Cell wall synthesis is necessary for membrane dynamics during sporulation of *B. subtilis*

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Supplemental Data

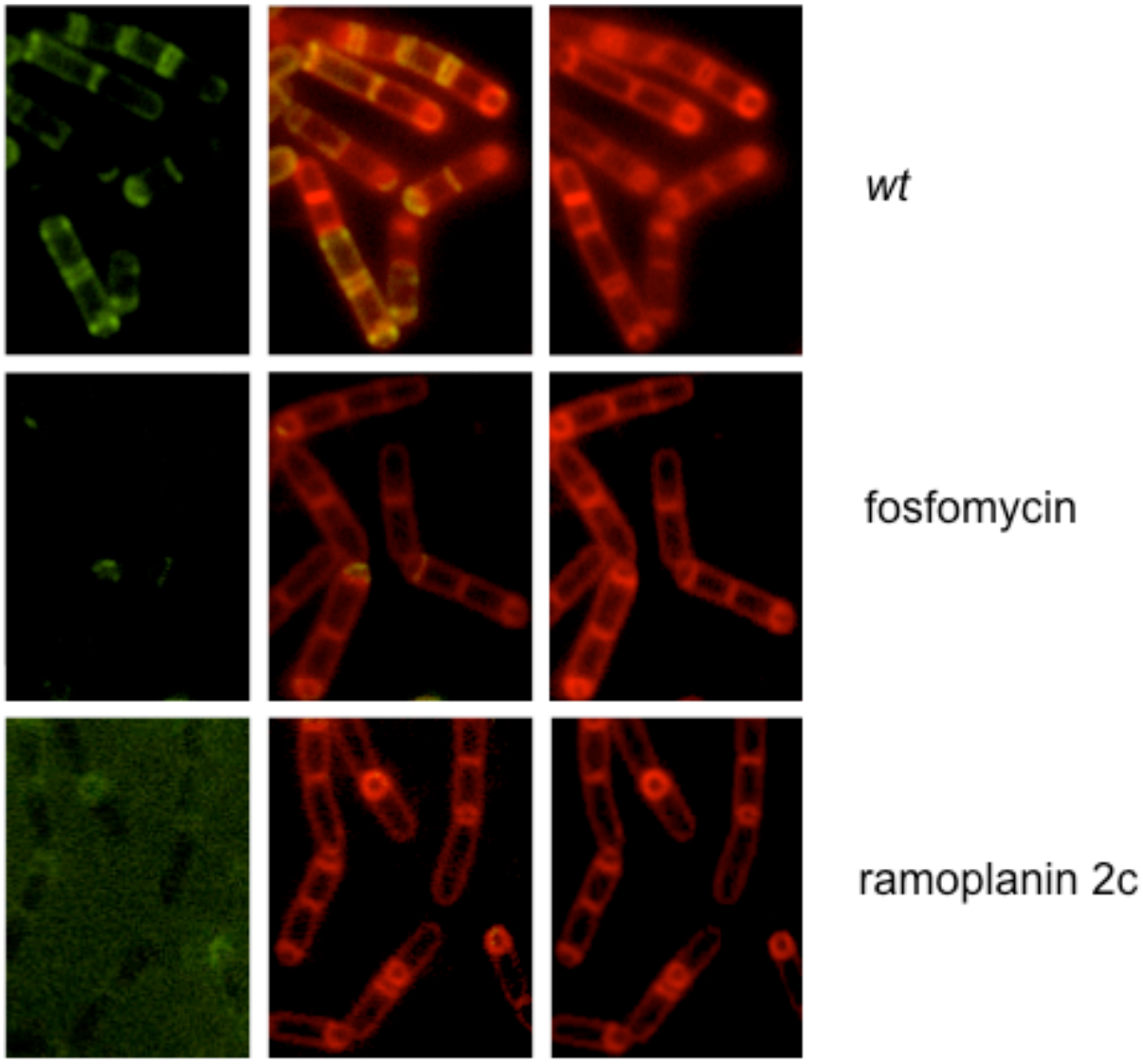

Figure S1

Figure S1. Ramoplanin labels specifically engulfing forespores. Ramoplanin-FL (left), FM4-64 (right) labeling of cells at T3.5 of sporulation. Middle panel is the overlay of both channels. Top, PY79 wild type cells; middle, 5mM fosfomycin was added at T1 to PY79 cells; bottom, wild type *B. subtilis* cells (PY79) were incubated with ramoplanin-2c (1µg/ml) and FM4-64 (1µg/ml) at T3.5 of sporulation.

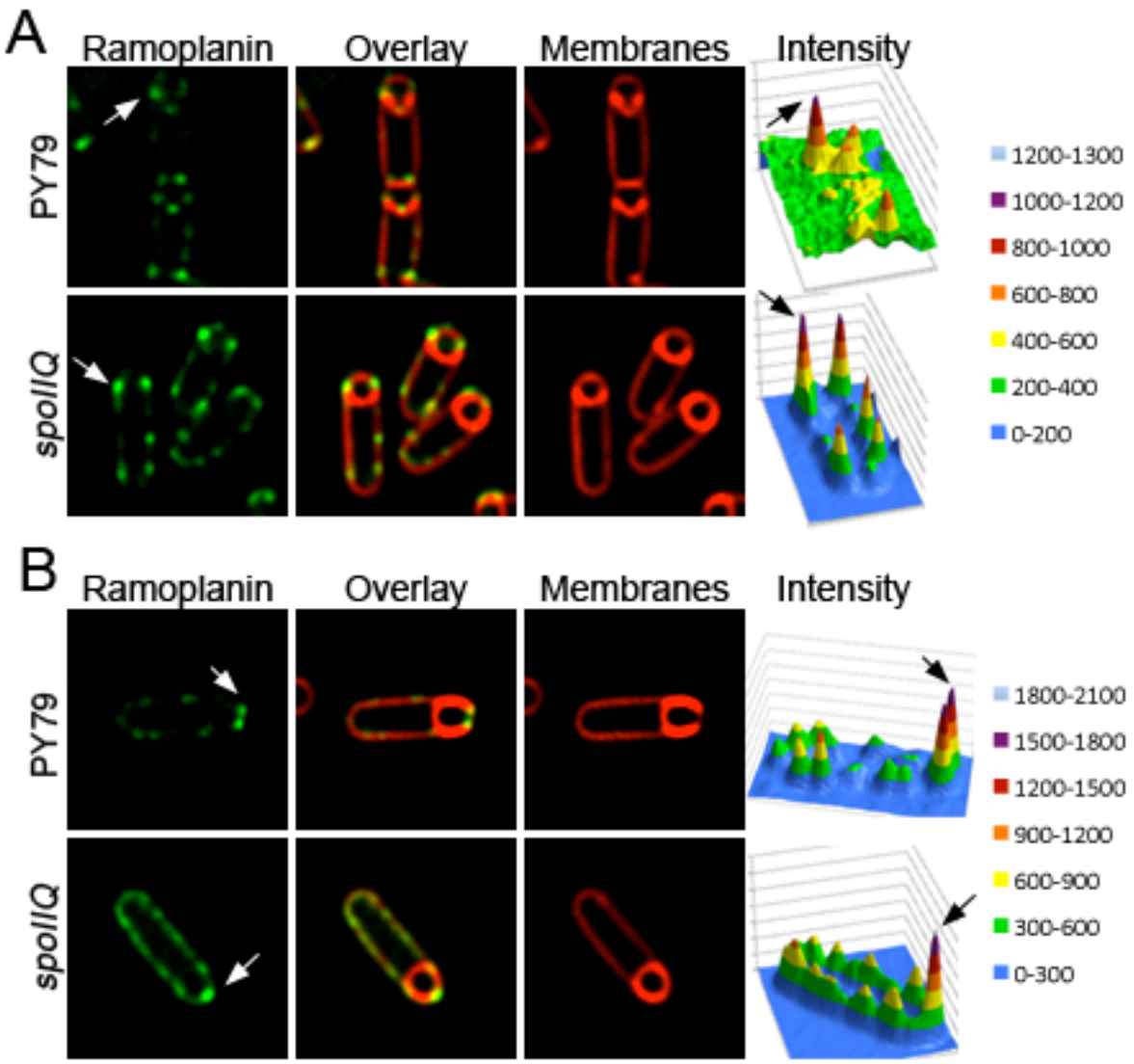

Figure S2

Figure S2. Ramoplanin labeling is not affected by a *spoIIQ* mutation Ramoplanin labelling is the same in wild type and *spoIIQ* mutant strain (KP575). Exposure times for all images were kept constant. Intensity plots were generated in Microsoft Excel using unaltered pixel intensity data and show the Ramoplanin-FL signal in a single cell for each panel. Arrows indicate alignment of the fluorescent image and the intensity plot. **A.** Images taken at T2.5, cells in early stages of migration. **B.** Images taken at T2.5 cells in late stages of migration.

Figure S3 Movies. Time-lapse movies from cells in Fig.3. **A**. Wild type (PY79). **B.** Wild type + 5mM fosfomycin added at T1.5 after initiation of sporulation. **C.** *spoIIQ (*KP575). **D.** *spoIIQ* + 5mM fosfomycin added at T1.5. **E.** *spoIIQ* strain expressing *malF*$_{12}$*-gfp* under P_{spoIIQ} (JFG488) + 5mM fosfomycin added at T1.5. Images show the expression of MalF$_{12}$-GFP superposed on FM4-64 staining. 12 out of 64 cells expressing MalF$_{12}$-GFP migrated, 37 out of 64 cells had a septa but no GFP.

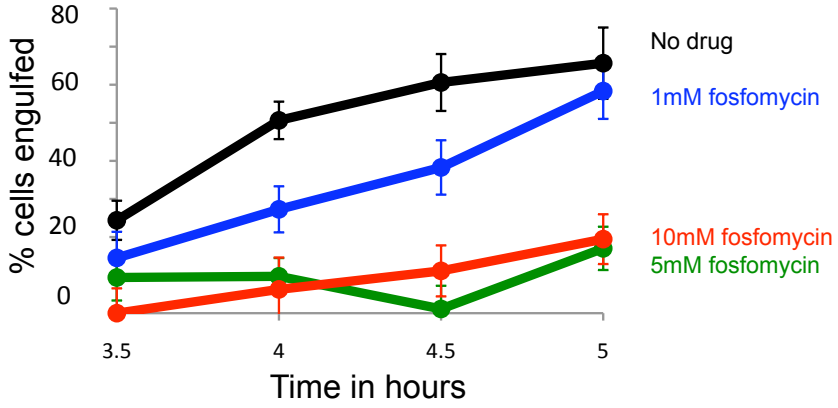

Figure S4

Figure S4. Fosfomycin dose response inhibition of engulfment. Fosfomycin was added at T2 in sporulating cells expressing CFP as a forespore marker (AES574). The fraction of cells showing a CFP signal and not surrounded by an FM4-64 signal was determined. Black, no drug; red, 10mM fosfomycin; green, 5mM; and blue, 1mM.

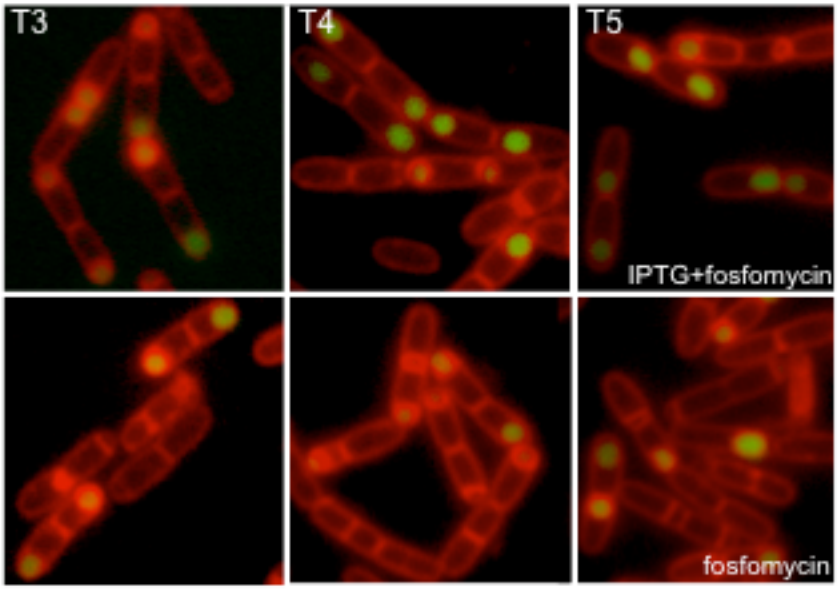

Figure S5

Figure S5. A strain carrying the fosfomycin resistant *murAA* mutant engulfs normally in the presence of fosfomycin. A merodiploid strain expressing inducible *C117D murAA* under IPTG control and *yfp* under (P_{spoIIQ}) control (JDB2426) was sporulated by resuspension. Top, both 1mM IPTG was added at T0 and 5mM fosfomycin at T2; bottom, 5mM fosfomycin was added at T2. Time after start of sporulation is indicated.

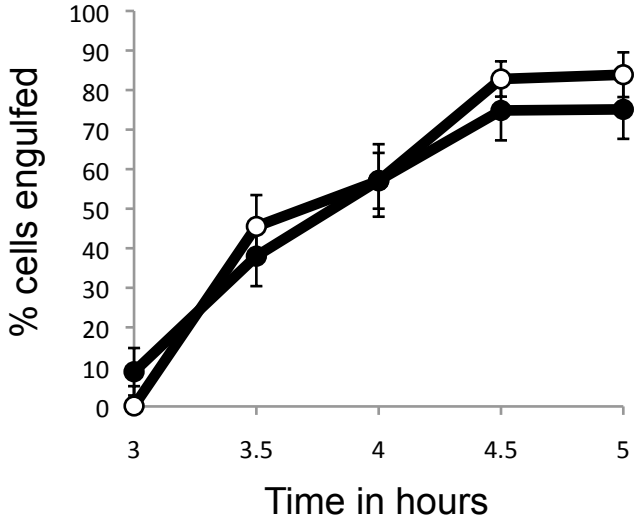

Figure S6

Figure S6. Cells expressing MurAA-C117D engulf normally

Engulfment of a merodiploid strain expressing inducible *C117D murAA* allele under P_{spank} control and *yfp* under (P_{spoIIQ}) control (JDB2426) was sporulated by resuspension and 1mM IPTG was added at T0 (open circles) was compared to a (AES574) strain (closed circles) expressing CFP under control of a forespore specific promoter (P_{spoIIQ}).

Figure S7. Time-lapse movie of bulge formation from cell shown in Fig.6A

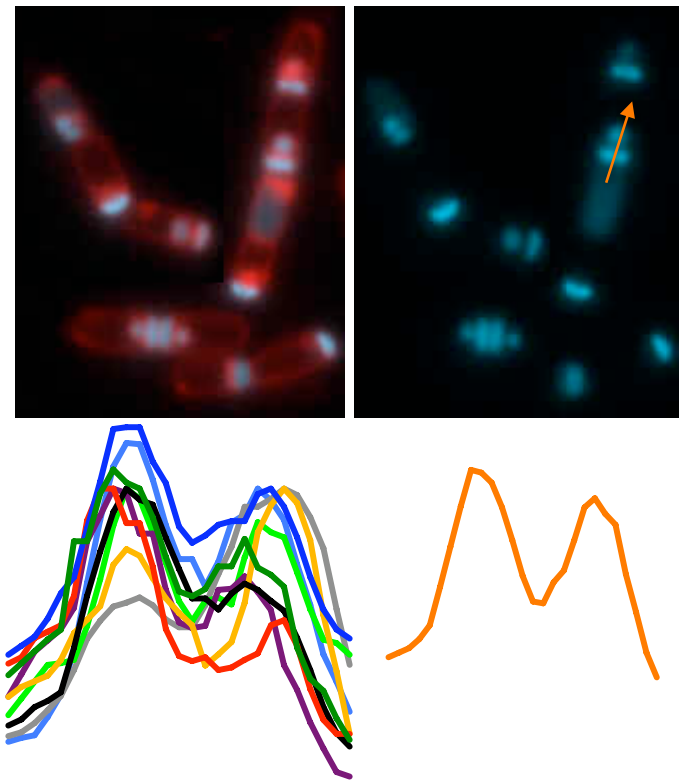

Figure S8

Figure S8. CFP fluorescence distribution in vesicles. A strain (JDB2494) lacking *spoIID* and expressing CFP (blue) under control of a forespore specific promoter (P_{spoIIQ}) as a forespore marker was imaged at T4 of sporulation. Left image is the overlay of CFP with FM4-64 dye in red, right image is CFP only. Bottom left, each trace represents the CFP fluorescence (arbitrary units) across the cell length in nine separate cells. Bottom right, orange trace shows CFP fluorescence (arbitrary units) as a function of distance along the length of the cell indicated by orange arrow.

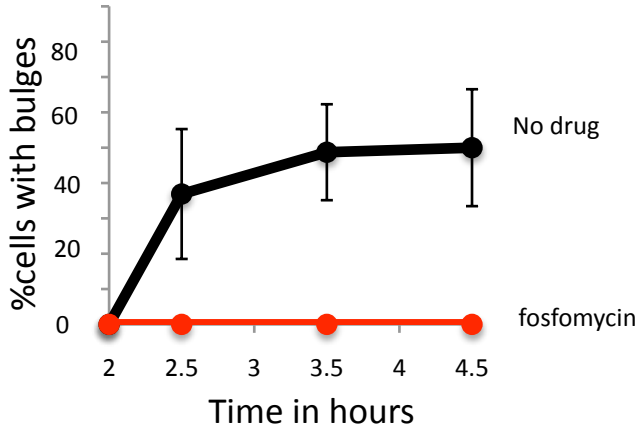

Figure S9

Figure S9. Fosfomycin blocks bulge formation in *spoIIP* cells. Bulges do not form in a strain (JDB2396) lacking *spoIIP* when 5mM fosfomycin was added at T2 of sporulation (red line) compared to cells where no fosfomycin was added (black line).

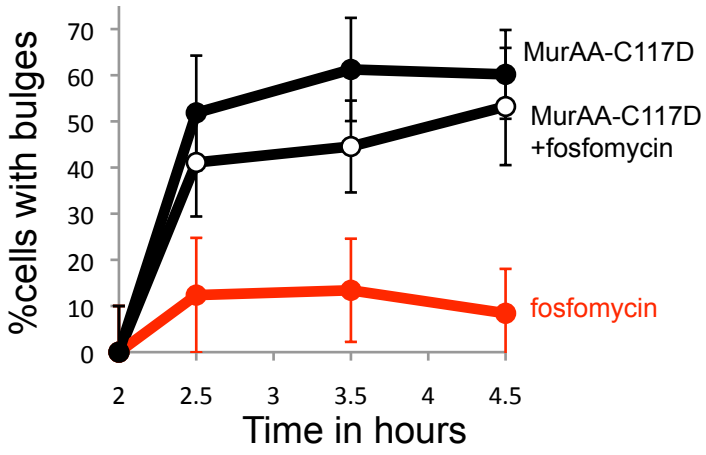

Figure S10

Figure S10. Fosfomycin does not block bulge formation in *spoIID* cells expressing MurAA-C117A. Percentage of bulges in a *spoIID* strain (JDB2535) expressing YFP under control of a forespore-specific promoter and carrying an MurAA-C117A allele under control of P_{spank} in the absence (black circles) or presence of 5 mM fosfomycin (red circles) or in the presence of 1 mM IPTG added at T0 and 5 mM fosfomycin (open circles).

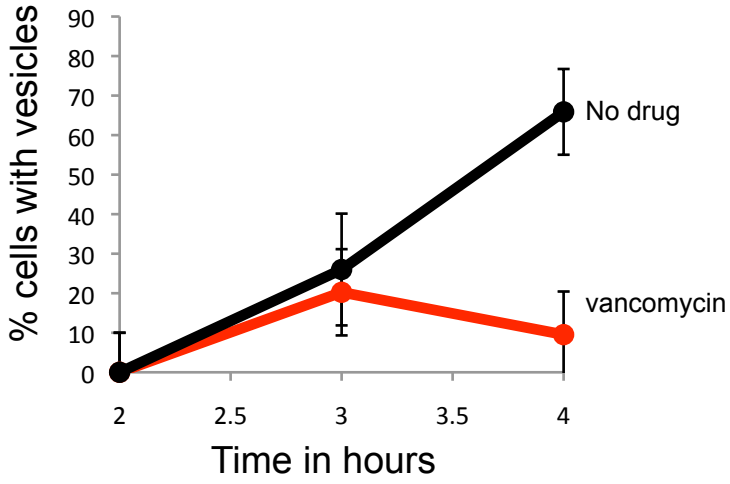

Figure S11

Figure S11. Vancomycin blocks vesicle formation. The percentage of cells containing vesicles of a strain (JDB2494) lacking *spoIID* and expressing CFP as a forespore marker in the absence (black) or presence of vancomycin (0.5 μg/ml) that was added at T2 (red). Vesicles were as defined in Fig. 1B.

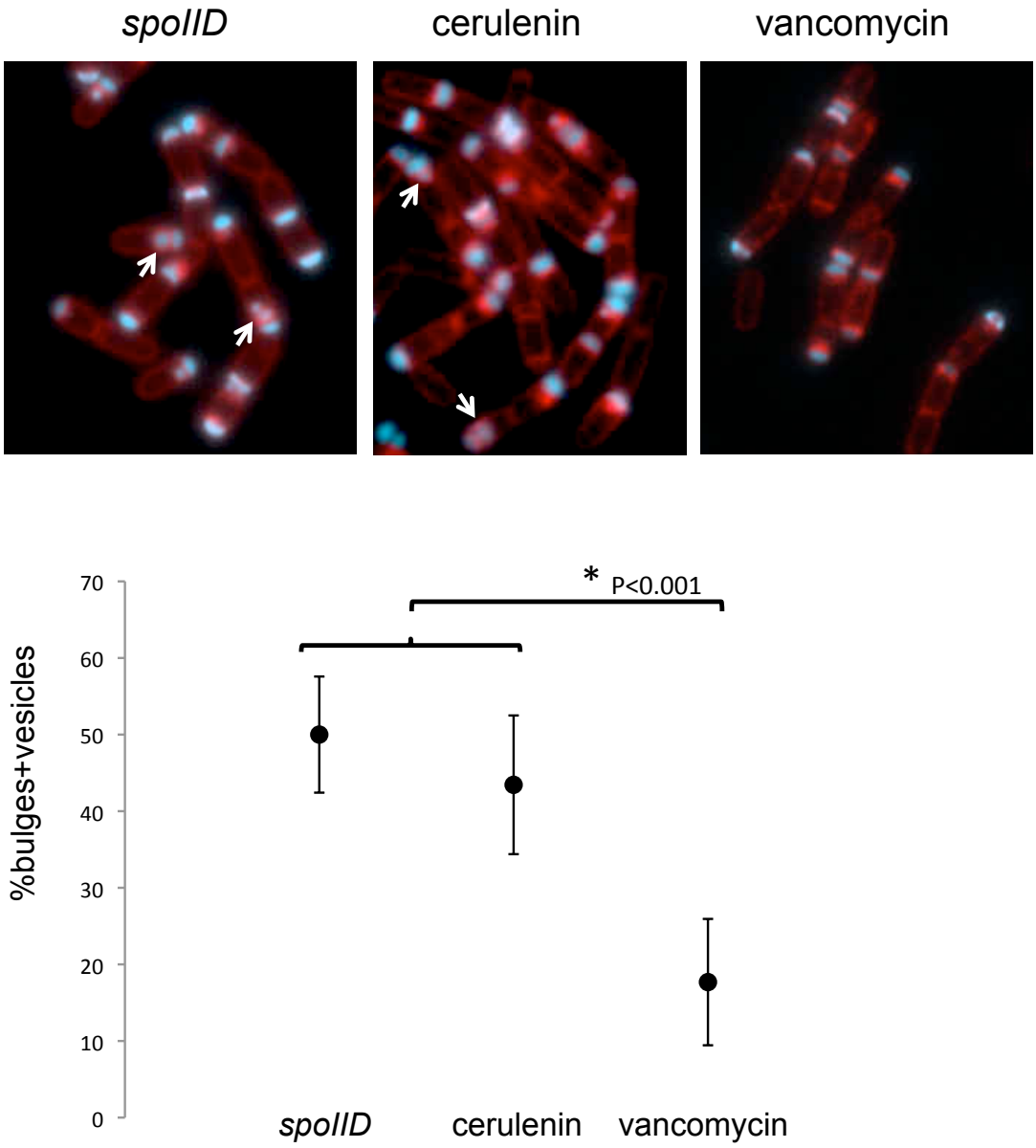

Figure S12

Figure S12. Inhibition of lipid synthesis does not block bulge formation. Images (top) and quantification (bottom) indicate that addition of 10μg/ml cerulenin at T3.5 to a strain (JDB2494) lacking *spoIID* and expressing CFP as a forespore marker does not affect bulge and vesicle formation compared to cells where no drug was added; parallel addition of 0.5μg/ml vancomycin to the same strain at T3.5 blocks bulge and vesicle

formation. All observations were made at T4.5 after resuspension. White arrows indicate cells with bulges. Asterisk indicates that for a Pearson's X^2 test with one degree of freedom, the independence hypothesis was true for the number of bulges in vancomycin treated as compared to untreated cells (long bracket, $P<0.001$), but was rejected for cerulenin treated as compared to untreated cells (short bracket, $P>0.1$).

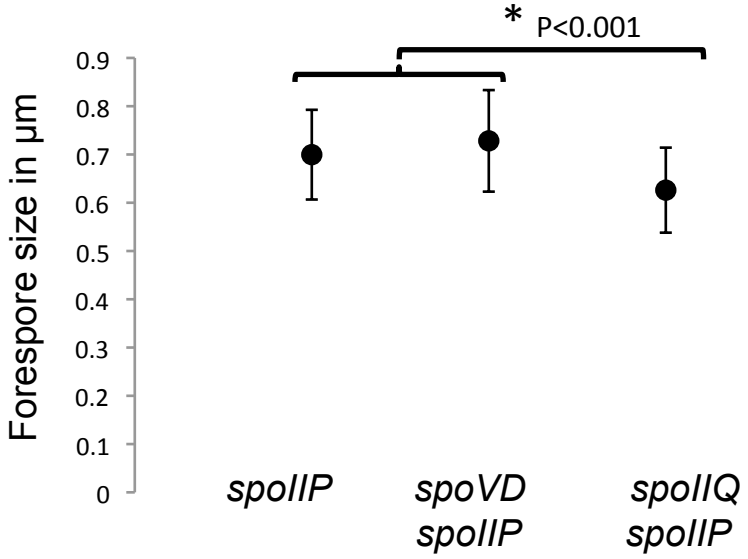

Figure S13

Figure S13. Forespore size measurements. Forespore size at T3 of sporulation was determined by measuring the length of a straight line traced between the forespore tip and the septum. The forespore size in *spoIIP* (KP575) mutant cells (0.69±.09µm) was not different than *spoIIPspoVD* (JDB2537) mutant cells (0.73±.1µm), but was statistically significantly larger than *spoIIPspoIIQ* (JDB2612) mutant cells (0.62±.09µm) by student t-test.

Table S1. Bacterial strains.

Strain	*Genotype*	Source
B. subtilis		
PY79	*wt*	Laboratory stock
JGF488	*spoIIQ::spec amyE::P_{spoIIQ}-malF12-gfp*	this work
KP575	*spoIIQ::spec*	(Sun et al., 2000)
KP1102	*amyE::spoIID(D210A) kan; spoIID298*	Gutierrez *et al. submitted*
PE177	*spoIID::cm spoIIP::tet spoIIM::erm*	(Eichenberger et al., 2001)
AES240	*sacA::P_{spoIIQ}-yfp cm*	Elowitz lab
AES574	*amyE:P_{spoIIQ}-cfp spec*	Elowitz lab
JDB1213	*spoVD::kan*	(Daniel et al., 1994)
JDB1448	*gltA:P_{spoVE}-yfp-spoVD cm*	(Fay et al., 2009)
JDB2351	*amyE:P_{spank} murAA(C117D) spec*	this work
JDB2395	*spoIID::cm*	this work
JDB2396	*spoIIP::tet*	this work
JDB2426	*amyE:P_{spank}-murAA(C117D) spec sacA:P_{spoIIQ}-yfp cm*	this work
JDB2494	*amyE::P_{spoIIQ}-cfp spec spoIID::cm*	this work
JDB2513	*amyE::P_{spank}-murAA(C117D) spec spoIID::cm*	this work
JDB2535	*amyE::P_{spank}-murAA(C117D) spec spoIID::cm sacA:P_{spoIIQ}-yfp kan*	this work
JDB2537	*spoVD::kan spoIIP::tet*	this work
JDB2546	*sacA::P_{spoIIQ}-yfp kan*	this work
JDB2553	*gltA::P_{spoVE}-yfp-spoVD cm spoIIP::tet*	this work
JDB2612	*spoIIQ::spec spoIIP::tet*	this work
E. coli	*Plasmid*	
BL21		Laboratory stock
	pAF54	this work
	pDG1662	(Guerout-Fleury et al., 1996)
	pMR15	this work
	pKL147	(Lemon and Grossman, 1998)
	pSac-Kan	(Middleton and Hofmeister, 2004)

Plasmid Construction

pMR15. *murAA* was subjected to site-directed mutagenesis with primers designed to substitute cysteine at position 117 with an aspartic acid. Two PCR products obtained

from PY79 gDNA using the 5' *murAA* primer and (C117D) reverse primer, as well as from C117D forward primer and 3' *murAA* primer were gel-purified and used as templates for PCR-SOEing using 5'*murAA* and 3' *murAA* primers. The product was digested with *NheI* and *SalI* and ligated to pDR111 digested with *NheI* and *SalI*.

pAF54. A PCR product obtained from a PY79 gDNA template containing 447bp upstream of *spoIIQ* was digested with *EcorI* and *HindIII* and ligated to plasmid pSac-Kan digested with *EcoRI* and *HindIII* (pSac-Kan-*PspoIIQ*). Then a PCR product from the plasmid pKL147 containing YFP and an optimal RBS was digested with the restriction enzymes *BamHI* and *HindIII* and ligated to pSac-Kan-*PspoIIQ* digested with the restriction enzymes *BamHI* and *HindIII*.

Strain Construction

JDB2351 pMR15 was transformed into PY79, selecting for spec^R, and screening for amy-

JDB2395 (*spoIID::cm*) transform gDNA from PE177 into PY79 select for cm^R

JDB2396 (*spoIIP::tet*) transform gDNA from PE177 into PY79 select for tet^R

JDB2426 (*amyE:Pspank murAA(C117D) spec sacA:PspoIIQ-yfp cm*) transform DNA from AES240 into JDB2351 select for cm^R, screen for spec^R ,amy-, sac-.

JDB2494 (*amyE:PspoIIQ-cfp spec, spoIID::cm*) transform DNA from PE177 in AES574 select for cm^R, screen for amy- , spec^R.

JDB2513 (*amyE:Pspan-murAA(C117D) spec, spoIID::cm*) transform DNA from PE177 in JDB2351 select for cm^R, screen for amy- , spec^R.

JDB2535 (*amyE:Pspank murAA(C117D) spec spoIID::cm sacA:PspoIIQ-yfp kan)* tansform DNA from JDB2546 into JDB2513 select for kan^R screen for amy-,sac-, cm^R and spec^R.

JDB2537 (*spoVD:kan spoIIP::tet*) transform DNA from JDB1213 into JDB2396 select for kan^R, screen for tet^R.

JDB2546 *(sacA:P_{spoIIQ}-yfp kan)* transform pAF54 into PY79 select for kan^R, screen for sac-.

JDB2553 *(gltA:P_{spoVE}-spoVD-yfp cm spoIIP:tet)* transform DNA from JDB1448 into JDB2396 select for cm, screen for tet^R, glt-

JDB2612 *(spoIIQ::spec spoIIP::tet)* transform DNA from KP575 into JDB2396 select for spec, screen for tet^R.

References

Daniel, R.A., Drake, S., Buchanan, C.E., Scholle, R., and Errington, J. (1994). The Bacillus subtilis spoVD gene encodes a mother-cell-specific penicillin-binding protein required for spore morphogenesis. J Mol Biol *235*, 209-220.

Eichenberger, P., Fawcett, P., and Losick, R. (2001). A three-protein inhibitor of polar septation during sporulation in Bacillus subtilis. Mol Microbiol *42*, 1147-1162.

Fay, A., Meyer, P., and Dworkin, J. (2009). Interactions between late acting proteins required for peptidoglycan synthesis during sporulation. submitted.

Guerout-Fleury, A.M., Frandsen, N., and Stragier, P. (1996). Plasmids for ectopic integration in Bacillus subtilis. Gene *180*, 57-61.

Gutierrez, J., R. Smith and Pogliano, K. (2010) SpoIID peptidoglycan hydrolase activity is required throughout engulfment during *Bacillus subtilis* sporulation, submitted.

Lemon, K.P., and Grossman, A.D. (1998). Localization of bacterial DNA polymerase: evidence for a factory model of replication. Science *282*, 1516-1519.

Middleton, R., and Hofmeister, A. (2004). New shuttle vectors for ectopic insertion of genes into Bacillus subtilis. Plasmid *51*, 238-245.

Sun, Y.L., Sharp, M.D., and Pogliano, K. (2000). A dispensable role for forespore-specific gene expression in engulfment of the forespore during sporulation of Bacillus subtilis. J Bacteriol *182*, 2919-2927.