Peptidoglycan hydrolysis is required for assembly and activity of the transenvelope secretion complex during sporulation in *Bacillus subtilis*

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SUPPLEMENTARY MATERIAL

Supplementary methods

Strain construction

yrnV::P$_{hyperspank}$-spoIIM (spec) in BCR299 was obtained by direct transformation of an isothermal assembly product (Gibson, 2011) into *B. subtilis*. The isothermal assembly reaction contained three PCR products: 1) *yrvN (back)*-P$_{hyperspank}$ (oligonucleotides primers oCR082 and oCR087 and pCR046 plasmid as template) 2) *optRBS-spoIIM* (oligonucleotide primers oCR090 and oCR091 and PY79 genomic DNA as template) and 3) *lacI-spec-yrvN* (front) (oligonucleotide primers oCR088 and oCR89 and pCR046 as template). pCR046 [*yrvN*::P$_{hyperspank}$ (*spec*)] is an IPTG-inducible expression plasmid for ectopic integration at the *yrvN* locus (C.R. and D.Z.R. unpublished).

Plasmid construction

pKM392 [*ycgO::P$_{spoIIQ}$-optRBS-gfp-spoIIQ (kan)*] was generated in two-way ligation in which a *Hind*III-*Xho*I fragment containing *gfp* was inserted into pDT68 [ycgO::PspoIIQ-*optRBS*-cfp-spoIIQ(kan)] (T.Doan and D.Z.R, unpublished) between *Hind*III and *Xho*I.

pKM399 [*yhdG::spoIIP, spoIID (spec)*] was generated in a three-way ligation with an *Eco*RI-*Nhe*I PCR product containing the *spoIID* gene (oligonucleotide primers oDR065 and oDR810 and PY79 genomic DNA) and a *Nhe*I-*Bam*HI fragment containing the *spoIIP* gene (oligonucleotide primers oDR0811 and oDR0812 and PY79 genomic DNA) and plasmid pBB278 cut with *Eco*RI and *Bam*HI. pBB278 [*yhdG::spec*] is an ectopic integration vector for double-crossover insertions into the *yhdG* gene (B. Burton and DZR, unpublished).

pLW003 [*yhdG::spoIIP*^{E359A}, *spoIID*^{E88A}*(spec)*] was generated by site-directed mutagenesis using oligonucleotide primers oDR0821 and oDR0734 and pKM399 plasmid DNA.

pCR009 [*ycgO::*P_{spoIIQ}*-optRBS-gfp-spoIIQ*^{R208A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0029 and plasmid pKM392.

pCR010 [*ycgO::*P_{spoIIQ}*-optRBS-gfp-spoIIQ*^{S164A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0030 and plasmid pKM392.

pCR011 [*ycgO::*P_{spoIIQ}*-optRBS-gfp-spoIIQ*^{V166A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0031 and plasmid pKM392.

pCR015 [*ycgO::*P_{spoIIQ}*-optRBS-gfp-spoIIQ*^{E206A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0033 and plasmid pKM392.

pCR016 [*ycgO::*P_{spoIIQ}*-optRBS-gfp-spoIIQ*^{D123A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0034 and plasmid pKM392.

pCR017 [*ycgO::*P_{spoIIQ}*-optRBS-gfp-spoIIQ*^{S119A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0035 and plasmid pKM392.

pCR018 [*ycgO::*P_{spoIIQ}*-optRBS-gfp-spoIIQ*^{E156A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0036 and plasmid pKM392.

pCR019 [*ycgO::*P_{spoIIQ}*-optRBS-gfp-spoIIQ*^{Q168A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0037 and plasmid pKM392.

pCR020 [*ycgO::*P_{spoIIQ}*-optRBS-gfp-spoIIQ*^{H202A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0032 and plasmid pKM392.

pCR026 [*ycgO::*P_{spoIID}*-mYpet-spoIID (spec)*] was generated in two-way ligation with an *Eco*RI-*Bam*HI isothermal assembly product (Gibson, 2011) containing P_{spoIID}*-mYpet-spoIID* and pKM083 [*ycgO::spec*] cut with *Eco*RI and *Bam*HI. The isothermal assembly product was generated with three PCR products 1) P_{spoIID} (oligonucleotide primers oDR0065 and oCR0046 and PY79 genomic DNA as template), 2) *mYpet* (oligonucleotide primers oCR0044 and oCR0045 and pROD50 (R. Reyes-Lamothe, unpublished) as template, 3) *spoIID* (oligonucleotide primers oCR47 and oDR0728 and PY79 genomic DNA template). pKM083 [*ycgO::spec*] is an ectopic integration vector for double crossover insertions into the *ycgO* locus (K.A.M. and D.Z.R. unpublished).

pCR027 [*ycgO::*P_{spoIID}*-mYpet-spoIID*^{E88A} *(spec)*] was generated in two-way ligation with an *Eco*RI-*Bam*HI isothermal assembly product (Gibson, 2011) containing P_{spoIID}*-yfp-spoIID*^{E88A} and pKM083 [*ycgO::spec*] cut with *Eco*RI and *Bam*HI. The isothermal assembly product was generated with three PCR products 1) P_{spoIID} (oligonucleotide primers oDR0065 and oCR0046 and PY79 genomic DNA as template), 2) *mYpet* (oligonucleotide primers oCR0044 and oCR0045 and pROD50 as template), 3) *spoIID*^{E88A} (oligonucleotide primers oCR0047 and oDR0728 and pLW003 plasmid DNA as template).

pCR028 [*yhdG::*P_{spoIIP}*-mYpet-spoIIP*^{E359A} *(kan)*] was generated in a two-way ligation with an *Eco*RI-*Bam*HI isothermal assembly product (Gibson, 2011) containing P_{spoIIP}*-mYpet-spoIIP*^{E359A} and plasmid pCR022 [*yhdG::kan*] cut with *Eco*RI and *Bam*HI. The isothermal assembly product was generated with three PCR products 1) P_{spoIIP} (oligonucleotide primers oCR0040 and oCR0042 and PY79 genomic DNA as template), 2) *mYpet* (oligonucleotide primers oCR0041 and oCR0044 and pROD50 as template), 3) *spoIIP*^{E359A} (oligonucleotide primers oCR0043 and oDR0812 and pLW003 plasmid as template). pCR022 [*yhdG::kan*]

is an ectopic integration vector for double crossover insertions into the *yhdG* locus (C.R. and D.Z.R, unpublished).

pCR029 [*yhdG::spoIIP*^{E359A} *(kan)*] was generated in a two-way ligation with an *Nhe*I-*Bam*HI fragment containing *spoIIP*^{E359A} obtained from pLW003 and pBB283 [*yhdG::kan*] cut with *Nhe*I and *Bam*HI. pBB283 [*yhdG::kan*] is an ectopic integration vector for double crossover insertions into the *yhdG* locus (B. Burton and D.Z.R. unpublished).

pCR030 [*ycgO::spoIID*^{E88A} *(spec)*] was generated in a two-way ligation with an *Nhe*I-*Eco*RI fragment containing *spoIID*^{E88A} obtained from pLW003 and plasmid pKM083 [*ycgO::spec*] cut with *Nhe*I and *Eco*RI.

pCR035 [*His-SUMO-sfGFP-spoIIQ*^{ECD}] was generated in a two-way ligation with a *Bam*HI-*Hind*III PCR product containing the extracytoplasmic domain of *spoIIQ* (oligonucleotide primers oCR0059 and oCR0060 and PY79 genomic DNA as template) and plasmid pTB260 (His-SUMO-GFP) cut with *Bam*HI and *Hind*III. pTB260 (His-SUMO-GFP) is an expression vector for production of GFP-tagged purified proteins (T. Bernhardt, unpublished).

pCR037 [*ycgO::spoIIQ*^{Q168A} *(kan)*] was generated by site-directed mutagenesis using oligonucleotide primer oCR0037 and plasmid pDT139 [*ycgO::spoIIQ (kan)*] (T. Doan and D.Z.R, unpublished).

pCR041 [*ycgO::*P$_{spoIIQ}$*-optRBS-mCherry(B.s)-spoIIQ (cat)*] was generated in a two-way ligation with an *Xho*I-*Hind*III PCR product containing *mCherry* (oligonucleotide primers oCR0073 and oCR0074 and pDR201 plasmid as template) and plasmid pCR007 [*ycgO::PspoIIQ-optRBS-GFP-spoIIQ (cat)*] cut with *Xho*I and *Hind*III. pDR201 contains mCherry with codons optimized for *B. subtilis* (D.Z.R. unpublished). pCR007 [*ycgO::PspoIIQ-optRBS-GFP-spoIIQ (cat)*] was generated in a two-way ligation with an *Eco*RI-*Bam*HI fragment from pKM392 and pKM077. pKM077 [*ycgO::cat*] is an ectopic integration vector for

double crossover integrations into the *ycgO* locus (K.A.M. and D.Z.R, unpublished).

pCR046 [*yvrN::*$P_{hyperspank}$ *(spec)]* was generated by isothermal assembly with $P_{hyperspank}$-*MCS-lacI* (oligonucleotide primers oCR0061 and oCR0062 and plasmid pER067 DNA as template) and pWX110 [*yvrN::spec*] digested with *Bam*HI and *Hind*III. pER067 is an ectopic integration vector containing a multiple cloning site downstream of the $P_{hyperspank}$ promoter (E. Riley and D.Z.R, unpublished). pWX110 is an ectopic integration vector for double cross-over at the *yvrN* locus (X. Wang and D.Z.R, unpublished).

pCR047 [*pelB::*$P_{hyperspank}$*-optRBS-spoIID (cat)*] was generated in a two-way ligation with an *Xma*I-*Sph*I PCR product containing *spoIID* (oligonucleotide primers oCR0075 and oCR0076 and PY79 genomic DNA) and plasmid pCR044 [*pelB::*$P_{hyperspank}$ *(cat)*]. pCR044 was generated in a two-ligation with an *Eco*RI-*Bam*HI fragment from pER067 [*ycgO::*$P_{hyperspank}$ *(erm)*] and pKM020 [*pelB::cat*]. pKM020 [*pelB::cat*] is an ectopic integration vector for double crossover integrations into the *pelB* locus (K. A. M. and D.Z.R, unpublished).

pCR050 [*His-SUMO-sfGFP-spoIIQ*$^{ECD(H202A\ H204A)}$] was generated in a two-way ligation with a *Bam*HI-*Hind*III PCR product containing the extracytoplasmic domain of *spoIIQ*$^{H202A\ H204A}$ (oligonucleotide primers oCR0059 and oCR0060 and pCR021 plasmid as template) and plasmid pTB260 (His-SUMO-GFP) cut with *Bam*HI and *Hind*III. pCR021 is an ectopic integration vector for double crossover integration at the *ycgO* locus that contains GFP-SpoIIQ$^{H202A\ H204A}$ (C.R. and D.Z.R, unpublished).

pCR052 [*ykoW::*$P_{hyperspank}$*-optRBS-spoIIP (phleo)*] was generated in a two-way ligation with a *Hind*III-*Nhe*I PCR product containing *spoIIP* (oligonucleotide primers oCR94 and oCR78 and PY79 genomic DNA) and plasmid pCR051 [ykoW::$P_{hyperspank}$ (phleo)]. pCR051 was generated by two-way ligation with an *Eco*RI-*Bam*HI fragment from pER067 and pWX112 [*ykoW::phleo*] cut with *Eco*RI

and *Bam*HI. pWX112 is a an ectopic integration vector for double crossover integrations into the *ykoW* locus (X. Wang and D.Z.R, unpublished).

Protein purification

His-SpoIID, His-SpoIIP and His-SpoIIQ were purified as described previously (Morlot *et al.*, 2010). Purified His-EnvC$^{35\text{-}419}$ was a gift from T. Uehara and T. Bernhardt.

GFP-SpoIIQ^{ECD}, GFP-SpoIIQ$^{ECD(H202A\ H204A)}$, and GFP-EnvC$^{35\text{-}419}$ fusion proteins were expressed in *E. coli* BL21(DE3), as His-SUMO tagged proteins (Bendezu *et al.*, 2009) following the procedure described in Uehara *et al.,* 2010 (Uehara *et al.*, 2010). All purification steps were carried out at 4°C. Cells were grown in 1 L LB supplemented with ampicillin (50 mg/mL) and 0.04% glucose at 37°C to an OD600 of 0.6. Protein overproduction was induced by the addition of IPTG to 1 mM. The cells were harvested (after 2.5 h) by centrifugation and resuspended in 12 ml of buffer A (50 mM Tris-HCl at pH 8, 300 mM NaCl, 20 mM imidazole, 10% glycerol). The cells were lysed by two passages through a French pressure cell. A soluble fraction was prepared by a 100,000Xg spin and was loaded on a 5-mL HisPur Ni-NTA spin column (Thermo Scientific) equilibrated with buffer A. Bound protein was washed with buffer B (20 mM Tris-HCl at pH 8, 300 mM NaCl, 50 mM imidazole, 10% glycerol) and eluted in buffer C (20 mM Tris-HCl at pH 8, 300 mM NaCl, 300 mM imidazole, 10% glycerol). Elution fractions were dialyzed overnight in buffer D (20 mM Tris-HCl at pH 8, 300 mM NaCl, 10% glycerol) using a Slide-A-Lyzer cassette (7-kDA cutoff, Thermo Scientific). The SUMO protease, UlpI, was added prior to dialysis. The dialyzed material was passed through HisPur Ni-NTA spin columns to remove free His-SUMO and His-UlpI yielding a pure preparation of the desired protein. The buffer was exchanged using Millipore filter units (10-kDa cutoff) into buffer E [(20 mM Tris-HCl (pH 8), 150 mM NaCl, and 10% glycerol)]. Protein concentration was determined by Comassie Plus protein reagent protocol (Thermo Scientific) and SDS-PAGE followed by Coomassie staining.

Preparation of *B. subtilis* sacculi

Vegetative and sporulating sacculi from the *B. subtilis* prototrophic strain PY79 were purified as described previously (McPherson & Popham, 2003), with some modifications. For vegetatively-growing cells, 200 mL of cells grown in CH medium were harvested at an OD600 of 0.5, resuspended in 10 ml ddH_2O and boiled in 40 mL of 5% SDS with vigorous stirring for 30 min. Sacculi were then harvested by centrifugation (8000g, 6 min, room temperature) and washed eight times with ddH_2O to remove SDS. Isolated sacculi were treated with ~ 1000 units of Benzonase (Sigma-Aldrich) for 24 h at 37°C in 1 ml buffer T (100 mM Tris HCl at pH 7.5, 20 mM $MgSO_4$) with vigorous agitation. Sacculi were then harvested by centrifugation (8000g, 6 min, room temperature) and resuspended in 1 ml buffer T containing 10 mM $CaCl_2$ and 100 mg/mL trypsin (Worthington Biochemical) and incubated overnight at 37°C. The sacculi were boiled again in 1% SDS for 15 min, diluted in 7 mL of ddH2O, and centrifuged at 12,000g for 10 min at room temperature. The pelleted sacculi were then washed twice in 20 mL of ddH_2O, once in 20 mL of 8 M LiCl, and twice again in 20 mL of ddH2O. The pellet was then incubated in 2 ml 10% TCA with constant rocking at 4°C for 48 h, after which the pellet was washed by centrifugation (8000g) with ddH_2O. The pellet was then resuspended in 1 ml alkaline phosphatase buffer with 5 units of alkaline phosphatase (calf intestinal, Boehringer Mannheim) and incubated overnight at 37°C. The pellet was boiled for 5 min and washed by centrifugation in ddH_2O three times. The final pellet was resuspended in 2 mL of ddH_2O containing 0.02% sodium azide and stored at 4°C.

Sporulating cells (100 mL) were harvested at hour 2 after transfer to resuspension medium and prepared as above.

Preparation of *E. coli* sacculi

Sacculi were prepared from strain TU163 [Δ*lpp*] as described by Uehara et al. (Uehara *et al.*, 2009).

Peptidoglycan pull-down assay

The extracellular domains of purified proteins were incubated separately with (+PG) and without (-PG) sacculi from *B. subtilis* (saculi of vegetatively-growing or sporulating cells) or *E. coli* in a 100 µL reaction mix. The reaction mix contained 4 µM of purified protein and 10 µl of purified sacculi. When sacculi was absent, an identical amount of 1XPBS buffer was added. Reactions were incubated for 30 min at 37°C. All reactions were then centrifuged at 13000g for 5 min to pellet the sacculi, after which 90 µL of supernatant was carefully removed and kept aside. This procedure was performed even in the sample that did not contain sacculi. The 10 µL pellet fraction was then resuspended in 90 µL 1XPBS. SDS-page sample buffer was added to the pellet fraction and the supernatant fraction to a final volume of 200 µl. Each fraction (20 µL) was separated by SDS-PAGE and stained with Comassie.

For the assay containing sacculi pre-digested by SpoIID, SpoIIP or both, the saculli (20 µL) were digested for 30 min at 37°C in 200 µL 1XPBS containing 1 µM of each protein. After 30 min, the reactions were quenched by boiling the samples for 5 min. The pre-digested sacculi were then pelleted by centrifugation at 13000 g for 5 min, and the pellet was used in the pull-down assay described above.

Visualization of purified GFP-tagged proteins on sacculi

For the visualization of GFP proteins on purified sacculi, 4 µM of protein was incubated with 1 µL of purified saculli in a final volume of 10 µL 1XPBS at room temperature for 15 min. The sample was then centrifuged at 13000g for 5 min, 5 µL of supernatant were removed and an equal volume of 1XPBS was added to the remaining 5 µL; the mixture was mixed and the above repeated three times to remove unbound protein. 5 µL of the washed sample were applied to poly L-lysine-coated slides and imaged as described in the Materials and Methods.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Localization of GFP-SpoIIQ to the septal membrane requires SpoIID, SpoIIP and SpoIIIAH. Representative images of GFP-SpoIIQ localization in sporulating cells at hour 2 of sporulation. Images are from a Δ*spoIID* Δ*spoIIP* double mutant (BKM1898), a Δ*spoIID* Δ*spoIIP* Δ*spoIIIAH* triple mutant (BKM1929), and Δ*sigE* (BKM1930). Sporulating cells that have uniformaly localized GFP-SpoIIQ throughout the forespore membranes (yellow carets) and those that have retained some localization GFP-SpoIIQ at the forespore septal membrane (white carets) are highlighted. We note that in a small subset of Δ*sigE* mutants, some GFP-SpoIIQ localized as a focus that could be found in either the septal membranes or the peripheral membranes. Images are GFP-SpoIIQ (left) and merge of GFP-SpoIIQ with membranes stained with TMA-DPH. Scale bar is 2 μm.

Figure S2: mCherry-SpoIIQ localizes in the wake of GFP-SpoIID and GFP-SpoIIP. Double labeling of mCherry-SpoIIQ and GFP-SpoIID (or GFP-SpoIIP) in sporulating cells (BCR189 or BCR188) imaged at hour 2 of sporulation. (A) Representative fields showing mCherry-SpoIIQ, GFP-SpoIID, a merged image of the two fluorescent fusion proteins and a merged image including the membranes stained with TMA-DPH (left). Larger images highlighting examples where mCherry-SpoIIQ and GFP-SpoIID do not completely overlap (right). Schematic representations of mCherry-SpoIIQ (red), GFP-SpoIID (green) and membranes (blue) are shown below. Scale bar is 2 μm. We note that mCherry-SpoIIQ localization is slightly delayed compared to GFP-SpoIIQ. We attribute this lag to the maturation time of the mCherry compared to GFP (Cormack *et al.*, 1996, Shaner *et al.*, 2005, Merzlyak *et al.*, 2007). (B) Larger representative images of mCherry-SpoIIQ and GFP-SpoIIP (top) and mCherry-SpoIIQ and GFP-SpoIID (bottom).

Figure S3: The extracytoplasmic domain of SpoIIQ does not bind peptidoglycan. Biochemical and cytological assays probling the interaction of SpoIIQ with purified peptidoglycan. **(A)** The extracytoplasmic domain of SpoIIQ

fused to superfolder GFP (sfGFP) (sfGFP-SpoIIQ^{ECD}) was purified and tested for binding to purified sacculi from sporulating and vegetatively grown *B. subtilis* cells. No interaction was observed by fluorescence microscopy. Scale bar is 2 µm. **(B)** The purified extracytoplasmic domain of SpoIIQ (lacking GFP) (IIQ^{ECD}) also failed to co-pellet (P) with B. subtilis sacculi. **(C)** Purified sfGFP-SpoIIQ and a mutant (sfGFP-SpoIIQ$^{H202A\ H204A}$), in which two highly conserved histidines in the LytM domain that have been implicated in PG binding in other LytM proteins (Sabala *et al.*, 2012) were mutated to alanine, displayed insignificant binding to *E. coli* sacculi. By contrast, labeling of *E. coli* sacculi by sfGFP-EnvC$^{34-419}$ was readily detectable. **(D)** Purified EnvC$^{34-419}$ but not the extracytoplasmic domain of SpoIIQ co-pelleted with *E. coli* saculli. **(E)** The extracytoplasmic domain of SpoIIQ did not pellet with *E. coli* saculli that were pre-treated with purified SpoIID and/or SpoIIP. These observations are consistent with those of Meisner and Moran (Meisner & Moran, 2011) who also failed to detect binding of SpoIIQ to purified sacculi.

Figure S4: Point mutants in the LytM groove of SpoIIQ impair its localization to the forespore septal membrane. (A) Surface representation of the SpoIIQ crystal structure highlighting the SpoIIIAH-SpoIIQ interaction interface (cyan), the degenerate active site (magenta) and the characteristic LytM groove (yellow). Residues that were mutated are highlighted. **(B)** Representative images of GFP-SpoIIQ mutants in a *spoIIIAH* null background at hour 2 of sporulation. From top to bottom, GFP-SpoIIQ^{D123A} (BCR77), GFP-SpoIIQ^{R208A} (BCR70), GFP-SpoIIQ^{H202A} (BCR81), GFP-SpoIIQ^{E206A} (BCR76), GFP-SpoIIQ^{E156A} (BCR79), GFP-SpoIIQ^{S119A} (BCR78) and GFP-SpoIIQ^{V166A} (BCR72). Images are GFP-SpoIIQ (left) and merge with TMA-DPH stained membranes (right). Scale bar is 2 µm.

Figure S5: Loss of GFP-SpoIIQ compartmentalization in a subset of cells experssing SpoIID, SpoIIP, and SpoIIM. Representative images of cells (strain BCR299) at hour 2 of sporulation from cultures with (+IPTG) and without (-IPTG) inducer added at hour 1.5. In a subset of sporulating cells in which IPTG was

added, GFP-SpoIIQ was present in both mother-cell and forespore membranes (white carets). It is not clear what accounts for this loss of forespore-specific compartmentalization but is likely related to the timing and/or extent of SpoIID, SpoIIP, and SpoIIM expression. Images are TMA-DPH-stained membranes (left), GFP-SpoIIQ (middle) and a merge (right). Scale bar is 2 μm.

Figure S6: The interaction mediated by the LytM groove in SpoIIQ is required for σ^G activity. Representative images of strains harboring the σ^G activity reporter P_{sspB}-*cfp* fluorescent-reporter. From top to bottom: wild-type (BCR185), Δ*spoIIQ* (BCR151), Δ*spoIIIAH* (BCR193), *spoIIQ(Q168A)* (BCR186) and a Δ*spoIIIAH spoIIQ(Q168A)* double mutant (BCR191). Forespores that display σ^G activity and are scored as positive (yellow carets) and those with weak activity and a collapsed forespore that are scored as negative (white carets) are highlighted (see Experimental Procedures). Images are CFP-SpoIIQ (left) and merge with TMA-DPH stained membranes (right). Scale bar is 2 μm.

Figure S7: Septal peptidoglycan thinning by SpoIID and SpoIIP is required for σ^G activity. Representative images of strains harboring the σ^G activity reporter P_{sspB}-*cfp* fluorescent-reporter. From top to bottom: wild-type (BTD2583), Δ*spoIID* (BTD2607), Δ*spoIIP* (BCR527), and a Δ*spoIID* Δ*spoIIP* double mutant (BCR529). Images are CFP (left) and merge with TMA-DPH-stained membranes (right). Scale bar is 2 μm.

SUPPLEMENTARY REFERENCES

Bendezu, F. O., C. A. Hale, T. G. Bernhardt & P. A. de Boer, (2009) RodZ (YfgA) is required for proper assembly of the MreB actin cytoskeleton and cell shape in E. coli. *EMBO J* **28**: 193-204.

Cormack, B. P., R. H. Valdivia & S. Falkow, (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**: 33-38.

Gibson, D. G., (2011) Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol* **498**: 349-361.

McPherson, D. C. & D. L. Popham, (2003) Peptidoglycan synthesis in the absence of class A penicillin-binding proteins in Bacillus subtilis. *J Bacteriol* **185**: 1423-1431.

Meisner, J. & C. P. Moran, Jr., (2011) A LytM domain dictates the localization of proteins to the mother cell-forespore interface during bacterial endospore formation. *J Bacteriol* **193**: 591-598.

Merzlyak, E. M., J. Goedhart, D. Shcherbo, M. E. Bulina, A. S. Shcheglov, A. F. Fradkov, A. Gaintzeva, K. A. Lukyanov, S. Lukyanov, T. W. Gadella & D. M. Chudakov, (2007) Bright monomeric red fluorescent protein with an extended fluorescence lifetime. *Nat Methods* **4**: 555-557.

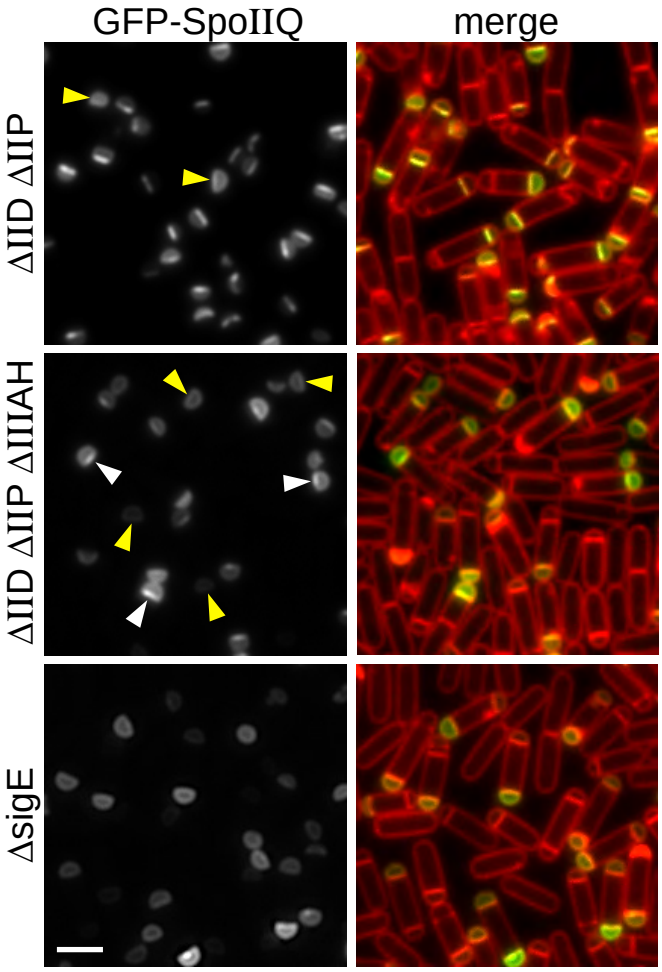
Morlot, C., T. Uehara, K. A. Marquis, T. G. Bernhardt & D. Z. Rudner, (2010) A highly coordinated cell wall degradation machine governs spore morphogenesis in Bacillus subtilis. *Genes Dev* **24**: 411-422.

Sabala, I., I. M. Jonsson, A. Tarkowski & M. Bochtler, (2012) Anti-staphylococcal activities of lysostaphin and LytM catalytic domain. *BMC Microbiol* **12**: 97.

Shaner, N. C., P. A. Steinbach & R. Y. Tsien, (2005) A guide to choosing fluorescent proteins. *Nat Methods* **2**: 905-909.

Uehara, T., T. Dinh & T. G. Bernhardt, (2009) LytM-domain factors are required for daughter cell separation and rapid ampicillin-induced lysis in Escherichia coli. *J Bacteriol* **191**: 5094-5107.

Uehara, T., K. R. Parzych, T. Dinh & T. G. Bernhardt, (2010) Daughter cell separation is controlled by cytokinetic ring-activated cell wall hydrolysis. *EMBO J* **29**: 1412-1422.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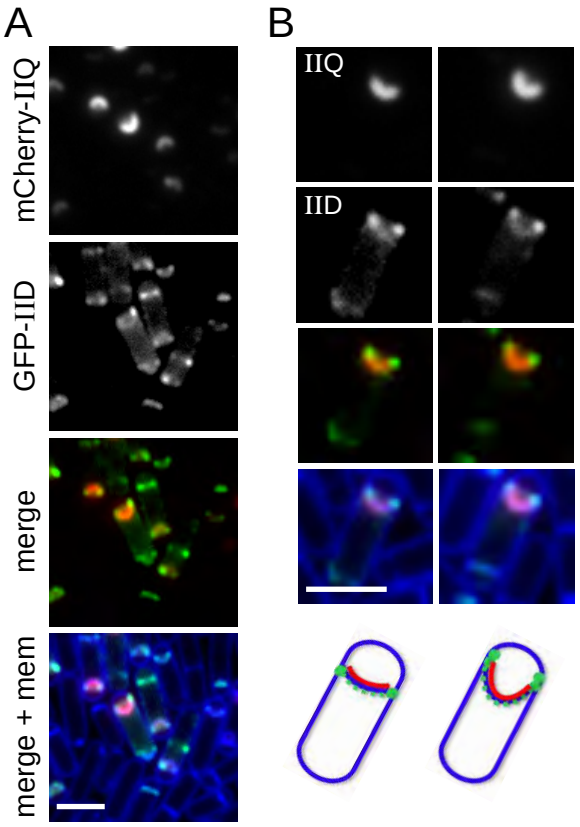

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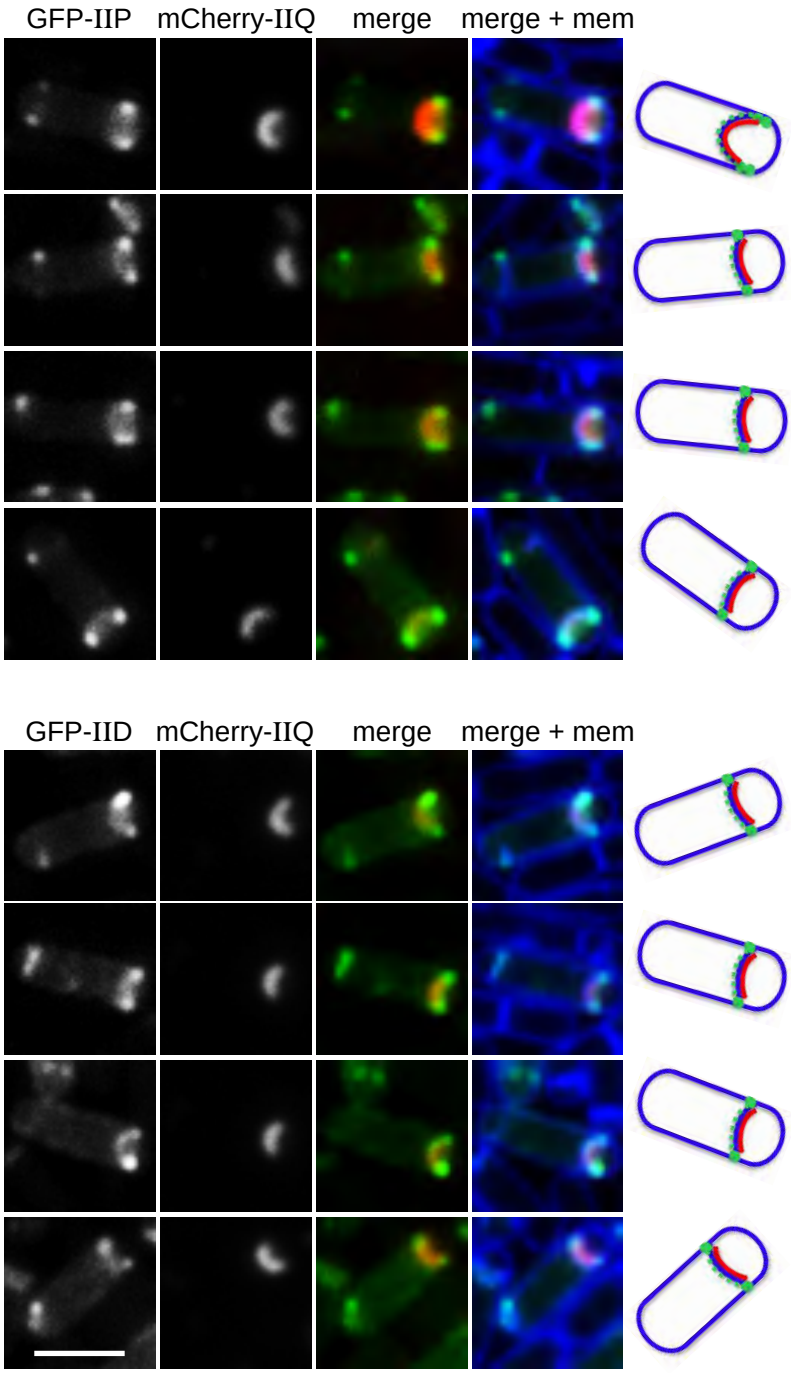

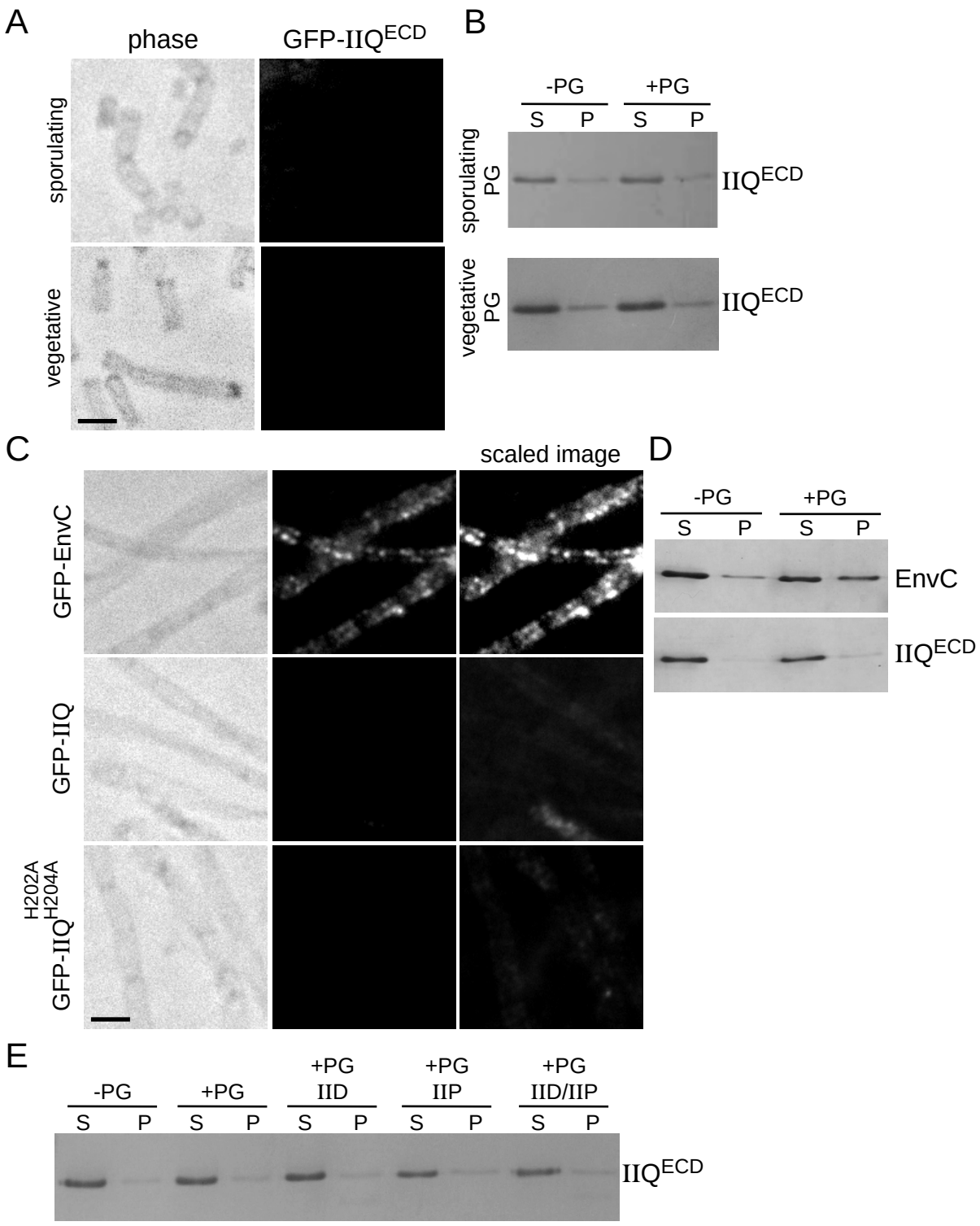
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P
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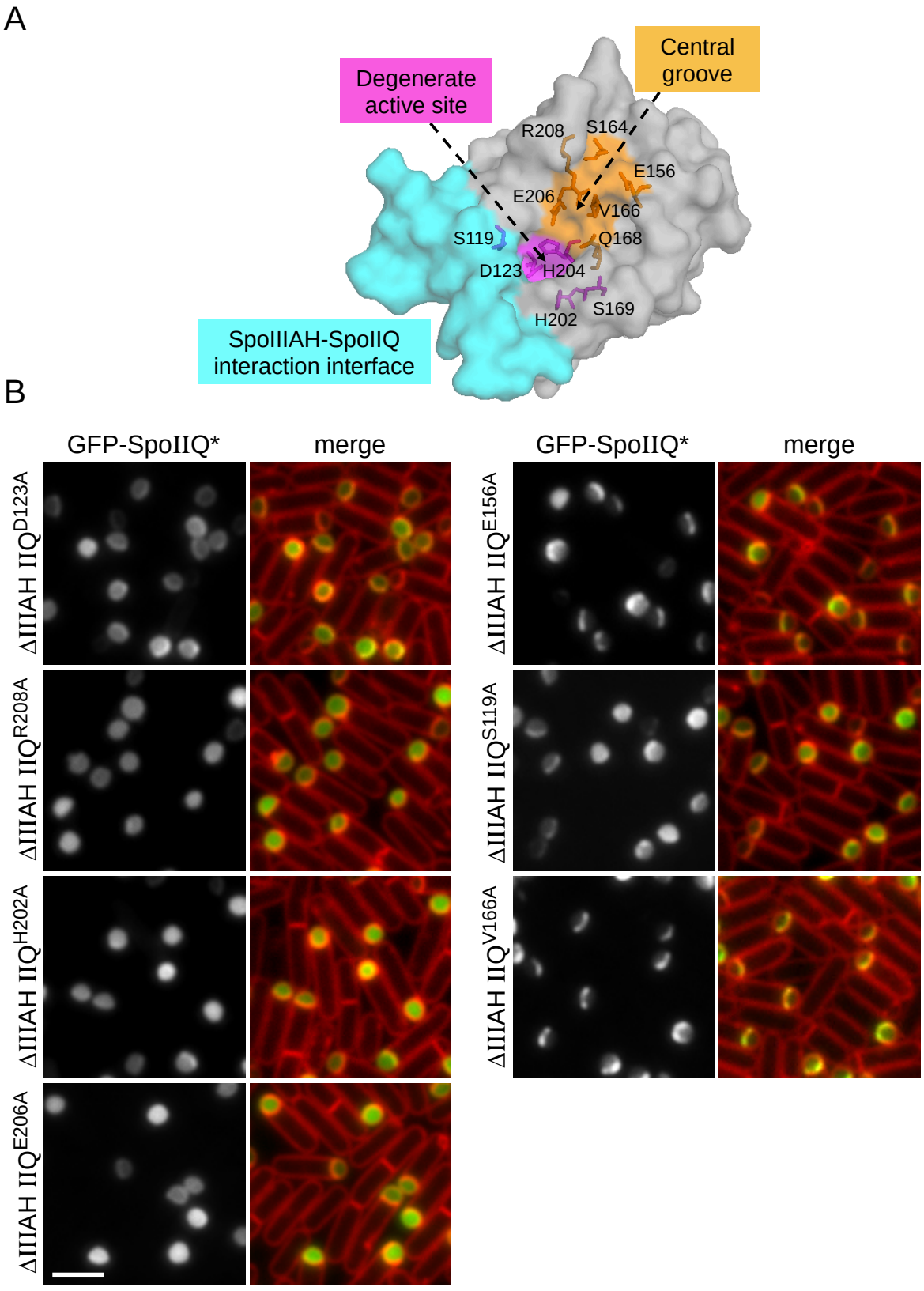
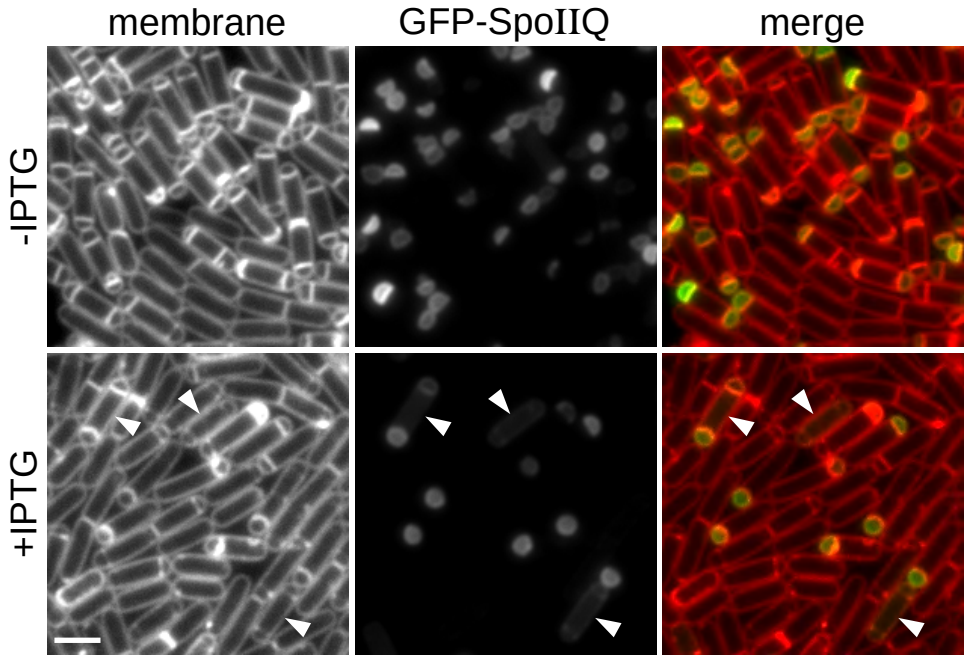
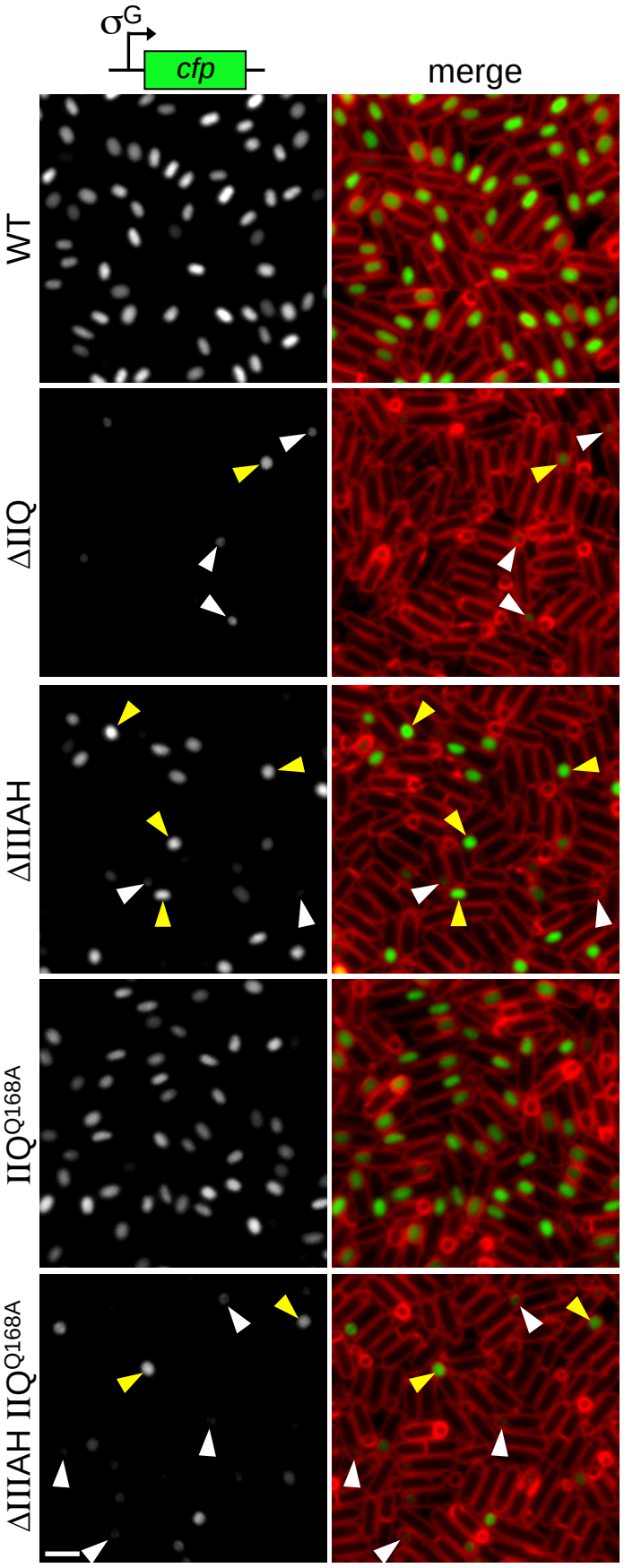
P
S
P
S
P
S
P
IIQ^ECD


A
Degenerate active site
Central groove
R208
S164
E156
E206
V166
S119
Q168
D123
H204
S169
H202
SpoIIIAH-SpoIIQ interaction interface
B
GFP-SpoIIQ*
merge
GFP-SpoIIQ*
merge
ΔIIIAH IIQD123A
ΔIIIAH IIQE156A
ΔIIIAH IIQR208A
ΔIIIAH IIQS119A
ΔIIIAH IIQH202A
ΔIIIAH IIQV166A
ΔIIIAH IIQE206A

Rodrigues_Fig S5



Rodrigues_Fig S6



Rodrigues_Fig S7

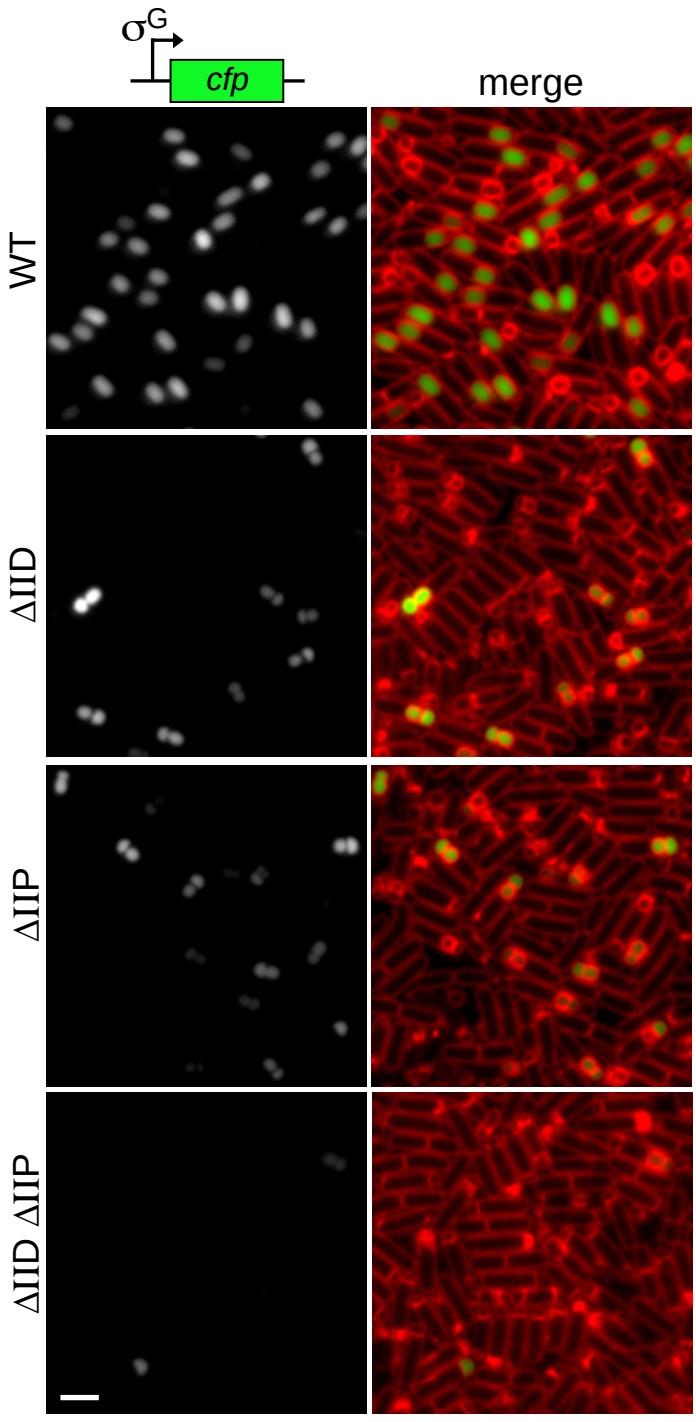


TABLE S1: Sporulation Efficiency

Strain	Genotype	Total CFU	Heat-resistant CFU	Spores/CFU (%)	Spores/WT (%)
BCR163	WT	3.9E+08	3.20E+08	82	100
BTD1541	Δ*spoIIQ*	6.5E+07	1.0E+05	0.2	0.03
BCR152	*spoIIQ*^{Q168A}	4.1E+08	3.0E+08	73	94
BCR161	Δ*spoIIIAH*	5.6E+07	6.2E+06	11	2
BCR153	Δ*spoIIIAH, spoIIQ*^{Q168A}	5.8E+07	1.1E+05	0.2	0.03
BCR162	Δ*spoIIB*	2.4E+08	1.9E+08	79	59
BCR196	Δ*spoIIB, spoIIQ*^{Q168A}	1.7E+08	1.6E+08	94	50

TABLE S2: *B. subtilis* strains used in this study

strain	genotype	source
PY79	Prototrophic wild-type strain	Youngman, 1983
ABS49	*spoIIP::tet Ω PspoIIP-gfp–spoIIP (erm)*	Chastanet & Losick, 2007
ABS325	*spoIID::kan Ω PspoIID-gfp-spoIID (erm)*	Chastanet & Losick, 2007
AHB1158	Δ*spoIIIAH::erm*	Gift from A. Camp
KP977	Δ*spoIIP::tet*	Gift from K. Pogliano
RL324	Δ*spoIID::cat*	Gift from R. Losick
RL873	Δ*spoIIB::erm*	Margolis et al., 1993
RL2022	Δ*spoIIQ::spec*	Londono-Vallejo et al, 1997
RL2036	Δ*spoIIIA::erm*	Gift from R. Losick
BTD1541	Δ*spoIIQ::phleo*	Doan et al., 2009
BTD2583	*yycR::P_{sspE}-optRBS-cfp (phleo)*	Doan et al., 2009
BTD2607	*yycR::P_{sspE}-optRBS-cfp (phleo),* Δ*spoIID::cat*	Doan et al., 2009
BLW2	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan),* Δ*spoIIP::tet*	This work
BKM1897	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan)*	This work
BKM1898	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan),* Δ*spoIID::cat*	This work
BKM1902	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan), spoIID::cat, amyE::spoIID(E88A)(spec)*	This work
BKM1905	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan),* Δ*spoIIIAH::erm*	This work
BKM1928	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan),* Δ*spoIID::cat,* Δ*spoIIP::tet*	This work
BKM1929	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan),* Δ*spoIID::cat,* Δ*spoIIP::tet,* Δ*spoIIIAH::erm*	This work
BKM1930	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan),* Δ*spoIIGB::erm,*	This work
BCR12	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan),* Δ*spoIID::cat,* Δ*spoIIP::tet,* Δ*spoIIIAH::erm, yhdG::spoIID(E88A),spoIIP(E359A) (spec)*	This work
BCR13	Δ*spoIIQ::phleo, P_{spoIIQ}-optRBS-gfp-spoIIQ(kan),* Δ*spoIID::cat,* Δ*spoIIP::tet,* Δ*spoIIIAH::erm, yhdG::spoIID,spoIIP (spec)*	This work
BCR24	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan), spoIIA:erm*	This work
BCR46	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (tet)*	This work
BCR70	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (R208A) (kan),* Δ*spoIIIAH::erm*	This work
BCR71	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (S164A) (kan),* Δ*spoIIIAH::erm*	This work
BCR72	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (V166A) (kan),* Δ*spoIIIAH::erm*	This work
BCR76	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (E206A) (kan),* Δ*spoIIIAH::erm*	This work
BCR77	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (D123A) (kan),* Δ*spoIIIAH::erm*	This work
BCR78	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (S119A) (kan),* Δ*spoIIIAH::erm*	This work
BCR79	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (E156A) (kan),* Δ*spoIIIAH::erm*	This work
BCR80	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (Q168A) (kan),* Δ*spoIIIAH::erm*	This work
BCR81	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (H202A) (kan),* Δ*spoIIIAH::erm*	This work
BCR87	Δ*spoIIQ::phleo, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (Q168A) (kan)*	This work
BCR92	*ycgO::P_{spoIID}-mYpet-spoIID (spec)*	This work
BCR100	*ycgO::P_{spoIID}-mYpet-spoIID (spec),* Δ*spoIID::cat*	This work
BCR103	*yhdG::P_{spoIIP}-mYpet-spoIIP (kan)*	This work
BCR110	*yhdG::P_{spoIIP}-mYpet-spoIIP (kan),* Δ*spoIIP::tet*	This work
BCR121	*yhdG:: P_{spoIIP}-mYpet-spoIIP(E359A) (kan),* Δ*spoIIP::tet,* Δ*spoIIIAH::erm, ycgO::spoIID(E88A) (spec),* Δ*spoIID::cat*	This work
BCR143	*ycgO::P_{spoIID}-mYpet-spoIID(E88A) (spec), yhdG::spoIIP(E359A) (kan),* Δ*spoIIP::tet,* Δ*spoIID::cat,* Δ*spoIIIAH::erm*	This work
BCR151	*yycR::P_{sspB}-optRBS-cfp (phleo),* Δ*spoIIQ::spec*	This work
BCR152	*ycgO::spoIIQ(Q168A) (kan),* Δ*spoIIQ::phleo*	This work
BCR153	*ycgO::spoIIQ(Q168A) (kan),* Δ*spoIIIAH::erm,* Δ*spoIIQ::phleo*	This work
BCR161	*ycgO::spoIIQ (kan),* Δ*spoIIIAH::erm,* Δ*spoIIQ::phleo*	This work
BCR162	*ycgO::spoIIQ (kan),* Δ*spoIIB::erm,* Δ*spoIIQ::phleo*	This work
BCR163	*ycgO::spoIIQ (kan),* Δ*spoIIQ::phleo*	This work
BCR185	*yycR::P_{sspB}-optRBS-cfp (phleo),* Δ*spoIIQ::spec, ycgO::spoIIQ(Q168A) (kan)*	This work
BCR186	*yycR::P_{sspB}-optRBS-cfp (phleo),* Δ*spoIIQ::spec, ycgO::spoIIQ (kan)*	This work
BCR188	Δ*spoIIP::tet ΩP$_{spoIIP}$-gfp–spoIIP (erm), ycgO::P_{spoIIQ}-optRBS-mCherry-spoIIQ (cat),* Δ*spoIIQ::phleo*	This work
BCR190	Δ*spoIID::kan ΩP$_{spoIID}$-gfp-spoIID (erm),ycgO::P_{spoIIQ}-optRBS-mCherry-spoIIQ (cat),* Δ*spoIIQ::phleo*	This work
BCR191	*yycR::P_{sspB}-optRBS-cfp (phleo),* Δ*spoIIQ::spec, ycgO::spoIIQ(Q168A) (kan),* Δ*spoIIIAH::erm*	This work
BCR193	*yycR::P_{sspB}-optRBS-cfp (phleo),* Δ*spoIIQ::spec, ycgO::spoIIQ (kan),* Δ*spoIIIAH::erm*	This work
BCR196	*ycgO::spoIIQ(Q168A) (kan),* Δ*spoIIQ::phleo,* Δ*spoIIB::erm,*	This work
BCR299	Δ*spoIIGB::erm,* Δ*spoIIQ::tet, ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ(kan), pelB::$_{hyperspank}$-optRBS-spoIID (cat), yrvN::$P_{hyperspank}$-optRBS-spoIIM (spec), ykoW::$_{hyperspank}$-optRBS-spoIIP (phleo)*	This work
BCR527	*yycR::P_{sspE}-optRBS-cfp (phleo),* Δ*spoIIP::tet*	This work
BCR529	*yycR::$_{sspE}$-optRBS-cfp (phleo),* Δ*spoIID::cat,* Δ*spoIIP::tet*	This work

TABLE S3: Plasmids used in this study

plasmids	description	source
pKM392	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ (kan)*	This work
pKM399	*yhdG::spoIIP, spoIID (spec)*	This work
pLW003	*yhdG::spoIIP^{E359A}, spoIID^{E88A}(spec)*	This work
pCR009	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ^{R208A} (kan)*	This work
pCR010	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ^{S164A} (kan)*	This work
pCR011	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ^{V166A} (kan)*	This work
pCR015	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ^{E206A} (kan)*	This work
pCR016	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ^{D123A} (kan)*	This work
pCR017	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ^{S119A} (kan)*	This work
pCR018	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ^{E156A} (kan)*	This work
pCR019	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ^{Q686A} (kan)*	This work
pCR020	*ycgO::P_{spoIIQ}-optRBS-gfp-spoIIQ^{H202A} (kan)*	This work
pCR026	*ycgO::P_{spoIID}-mYpet-spoIID (spec)*	This work
pCR027	*ycgO::P_{spoIID}-mYpet-spoIID^{E88A} (spec)*	This work
pCR028	*yhdG::P_{spoIIP}-mYpet-spoIIP ^{E359A} (kan)*	This work
pCR029	*yhdG::spoIIP^{E359A} (kan)*	This work
pCR030	*ycgO::spoIID^{E88A} (spec)*	This work
pCR035	*His-SUMO-sfGFP-spoIIQ^{ECD}*	This work
pCR037	*ycgO::spoIIQ^{Q168A} (kan)*	This work
pCR041	*ycgO::P_{spoIIQ}-optRBS-mCherry(B.s.)-spoIIQ (cat)*	This work
pCR046	*yvrN::*$P_{hyperspank}$ *(spec)*	This work
pCR047	*pelB::$P_{hyperspank}$-optRBS-spoIID (cat)*	This work
pCR050	*His-SUMO-sfGFP-spoIIQ$^{ECD\ (H202AH204A\ )}$*	This work
pCR052	*ykoW::$P_{hyperspank}$-optRBS-spoIIP (phleo*	This work
pTU130	*His-SUMO-sfGFP-envC$^{35-419}$*	T. Uehara

TABLE S4: Oligonucleotide primers used in this study

primer	sequence*
oCR0029	ccacgtgcactttgaaatcgctaaagatggggttgcaatgaa
oCR0030	gagattgatacacagtcgctaaaccgtcggcatgttcta
oCR0031	ccgacggtttatcgactgcgtatcaatctctttccgaagtaag
oCR0033	gaaaccacgtgcactttgcaatccgtaaagatggggttg
oCR0034	gcctaagcaaaggaattgccttagctgagaaagacggaa
oCR0035	ctataataacacgtacagcctagccaaaggaattgacttagctg
oCR0036	gtgctgggatatgttgtggcagtagaacatgccgacggtt
oCR0037	ggtttatcgactgtgtatgcatctctttccgaagtaagcg
oCR0040	cgcGAATTCgggcacaacttaatggttac
oCR0041	caagcgctggagggattcaatgtcaaaaggcgaagagctg
oCR0042	cagctcttcgccttttgacattgaatccctccagcgcttg
oCR0044	gccgctagagcctgatcccttgtaaagttcattcatccc
oCR0045	cgagcaggaggcagctgaatatgtcaaaaggcgaagagctg
oCR0046	cagctcttcgccttttgacatattcagctgcctcctgctcg
oCR0047	ggatcaggctctagcggcatgaaacaattcgcaatcacac
oCR0059	ggcGGATCCagccaatcagtatcaaatgatgagg
oCR0060	gccAAGCTTttaagactgttcagtgtcttc
oCR0061	caccatctttatcgtgagaaagaattcgacctctagcttgag
oCR0062	gaaaatcgccattcgccaggggatcctaactcacattaattgcgttgc
oCR0073	ggcAAGCTTacataaggaggaactactatggtcagcaagggagagg
oCR0074	cggCTCGAGtttgtataattcgtccattccacc
oCR0075	ggccgCCCGGGacataaggaggaactactatgaaacaattcgcaatcacac
oCR0076	ggcgcGCATGCcgacaaatgtggatgactttac
oCR0078	ggcgcGCTAGCctagcttagattatatcaatagg
oCR0082	tagttcctccttatgtcccgggacaagcttaattgttatccgctcac
oCR0087	cggacaggtatccggtaag
oCR0088	agttggaactctgcgcggctagctcgcatgcaagctaattcggtgg
oCR0089	atagggttgagtgttgttcc
oCR0090	cccgggacataaggaggaactactatgcgaaaaatctcttataaggac
oCR0091	gctagccgcgcagagttccaactata
oCR0094	cgcAAGCTTacataaggaggaactactatgatgagaaataaacgcagaaacag
oDR0065	ggcGAATTCgccgctctgggcgcagac
oDR0320	acagtgaagacagcggaaacgccgtgcactttgaaatccgtaa
oDR0728	cgcGGATCCgacaaatgtggatgactttacc
oDR0734	gattggagtcgtcgcctccgcaatgccggcaacctttaaacc
oDR0810	gccGCTAGCgacaaatgtggatgactttacc
oDR0811	ccgGCTAGCacaacttaatggttacaccg
oDR0812	cgcGGATCCactagcttagattatatcaatagg
oDR0821	gatagagcgcttttgctcgcatttggcggagtggataataat

***** capital letters indicate the recognition sites of restriction enzymes