# Supplementary Material

## Forespore targeting of SpoVD in *Bacillus subtilis* is mediated by the N-terminal part of the protein

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**Construction of Plasmids**

Plasmids used in the work are presented in Table S2.

pLEB26 and pLEB28

Primers Ewa40 and Ewa41 were used in PCR to amplify a 1641 bp fragment of 1617 bp encoding the extra-membraneous part of PBP4b using *B. subtilis* 1A1 chromosomal DNA as template. This fragment was cloned into pCR®-Blunt II-TOPO®, resulting in pLEB26. The fragment was cleaved out from pLEB26 using BamHI and EcoRI and ligated into pGEX-2T, resulting in pLEB28. *E. coli* TOP10 and *E. coli* Tuner (DE3) were transformed with pLEB28 by electroporation and chemical transformation, respectively.

pLEB27

A 972 bp DNA fragment was synthesized at MWG Eurofins, Germany. This fragment contained 477 bp upstream region of *spoVE*, the three-first and the three-last nucleotides of the *spoVE* open reading frame, and 477 bp downstream region of *spoVE*. The fragment was flanked by BamHI and PstI restriction sites at the 5' and 3' end, respectively. The synthetic DNA fragment was cut out from pEX-A-ΔspoVE (plasmid delivered by MWG Eurofins) using BamHI and PstI and cloned into BamHI/PstI-digested pJM103-I-SceI, resulting in pLEB27.

pLEB33

A 1160 bp fragment containing the *spoVE* gene without the translation start codon was amplified by PCR using primers Ewa47 and Ewa48 and *B. subtilis* 1A1 chromosomal DNA as template. The primers generated restriction sites for BamHI and EcoRI, respectively. After

restriction enzyme digestion, the PCR product was ligated into BamHI/EcoRI-digested pSG1729, resulting in pLEB33.

pLMS1 and pLMS2

The plasmids were constructed in two steps. First, 462 bp of the upstream region of *pbpI* and the three-first nucleotides of the *pbpI* open reading frame were amplified by PCR using primers MSI001 and MSI002 and *B. subtilis* 1A1 chromosomal DNA as template. The primers generated restriction sites for XmaI and BamHI, respectively. After restriction enzyme digestion, the PCR product was ligated into pJM103-I-SceI (obtained by cleaving pLEB1 with XmaI and BamHI), resulting in pLMS1. Next, a fragment containing the three last nucleotides of *pbpI* and 529 bp downstream of *pbpI* was amplified using primers MSI003 and MSI004. The PCR product was digested with BamHI and SphI and inserted into pLMS1 cut with the same enzymes, resulting in pLMS2.

pLMS3

Removal of the *strep-tag* at the C-terminal end of *spoVD* in pLEB34 was accomplished using the inverted PCR method. Phosphorylated primers MSI005 and MSI006 were used to amplify pLEB34 except for the 24 bp *strep-tag*. The PCR product was self-ligated, resulting in pLMS3.

pLEB47, pLEB48, pLMS4, pLMS5, pLMS6, pLMS7, and pLMS8

The plasmids were constructed using the overlap extension PCR cloning method (1). Briefly, chimeric primers complementary to plasmid vector sequence at the 5' end and an insert sequence at the 3' end were synthesized. In the first step, the desired insert was amplified in a standard PCR reaction using the chimeric primers and *B. subtilis* 1A1 chromosomal DNA as a template. Purified PCR products were then used as a pair of megaprimers in overlap extension PCR with the vector. The following vectors and synthetic primers were used: plasmid pLEB33 was used to obtain pLEB47 (primers Ewa77/Ewa78) and pLEB48 (primers Ewa79/Ewa80); pLMS3 was used to obtain pLMS4 (primers MSI007/MSI008), pLMS5 (primers MSI009/MSI010), pLMS6 (primers MSI008/MSI009), pLMS7 (primers MSI009/MSI013); and pLMS6 was used to obtain pLMS8 (primers MSI014/MSI015). Subsequently, the original plasmid DNA was destroyed by cleavage with DpnI. The DpnI treated DNA was used to transform *E. coli*. It should be noted that in the chimeric protein encoded by pLMS4 residue 236 of PBP4b is a Gly instead of a Pro.

pLMS9, pLMS10, pLMS11, and pLMS12

Replacement of the xylose-inducible promoter by the $\sigma^E$-dependent *spoVD* promoter in pLMS3, pLMS6, pLMS7, and pLMS8 was achieved by using the inverted PCR method. Phosphorylated primers MSI018 (containing the vector complementary part and -10 $\sigma^E$ promoter region) and MSI019 (containing the vector complementary part and -35 $\sigma^E$ promoter region) were used to amplify the entire plasmids except for the 110 bp xylose-inducible promoter region. Upon completion of reaction, DpnI was added to digest the parental plasmids and the PCR products were purified by agarose gel electrophoresis. The PCR fragments were self-ligated, resulting in pLMS9, pLMS10, pLMS11, and pLMS12, respectively.

**Production and purification of sPBP4b**

*E. coli* Tuner (DE3)/pLEB28 cells were grown at 37ºC in 2 L 2-YTA medium (1 L per 5 L baffled E-flask) on a rotary shaker (200 rpm). At an optical density of 0.4 at 600 nm, the culture was shifted to 18ºC and *gst-pbpI* gene expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.05 mM. After incubation overnight (~16 h) at 18ºC the cells were collected by centrifugation at 6,000 × g for 20 min at 4ºC and washed in PBS (1.8 mM $KH_2PO_4$, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 140 mM NaCl, pH 7.4). The cells were suspended in 15 ml PBS and disrupted using a French Pressure cell operated at 18,000 Psi. The cell lysate was centrifuged at 48,000 ×g for 30 min at 4ºC to remove unbroken cells and the supernatant was then centrifuged at 100,000 ×g for 1 h at 4ºC. The resulting supernatant was passed through a 0.45 μm filter and incubated with 2 ml of a 50% slurry of Glutathione Sepharose 4B (GE Healthcare) under constant mixing for 30 min at room temperature. The mixture was then added to a 10 ml Polyprep chromatography column (BioRad) and after washing with PBS GST-sPBP4b was eluted with 3 ml of reduced glutathione according to the manufacturer's protocol. The eluted protein was incubated with 10 units of thrombin (GE Healthcare) per mg of fusion protein for 2 h at room temperature and applied on to a Sephacryl S-100 HR gel filtration column that was eluted with PBS at 4ºC. Fractions containing sPBP4b were traced by the absorbance at 280 nm and the purity of these fractions was analyzed by SDS-PAGE. Fractions containing mainly sPBP4b were pooled and remaining GST was removed by affinity chromatography using Glutathione Sepharose 4B. The flow through, which contained purified sPBP4b, was concentrated using a Centriplus concentrator 10 (Amicon). See Figure S2.

**Penicillin-binding assay**

Purified sPBP4b was incubated at 35ºC for 30 min with a molar excess of 50 µM Penicillin G or Ampicillin or without addition of a penicillin. Bocillin FL at 50 µM or 25 µM was then added to covalently label protein with transpeptidase activity (2). The three reaction mixtures were incubated for another 30 min at the same temperature before they were incubated with SDS-PAGE sample buffer at 40ºC for 15 min and subjected to SDS-PAGE with a precast Bis-Tris 10% acrylamide gel (Invitrogen). After electrophoresis binding of Bocillin FL to PBP4b was determined by fluorometry using a ChemiDocTM Imaging System (BioRad) (excitation at 455-485 nm and emission light filter 5532AE28).

**Production of PBP4b antiserum**

One rabbit was on 4 occasions subcutaneously injected with 40 µg of purified sPBP4b. At the first injection the antigen was in complete Freunds adjuvant while in the remaining immunizations incomplete Freunds was used. The rabbit was immunized on day 1, 14, 28 and 49. The rabbit was bled before the first injection (pre-immune serum) and the first sample of antiserum was collected 2 weeks after the third injection. The production of antiserum was at Innovagen, Lund.

**Table S1.** Oligonucleotides (primers) used in this work.

| Name | Sequence (5' to 3') | Restriction sites |
|---|---|---|
| Ewa1 | ggccatcccgggagaagagaggttct | XmaI |
| Ewa4 | ggccatggatccaagagaccgttcac | BamHI |
| Ewa32 | ggccatcccgggatcgcggaaatggt | XmaI |
| Ewa35 | ggccatgcatgcaattgaacgaaacggt | SphI |
| Ewa40 | ggccatggatccagtgtgaaacagcgga | BamHI |
| Ewa41 | ggccatgaattcctatttctgattgatttcaa | EcoRI |
| Ewa47 | ggccatggatccctactaaaaaaacatcgcc | BamHI |
| Ewa48 | ggccatgaattccaagcttcatgttagacag | EcoRI |
| Ewa54 | agagagaccacatggtc | |
| Ewa56 | ggacacatggaaacacac | |
| Ewa77 | ctgctgaatgtaagccgctactcgaggtatggctcgggctcgggctcgat gaagatatcgaaacgaatgaagc | |
| Ewa78 | ccggttattattatttttgacaccagaccaactggctatttctgattgatttcaat ttcg | |
| Ewa79 | ctgctgaatgtaagccgctactcgaggtatggctcgggctcgggctcgttg cgcgtctcgaatgtaacggtta | |
| Ewa80 | ccggttattattatttttgacaccagaccaactggtcaatcggctgcctccttt tcatc | |
| MSI001 | ggccatcccgggagaagtagtagagcg | XmaI |

| | | |
|---|---|---|
| MSI002 | ggccatggatcccataaaaaaacacctc | BamHI |
| MSI003 | ggccatggatcctagaacaacttctcag | BamHI |
| MSI004 | ggccatgcatgctgctttccagtcagg | SphI |
| MSI005 | P-atcggctgcctccttttcatc | |
| MSI006 | P-tgagaattctagttctagagcggccg | |
| MSI007 | gcggatgactatacgccgcctaaggatgggctccagatcaaaacaacgatcg | |
| MSI008 | taattcgcggccgctctagaactagaattcctatttctgattgatttcaatttcg | |
| MSI009 | accatacaaaaaaatgtccagacttcggatcctgatgaagatatcgaaacgaatgaagc | |
| MSI010 | ctttgaatcaactgtcagcttcatatcaagcccataaaatgtattggcttcagctgtatatttg | |
| MSI011 | gagggacaaccagaaggag | |
| MSI012 | tttggcatcctgagggctg | |
| MSI013 | gctccaggaatctttcgctagcgaagtcagtttctttttagaaaatgattctgtgaaaaac | |
| MSI014 | accatacaaaaaaatgtccagacttcggatcctgcgcgtctcgaatgtaacggttag | |
| MSI015 | cctccgtccgctgtttcacactttcttgaatcaaattaatttttttcgcccatcacaaactgaac | |
| MSI018 | P-ggcataaaatgaaacaagccgacataaaatgcatctagaaagg | |
| MSI019 | P-tgaatttggacaggtagaacgatgctcctcccgatcttttgtttataagtg | |

**Table S2.** Plasmids used in this work

| **Strain** | **Description**[a] | **Origin/Reference** |
|---|---|---|
| pBKJ223 | I-SceI expression vector; $Amp^R$ $Tet^R$ | (3) |
| pCR®-Blunt II-TOPO® | Cloning vector; $Kan^R$ | Invitrogen |
| pEX-A-ΔspoVE | pEX-A (MWG Eurofins) with a custom 972 bp synthetic DNA fragment containing *spoVE* markerless inframe deletion; $Amp^R$ | MWG Eurofins |
| pGEX-2T | Expression vector for GST-tagged proteins; $Amp^R$ | GE Healthcare |
| pJM103-I-SceI | Suicide integration vector pJM103 with I-SceI restriction site; $Amp^R$ $Cml^R$ | (4) |
| pSG1729 | *amyE* integration vector designed to generate N-terminal GFP fusions under the xylose-inducible promoter | BGSC[b] |
| pLEB1 | 289 bp upstream region of *spoVD* cloned into pJM103-I-SceI; $Amp^R$ $Cml^R$ | (5) |
| pLEB2 | 336 bp downstream region of *spoVD* cloned into pLEB1; $Amp^R$ $Cml^R$ | (5) |
| pLEB26 | 1641 bp PCR fragment containing part of the *pbpI* gene cloned into pCR®-Blunt II-TOPO®; $Kan^R$ | This work |

| | | |
|---|---|---|
| pLEB27 | 972 bp fragment containing *spoVE* markerless inframe deletion cloned into pJM103-I-SceI; $Amp^R$ $Cml^R$ | This work |
| pLEB28 | pGEX-2T with a 1617 bp fragment containing *pbpI*; $Amp^R$ | This work |
| pLEB33 | Plasmid containing *Pxyl-gfp-spoVE*; $Amp^R$ $Spc^R$ (derivative of pSG1729) | This work |
| pLEB34 | Plasmid containing *Pxyl-gfp-spoVD-strepII-tag*; $Amp^R$ $Spc^R$ (derivative of pSG1729) | (6) |
| pLEB47 | Plasmid containing *Pxyl-gfp-spoVE-pbpI*; $Amp^R$ $Spc^R$ (derivative of pSG1729) | This work |
| pLEB48 | Plasmid containing *Pxyl-gfp-spoVE-spoVD*; $Amp^R$ $Spc^R$ (derivative of pSG1729) | This work |
| pLMS1 | 462 bp upstream region of *pbpI* cloned into pJM103-I-SceI; $Amp^R$ $Cml^R$ | This work |
| pLMS2 | 529 bp downstream region of *pbpI* cloned into pLMS1; $Amp^R$ $Cml^R$ | This work |
| pLMS3 | pLEB34 without *strepII-tag* sequence; $Amp^R$ $Spc^R$ | This work |
| PLMS4 | Plasmid containing *Pxyl-gfp-spoVD*$^{2\text{-}214}$*-pbpI*$^{236\text{-}584}$; $Amp^R$ $Spc^R$ (derivative of pSG1729) | This work |
| pLMS5 | Plasmid containing *Pxyl-gfp-pbpI*$^{1\text{-}235}$*-spoVD*$^{215\text{-}646}$; $Amp^R$ $Spc^R$ (derivative of pSG1729) | This work |
| pLMS6 | Plasmid containing *gfp-pbpI*; $Amp^R$ $Spc^R$ (derivative of pSG1729) | This work |
| pLMS7 | Plasmid containing *Pxyl-gfp-pbpI*$^{1\text{-}40}$*-spoVD*$^{41\text{-}646}$*;* $Amp^R$ $Spc^R$ (derivative of pSG1729) | This work |
| pLMS8 | Plasmid containing *Pxyl-gfp-spoVD*$^{2\text{-}40}$*-pbpI*$^{41\text{-}584}$; $Amp^R$ $Spc^R$ (derivative of pSG1729) | This work |
| pLMS9 | pLMS3 with *spoVD* native promoter; $Amp^R$ $Spc^R$ | This work |
| pLMS10 | pLMS6 with *spoVD* native promoter; $Amp^R$ $Spc^R$ | This work |
| pLMS11 | pLMS7 with *spoVD* native promoter; $Amp^R$ $Spc^R$ | This work |
| pLMS12 | pLMS8 with s*poVD* native promoter; $Amp^R$ $Spc^R$ | This work |

[a]$Amp^R$, $Tet^R$, $Kan^R$, $Cml^R$, and $Spc^R$ indicate resistance to ampicillin, tetracycline, chloramphenicol, kanamycin, and spectinomycin respectively. The superscript numbers in chimeras refer to amino acid positions in the wild-type protein.

[b]Bacillus Genetic Stock Center, Columbus, Ohio, USA.

## References


1. Bryskin A, Matsumura I. 2013. Overlap extension PCR cloning. Methods Mol Biol 1073:31-42.
2. Zhao G, Meier T, Kahl S, Gee K, Blaszczak L. 1999. BOCILLIN FL, a sensitive and commercially available reagent for detection of penicillin-binding proteins. Antimicrob Agents Chemotherapy 43:1124-1128.
3. Janes B, Stibitz S. 2006. Routine markerless gene replacement in *Bacillus anthracis*. Infect Immun 74:1949-1953.
4. Perego M. 1993. Integrational vectors for genetic manipulation in *Bacillus subtilis*., p 615-624. *In* Sonenshein AL, Hoch JA, Losick R (ed), *Bacillus subtilis* and other Gram-positive bacteria: biochemistry, physiology and molecular genetics. American Society for Microbiology, Washington, DC.
5. Bukowska-Faniband E, Hederstedt L. 2013. Cortex synthesis during *Bacillus subtilis* sporulation depends on the transpeptidase activity of SpoVD. FEMS Microbiol Lett 346:65-72.
6. Bukowska-Faniband E, Hederstedt L. 2015. The PASTA domain of penicillin-binding protein SpoVD is dispensable for endospore cortex peptidoglycan assembly in *Bacillus subtilis*. Microbiology 161:330-40.

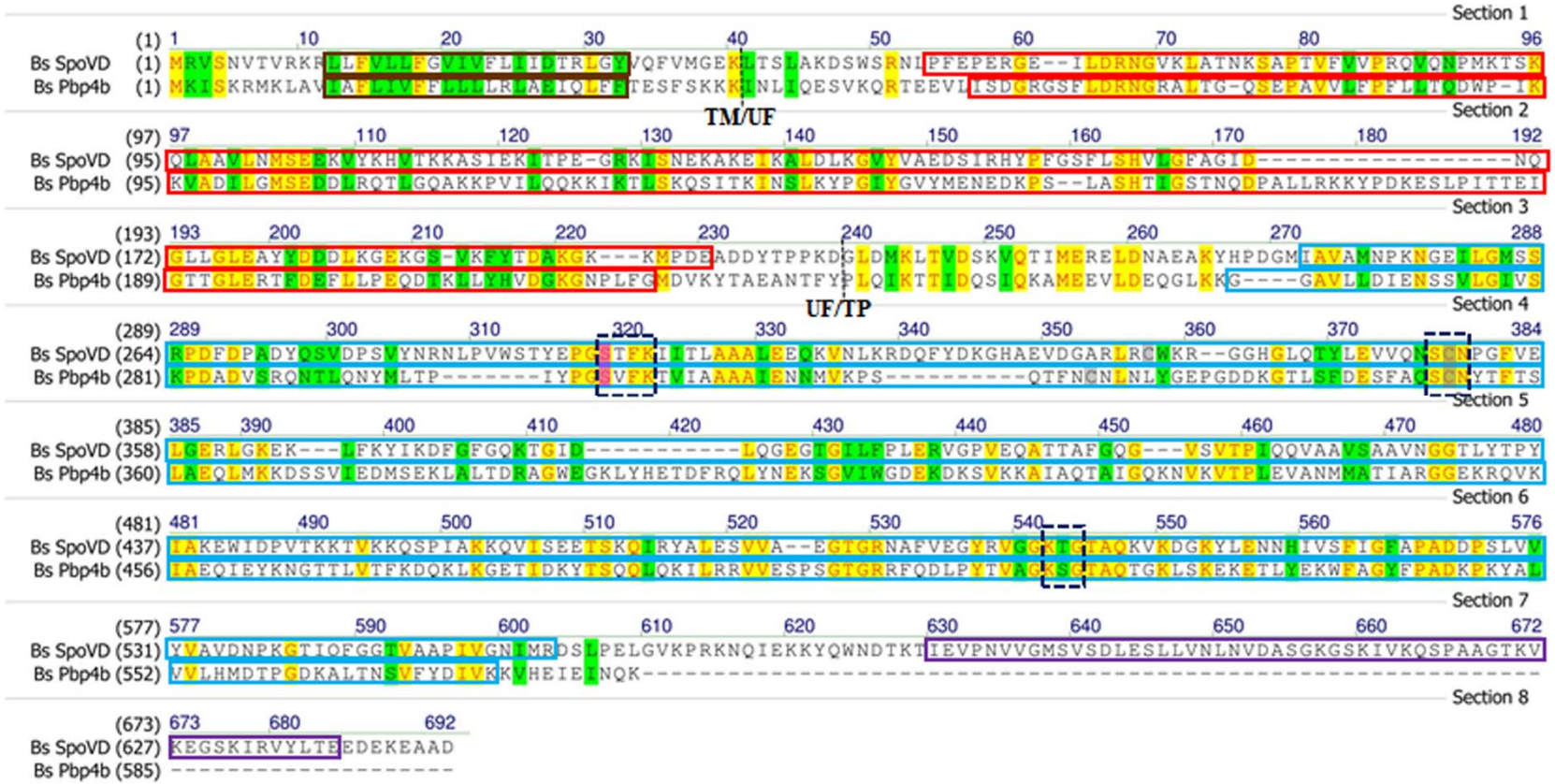



**Figure S1.** Amino acid sequence alignment of *B. subtilis* SpoVD (71 kDa) and PBP4b (65 kDa). The borders of the TM (transmembrane), UF (unknown function), and TP (transpeptidase) domains in the chimeric proteins studied in this work (Fig. 1) are marked with a vertical dashed line. The predicted membrane spanning sequence is marked with a brown box. The UF and TP regions are marked with a red and blue box, respectively. The PASTA region of SpoVD is marked with a purple box. Conserved active site sequence motifs in the TP domain are indicated with dashed lined boxes and two conserved cysteine residues are indicated in gray. The active site serine in the TP domain is highlighted in red. Identical and similar amino acids are highlighted in yellow and green, respectively. The alignment was carried out using Vector NTI software. The amino acid sequences were obtained from the UNIPROT database (*http://www.uniprot.org/*) under the name Q03524 for SpoVD and O32032 for PBP4b.

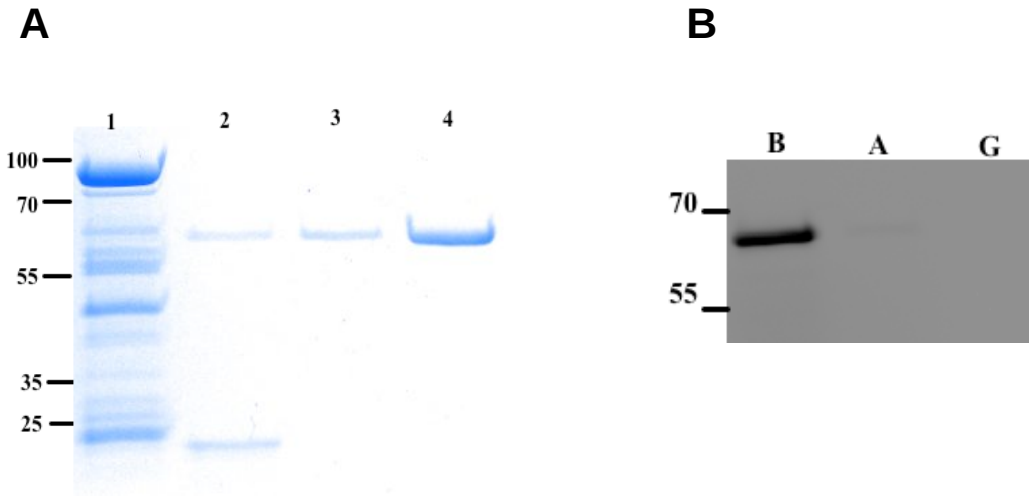
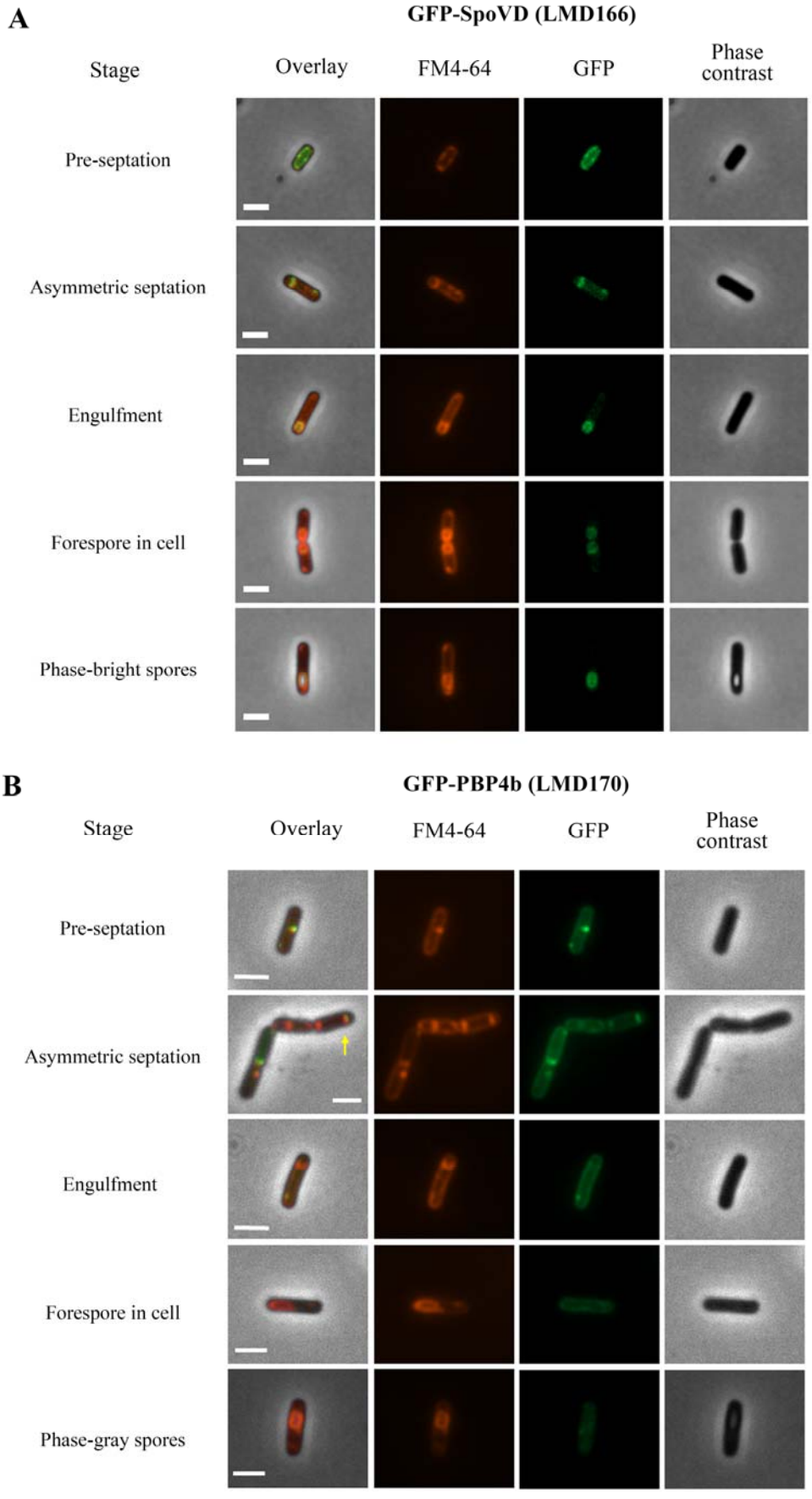



**Figure S2.**

Panel A. Purification of sPBP4b produced in *E. coli*. Shown is a SDS polyacrylamide electrophoresis gel stained for protein using Coomassie Brilliant Blue. Lane 1, GST-sPBP4b (~86 kDa, 18 µg) eluted from Glutathione Sepharose 4B; lane 2, sPBP4b (~60 kDa) and GST(~26 kDa) (0.8 µg protein) after cleavage by thrombin and purified by gel filtration; lane 3. sPBP4b (0.8 µg) after 2$^{nd}$ Glutathione Sepharose 4B affinity chromatography; lane 4. concentrated sPBP4b (2.6 µg). Molecular mass markers, in kDa, are indicated.

Panel B. Penicillin binding properties of sPBP4b. Purified sPBP4b at 0.1 mg/ml was incubated with 50 µM Bocillin FL (lane B), ampicillin (lane A) or penicillin G (lane G) for 30 min. Then Bocillin FL (25 µM) was added to the samples preincubated with ampicillin and penicillin G. The reaction mixtures were subjected to SDS polyacrylamide gel electrophoresis as in Panel A (1.6 µg of sPBP4b was loaded per lane). Labeled sPBP4b was detected as fluorescence.


A
GFP-SpoVD (LMD166)
Stage
Overlay
FM4-64
GFP
Phase contrast
Pre-septation
Asymmetric septation
Engulfment
Forespore in cell
Phase-bright spores
B
GFP-PBP4b (LMD170)
Stage
Overlay
FM4-64
GFP
Phase contrast
Pre-septation
Asymmetric septation
Engulfment
Forespore in cell
Phase-gray spores

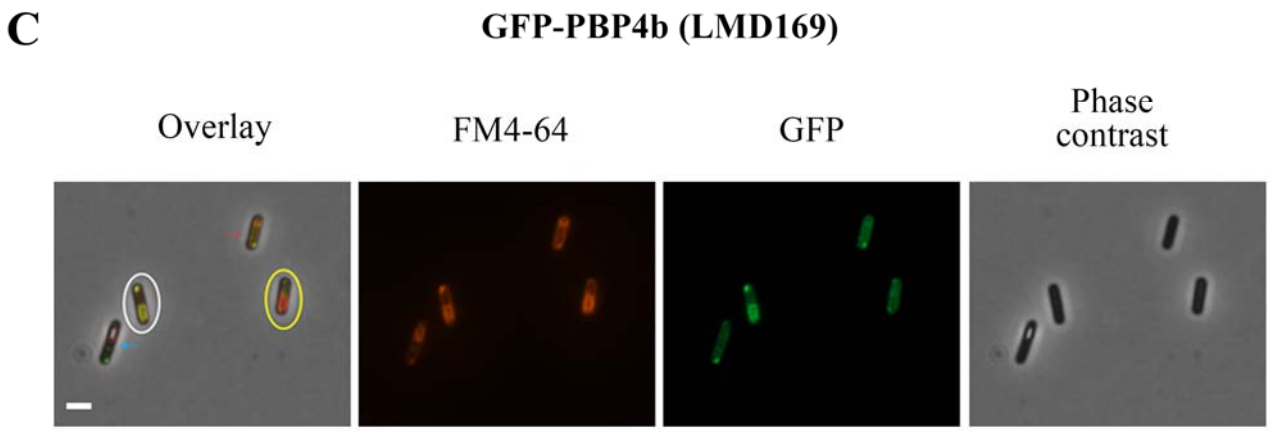



**Figure S3.** Fluorescence and phase contrast microscopy images of sporulating *B. subtilis ΔspoVD ΔpbpI* cells containing a gene, under the control of the xylose promoter, encoding GFP fused to SpoVD or PBP4b. The scale bar indicates 2 µm. Panel A, GFP-SpoVD in strain LMD166. Panel B, GFP-PBP4b in strain LMD170. Panel C, GFP-PBP4b in strain LMD169. FM4-64 membrane dye fluorescence (red), GFP fluorescence (green), and phase contrast images were taken at hourly intervals after induction of sporulation by resuspension. The sporulation stage of individual cells was classified based on the FM4-64 fluorescence and phase contrast images; pre-septated cells (ca. 1 h after induction), asymmetric septation and forespore engulfment (2-3 h after induction), forespore in cell (ca. 4 h after induction), phase-bright or phase-gray spores (4-5 h after induction). The yellow arrow in panel B indicates an asymmetric septum in one cell. In panel C, the red arrow indicates engulfment of forespore, the blue arrow indicates a cell with a phase-gray spore, the white circle indicates a forespore in a cell with GFP fluorescence from both the mother cell and forespore membrane, and the yellow circle indicates a forespore in a cell with GFP fluorescence from only the mother cell membrane. The pattern shown for the forespore in cell stage was for LMD166 found in 86% (N=139) of the cases and for LMD170 found in 90% (N=29) of the cases.

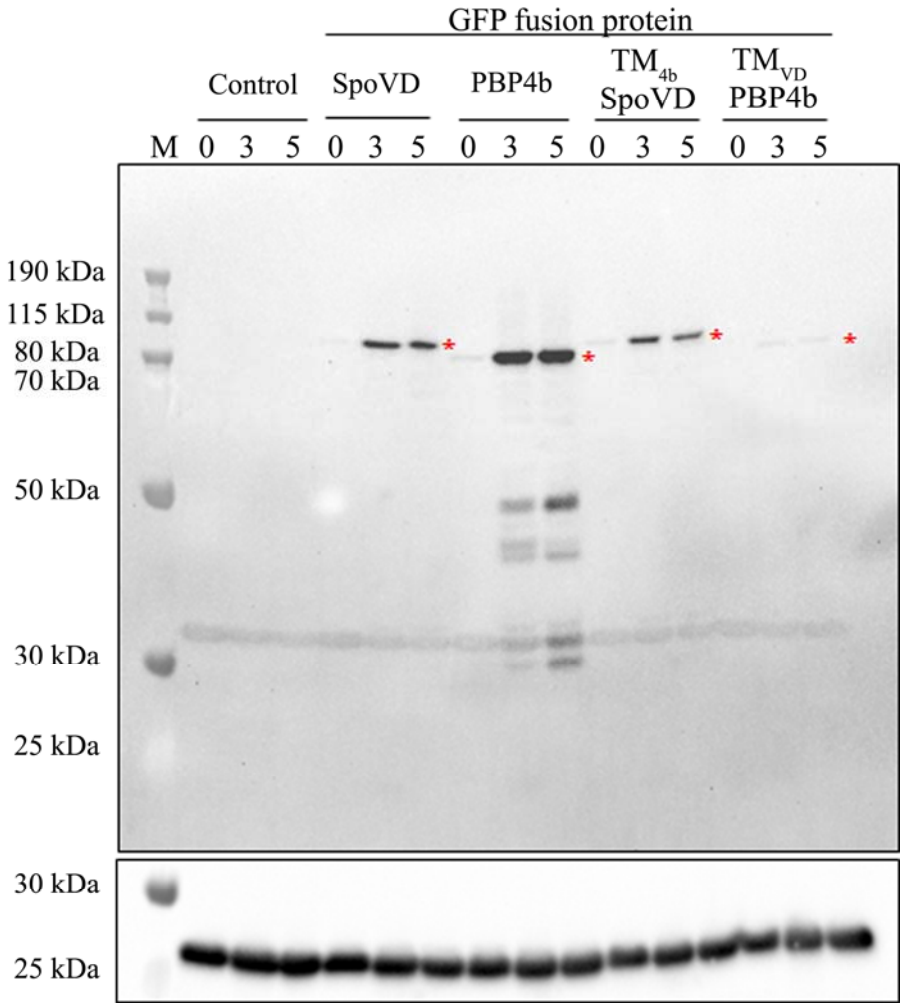
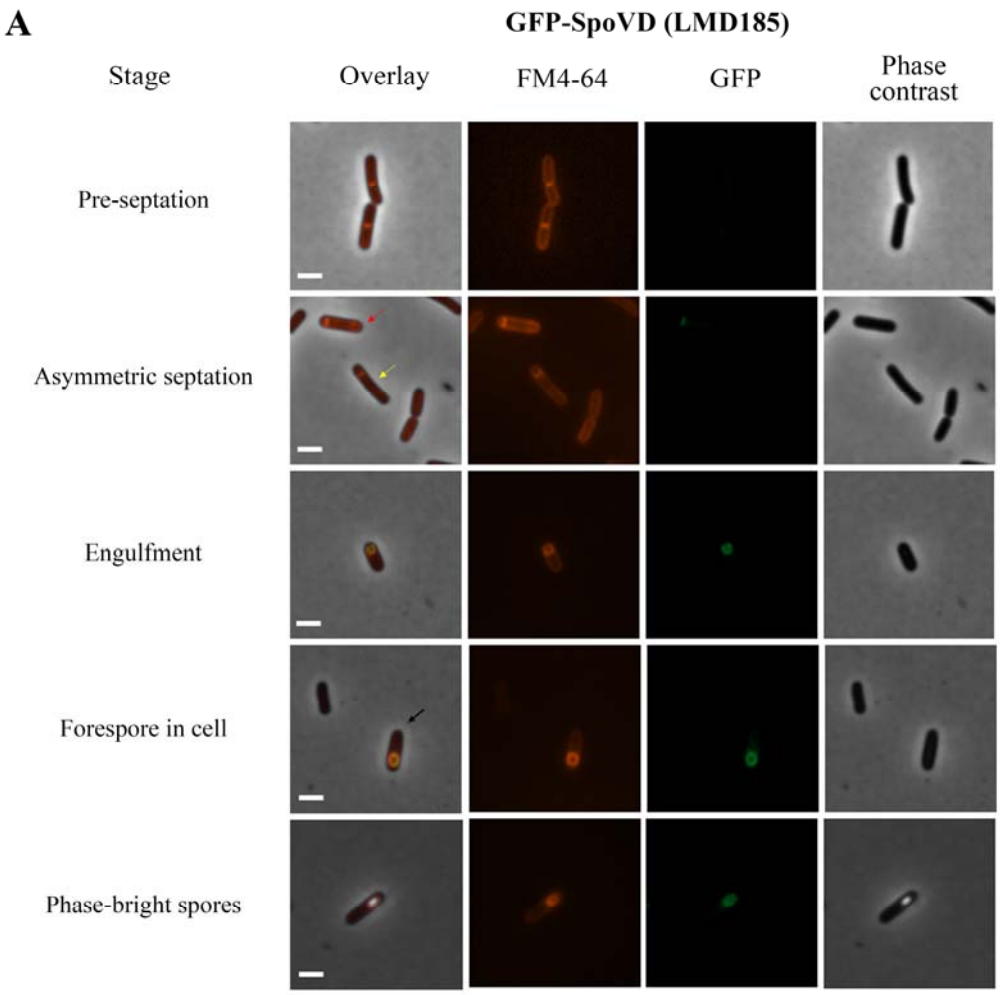



**Figure S4**. Immuno-blot for GFP-SpoVD, GFP-PBP4b, and two GFP-PBP4b/SpoVD chimeric proteins in the membrane fraction of strains grown for sporulation. Samples for analysis were taken at 0, 3, and 5h after induction of sporulation by resuspension. The strains analyzed are deleted for *spoVD* and *pbpI* and contain the gene for a GFP fusion protein expressed under control of the *spoVD* promoter. The strains used in this experiment were LMD162 (control lacking GFP fusion protein), LMD185, LMD186, LMD187, and LMD188. An equal amount of protein (11 μg) was loaded in each lane. The upper panel was probed with anti-GFP serum. GFP fusion protein bands are indicated by an asterisk. The lower panel was probed with anti-BdbD serum. The size of molecular mass markers (lane M) are indicated.


A
GFP-SpoVD (LMD185)
Stage
Overlay
FM4-64
GFP
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Pre-septation
Asymmetric septation
Engulfment
Forespore in cell
Phase-bright spores

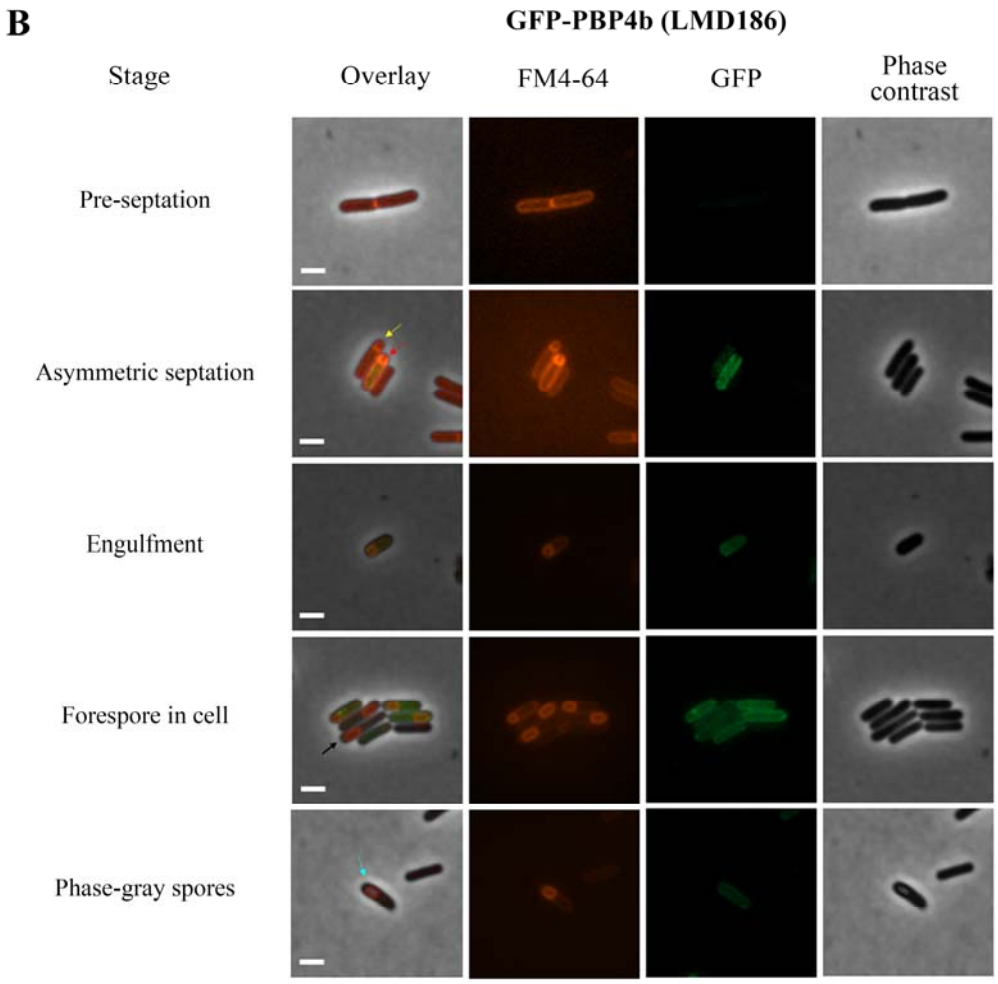



**Figure S5.** Fluorescence and phase contrast microscopy images of sporulating *B. subtilis ΔspoVD ΔpbpI* cells containing a gene, under control of the *spoVD* promoter, encoding a GFP fusion protein. The scale bar indicates 2 µm. Panel A, GFP-SpoVD in strain LMD185. Panel B, GFP-PBP4b in strain LMD186. FM4-64 membrane dye fluorescence (red), GFP fluorescence (green), and phase contrast images were taken at hourly intervals after induction of sporulation by resuspension. The sporulation stage of individual cells was classified based on the FM4-64 fluorescence and phase contrast images. The yellow arrows indicate cells with an asymmetric septum, a red arrow indicates a cell undergoing engulfment of the forespore, a black arrow indicates a cell with engulfed forespore, and a blue arrow indicates a cell with a phase-gray spore. The pattern shown for the indicated cell in the forespore in cell stage was for LMD185 found in 98% (N=259) of the cases and for LMD186 found in 93% (N=443) of the cases.

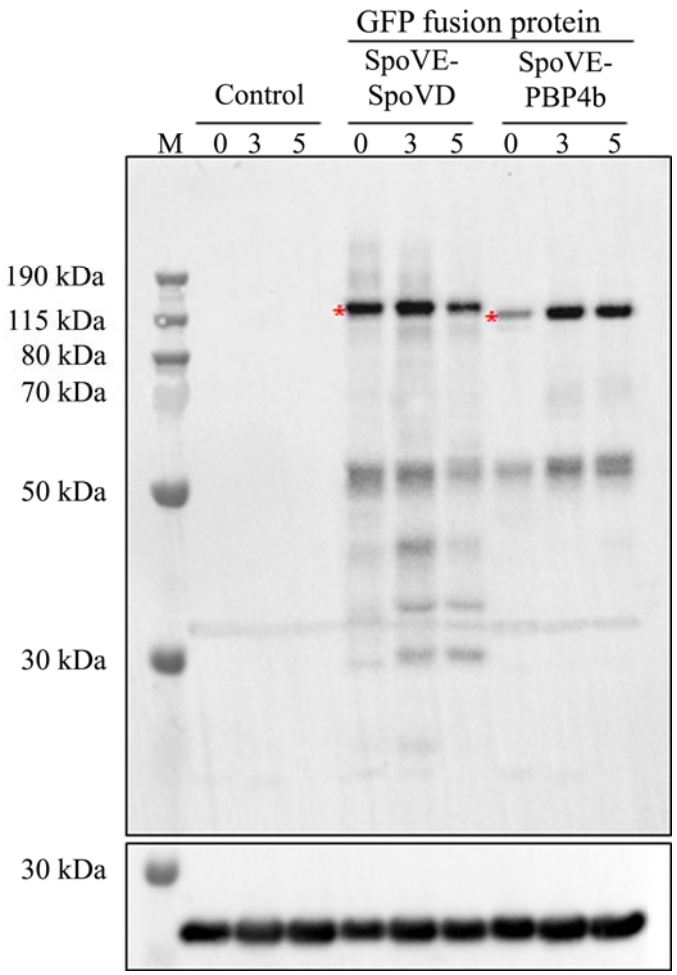
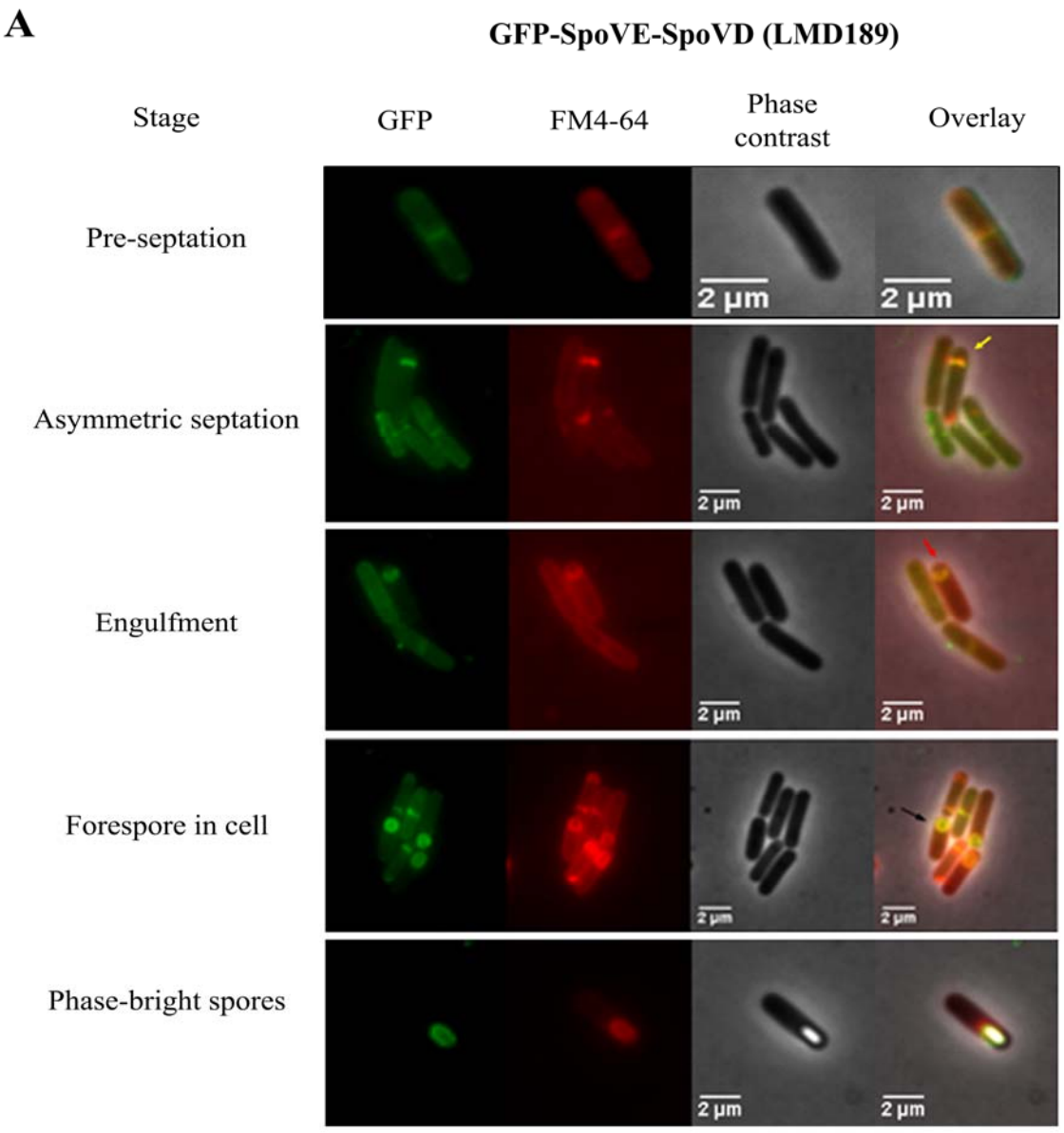



**Figure S6**. Immuno-blot for GFP-SpoVE-SpoVD and GFP-SpoVE-PBP4b fusion protein in membranes isolated from sporulating *B. subtilis* strains. Samples for analysis was taken at 0, 3, and 5 h after induction of sporulation by resuspension. The strains used in the experiment were LMD101 (control lacking GFP fusion protein), LMD189, and LMD190. The strains are deleted for *spoVD* and two of them contain a gene encoding a triple fusion protein expressed under the control of the xylose-inducible promoter. The upper panel was probed with anti-GFP serum. GFP fusion proteins are indicated by an asterisk. The lower panel was probed with anti-BdbD serum. An equal amount of protein (15 µg) was loaded in each lane. The size of molecular mass markers (lane M) are indicated.


A
GFP-SpoVE-SpoVD (LMD189)
Stage
GFP
FM4-64
Phase contrast
Overlay
Pre-septation
Asymmetric septation
Engulfment
Forespore in cell
Phase-bright spores
2 µm

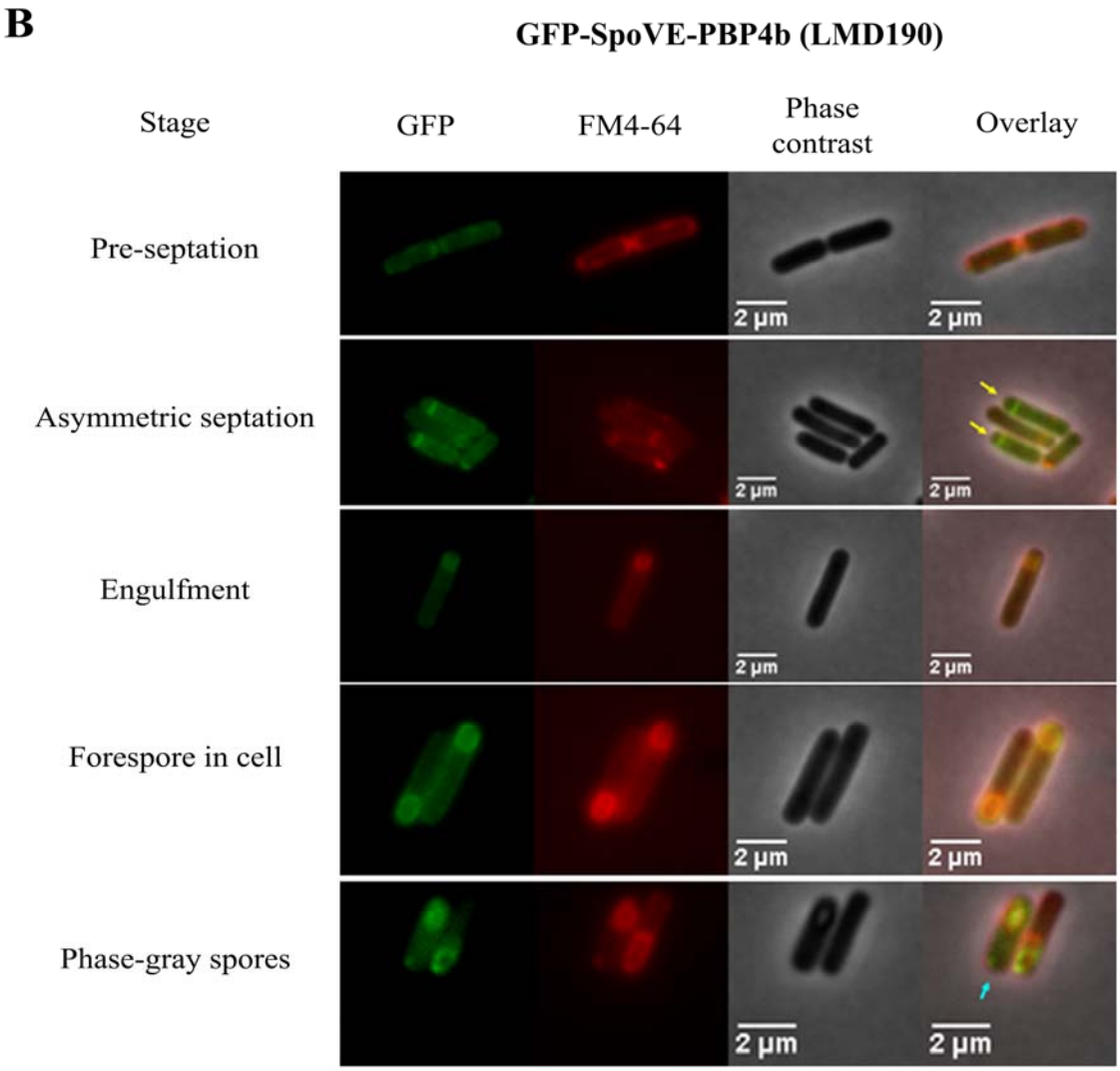



**Figure S7.** Fluorescence and phase contrast microscopy images of sporulating *B. subtilis ΔspoVD* cells containing a gene encoding GFP-SpoVE-SpoVD or GFP-SpoVE-PBP4b expressed under the control of the xylose promoter. The scale bar indicates 2 µm. Panel A, GFP-SpoVE-SpoVD in strain LMD189. Panel B, GFP-SpoVE-PBP4b in strain LMD190. FM4-64 membrane dye fluorescence (red), GFP fluorescence (green), and phase contrast images were taken at hourly intervals after induction of sporulation by resuspension. The sporulation stage of individual cells was classified based on the FM4-64 fluorescence and phase contrast images. The yellow arrows indicate cells with an asymmetric septum, a red arrow indicate a cell undergoing engulfment of the forespore, a black arrow indicate a cell with an engulfed forespore, and the blue arrow indicates a cell with a phase gray spore. The pattern shown for the forespore in cell stage was for LMD189 found in 93% (N=329) of the cases and for LMD190 in 96% (N=491) of the cases.

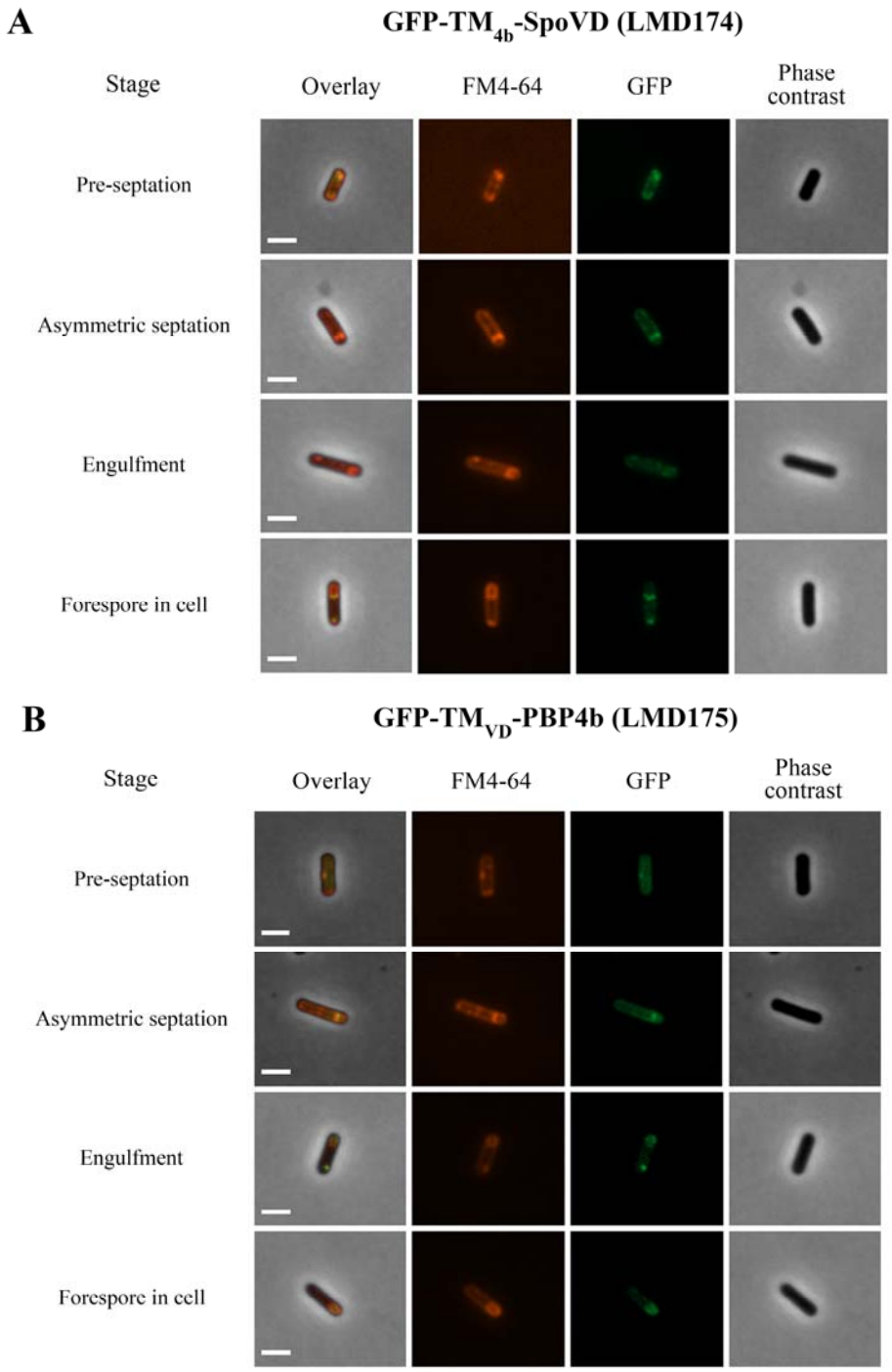



**Figure S8.** Fluorescence and phase contrast microscopy images of sporulating *B. subtilis* cells containing GFP fusion proteins. Panel A, GFP-TM$_{4b}$-SpoVD in strain LMD174. Panel B, GFP-TM$_{VD}$-PBP4b in strain LMD175. The scale bar indicates 2 µm. FM4-64 membrane dye fluorescence (red), GFP fluorescence (green), and phase contrast images were taken at hourly intervals after induction of sporulation by resuspension. The sporulation stage of individual cells was classified based on the FM4-64 fluorescence and phase contrast images. Phase-gray spores (5 h after induction) are not shown because of low GFP signal intensity from the cells. The pattern shown for the forespore in cell stage was for LMD174 found in 100% (N=48) of the cases and for LMD175 found in 80% (N=131) of the cases.

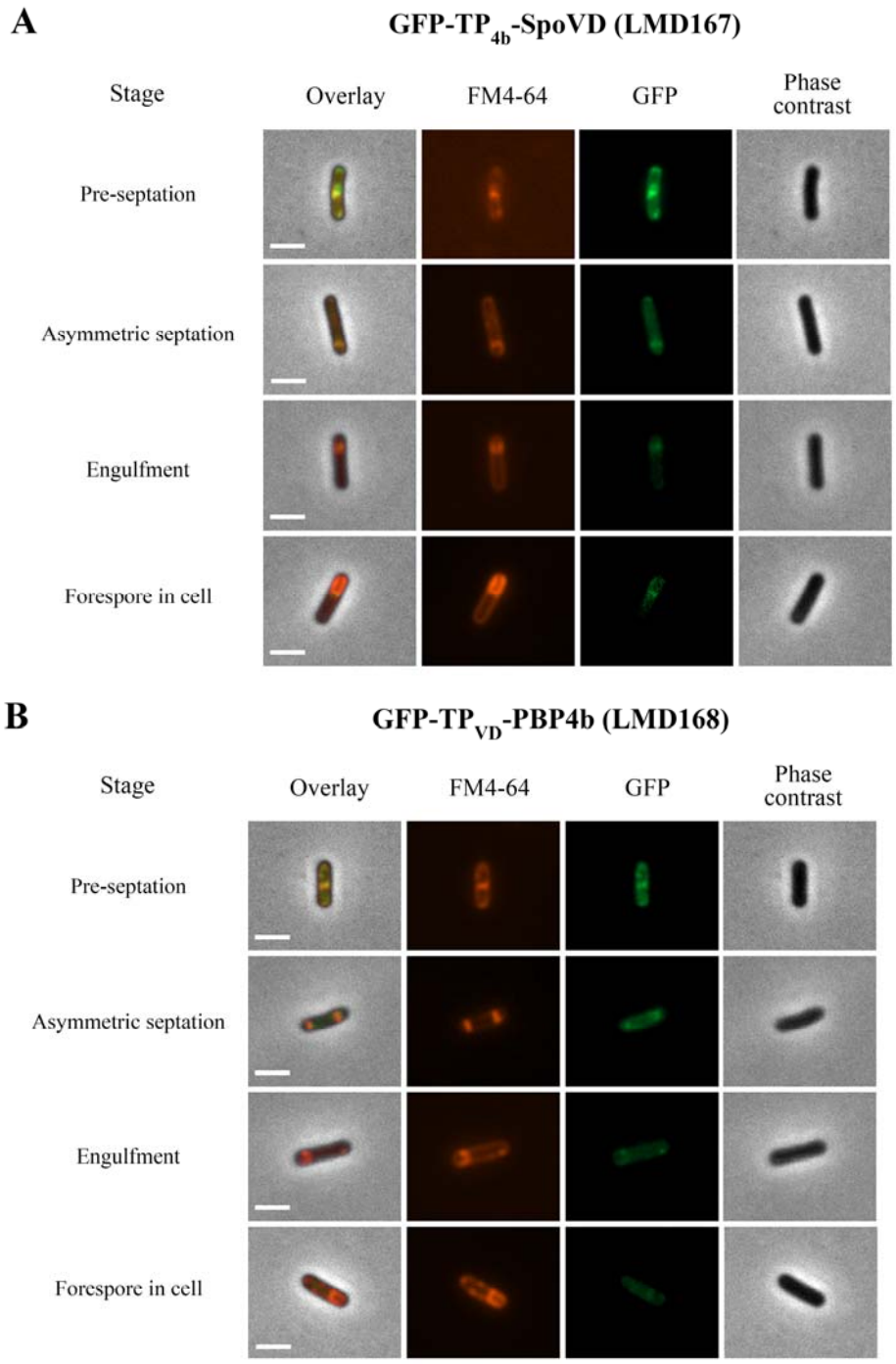



**Figure S9.** Fluorescence and phase contrast microscopy images of sporulating *B. subtilis* cells containing GFP fusion proteins. Panel A, GFP-TP$_{4b}$-SpoVD in strain LMD167. Panel B, GFP-TP$_{VD}$-PBP4b in strain LMD168. The scale bar indicates 2 μm. FM4-64 membrane dye fluorescence (red), GFP fluorescence (green), and phase contrast images were taken at hourly intervals after induction of sporulation by resuspension. Sporulation stage of cells was classified based on the FM4-64 fluorescence and phase contrast images.The pattern shown for the forespore in cell stage was for LMD167 found in 95% (N=41) of the cases and for LMD168 found in 100% (N=23) of the cases.