Supplement to: Expansion of the spore surface polysaccharide layer in *Bacillus subtilis* by deletion of genes encoding glycosyltransferases and glucose modification enzymes

Running title: Properties of *B. subtilis* spore surface polysaccharides

Bentley Shuster[1*], Mark Khemmani[2*], Yusei Nakaya[3*], Gudrun Holland[4], Keito Iwamoto[3], Kimihiro Abe[5], Daisuke Imamura[3], Nina Maryn[1], Adam Driks[2†], Tsutomu Sato[3,5] and Patrick Eichenberger[1#]

[1]Center for Genomics and Systems Biology, Department of Biology, New York University, New York, NY10003, USA

[2] Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, IL 60153, USA

[3] Department of Frontier Bioscience, Hosei University, Koganei, Tokyo, Japan

[4]Advanced Light and Electron Microscopy (ZBS 4), Robert Koch Institute, D-13353, Berlin, Germany

[5] Research Center for Micro-Nano Technology, Hosei University, Koganei, Tokyo, Japan

*equal contribution

†Deceased, June 7th, 2019

#Corresponding author: pe19@nyu.edu

Figure S1 – Putative enzymatic pathways involved in spore PS production. Left: The experimentally characterized pathway from glucose-1-phosphate to dTDP-rhamnose catalyzed by enzymes encoded by *spsIJKL*. **Center:** Putative pathway with enzymes encoded by *spsCDEF*. **Right:** Putative enzymatic pathways encoded by gene clusters *yfnH-D* and *ytdA-ytcABC*. All enzymes are color-coded based on their inferred or experimentally validated functions according to key on the right. All acronyms are explained in key on the left.

Figure S1

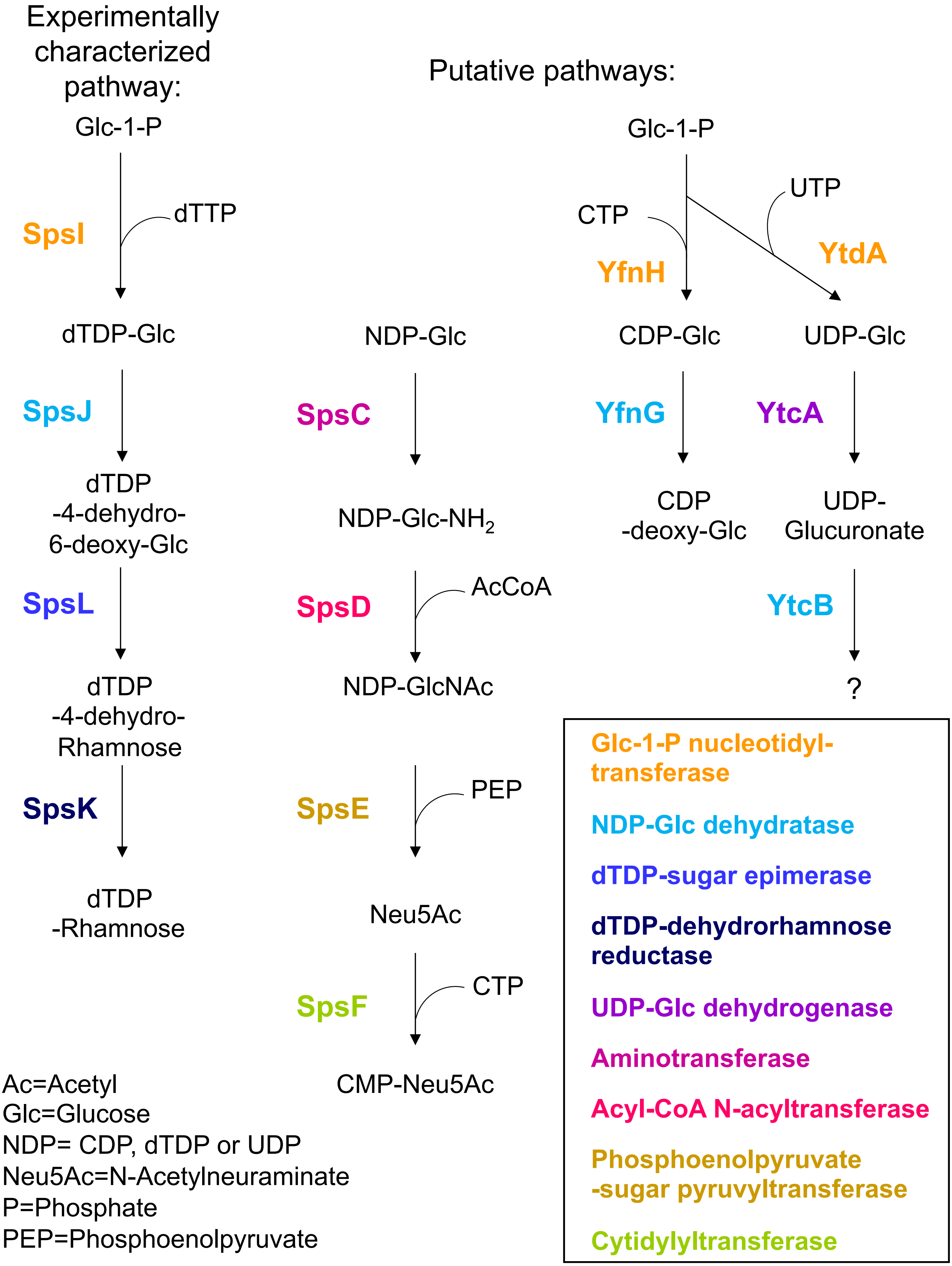

Experimentally characterized pathway:
Glc-1-P
SpsI
dTTP
dTDP-Glc
SpsJ
dTDP -4-dehydro- 6-deoxy-Glc
SpsL
dTDP -4-dehydro- Rhamnose
SpsK
dTDP -Rhamnose
Putative pathways:
NDP-Glc
SpsC
NDP-Glc-NH$_2$
SpsD
AcCoA
NDP-GlcNAc
SpsE
PEP
Neu5Ac
SpsF
CTP
CMP-Neu5Ac
Glc-1-P
CTP
UTP
YfnH
YtdA
CDP-Glc
UDP-Glc
YfnG
YtcA
CDP -deoxy-Glc
UDP- Glucuronate
YtcB
?
Glc-1-P nucleotidyl-transferase
NDP-Glc dehydratase
dTDP-sugar epimerase
dTDP-dehydrorhamnose reductase
UDP-Glc dehydrogenase
Aminotransferase
Acyl-CoA N-acyltransferase
Phosphoenolpyruvate -sugar pyruvyltransferase
Cytidylyltransferase
Ac=Acetyl
Glc=Glucose
NDP= CDP, dTDP or UDP
Neu5Ac=N-Acetylneuraminate
P=Phosphate
PEP=Phosphoenolpyruvate

Figure S2. Contributions of the *spsA-L* operon to spore surface properties. A. BATH assay of spores with the following gene deletions: Δ*spsA* (PE3341), Δ*spsB* (PE3342), Δ*spsC* (PE3343), Δ*spsD* (PE3344), Δ*spsE* (PE3345), Δ*spsF* (PE3346), Δ*spsG* (PE3347). Experiments were performed in triplicate; error bars represent standard deviation. Each individual deletion causes an increase in relative hydrophobicity profile as compared to wild type spores. **B.** India Ink staining reveals the presence of a PS layer (halo) by negative staining in wild type spores (168). The *spsA-L* mutant spores exhibit no halo, suggesting disruption of the PS layer: Δ*spsA* (BKE37910), Δ*spsB* (BKE37900), Δ*spsC* (BKE37890), Δ*spsD* (BKE37880), Δ*spsE* (BKE37870), Δ*spsF* (BKE37860), Δ*spsG* (BKE37850). Scale bars are 2.5 μm. Similar results are obtained with deletions downstream of *spsI* (Figure 2) or by deletion of the entire *sps* operon: Δ*spsA-L* (NY212). **C.** Analysis of the localization during sporulation of the crust proteins CotX-GFP, CotY-GFP, CotZ-GFP and CgeA-GFP in wild type (PE3151, PE3158, HS176, PE3260), Δ*spsI* (PE3245, PE3242, PE3248, PE3323) and Δ*yfnH* (PE3244, PE3243, PE3247, PE3322) strains. Localization is identical in the mutant and wild type strains, suggesting that a deletion of *spsI* or *yfnH* does not interfere with crust protein assembly but alters PS deposition.

Figure S2

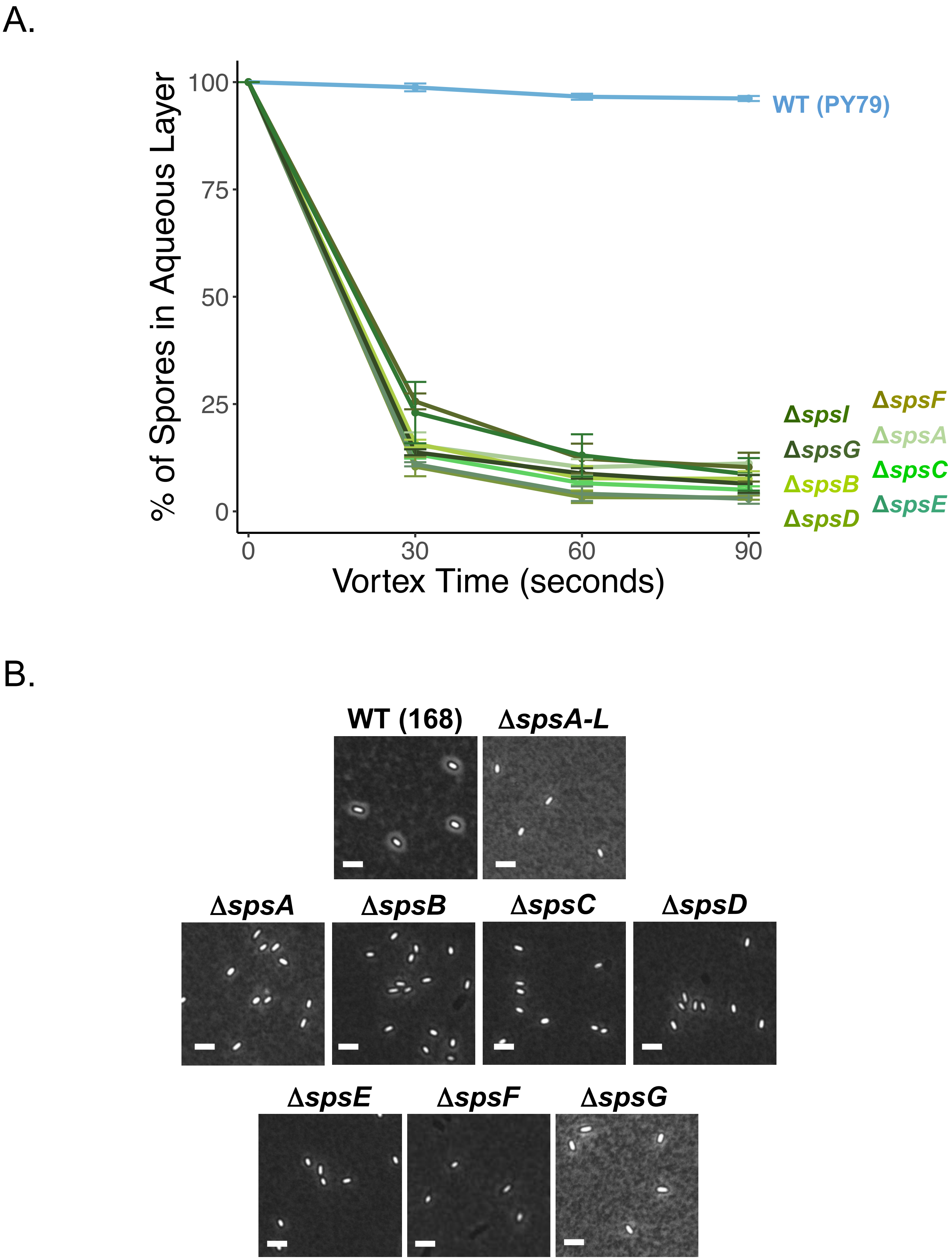

A.
% of Spores in Aqueous Layer
100
75
50
25
0
0
30
60
90
Vortex Time (seconds)
WT (PY79)
ΔspsI
ΔspsG
ΔspsB
ΔspsD
ΔspsF
ΔspsA
ΔspsC
ΔspsE
B.
WT (168)
ΔspsA-L
ΔspsA
ΔspsB
ΔspsC
ΔspsD
ΔspsE
ΔspsF
ΔspsG

Figure S2
(continued)

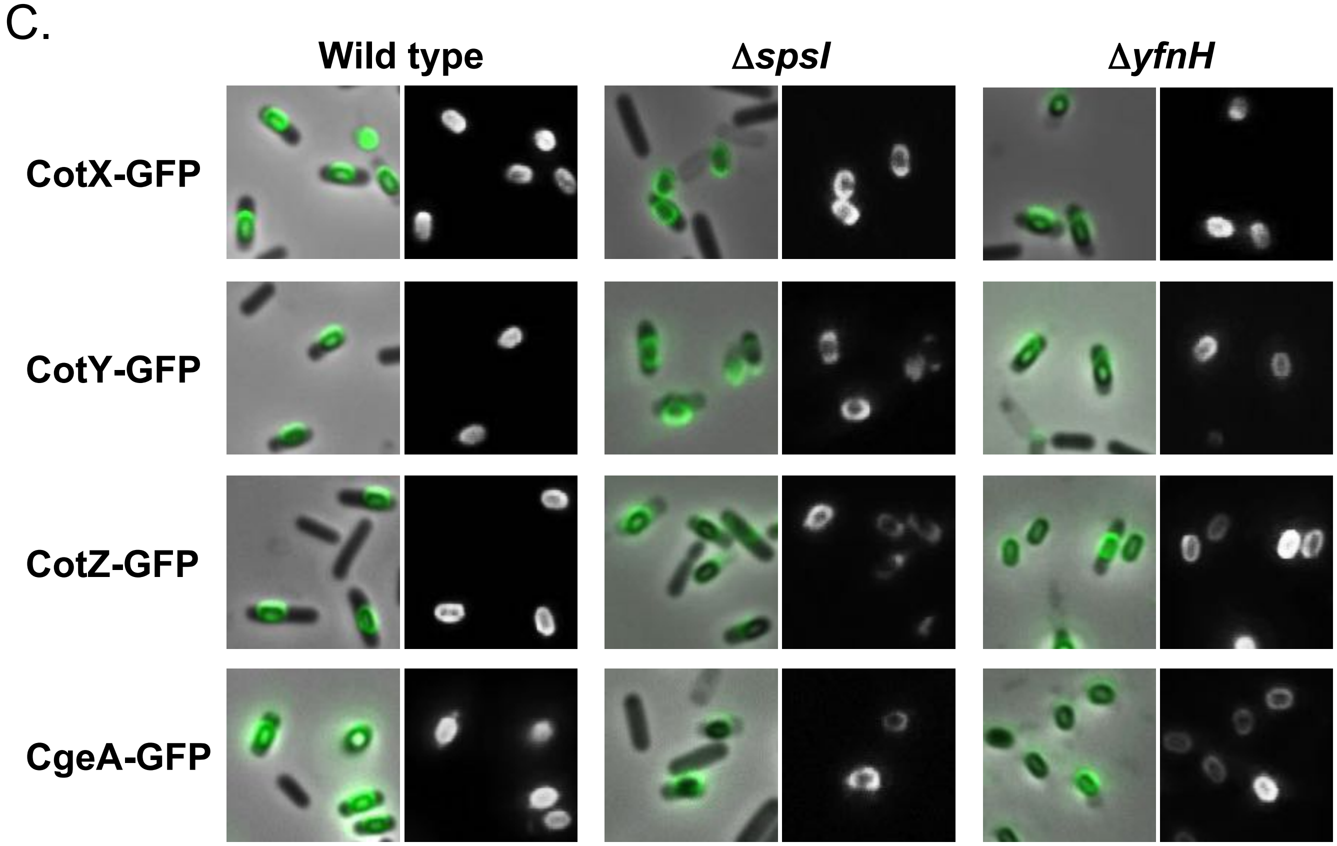


Figure S3. Putative glucose-1-phosphate nucleotidyl-transferases expressed during late sporulation play different roles in spore PS synthesis. A. Measurements of the halo areas (numbers of spores correspond to the number of spores considered for the measurements) in wild type spores (168) and Δ*ytdA* spores (BKE30850). The halo size is not significantly different between the two strains. **B.** Field of wild type (PY79) spores by TEM with Ruthenium red staining. All spore coat layers, including the crust, are visible. Scale bar is 500 nm. **C.** Field of Δ*ytdA* spores (PE2764) by TEM with Ruthenium red staining. All spore coat layers, including the crust, are visible. Scale bar is 500 nm. **D.** Field of Δ*yfnH* spores (PE2919) by TEM with Ruthenium red staining shows expansion and webbing of spore crust. Scale bars are 500 nm. **E.** Field of wild type spores (PY79) by SEM. Scale bar is 1 µm. **F.** Field of Δ*spsI* spores (PE2763) by SEM. Scale bar is 1 µm. **G**. Field of Δ*yfnH* spores (PE2919) by SEM. Scale bar is 1 µm. A web-like structure covers the surface of most spores, especially at the spore poles. **H.** BATH analysis of spores with the following gene deletions: Δ*ytdA* (PE2764), Δ*ytcA* (PE2767), Δ*ytcB* (PE2945), Δ*ytcC* (PE2946). Experiments were performed in triplicate; error bars represent standard deviation. All mutant spores are as hydrophilic as wild type (PY79) spores. **I.** India ink staining of gene deletions: Δ*ytcA* (BKE30860), Δ*ytcB* (BKE30870), Δ*ytdcC* (BKE30880). Halos are indistinguishable from wild type spores (168).

Figure S3

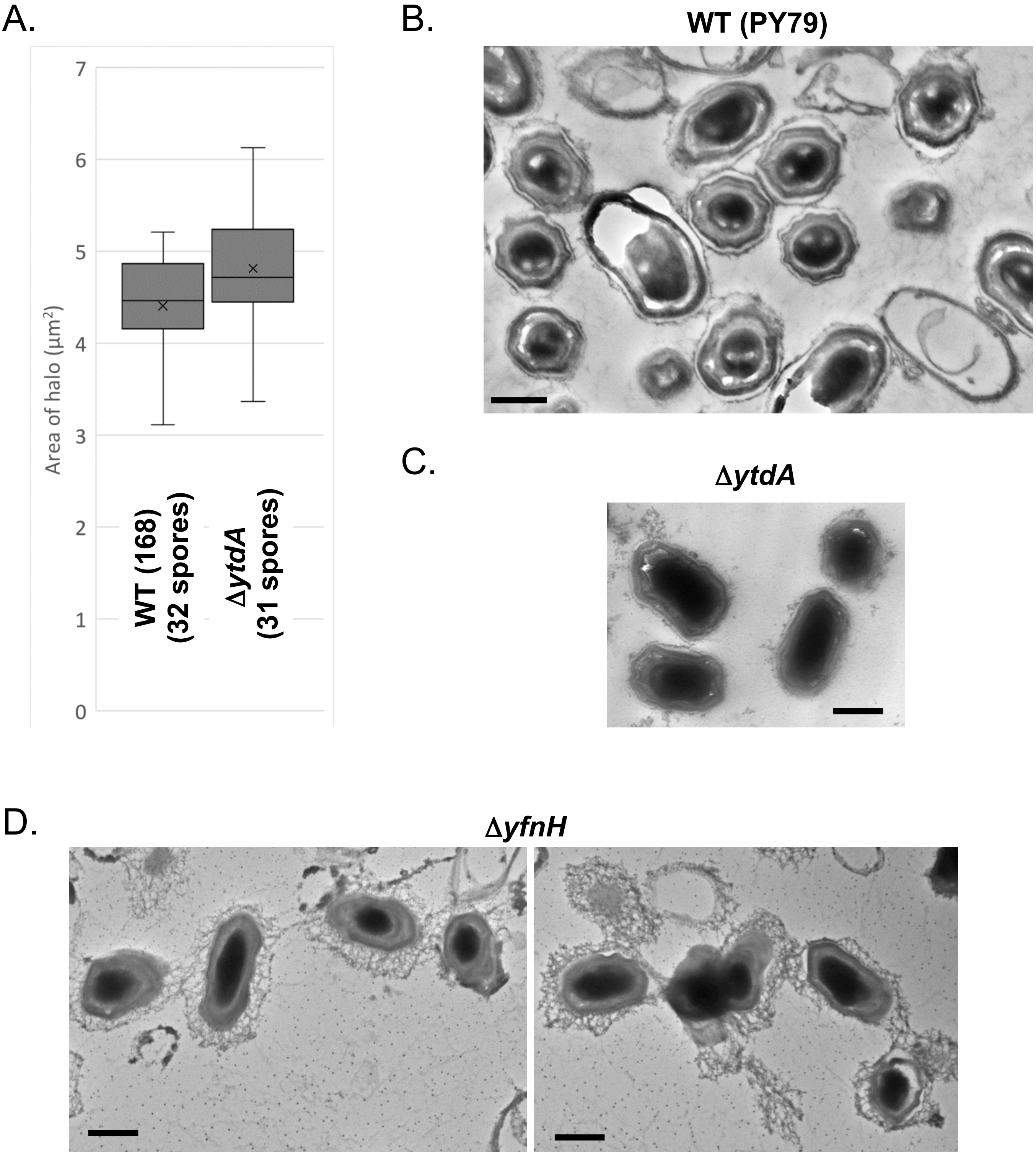
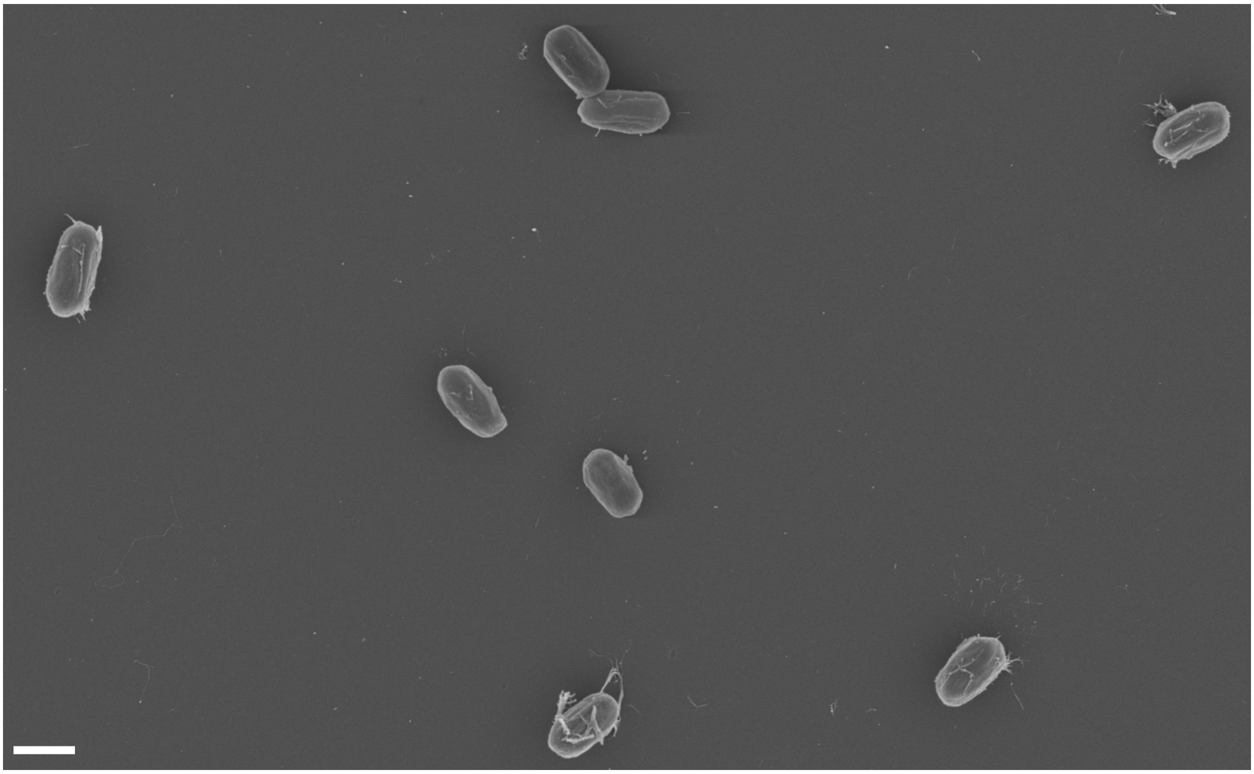

A.
7
6
5
4
3
2
1
0
Area of halo (μm²)
WT (168) (32 spores)
ΔytdA (31 spores)
B.
WT (PY79)
C.
ΔytdA
D.
ΔyfnH

Figure S3

(continued)

E.

WT (PY79)



F.

Δ*spsI*

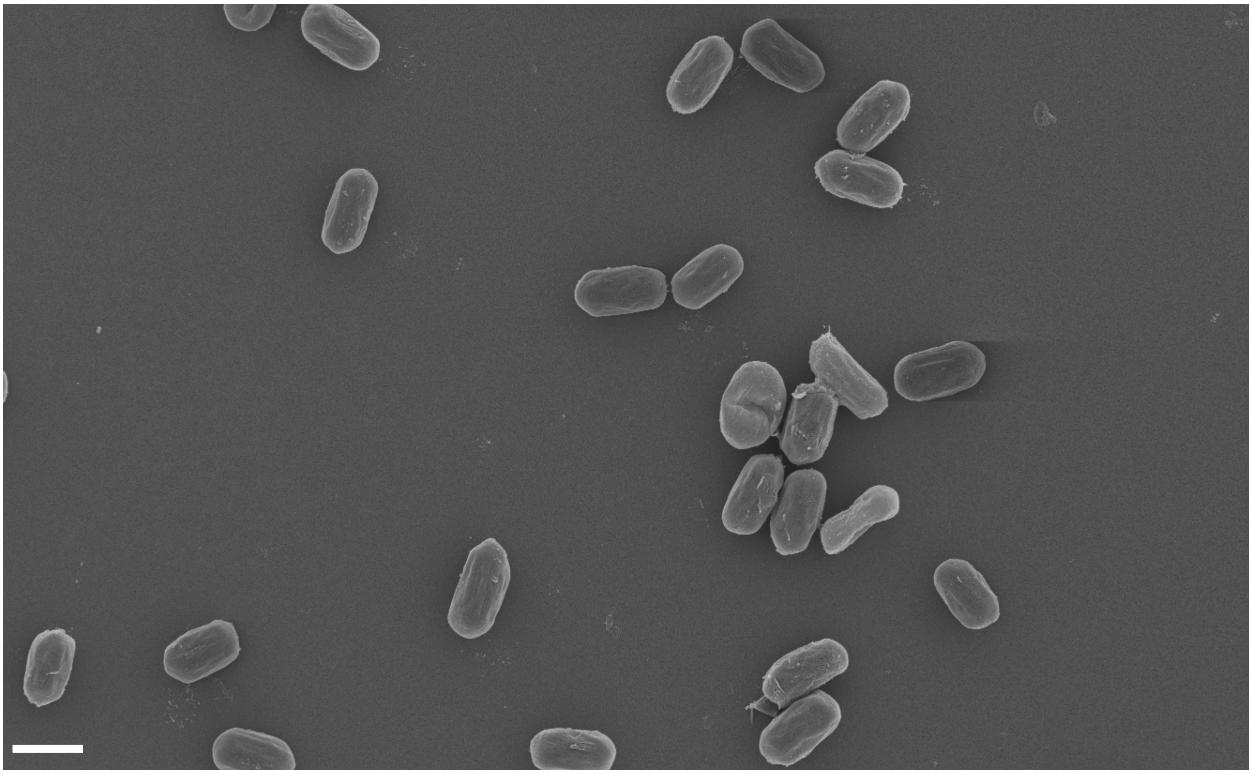
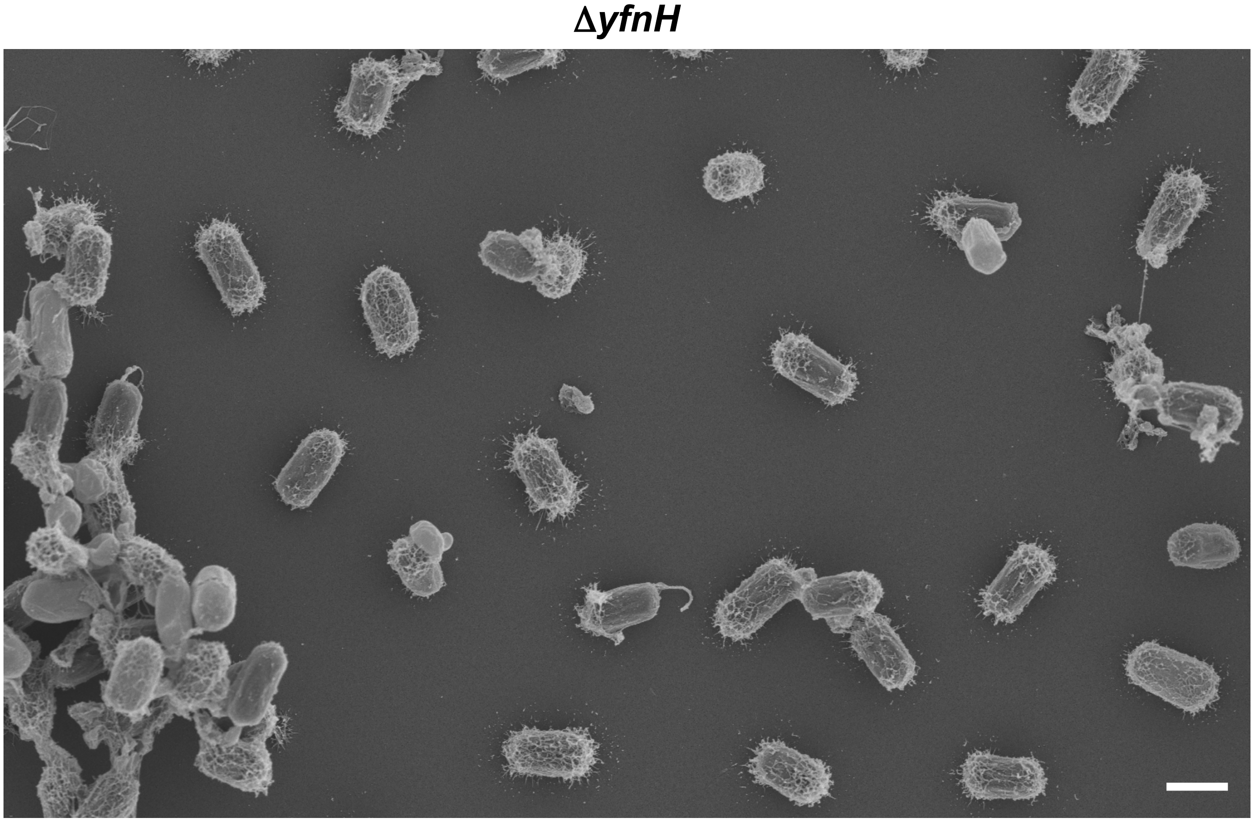
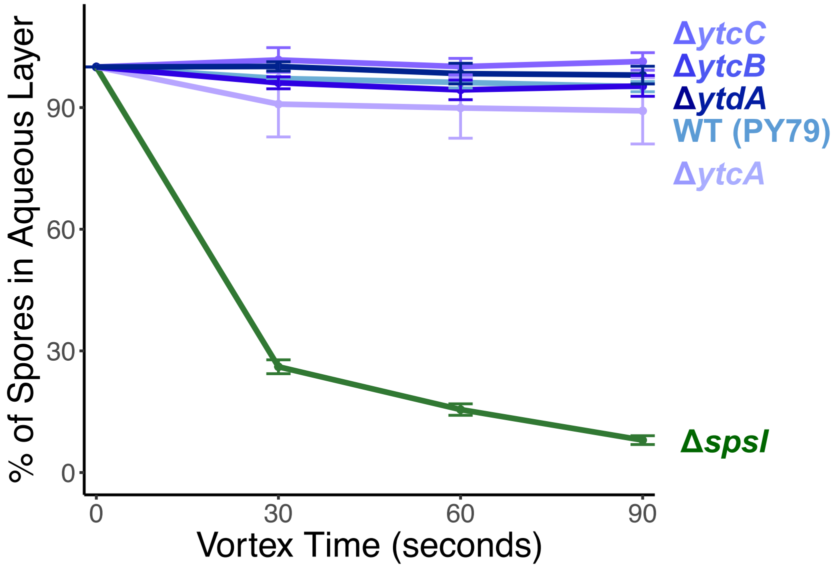


Figure S3
(continued)

G.



H.



I.

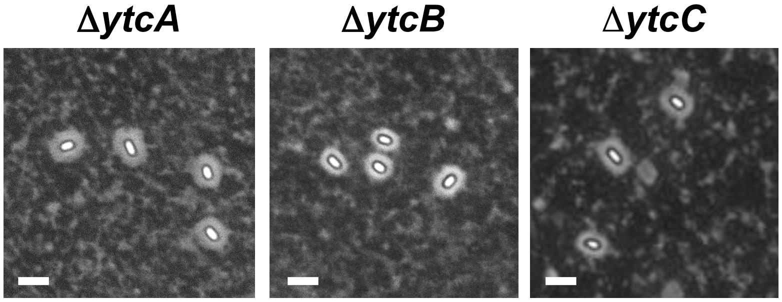


Figure S4. Gene deletions in the *yfnH-D* cluster result in expansion of the PS layer. A. Measurements of the halo areas (numbers of spores correspond to the number of spores considered for the measurements) for wild type (168), Δ*yfnH-D* (NY35), Δ*yfnD* (BKE07310), Δ*yfnE* (BKE07300), Δ*yfnF* (BKE07290), Δ*yfnG* (BKE07280) and Δ*yfnH* (BKE07270) spores. **B.** BATH analysis of spores with following gene deletions: Δ*yfnD* (PE3060), Δ*yfnE* (PE3063), Δ*yfnF* (PE3062), Δ*yfnG* (PE2961) and Δ*yfnH* (PE2919), which are as hydrophilic as wild type spores (PY79). Experiments were performed in triplicate; error bars represent standard deviation. Fields of spores imaged by TEM with Ruthenium red staining: **C.** Δ*yfnE* (PE3063), scale bar is 400 nm; **D.** Δ*yfnF* (PE3062), scale bar is 500 nm; **E.** Δ*yfnG* (PE2961), scale bar is 500 nm. All mutant spores exhibit expanded and web-like crust morphology. **F**. Field of Δ*yfnF* spores (PE3062) by SEM. Scale bar is 1 μm. A web-like structure covers the surface of most spore poles.

Figure S4

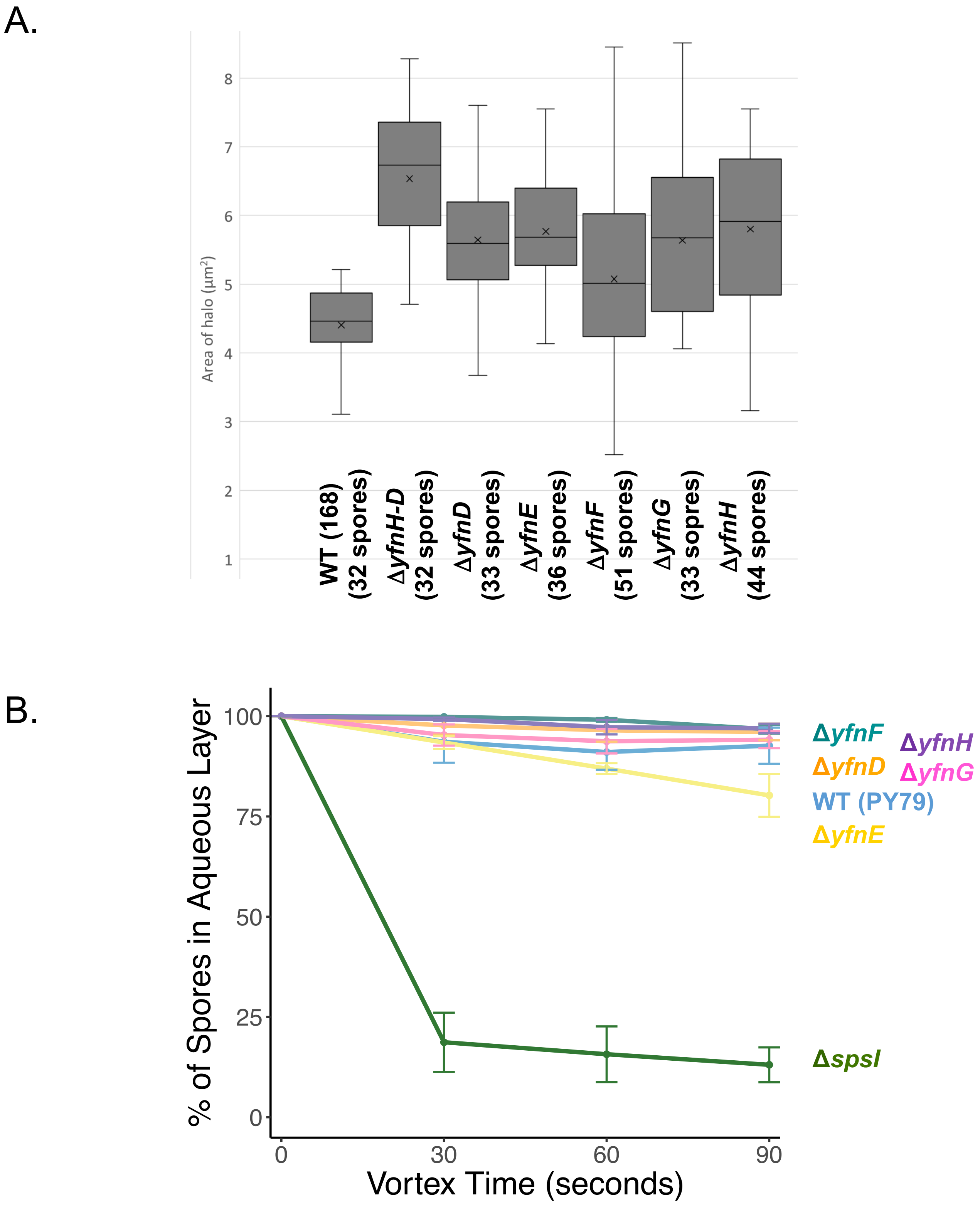
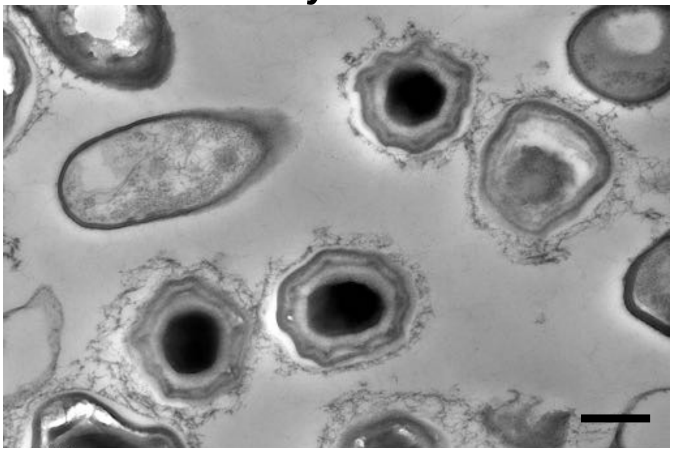

A.
Area of halo (µm²)
1
2
3
4
5
6
7
8
WT (168) (32 spores)
ΔyfnH-D (32 spores)
ΔyfnD (33 spores)
ΔyfnE (36 spores)
ΔyfnF (51 spores)
ΔyfnG (33 sopres)
ΔyfnH (44 spores)
B.
% of Spores in Aqueous Layer
0
25
50
75
100
Vortex Time (seconds)
0
30
60
90
ΔyfnF
ΔyfnH
ΔyfnD
ΔyfnG
WT (PY79)
ΔyfnE
ΔspsI

Figure S4
(continued)

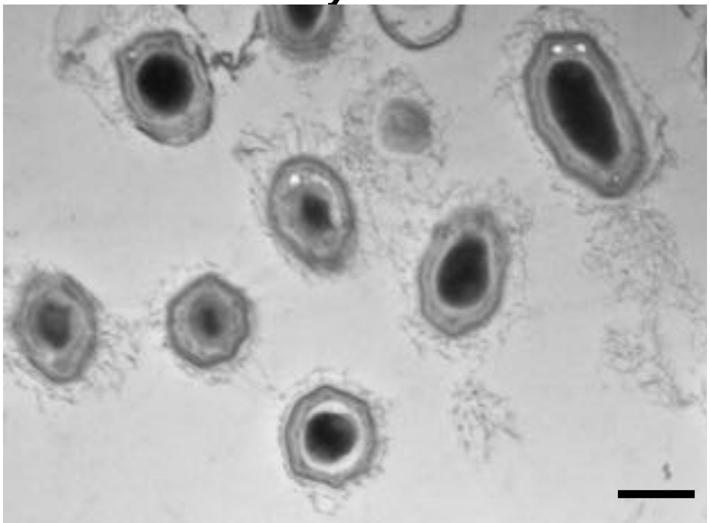
C.

Δ*yfnE*



D.

Δ*yfnF*



E.

Δ*yfnG*

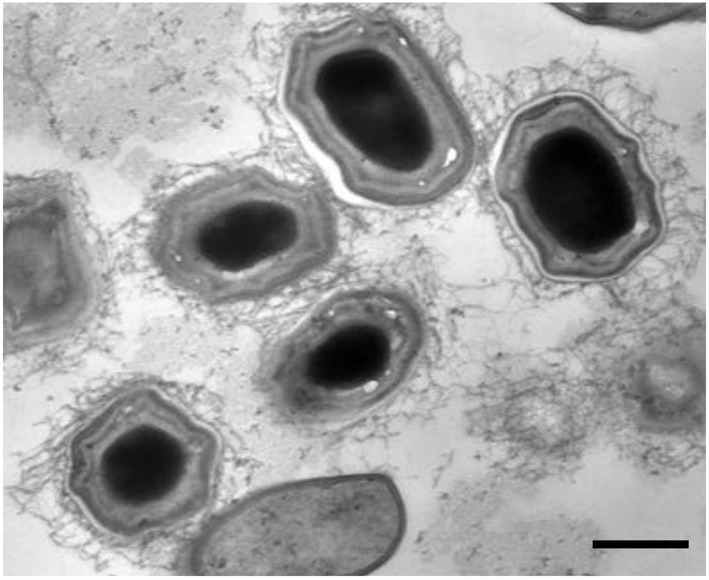


Figure S4
(continued)

F.

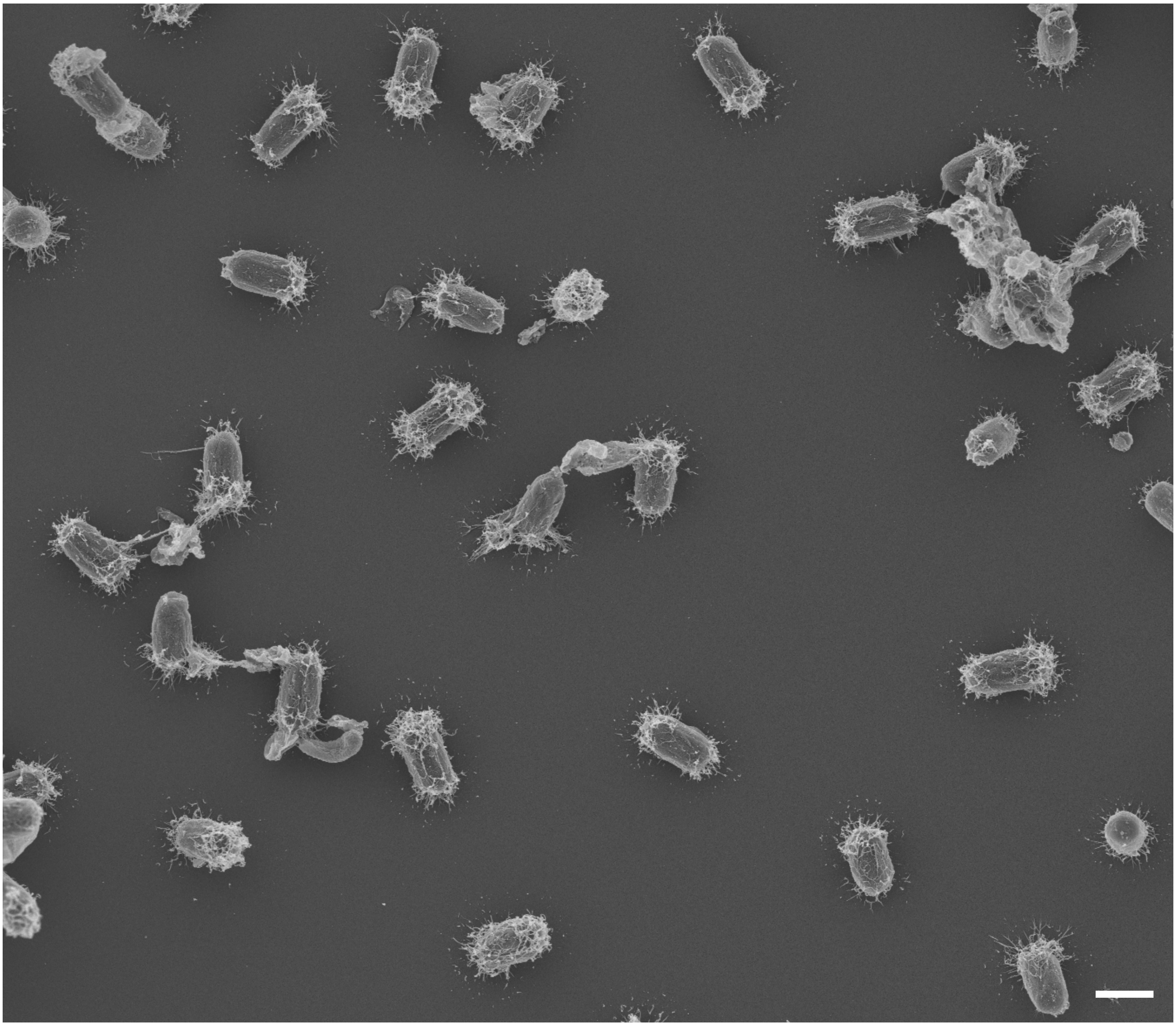


Figure S5. Partial complementation of Δ*spsI* spores by deletions in the *yfnH-D* cluster.

A. BATH analysis of spores with following gene deletions: Δ*yfnD* (PE3060), Δ*yfnE* (PE3063), Δ*yfnD* Δ*spsI* (PE3357) and Δ*yfnE* Δ*spsI* (PE3117). Unlike the partial rescue of Δ*spsI* by Δ*yfnH* or Δ*yfnF* (Figure 5), doubly mutant spores of Δ*yfnD* or Δ*yfnE* with Δ*spsI* exhibit the same levels of hydrophobicity as the single Δ*spsI* mutant spores. Experiments were performed in triplicate; error bars represent standard deviation. **B.** TEM with Ruthenium red of Δ*yfnF* (PE3062), Δ*spsI* (PE2763), and Δ*yfnF* Δ*spsI* (PE3118) spores. Scale bars are 200 nm. A webbed PS layer (red arrow) is characteristic of Δ*yfnF* spores, whereas Δ*spsI* spores have a greatly diminished crust. A return of a thin crust (red arrow) is observed in the double mutant. **C.** TEM with Ruthenium red of Δ*spsI* (PE2763), Δ*yfnE* (PE3063), Δ*yfnH* (PE2919), Δ*yfnE* Δ*spsI* (PE3117) and Δ*yfnH* Δ*spsI* (PE3119) spores. Scale bars are 200 nm. Both Δ*yfnH* and Δ*yfnE* spores present a webbed and expanded PS layer (red arrow) compared to a diminished crust layer in Δ*spsI*. The double mutants show a partially restored thin outermost layer (red arrow). **D.** SEM images for Δ*yfnH* (PE2919) and Δ*yfnH* Δ*spsI* (PE3119) spores. Scale bars are 500 nm. **E.** Field of Δ*yfnF* Δ*spsI* (PE3118) by SEM. Scale bar is 1 μm.

Figure S5

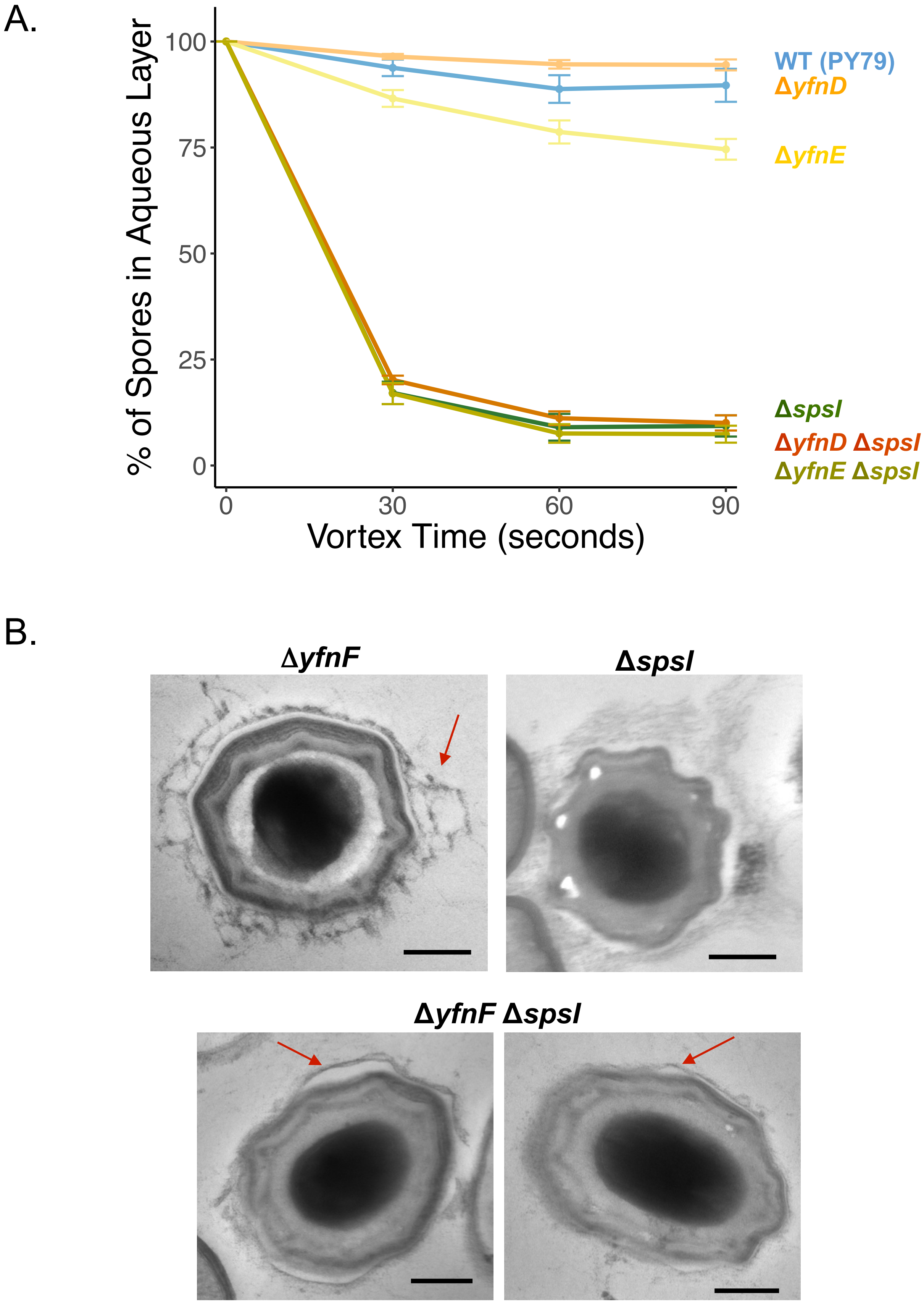

A.
% of Spores in Aqueous Layer
100
75
50
25
0
0
30
60
90
Vortex Time (seconds)
WT (PY79)
ΔyfnD
ΔyfnE
ΔspsI
ΔyfnD ΔspsI
ΔyfnE ΔspsI
B.
ΔyfnF
ΔspsI
ΔyfnF ΔspsI

Figure S5

(continued)

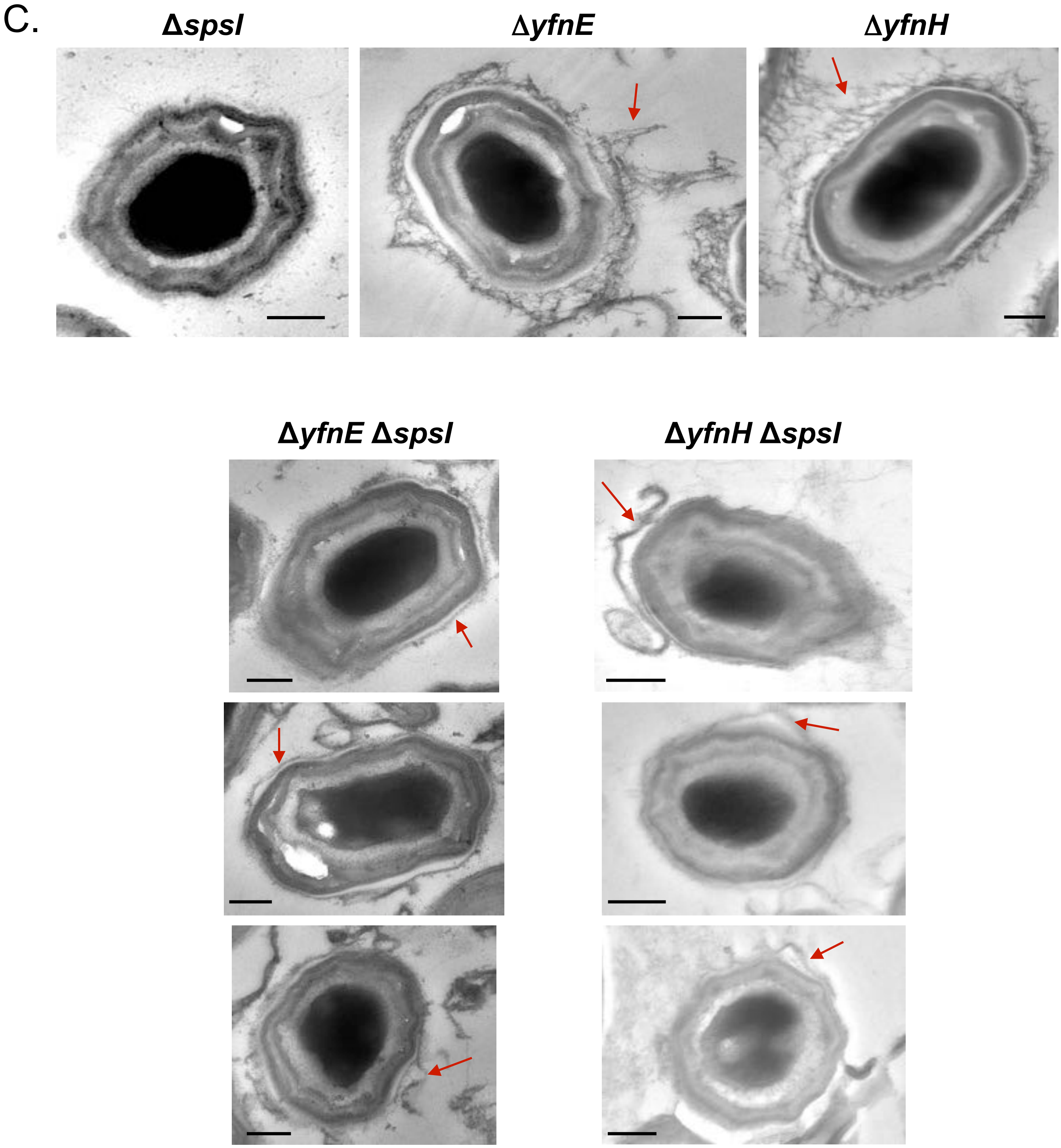
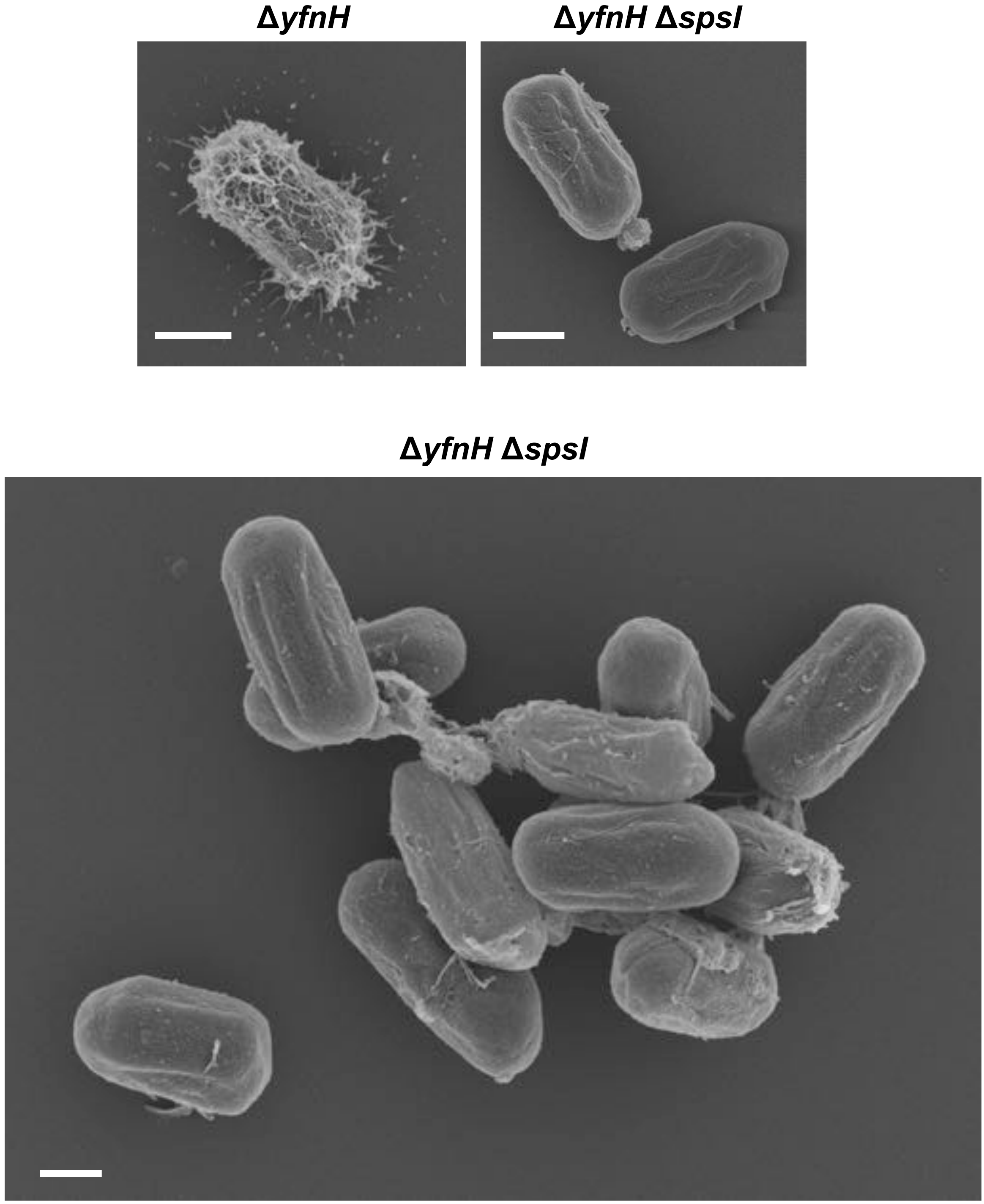


Figure S5

(continued)

D.



ΔyfnH

ΔyfnH ΔspsI

ΔyfnH ΔspsI

Figure S5
(continued)

E.

ΔyfnF ΔspsI

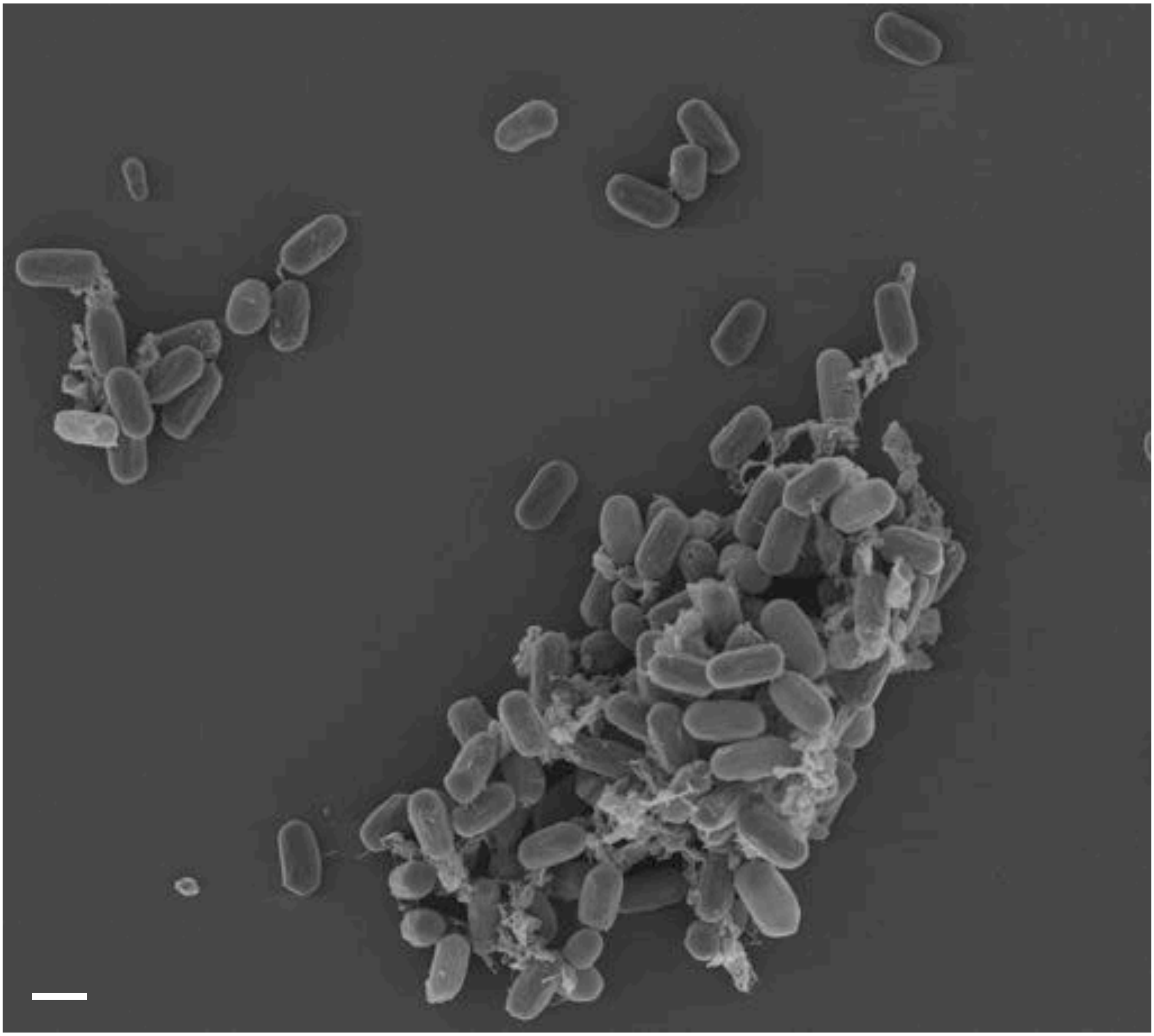


Figure S6. Analysis of spore surface properties in mutants with deletions in the *cgeCDE* operon. A. Measurements of the halo areas (numbers of spores correspond to the number of spores considered for the measurements) in wild type (168) and Δ*cgeD* spores (BKE19750). A large expansion of the halo area is observed for Δ*cgeD* spores. **B.** India ink staining images of the spores used for quantification (as displayed in **A**). **C.** Analyses of spore surface extracts by gel electrophoresis (5% polyacrylamide, TBE, stained with Stains-All). Δ*cgeD* mutants show an increase in size presumably caused by an expansion in PS content. **D.** BATH assay of Δ*cgeD* spores (RL1405) compared to wild type spores (PY79), Δ*spsI* spores (PE2763), and doubly mutant Δ*cgeD* Δ*spsI* spores (PE3078). Experiments were performed in triplicate; error bars represent standard deviation. Though Δ*cgeD* spores have an expanded PS layer by India ink staining and analyses of spore surface extracts, deletion of *cgeD* does not rescue the hydrophobic phenotype of Δ*spsI* spores. **E.** BATH assay of Δ*cgeD* spores (BKE19760) compared to wild type spores (168), Δ*ypqP* (Δ*spsM*-3') spores (BKK21670) and doubly mutant spores Δ*cgeD* Δ*ypqP* (PE3369). Experiments were performed in triplicate; error bars represent standard deviation. Deletion of *cgeD* does not rescue the hydrophobic phenotype of Δ*ypqP* (Δ*spsM*-3') spores.

Figure S6

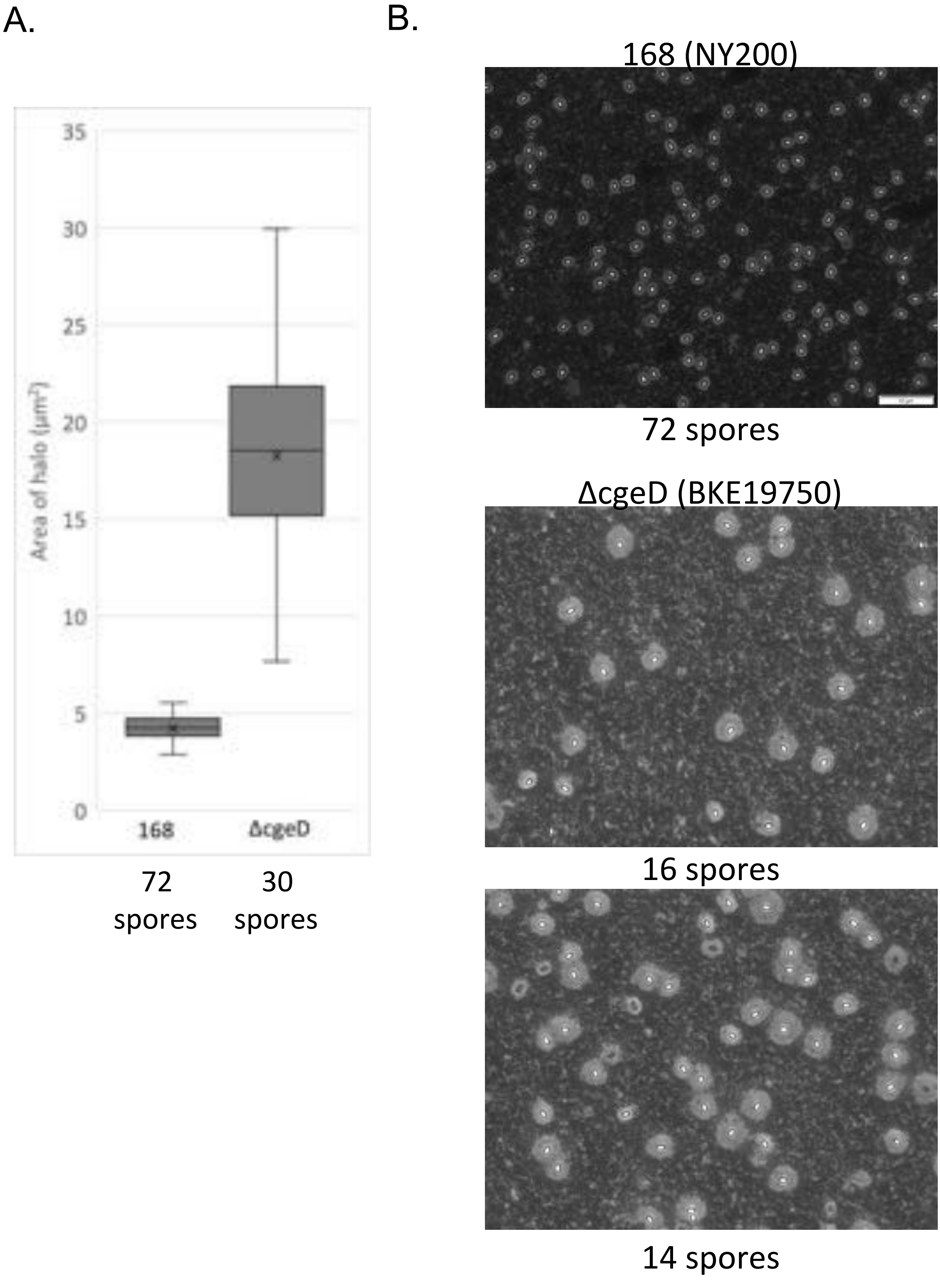
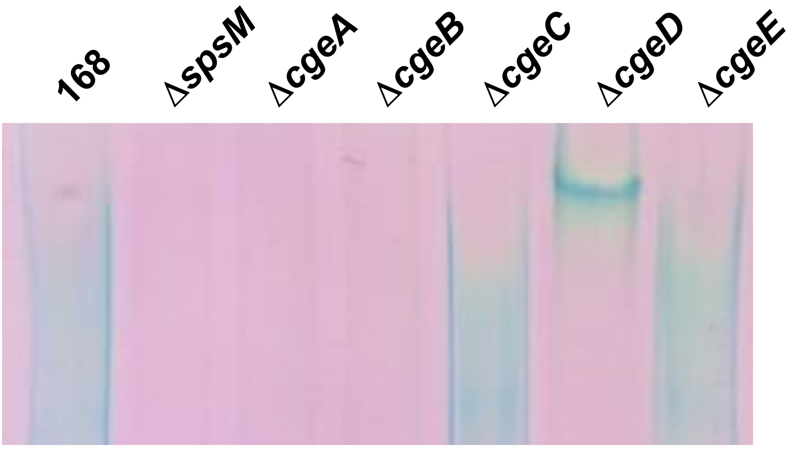

A.
35
30
25
20
15
10
5
0
Area of halo (μm²)
168
ΔcgeD
72 spores
30 spores
B.
168 (NY200)
72 spores
ΔcgeD (BKE19750)
16 spores
14 spores

Figure S6
(continued)

C.



5% polyacrylamide gel,
TBE
Stains All staining

D.

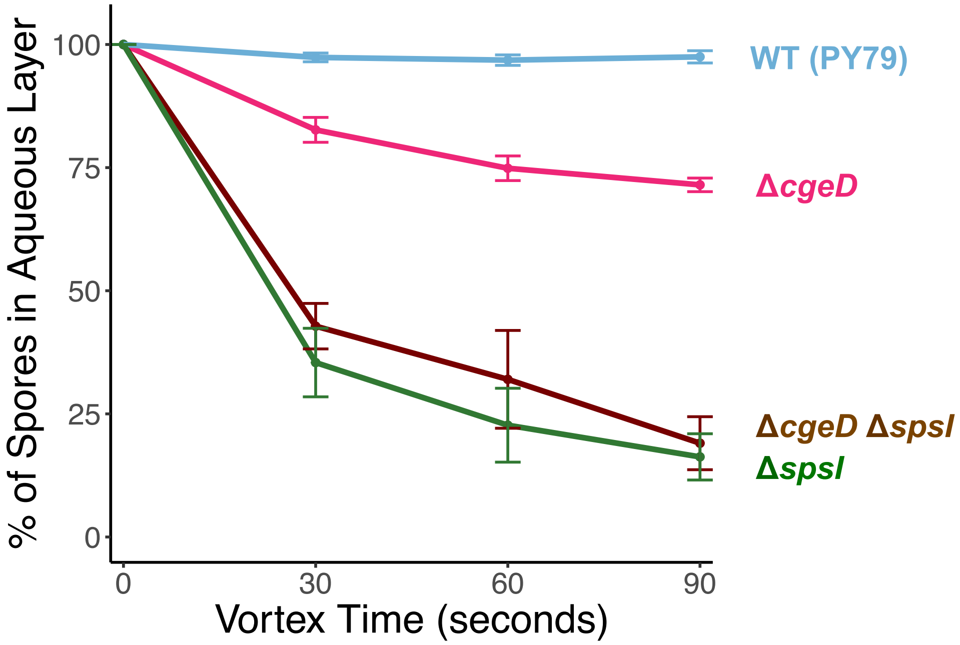


Figure S6
(continued)

E.

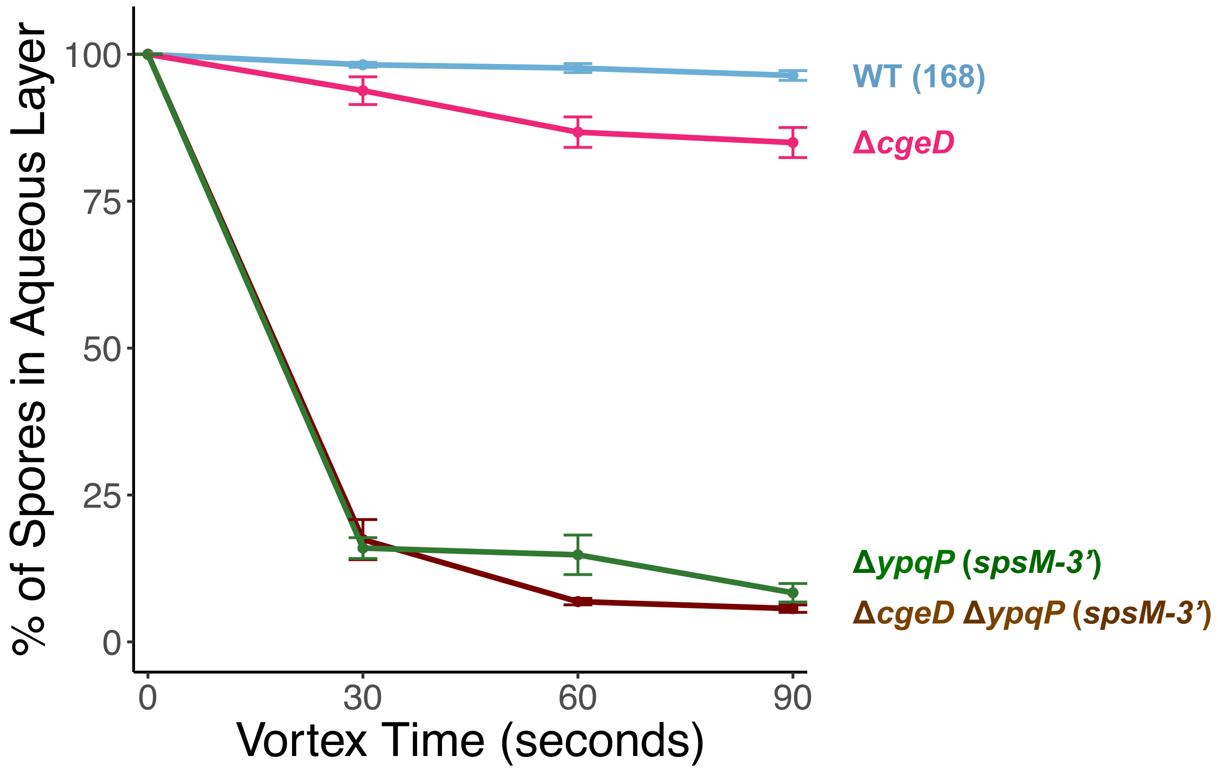


Figure S7. Construction of strains NY35, NY212, NY226, NY227and NY228. A. Construction of strain NY35 with a deletion of the entire *yfnH-D* cluster. **B.** Construction of strain NY212 with a deletion of the entire *spsA-L* operon. **C.** Construction of strains NY226 (deletion of *cgeC*) and NY227 (deletion of *cgeD*). D. Construction of strain NY228 (deletion of *cgeE*).

Figure S7

A.

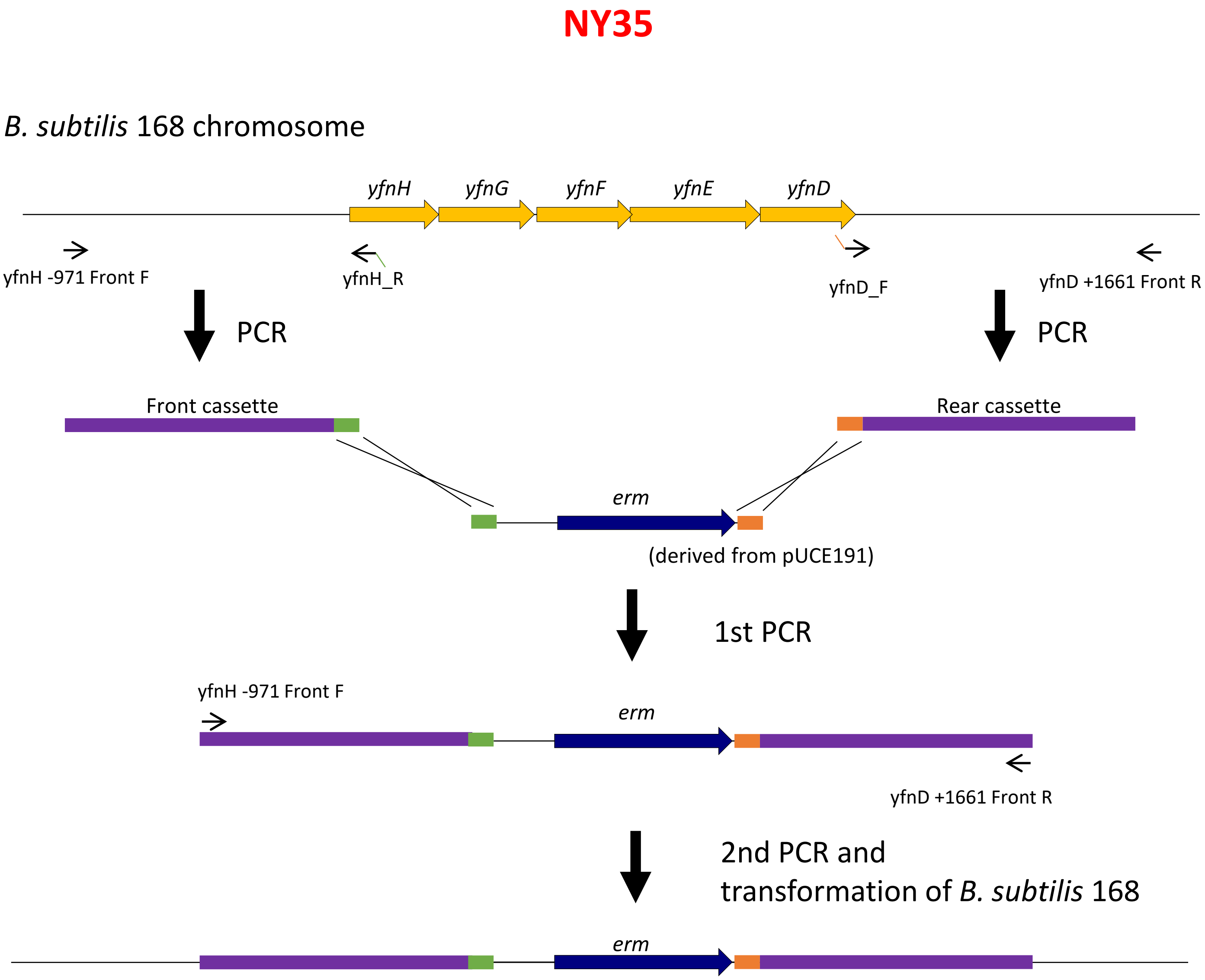

NY35
B. subtilis 168 chromosome
yfnH
yfnG
yfnF
yfnE
yfnD
yfnH -971 Front F
yfnH_R
yfnD_F
yfnD +1661 Front R
PCR
PCR
Front cassette
Rear cassette
erm
(derived from pUCE191)
1st PCR
yfnH -971 Front F
erm
yfnD +1661 Front R
2nd PCR and
transformation of *B. subtilis* 168
erm

Figure S7
(continued)

B.

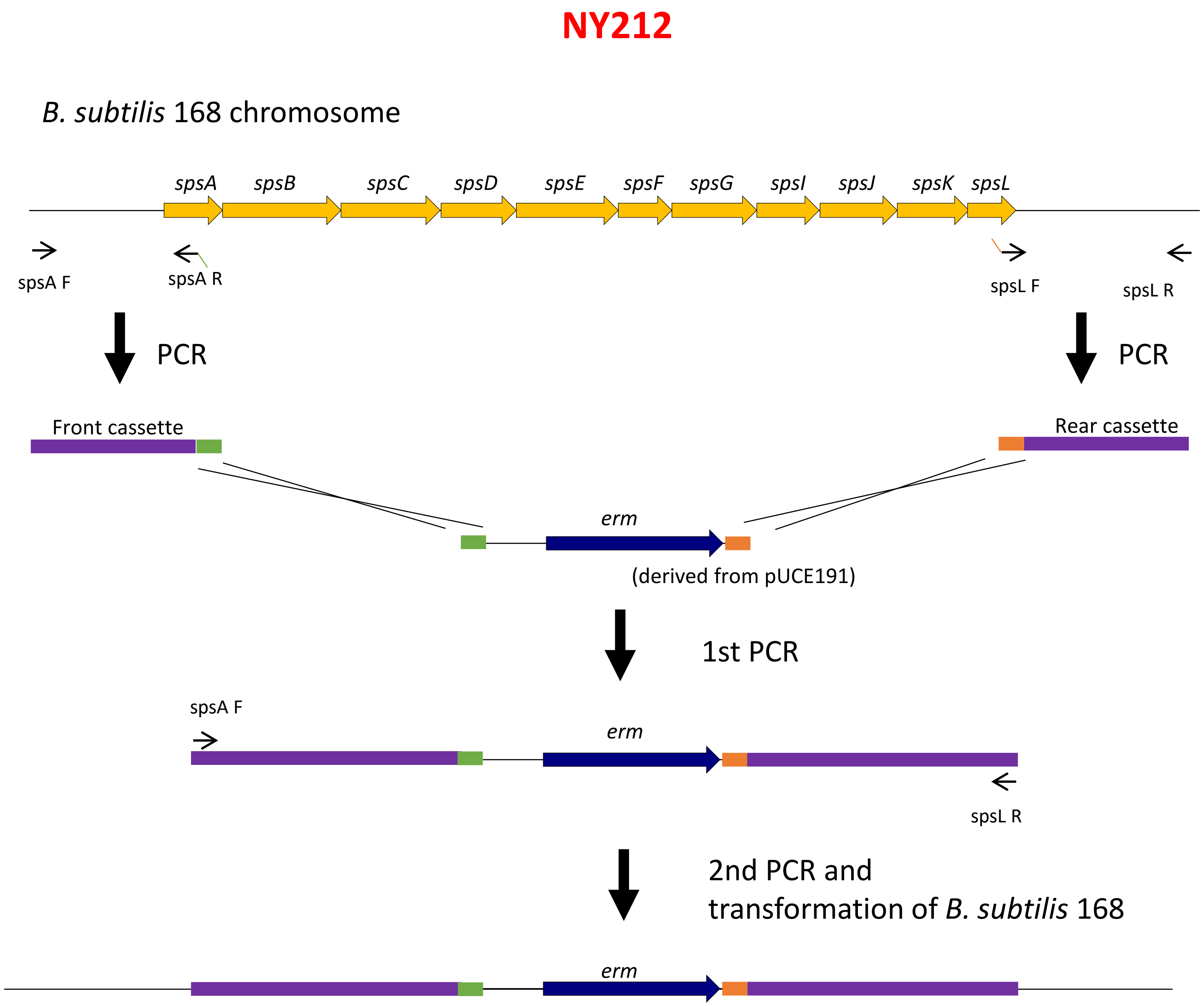


Figure S7
(continued)

C.

NY226, NY227

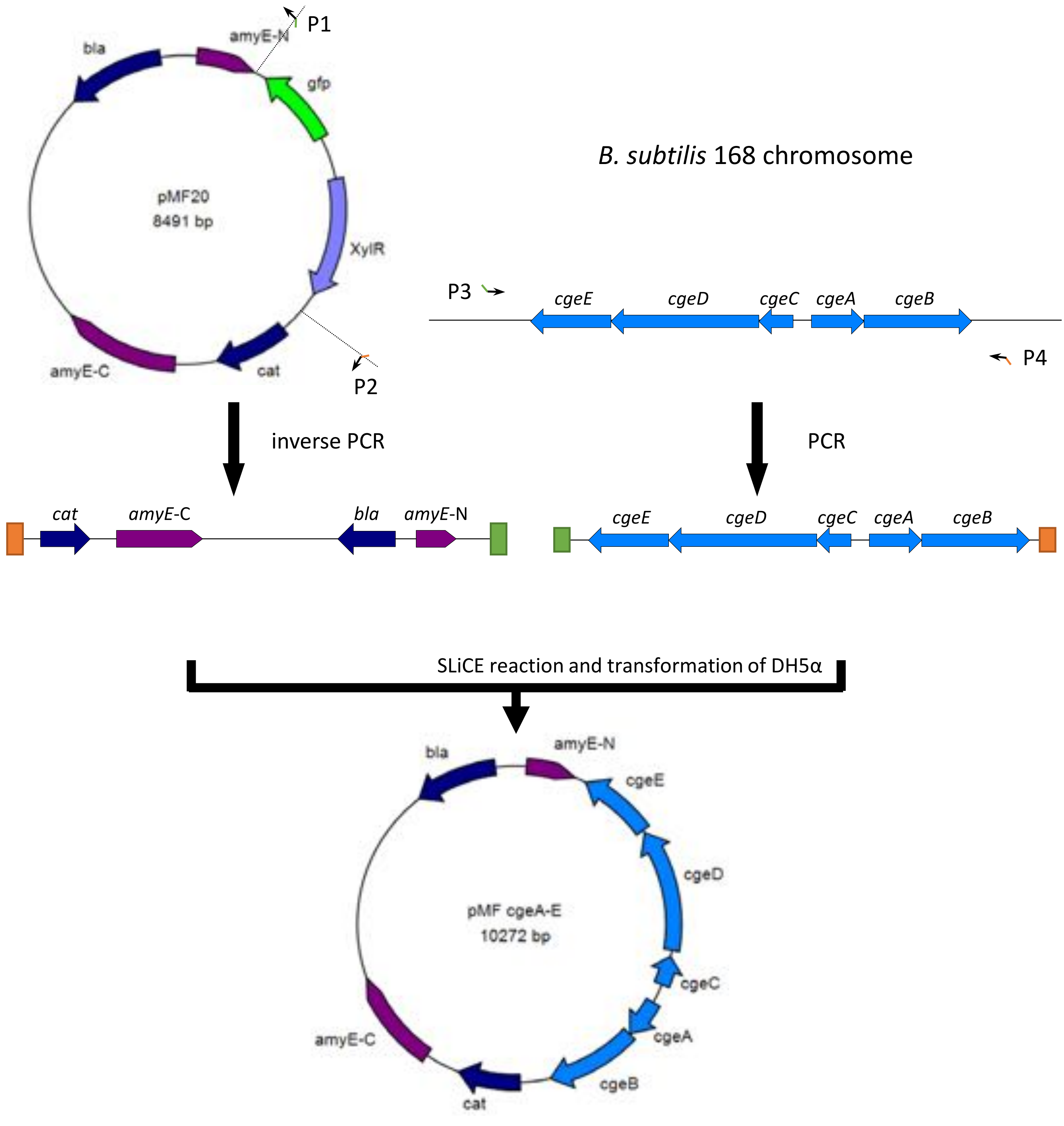
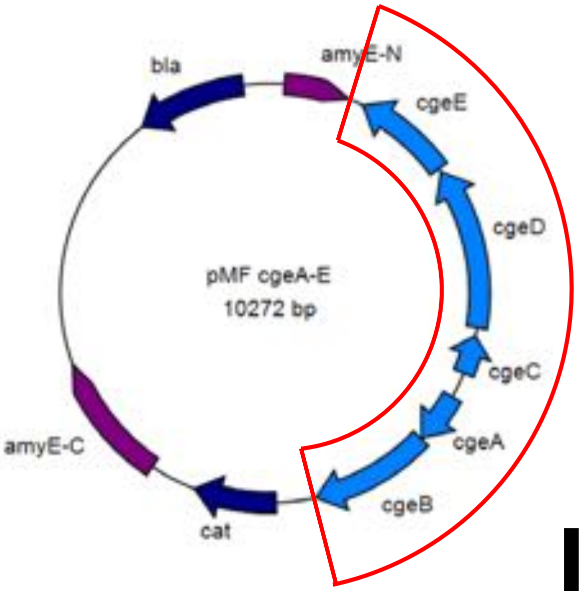
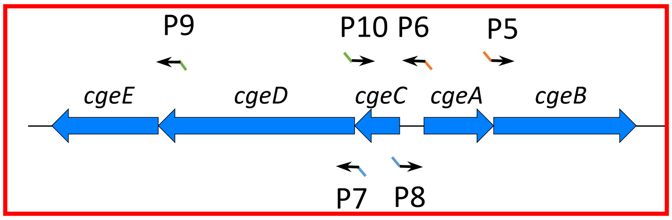


Figure S7
(continued)

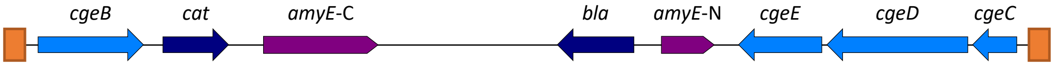
NY226, NY227



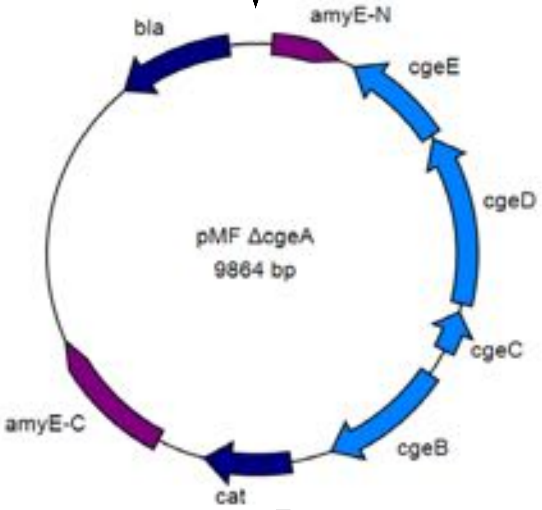


NY207	P5,P6
NY226	P7,P8
NY227	P9,P10

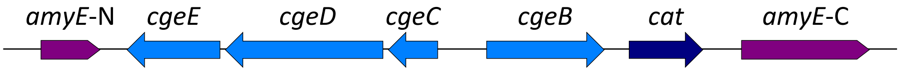
inverse PCR



SLiCE reaction
and transformation of DH5α



transformation of ΔcgeA-E (NY211)*
(double crossover)



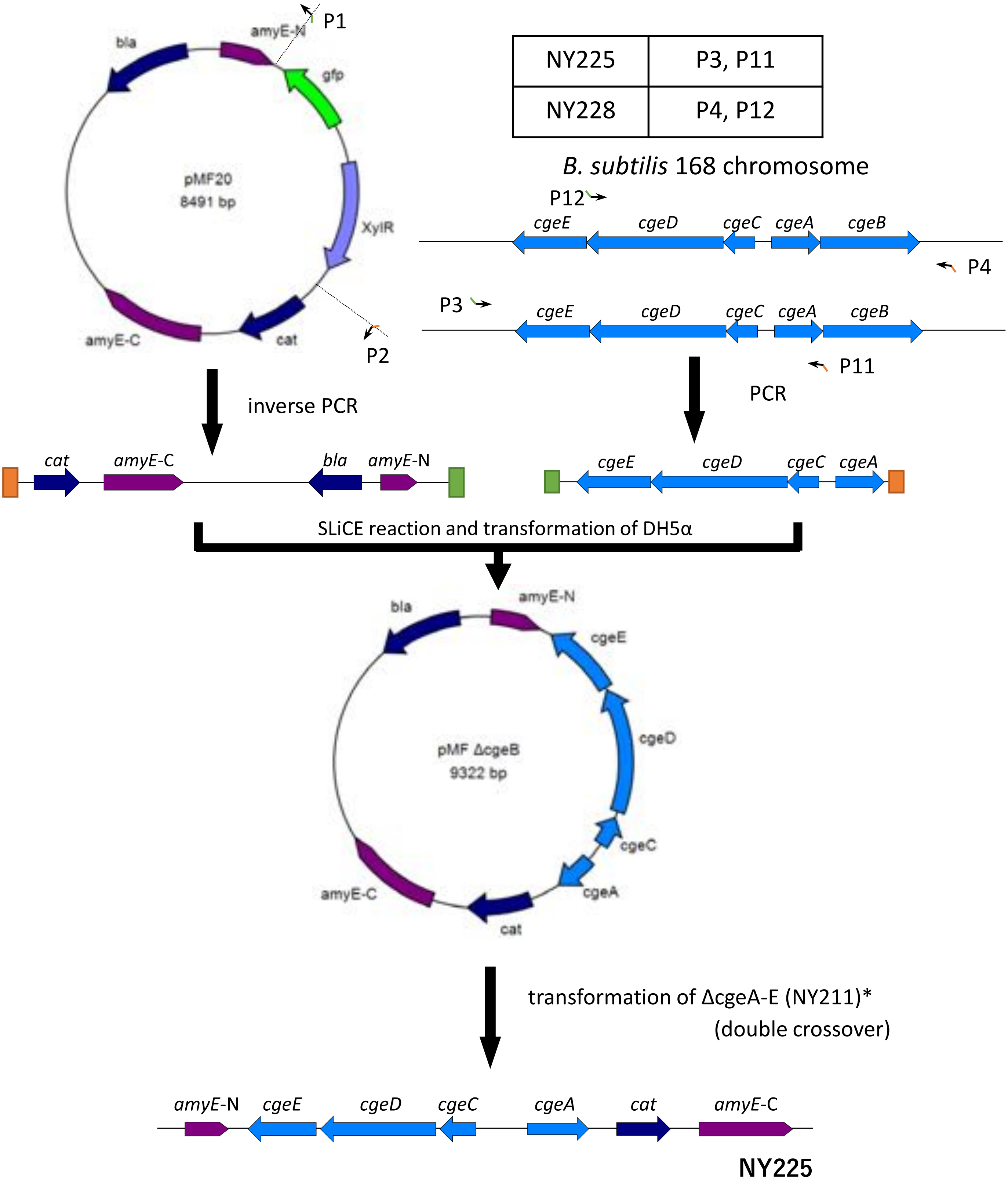
NY207

* selection for chloramphenicol and erythromycin resistance

Figure S7
(continued)

D.

NY228



NY225	P3, P11
NY228	P4, P12

* selection for chloramphenicol and erythromycin resistance

Table S1: Bacterial strains used in this study

Strain number	**Genotype**	**Background**	**Source**
168	*Bacillus subtilis*	Wild type	Laboratory stock
PY79	*Bacillus subtilis*	Wild type	(1)
BKE07270	Δ*yfnH*::*erm*	168	(2)
BKE07280	Δ*yfnG*::*erm*	168	(2)
BKE07290	Δ*yfnF*::*erm*	168	(2)
BKE07300	Δ*yfnE*::*erm*	168	(2)
BKE07310	Δ*yfnD*::*erm*	168	(2)
BKE19750	Δ*cgeE*::*erm*	168	(2)
BKE19760	Δ*cgeD*::*erm*	168	(2)
BKE19770	Δ*cgeC*::*erm*	168	(2)
BKE19810	Δ*yodU*::*erm*	168	(2)
BKE21670	Δ*ypqP*::*erm*	168	(2)
BKE30850	Δ*ytdA*::*erm*	168	(2)
BKE30860	Δ*ytcA*::*erm*	168	(2)
BKE30870	Δ*ytcB*::*erm*	168	(2)
BKE30880	Δ*ytcC*::*erm*	168	(2)
BKE37810	Δ*spsL*::*erm*	168	(2)
BKE37820	Δ*spsK*::*erm*	168	(2)
BKE37830	Δ*spsJ*::*erm*	168	(2)
BKE37840	Δ*spsI*::*erm*	168	(2)

BKE37850	Δ*spsG*::*erm*	168	(2)
BKE37860	Δ*spsF*::*erm*	168	(2)
BKE37870	Δ*spsE*::*erm*	168	(2)
BKE37880	Δ*spsD*::*erm*	168	(2)
BKE37890	Δ*spsC*::*erm*	168	(2)
BKE37900	Δ*spsB*::*erm*	168	(2)
BKE37910	Δ*spsA*::*erm*	168	(2)
HS176	*cotZ*ΩpHS2 (*cotZ-gfp spc*)	PY79	(3)
NY6	*amyE*::*cotX-gfp cat*	168	(4)
NY10	*amyE*::*cotY-gfp cat*	168	(4)
NY17	*amyE*::*cgeA-gfp cat*	168	(4)
NY35	Δ*yfnH-D*::*erm*	168	This study
NY211	Δ*cgeA-E*::*erm*	168	This study
NY212	Δ*spsA-L*::*erm*	168	This study
NY226	Δ*cgeA-E*::*erm* *amyE*::*cgeAB-cgeDE cat*	168	This study
NY227	Δ*cgeA-E*::*erm* *amyE*::*cgeAB-cgeCE cat*	168	This study
NY228	Δ*cgeA-E*::*erm* *amyE*::*cgeAB-cgeCD cat*	168	This study
PE2763	Δ*spsI*::*erm*	PY79	Derived from BKE37840
PE2764	Δ*ytdA*::*erm*	PY79	Derived from BKE30850
PE2767	Δ*ytcA*::*erm*	PY79	Derived from BKE30860
PE2777	Δ*spsI*::*erm*::*spc*	PY79	Derived from PE2763, resistance switched by insertion of pEr::Sp (5)
PE2914	Δ*cgeD*::*erm*	168	Derived from BKE19760
PE2917	Δ*cgeC*::*erm*	PY79	Derived from BKE19770
PE2918	Δ*cgeD*::*erm*	PY79	Derived from BKE19760
PE2919	Δ*yfnH*::*erm*	PY79	Derived from BKE07270

PE2945	Δ*ytcB*::*erm*	PY79	Derived from BKE30870
PE2946	Δ*ytcC*::*erm*	PY79	Derived from BKE30880
PE2958	Δ*spsJ::erm*	PY79	Derived from BKE37830
PE2959	Δ*spsK*::*erm*	PY79	Derived from BKE37820
PE2960	Δ*spsL::erm*	PY79	Derived from BKE37810
PE2961	Δ*yfnG*::*erm*	PY79	Derived from BKE07280
PE3060	Δ*yfnD*::*erm*	PY79	Derived from BKE07310
PE3062	Δ*yfnF*::*erm*	PY79	Derived from BKE07290
PE3063	Δ*yfnE*::*erm*	PY79	Derived from BKE07300
PE3065	Δ*cgeE*::*erm*	PY79	Derived from BKE19750
PE3078	Δ*cgeD*::*kan* Δ*spsI*::*erm*	PY79	PE2763→RL1405
PE3111	Δ*spsI*::*kan*	PY79	Derived from BKK37840
PE3115	Δ*yfnH*::*kan*	PY79	Derived from BKK07270
PE3117	Δ*yfnE*::*erm* Δ*spsI*::*kan*	PY79	PE3063→PE3111
PE3118	Δ*yfnF*::*erm* Δ*spsI*::*kan*	PY79	PE3062→PE3111
PE3119	Δ*yfnH*::*erm* Δ*spsI*::*kan*	PY79	PE2919→PE3111
PE3151	*amyE*::*cotX-gfp cat*	PY79	(4)
PE3158	*amyE*::*cotY-gfp cat*	PY79	(4)
PE3216	Δ*yodU*::*erm*	168	Derived from BKE19810
PE3218	Δ*ypqP*::*erm*	168	Derived from BKE21670
PE3220	Δ*yfnH*::*kan* Δ*spsA*::*erm*	168	PE3115→PE3203
PE3221	Δ*yfnH*::*kan* Δ*spsB*::*erm*	168	PE3115→PE3204
PE3222	Δ*yfnH*::*kan* Δ*spsC*::*erm*	168	PE3115→PE3205
PE3223	Δ*yfnH*::*kan* Δ*spsD*::*erm*	168	PE3115→PE3206
PE3224	Δ*yfnH*::*kan* Δ*spsE*::*erm*	168	PE3115→PE3207
PE3225	Δ*yfnH*::*kan* Δ*spsF*::*erm*	168	PE3115→PE3208
PE3226	Δ*yfnH*::*kan* Δ*spsJ*::*erm*	168	PE3115→PE2958
PE3227	Δ*yfnH*::*kan* Δ*spsK*::*erm*	168	PE3115→PE2959
PE3228	Δ*yfnH*::*kan* Δ*spsL*::*erm*	168	PE3115→PE2960
PE3229	Δ*yfnH*::*kan* Δ*ypqP*::*erm*	168	PE3115→PE3218
PE3232	Δ*yfnG*::*kan* Δ*spsI*::*erm*	PY79	PE3111→PE3060
PE3233	Δ*yfnH*::*kan* Δ*cgeD*::*erm*	PY79	PE3115→PE2918
PE3235	Δ*spsI*::*spc* Δ*yfnH*::*kan* Δ*cgeD*::*erm*	PY79	PE2777→PE33233
PE3242	Δ*spsI*::*erm amyE*::*cotY-gfp cat*	PY79	NY10→PE2763
PE3243	Δ*yfnH*::*erm amyE*::*cotY-gfp cat*	PY79	NY10→PE2919
PE3244	Δ*yfnH*::*erm amyE*::*cotX-gfp cat*	PY79	NY6→PE2919
PE3245	Δ*spsI*::*erm amyE*::*cotX-gfp cat*	PY79	NY6→PE2763
PE3247	Δ*yfnH*::*erm cotZ*ΩpHS2 (*cotZ-gfp spc*)	PY79	HS176→PE2919
PE3248	Δ*spsI*::*erm cotZ*ΩpHS2 (*cotZ-gfp spc*)	PY79	HS176→PE2763

PE3260	*amyE*::*cgeA-gfp cat*	PY79	(4)
PE3303	Δ*spsA*::*erm*	168	Derived from BKE37910
PE3304	Δ*spsB*::*erm*	168	Derived from BKE37900
PE3305	Δ*spsC*::*erm*	168	Derived from BKE37890
PE3306	Δ*spsD*::*erm*	168	Derived from BKE37880
PE3307	Δ*spsE*::*erm*	168	Derived from BKE37870
PE3308	Δ*spsF*::*erm*	168	Derived from BKE37860
PE3309	Δ*spsG*::*erm*	168	Derived from BKE37850
PE3310	Δ*spsJ*::*kan*	168	Derived from BKE37830
PE3311	Δ*spsK*::*kan*	168	Derived from BKE37820
PE3312	Δ*spsL*::*kan*	168	Derived from BKE37810
PE3313	Δ*spsI*::*kan*	168	Derived from BKK37840
PE3314	Δ*ypqP*::*kan*	168	Derived from BKK21670
PE3322	Δ*yfnH*::*erm amyE*::*cgeA-gfp cat*	PY79	NY17→PE2919
PE3323	Δ*spsI*::*erm amyE*::*cgeA-gfp cat*	PY79	NY17→PE2763
PE3341	Δ*spsA*::*erm*	PY79	Derived from BKE37910
PE3342	Δ*spsB*::*erm*	PY79	Derived from BKE37900
PE3343	Δ*spsC*::*erm*	PY79	Derived from BKE37890
PE3344	Δ*spsD*::*erm*	PY79	Derived from BKE37880
PE3345	Δ*spsE*::*erm*	PY79	Derived from BKE37870
PE3346	Δ*spsF*::*erm*	PY79	Derived from BKE37860
PE3347	Δ*spsG*::*erm*	PY79	Derived from BKE37850
PE3348	Δ*spsJ*::*kan*	PY79	Derived from BKK37830
PE3349	Δ*spsK*::*kan*	PY79	Derived from BKK37820
PE3350	Δ*spsL*::*kan*	PY79	Derived from BKK37810
PE3352	Δ*spsA*	PY79	PE3341 with *erm* resistance removed (using pDR244a)
PE3353	Δ*spsB*	PY79	PE3342 with *erm* resistance removed (using pDR244a)
PE3354	Δ*spsC*	PY79	PE3343 with *erm* resistance removed (using pDR244a)
PE3379	Δ*spsD*	PY79	PE3344 with *erm* resistance removed (using pDR244a)
PE3355	Δ*spsE*	PY79	PE3345 with *erm* resistance removed (using pDR244a)
PE3356	Δ*spsG*	PY79	PE3347 with *erm* resistance removed (using pDR244a)
PE3357	Δ*yfnD*::*erm* Δ*spsI*::*kan*	PY79	PE2919→PE3060
PE3358	Δ*ytdA*	PY79	PE2764 with *erm* resistance removed (using pDR244a)
PE3359	Δ*ytcA*	PY79	PE2767 with *erm* resistance removed (using pDR244a)

PE3360	Δ*ytcB*	PY79	PE2945 with *erm* resistance removed (using pDR244a)
PE3361	Δ*yfnE*	PY79	PE3063 with *erm* resistance removed (using pDR244a)
PE3362	Δ*yfnF*	PY79	PE3062 with *erm* resistance removed (using pDR244a)
PE3363	Δ*yfnG*	PY79	PE2961 with *erm* resistance removed (using pDR244a)
PE3364	Δ*yfnH*	PY79	PE2919 with *erm* resistance removed (using pDR244a)
PE3365	Δ*cgeD*	PY79	PE2918 with *erm* resistance removed (using pDR244a)
PE3369	Δ*cgeD*::*erm* Δ*ypqP*::*kan*	168	BKE19760→PE2918
RL1405	Δ*cgeD*::*kan*	PY79	(6)

REFERENCES

1. Youngman P, Perkins JB, Losick R. 1984. Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. Plasmid 12:1-9.
2. Koo BM, Kritikos G, Farelli JD, Todor H, Tong K, Kimsey H, Wapinski I, Galardini M, Cabal A, Peters JM, Hachmann AB, Rudner DZ, Allen KN, Typas A, Gross CA. 2017. Construction and Analysis of Two Genome-Scale Deletion Libraries for *Bacillus subtilis*. Cell Syst 4:291-305 e7.
3. Kim H, Hahn M, Grabowski P, McPherson DC, Otte MM, Wang R, Ferguson CC, Eichenberger P, Driks A. 2006. The *Bacillus subtilis* spore coat protein interaction network. Mol Microbiol 59:487-502.
4. Shuster B, Khemmani M, Abe K, Huang X, Nakaya Y, Maryn N, Buttar S, Gonzalez AN, Driks A, Sato T, Eichenberger P. 2018. Contributions of crust proteins to spore surface properties in *Bacillus subtilis*. Mol Microbiol doi:10.1111/mmi.14194.
5. Steinmetz M, Richter R. 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through in vivo recombination. Gene 142:79-83.
6. Roels S, Losick R. 1995. Adjacent and divergently oriented operons under the control of the sporulation regulatory protein GerE in *Bacillus subtilis*. J Bacteriol 177:6263-75.

Table S2: Primers used in this study

Primer name	Sequence (5’→3’)	Location[1]
spsA F	ATTACCATCTTGCTGATCGT	*spsA* sense sequence -1950
spsA R	ccatgcgtttgggccTACATTCAGCGTCTC	*spsA* anti-sense sequence +145
spsL F	ccaaggagatggccgCGTGGGATGATGAGA	*spsF* sense sequence +407
spsL R	ACGACGATTGACGCCTGAAT	*spsF* anti-sense sequence +2386
yfnH -971 Front F	AAGCCGTTCTTTACAGAATCGATTCCAAAG	*yfnH* sense strand -971
yfnH_R	ccatgcgtttgggccTCATAATATGCCATA	*yfnH* anti-sense strand +101
yfnD_F	ccaaggagatggccgGACAATTAAGATGAT	*yfnD* sense strand +809
yfnD+1661 Front R	GCCGCTCGGGCAATTCGGCGCTGTTTGGTT	*yfnD* anti-sense strand +1661
ermC bstBF	ggcccaaacgcatggTAAACGTATATAGAT	pUCE191 *ermC* sense strand −370
ermC bstAR	cggccatctccttggTCGCGCGTTTCGGTG	pUCE191 *ermC* anti-sense strand +973
P1 (SLiCE pMF20 BamHI F)	GGATCCTGAGCGCCGGTCGCTACCATTACCAGTTG	pMF20 *gfp* sense strand +752
P2 (SLiCE pMF20 EcoRI R)	GAATTCTCATGTTTGACAGCTTATCATCGGCAATA	pMF20 *xylR* sense strand +1180
P3 (cgeE +765 BR)	cggcgctcaggatccTATTTCATGTAAGGAAATAA	*cgeE* anti-sense strand +765
P4 (cgeB +1027 ER)	caaacatgagaattcGAAAGACCGTTCTGACGGCT	*cgeB* anti-sense strand +1027
P5 (cgeA +408 F)	tgtgtgtaatgaaagTCTTGTATATCCAGTCGGGA	*cgeA* sense strand +408

P6 (cgeA -1 R)	`ctttcattacacacaCCTCCTATTCGATAGTGAAC`	*cgeA* anti-sense strand -1
P7 (cgeC +370 F)	`gggatgatatgggagAGAAAGTGTCTATTATTTTA`	*cgeC* sense strand +370
P8 (cgeC -1 R)	`ctcccatatcatcccTCTCCCTTATCCTTATCTCT`	*cgeC* anti-sense strand -1
P9 (cgeD +1309 F)	`cggtatagatgacagGACATTATTCAAAAACGGAA`	*cgeD* sense strand +1309
P10 (cgeD -1 R)	`ctgtcatctataccgCCTCCCGCCGTCAAACATAA`	*cgeD* anti-sense strand -1
P11 (cgeB -1 R SLiCE)	`atgagaattcCGGATGTTATGAAAAGAACGTAACG`	*cgeB* anti-sense strand -1
P12 (cgeE -1 R SLiCE)	`ctcaggatccGAACGTCTCCTTTTTATGACCTATA`	*cgeE* anti-sense strand -1

[1] Indicates the 3'end position of the primer relative to the first nucleotide of the coding sequence