIID::Tn917 (erm), yvbJ::$P_{spoIVCB}$-spoIID (spec), lacA::$P_{spoIVCB}$-spoIID (tet)*	This work
BDR103	*bofA::cat*	Ricca *et al.*, 1992.
BTD2021	*bofA::cat, spoIIQ::spec*	This work
BTD2023	*bofA::cat, spoIID::Tn917 (erm)*	This work
BTD2025	*bofA::cat, spoIVFBΩpdr68 [spoIVFB-gfp(mut2) (tet)]*	This work
BTD2029	*bofA::cat, spoIVFBΩpdr68 [spoIVFB-gfp(mut2) (tet)], spoIIQ::spec*	This work
BTD2031	*bofA::cat, spoIVFBΩpdr68 [spoIVFB-gfp(mut2) (tet)], spoIID::Tn917 (erm)*	This work
BDR347	*spoIVFBΩpdr68 [spoIVFB-gfp(mut2) (tet)]*	Rudner and Losick, 2002
BDR94	*spoIVB::spec*	This work
BTD2027	*spoIVB::spec, spoIVFBΩpdr68 [spoIVFB-gfp(mut2) (tet)]*	This work

Bs: *Bacillus subtilis*
JF: Jelly Fish

Supplemental Table 2. *Plasmids used in this study*

plasmid	Description	Source or Reference
pDT47	*amyE::P_{spoIIQ}-spoIVB (cat)*	This work
pDT75	*lacA::P_{spoIIQ}-spoIVB (erm)*	This work
pDT28	*amyE::P_{spoIIQ}-cfp(Bs) (cat)*	Doan et al., 2005.
pNS58	*yycR::P_{spoIIQ}-cfp(Bs) (spec)*	This work
pDR203	*amyE::P_{spoIIQ}-bofC (cat)*	This work
pDT83	*ycgO::P_{spoIIQ}-SS(amyE)-malE (erm)*	This work
pDT104	*ycgO::P_{spoIIQ}-SS(amyE)-dsbA (erm)*	This work
pDT74	*amyE::P_{spoIIQ}-spoIVB(S378A) (cat)*	This work
pKM48	*pelB::P_{spoIVF}-yfp(JF)-spoIVFA (tet)*	This work
pNS50	*yycR::P_{spoIIQ}-cfp(Bs) (phleo)*	This work
pDT117	*yvbJ::$P_{spoIVCB}$-spoIID (spec)*	This work
pDT124	*lacA::$P_{spoIVCB}$-spoIID (tet)*	This work
pDR199	*[his_6-spoIID (extracellular domain)]*	This work
pKM67	*[his_6-bofC]*	This work

Bs: *Bacillus subtilis*
JF: Jelly Fish

Supplemental Table 3. *Oligonucleotides used in this study*

primer	sequence
oTD32	ggcAAGCTTacataaggaggaactactatgcccgataacatcagaaaa
oTD33	gccGGATCCttagcttgctttttctttccata
oDR394	cgcGGATCCgttaacatatgttcgcttctctc
oDR395	gcgAAGCTTcgtgtgcaaagaggtgtagag
oTD69	gttAAGCTTacataaggaggaactactatgtttgcaaaacgattcaaa
oTD70	gtgCTCGAGtgaagcactcgcagccgccggtcc
oTD71	gtgCTCGAGaaaatcgaagaaggtaaactg
oTD72	gttGGATCCttacttggtgatacgagtctgcgc
oTD87	gtgCTCGAGgcgcagtatgaagatggtaaa
oTD88	gttGGATCCttatttttctcggacagatatttcac
oTD97	tccGCTAGCacagctaaggaggaataaattatgaaacaattcgcaatcaca
oDR385	cgcGGATCCctacttttttcgccatatatttattc
oTD102	gttGAATTCagattcctcccgccttttgtc
oTD103	tccGCTAGCgagctctaagtatttttttcttc
oDR379	gccGCTAGCcataataaggaagcgggggcc
oDR380	cggCTCGAGctacttttttcgccatatatttattc
oKM2	gccGCTAGCggagctccttcccgcgcac
oKM4	cggCTCGAGctacccgctgtatgtcttcat

Restriction endonuclease sites are in capital letters.