SUPPLEMENTAL INFORMATION

The SpoVA membrane complex is required for dipicolinic acid import during sporulation and export during germination

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Supplemental Methods

Plasmid constructions

pCB179 [*yhdG::PVA(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing promoter region of *spoVA* promoter(*PVA*), (primers oCB55 and oCB110 and *B. subtilis* 168 gDNA) and pCB33 cut with EcoRI and HindIII. pCB33 is a double crossover integration vector at the *yhdG* locus with a *spec* cassette (laboratory stock).

pYG01 [*yhdG::PVA-spoVAA(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing the *PVA-spoVAA* cassette (primers oCB55 and oYG28 and *B. subtilis 168* gDNA) and pCB33 cut with EcoRI and XhoI. pCB33 is a double crossover integration vector at the *yhdG* locus with a *spec* cassette (laboratory stock).

pYG21 [*yhdG::PVA-optRBS-spoVAB(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing *optRBS-spoVAB*, (primers oYG55 and oYG30 and *B. subtilis 168* gDNA) and pCB179 cut with HindIII and XhoI.

pYG24 [*yhdG::PVA-optRBS-spoVAC(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing *optRBS-spoVAC*, (primers oYG61 and oYG32 and *B. subtilis 168* gDNA) and pCB179 cut with HindIII and XhoI.

pYG04 [*yhdG::PVA-spoVAD(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing the *spoVAD* (primers oYG322 and oYG323 and *B. subtilis 168* gDNA) and pCB179 cut with HindIII and XhoI.

pYG22 [*yhdG::PVA-optRBS-spoVAEb(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing *optRBS-spoVAEb* (primers oYG56 and oYG34 and *B. subtilis 168* gDNA) and pCB179 cut with HindIII and XhoI.

pYG06 [*yhdG::PVA-spoVAEa(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing *spoVAEa (*primers oYG35 and oYG36 and *B. subtilis 168* gDNA) and pCB179 cut with SpeI and XhoI.

pYG07 [*yhdG::PVA-spoVAF(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing *spoVAF* (primers oYG56 and oYG34 and *B. subtilis 168* gDNA) and pCB179 cut with SpeI and XhoI.

pCB175 [*yhdG::PVA-spoVAC-spoVAD-spoVAEb(spec)(amp)*] was constructed in a 2-way ligation with a PCR product containing *spoVAC-spoVAD-spoVAEb* (primers oCB106 and oCB107 and *B. subtilis 168* gDNA) and pCB179 cut with SpeI and XhoI.

pYG30 [*yhdG::PVA-optRBS-spoVAA-His8(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing the *optRBS-spoVAA-His8* and pCB179. Both were cut with HindIII and XhoI. The PCR product was first amplified with oYG72 and oYG21 using gDNA of *B. subtilis 168*, and subsequently the PCR product was used as a template with primers oYG72 and oYG73 to generate *optRBS-spoVAA-His8.*

pYG25 [*yhdG::PVA-optRBS-His8-spoVAC(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing *optRBS-His8-spoVAC* and pCB179. Both of them were cut with HindIII and XhoI. The PCR product was first amplified with primers oYG45 and oYG32 using gDNA of *B. subtilis 168,* and subsequently the PCR product was used as a template with primers oYG62 and oYG32 to generate *optRBS-His8-spoVAC.*

pYG11 [*yhdG::PVA-spoVAD-His8(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *spoVAD-His8* amplified with primers oYG09 and oYG24 and gDNA of *B. subtilis 168* as template, and pCB179 amplified with primers oYG19 and oYG20.

pYG27 [*yhdG::PVA-optRBS-spoVAEb-(GGS)3-His8(spec) (amp)*] was constructed in a 2-way ligation with a PCR product containing *optRBS-spoVAEb* and pCB179. Both were cut with HindIII and XhoI. The PCR product was first amplified with primers oYG56 and oYG64 using gDNA of *B. subtilis 168* as template, and subsequently the PCR product was used as a template with primers oYG56 and oYG65 to generate *optRBS-His8-spoVAC*.

pYG13 [*yhdG::PVA-spoVAEa-His8(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *spoVAEa-His8* amplified with primers oYG13 and oYG26 using gDNA of *B. subtilis 168* as template, and pCB179 amplified with primers oYG19 and oYG20.

pYG14 [*yhdG::PVA-spoVAF-His8(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *spoVAF-His8* amplified with primers oYG15 and oYG27 and *B. subtilis 168* gDNA as template and plasmid pCB179 amplified with primers oYG19 and oYG20.

pYG48 [*ycgO::PVA-optRBS-spoVAC(cat) (amp)*] was constructed in a 2-way ligation with a PCR product containing *PVA-optRBS-spoVAC,* amplified from plasmid pYG24 with primers oCB55 and oYG32, and pCB42 a double crossover integration vector at the *ycgO* locus with a *cat* cassette (laboratory stock). Both were cut with EcoRI and XhoI.

pYG47 [*ycgO::PVA-optRBS-spoVAEb(cat) (amp)*] was constructed in a 2-way ligation with a PCR product containing *PVA-optRBS-spoVAEb,* amplified from plasmid pYG22 with primers oCB55 and oYG34 and pCB42 cut with EcoRI and XhoI.

pYG50 [*yhdG::PVA-optRBS-gfp-(GGS)3-spoVAC(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *gfp* amplified with primers

oYG114 and oYG115 using plasmid pHCL132 (laboratory stock) and pYG25 amplified with primers oYG112 and oYG113.

pYG49 [*yhdG::PVA-optRBS-spoVAEb-(GGS)3-gfp(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *gfp* amplified with primers oYG110 and oYG111 using plasmid pHCL132 (laboratory stock), and pYG27 amplified with primers oYG64 and oYG109.

pYG103 [*yhdG::PVA-spoVAD-gfp(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *gfp* amplified with primers oYG110 and oYG111 using plasmid pHCL132 (laboratory stock) and pYG11 amplified with primers oYG275 and oYG109.

pYG75 [*ycgO::PVA-optRBS-spoVAC(K11E)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG176 and oYG177, and pYG48 as template.

pYG100 [*ycgO::PVA-optRBS-spoVAC(N7A K11E)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG269 and oYG270, and pYG75 as template.

pYG101 [*ycgO::PVA-optRBS-spoVAC(N7A K11E Q16A)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG271 and oYG272, and pYG100 as template.

pYG102 [*ycgO::PVA-optRBS-spoVAC(N7A K11E Q16A Y15A)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG273 and oYG274, and pYG101 as template.

pYG121 [*ycgO::PVA-optRBS-spoVAC(Y8A)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG325 and oYG326, and pYG48 as template.

pYG123 [*ycgO::PVA-optRBS-spoVAC(C36A)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG329 and oYG330, and pYG48 as template.

pYG124 [*ycgO::PVA-optRBS-spoVAC(Q40A)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG331 and oYG332, and pYG48 as template.

pYG127 [*ycgO::PVA-optRBS-spoVAC(N98A)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG337 and oYG338, and pYG48 as template.

pYG158 [*yhdG::PVA-spoVAD(I242A)(spec) (amp)*] was constructed by site-directed mutagenesis using primers oYG399 and oYG400, and pYG04 as template.

pYG160 [*yhdG::PVA-spoVAD(I242A D245A)(spec) (amp)*] was constructed by site-directed mutagenesis using primers oYG403 and oYG404, and pYG04 as template.

pYG176 [*yhdG::PVA-spoVAD(I242A D245A T160A)(spec) (amp)*] was constructed by site-directed mutagenesis using primers oYG405 and oYG406, and pYG160 as template.

pYG169 [*yhdG::PVA-spoVAD(I242A D245A T160A D200A)(spec) (amp)*] was constructed by site-directed mutagenesis using primers oYG407 and oYG408, and pYG176 as template.

pYG180 [*yhdG::PVA-spoVAD(I242A D245A T160A)-gfp(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *gfp* amplified with primers oYG110 and oYG111 using plasmid pHCL132 (laboratory stock) and pYG176 amplified with primers oYG275 and oYG109.

pYG172 [*yhdG::PVA-spoVAD(I242A D245A T160A D200A)-gfp(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *gfp* amplified with primers oYG110 and oYG111 using plasmid pHCL132 (laboratory stock) and pYG169 amplified with primers oYG275 and oYG109.

pYG250 [*yhdG::PVA-spoVAD(E152A)(spec) (amp)*] was constructed by site-directed mutagenesis using primers oYG584 and oYG584, and pYG04 as template.

pYG251 [*yhdG::PVA-spoVAD(Y153A)(spec) (amp)*] was constructed by site-directed mutagenesis using primers oYG585 and oYG586, and pYG04 as template.

pYG252 [*yhdG::PVA-spoVAD(E152A Y153A)(spec) (amp)*] was constructed by site-directed mutagenesis using primers oYG587 and oYG588, and pYG04 as template.

pYG256 [*yhdG::PVA-spoVAD(E152A)-gfp(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *gfp* amplified with primers oYG110 and oYG111 using plasmid pHCL132(laboratory stock), and plasmid backbone was derived from pYG250 with primers oYG275 and oYG109.

pYG258 [*yhdG::PVA-spoVAD(E152A Y153A)-gfp(spec) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *gfp* amplified with primers oYG110 and oYG111 using plasmid pHCL132(laboratory stock), and plasmid backbone was derived from pYG252 with primers oYG275 and oYG109.

pYG286 [*ycgO::PVA-optRBS-spoVAC(D79A)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG627 and oYG628, and pYG48 as template.

pYG290 [*ycgO::PVA-optRBS-spoVAC(D79A L123A)(cat) (amp)*] was constructed by site-directed mutagenesis using primers oYG633 and oYG634, and pYG286 as template.

pYG227 [*yvbJ::PsspB-optRBS-mScarlett(kan) (amp)*] was constructed in a 2-way isothermal assembly reaction with a PCR product containing *PsspB-optRBS-mScarlett* amplified with

primers oYG552 and oYG523 using plasmid pCB142 (laboratory stock), and pCB47[*yvbJ::kan*] (laboratory stock) amplified with primers oYG292 and oYG293.

pYG82 [*spoVAEb1-proC His-SUMO-FLAG-spoVAC1 (amp)*] and **pYG239 [*spoVAEb1-proC His-SUMO-spoVAC1(No FLAG) (amp)*]** were constructed in 2 steps: (1) *spoVAEb-1* was amplified with primers oYG194 and oYG195 using the *Bacillus cereus* ATCC 14579 gDNA as template, and pLA73(*MCS-proC His-SUMO-FLAG-MCS*) (Taguchi et al. 2019) amplified with primers oYG190 and oYG191 to generate pYG82-1. (2A) pYG82 was constructed in a 2-way isothermal assembly reaction with PCR product containing *spoVAC-1* amplified with primers oYG196 and oYG197 using the *Bacillus cereus* ATCC 14579 gDNA as template and pYG82-1 amplified with primers oYG276 and oYG193. (2B) pYG239 was also generated in a 2-way isothermal assembly reaction with PCR product containing *spoVAC-1* amplified with primers oYG564 and oYG197 using the *Bacillus cereus* ATCC 14579 gDNA as template and pYG82-1 amplified with primers oYG563 and oYG193.

pYG241 [*His8-spoVAB-1 spoVAD1-His8 (kan)*] was constructed in 2 steps: (1) *spoVAD-1* was amplified with primers oYG203 and oYG204 using the *Bacillus cereus* ATCC 14579 gDNA as template and pCOLADuet-1 (Novagen) amplified with primers oYG200 and oYG167 to generate pYG241-1. (2) pYG82 was constructed in a 2-way isothermal assembly reaction with PCR product containing *spoVAB-1* amplified with primers oYG567 and oYG202 using the *Bacillus cereus* ATCC 14579 gDNA as template and pYG241-1 amplified with primers oYG566 and oYG199.

The sequence of all plasmids was confirmed by Sanger sequencing.

Supplemental Table 1. Strains used in this study

Strains	Genotype	Source	Figures
bDR2414	*Wild-type Bacillus subtilis 168(trpC2)*	(Zeigler et al. 2008)	1, 2, 3, 4, 5, 6, S1, S2, S4, S9, S11, S12, S13, S14, S15, S16, S17, S18, S19
bAM984	Δ*sleB::erm*	(Koo et al. 2017)	
bDR3487	Δ*sleB::lox72*	This work	1C, S3B, S2
bYG793	Δ*gerA::cat*	This work	1B, S1, S9, S18
bAM786	Δ*gerAB::erm*	(Koo et al. 2017)	S12
bDR3871	Δ*sleB::lox72* Δ*gerAB::erm*	This work	1B, 1C, S2
bYG17	Δ*sleB::lox72* Δ*spoVFA::lox72*	This work	
bYG1013	Δ*sleB::lox72* Δ*spoVFA::lox72* Δ*gerAB::erm*	This work	1C
bYG03	Δ*spoVAA::lox72*	This work	1B, S3A
bYG04	Δ*spoVAB::lox72*	This work	1B, 5, S1, S3A, S13, S16
bYG05	Δ*spoVAC::lox72*	This work	1B, 4, S3A, 4, S7, S9
bYG06	Δ*spoVAD::lox72*	This work	4, 1B, S3A, S7
bYG07	Δ*spoVAEb::lox72*	This work	1B, S3A, S7
bYG08	Δ*spoVAEa::lox72*	This work	1B
bYG09	Δ*spoVAF::lox72*	This work	1B
bDR4019	Δ*spoVA::tet yhdG::PVA-spoVAC-spoVAD-spoVAEb(spec)*	This work	1B, S1
bYG37	Δ*spoVAA::lox72 yhdG::PVA-spoVAA(spec)*	This work	S3A, S4A
bYG58	Δ*spoVAB::lox72 yhdG::PVA-spoVAB(spec)*	This work	S3A
bYG60	Δ*spoVAC::lox72 yhdG::PVA-spoVAC(spec)*	This work	S3A, S4A
bYG40	Δ*spoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	4, 5, 6, S3A, S4A, S9, S11, S13, S16, S19, S15, S14
bYG59	Δ*spoVAEb::lox72 yhdG::PVA-spoVAEb(spec)*	This work	S3A, S4A
bYG42	Δ*spoVAEa::lox72 yhdG::PVA-spoVAEa(spec)*	This work	S4A
bYG43	Δ*spoVAF::lox72 yhdG::PVA-spoVAF(spec)*	This work	S4A
bYG138	Δ*spoVAA::lox72 yhdG::PVA-spoVAA-His8(spec)*	This work	S4A
bYG61	Δ*spoVAC::lox72 yhdG::PVA-His8-spoVAC(spec)*	This work	S4A
bYG54	Δ*spoVAD::lox72 yhdG::PVA-spoVAD-His8(spec)*	This work	S4A
bYG63	Δ*spoVAEb::lox72 yhdG::PVA-spoVAEb-His8(spec)*	This work	S4A
bYG56	Δ*spoVAEa::lox72 yhdG::PVA-spoVAEa-His8(spec)*	This work	S4A
bYG57	Δ*spoVAF::lox72 yhdG::PVA-spoVAF-His8(spec)*	This work	S4A
bYG10	Δ*sleB::lox72* Δ*spoVAA::lox72*	This work	S3B, S2, S4B
bYG11	Δ*sleB::lox72* Δ*spoVAB::lox72*	This work	S3B, S2
bYG12	Δ*sleB::lox72* Δ*spoVAC::lox72*	This work	S3B, S2, S4B
bYG13	Δ*sleB::lox72* Δ*spoVAD::lox72*	This work	S3B, S2, S4B
bYG14	Δ*sleB::lox72* Δ*spoVAEb::lox72*	This work	S3B, S2, S4B
bYG15	Δ*sleB::lox72* Δ*spoVAEa::lox72*	This work	S3B, S2, S4B
bYG16	Δ*sleB::lox72* Δ*spoVAF::lox72*	This work	S3B, S2, S4B
bDR3970	Δ*sleB::lox72* Δ*spoVA::tet*	This work	
bYG142	Δ*sleB::lox72* Δ*spoVAA::lox72 yhdG::PVA-spoVAA(spec)*	This work	S3B
bYG1014	Δ*sleB::lox72* Δ*spoVAB::lox72 yhdG::PVA-spoVAB(spec)*	This work	S3B
bYG261	Δ*sleB::lox72* Δ*spoVAC::lox72 yhdG::PVA-spoVAC(spec)*	This work	S3B
bYG279	Δ*sleB::lox72* Δ*spoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	S3B
bYG297	Δ*sleB::lox72* Δ*spoVAEb::lox72 yhdG::PVA-spoVAEb(spec)*	This work	S3B
bDR4023	Δ*sleB::lox72* Δ*spoVA::tet yhdG::PVA-spoVAC-AD-AEb(spec)*	This work	S2
bYG872	Δ*gerA::cat* Δ*spoVAA::lox72*	This work	1B, 1C
bYG873	Δ*gerA::cat* Δ*spoVAB::lox72*	This work	1B, 1C, S1
bYG874	Δ*gerA::cat* Δ*spoVAC::lox72*	This work	1B, 1C
bYG875	Δ*gerA::cat* Δ*spoVAD::lox72*	This work	1B, 1C

bYG876	*ΔgerA::cat ΔspoVAEb::lox72*	This work	1B, 1C
bYG877	*ΔgerA::cat ΔspoVAEa::lox72*	This work	1B, 1C
bYG878	*ΔgerA::cat ΔspoVAF::lox72*	This work	1B, 1C
bYG895	*ΔgerA::cat ΔspoVA::tet yhdG::PVA-spoVAC-AD-AEb(spec)*	This work	1B, 1C, S1
bYG921	*ΔsleB::erm ΔgerA::cat ΔspoVAA::lox72*	This work	1B, S2
bYG922	*ΔsleB::erm ΔgerA::cat ΔspoVAB::lox72*	This work	1B, S2
bYG923	*ΔsleB::erm ΔgerA::cat ΔspoVAC::lox72*	This work	1B, S2
bYG924	*ΔsleB::erm ΔgerA::cat ΔspoVAD::lox72*	This work	1B, S2
bYG925	*ΔsleB::erm ΔgerA::cat ΔspoVAEb::lox72*	This work	1B, S2
bYG926	*ΔsleB::erm ΔgerA::cat ΔspoVAEa::lox72*	This work	1B, S2
bYG927	*ΔsleB::erm ΔgerA::cat ΔspoVAF::lox72*	This work	1B, S2
bYG1015	*ΔsleB::lox72 ΔgerA::cat ΔspoVA::tet yhdG::PVA-spoVAC-AD-AEb(spec)*	This work	1B, S2
bYG199	*ΔsleB::lox72 ΔspoVAA::lox72 yhdG::PVA-spoVAA-His8(spec)*	This work	2A, S4B, S4C
bYG198	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-spoVAA-His8(spec)*	This work	2A, S4C
bYG200	*ΔsleB::lox72 ΔspoVAB::lox72 yhdG::PVA-spoVAA-His8(spec)*	This work	S4C
bYG201	*ΔsleB::lox72 ΔspoVAC::lox72 yhdG::PVA-spoVAA-His8(spec)*	This work	S4C
bYG202	*ΔsleB::lox72 ΔspoVAD::lox72 yhdG::PVA-spoVAA-His8(spec)*	This work	S4C
bYG203	*ΔsleB::lox72 ΔspoVAEb::lox72 yhdG::PVA-spoVAA-His8(spec)*	This work	S4C
bYG204	*ΔsleB::lox72 ΔspoVAEa::lox72 yhdG::PVA-spoVAA-His8(spec)*	This work	S4C
bYG205	*ΔsleB::lox72 ΔspoVAF::lox72 yhdG::PVA-spoVAA-His8(spec)*	This work	S4C
bYG110	*ΔsleB::lox72 ΔspoVAC::lox72 yhdG::PVA-His8-spoVAC(spec)*	This work	2A, 2B, 2C, S4B
bYG109	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-His8-spoVAC(spec)*	This work	2A, 2B, 2C
bYG134	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-His8-spoVAC(spec) ycgO::PVA-spoVAEb(cat)*	This work	2C
bYG118	*ΔsleB::lox72 ΔspoVAA::lox72 yhdG::PVA-His8-spoVAC(spec)*	This work	2B
bYG119	*ΔsleB::lox72 ΔspoVAB::lox72 yhdG::PVA-His8-spoVAC(spec)*	This work	2B
bYG120	*ΔsleB::lox72 ΔspoVAD::lox72 yhdG::PVA-His8-spoVAC(spec)*	This work	2B
bYG121	*ΔsleB::lox72 ΔspoVAEb::lox72 yhdG::PVA-His8-spoVAC(spec)*	This work	2B
bYG122	*ΔsleB::lox72 ΔspoVAEa::lox72 yhdG::PVA-His8-spoVAC(spec)*	This work	2B
bYG123	*ΔsleB::lox72 ΔspoVAF::lox72 yhdG::PVA-His8-spoVAC(spec)*	This work	2B
bYG112	*ΔsleB::lox72 ΔspoVAD::lox72 yhdG::PVA-spoVAD-His8(spec)*	This work	2A, S4B
bYG111	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-spoVAD-His8(spec)*	This work	2A
bYG114	*ΔsleB::lox72 ΔspoVAEb::lox72 yhdG::PVA-spoVAEb-His8(spec)*	This work	2A, 2B, 2C, S4B
bYG113	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-spoVAEb-His8(spec)*	This work	2A, 2B, 2C
bYG133	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-spoVAEb-His8(spec) ycgO::PVA-spoVAC(cat)*	This work	2C
bYG127	*ΔsleB::lox72 ΔspoVAA::lox72 yhdG::PVA-spoVAEb-His8(spec)*	This work	2B
bYG128	*ΔsleB::lox72 ΔspoVAB::lox72 yhdG::PVA-spoVAEb-His8(spec)*	This work	2B
bYG129	*ΔsleB::lox72 ΔspoVAC::lox72 yhdG::PVA-spoVAEb-His8(spec)*	This work	2B
bYG130	*ΔsleB::lox72 ΔspoVAD::lox72 yhdG::PVA-spoVAEb-His8(spec)*	This work	2B
bYG131	*ΔsleB::lox72 ΔspoVAEa::lox72 yhdG::PVA-spoVAEb-His8(spec)*	This work	2B
bYG132	*ΔsleB::lox72 ΔspoVAF::lox72 yhdG::PVA-spoVAEb-His8(spec)*	This work	2B
bYG116	*ΔsleB::lox72 ΔspoVAEa::lox72 yhdG::PVA-spoVAEa-His8(spec)*	This work	2A, S4B
bYG115	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-spoVAEa-His8(spec)*	This work	2A
bYG220	*ΔsleB::lox72 ΔspoVAF::lox72 yhdG::PVA-spoVAF-His8(spec)*	This work	2A, S4B
bYG221	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-spoVAF-His8(spec)*	This work	2A
bYG183	*ΔspoVAC::lox72 yhdG::PVA-gfp-spoVAC(spec)*	This work	S7
bYG223	*ΔspoVAEb::lox72 yhdG::PVA-spoVAEb-gfp(spec)*	This work	S7
bYG256	*ΔspoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	S7
bYG185	*ΔsleB::lox72 ΔspoVAC::lox72 yhdG::PVA-gfp-spoVAC(spec)*	This work	3
bYG184	*ΔsleB::lox72 ΔspoVAEb::lox72 yhdG::PVA-spoVAEb-gfp(spec)*	This work	3, 6, S20
bYG265	*ΔsleB::lox72 ΔspoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	3, 4, 6, S10, S20
bYG269	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-spoVAD-gfp(spec)*	This work	3
bYG262	*ΔsleB::lox72 ΔspoVAA::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	3
bYG263	*ΔsleB::lox72 ΔspoVAB::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	3, 4, S10
bYG264	*ΔsleB::lox72 ΔspoVAC::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	3, S10
bYG266	*ΔsleB::lox72 ΔspoVAEb::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	3

bYG267	*ΔsleB::lox72 ΔspoVAEa::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	3
bYG268	*ΔsleB::lox72 ΔspoVAF::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	3
bYG436	*ΔsleB::lox72 ΔspoVFA::erm ΔspoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	3
bYG231	*ΔspoVAC::lox72 ycgO::PVA-spoVAC(cat)*	This work	4
bYG232	*ΔspoVAC::lox72 ycgO::PVA-spoVAC(K11E)(cat)*	This work	4, S9
bYG240	*ΔspoVAC::lox72 ycgO::PVA-spoVAC(N7A K11E)(cat)*	This work	4, S9
bYG241	*ΔspoVAC::lox72 ycgO::PVA-spoVAC(N7A K11E Q16A)(cat)*	This work	4, S9
bYG242	*ΔspoVAC::lox72 ycgO::PVA-spoVAC(N7A K11E Q16A Y15A)(cat)*	This work	4, S9
bYG320	*ΔspoVAC::lox72 ycgO::PVA-spoVAC(Y8A)(cat)*	This work	4, S9
bYG326	*ΔspoVAC::lox72 ycgO::PVA-spoVAC(C36A)(cat)*	This work	S9
bYG324	*ΔspoVAC::lox72 ycgO::PVA-spoVAC(Q40A)(cat)*	This work	S9
bYG322	*ΔspoVAC::lox72 ycgO::PVA-spoVAC(N98A)(cat)*	This work	4, S9
bYG514	*spoVAC(N7A K11E Q16A Y15A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	5, S13, S16, S15, S14
bYG515	*spoVAC(Y8A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	5, S11, S13, S16, S15, S14
bYG651	*spoVAC(C36A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	S11
bYG652	*spoVAC(Q40A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	S11
bYG653	*spoVAC(N98A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	5, S11, S13, S16, S15, S14
bYG424	*ΔsleB::lox72 spoVAC(N7A K11E Q16A Y15A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	4, S10
bYG481	*ΔsleB::lox72 spoVAC(Y8A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	4, S10, S11
bYG663	*ΔsleB::lox72 spoVAC(C36A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	S11
bYG664	*ΔsleB::lox72 spoVAC(Q40A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	S11
bYG665	*ΔsleB::lox72 spoVAC(N98A)-spoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	4, S11
bYG487	*ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A)(spec)*	This work	4, S9
bYG489	*ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A D245A)(spec)*	This work	4, S9
bYG549	*ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A D245A T160A)(spec)*	This work	4, 5, S9, S13
bYG520	*ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A D245A T160A D200A)(spec)*	This work	4, 5, S9, S13, S14
bYG558	*ΔsleB::lox72 ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A D245A T160A)-gfp(spec)*	This work	S10
bYG553	*ΔsleB::lox72 ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A D245A T160A D200A)-gfp(spec)*	This work	4, S10
bYG959	*ΔgerAB::erm ΔspoVAC::lox72 ycgO::PVA-spoVAC(K11E)(cat)*	This work	S9
bYG960	*ΔgerAB::erm ΔspoVAC::lox72 ycgO::PVA-spoVAC(N7A K11E)(cat)*	This work	S9
bYG961	*ΔgerAB::erm ΔspoVAC::lox72 ycgO::PVA-spoVAC(N7A K11E Q16A)(cat)*	This work	S9
bYG962	*ΔgerAB::erm ΔspoVAC::lox72 ycgO::PVA-spoVAC(N7A K11E Q16A Y15A)(cat)*	This work	S9
bYG963	*ΔgerAB::erm ΔspoVAC::lox72 ycgO::PVA-spoVAC(Y8A)(cat)*	This work	S9
bYG964	*ΔgerAB::erm ΔspoVAC::lox72 ycgO::PVA-spoVAC(C36A)(cat)*	This work	S9
bYG965	*ΔgerAB::erm ΔspoVAC::lox72 ycgO::PVA-spoVAC(Q40A)(cat)*	This work	S9
bYG966	*ΔgerAB::erm ΔspoVAC::lox72 ycgO::PVA-spoVAC(N98A)(cat)*	This work	S9
bYG967	*ΔgerA::cat ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	S9, S18, S19
bYG968	*ΔgerA::cat ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A)(spec)*	This work	S9
bYG969	*ΔgerA::cat ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A D245A)(spec)*	This work	S9
bYG970	*ΔgerA::cat ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A D245A T160A)(spec)*	This work	S9
bYG971	*ΔgerA::cat ΔspoVAD::lox72 yhdG::PVA-spoVAD(I242A D245A T160A D200A)(spec)*	This work	S9

bYG637	*ΔgerAB::erm spoVAC(N7A K11E Q16A Y15A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	S12, S18, S19
bYG635	*ΔgerAB::erm spoVAC(Y8A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	S12, S18
bYG616	*ΔgerAB::erm ΔspoVAB::lox72*	This work	S12
bYG811	*ΔspoVAD::lox72 yhdG::PVA-spoVAD(E152A)(spec)*	This work	6, S17, S19
bYG813	*ΔspoVAD::lox72 yhdG::PVA-spoVAD(Y153A)(spec)*	This work	6, S17, S19
bYG814	*ΔspoVAD::lox72 yhdG::PVA-spoVAD(E152A Y153A)(spec)*	This work	6, S17
bYG973	*ΔgerA::cat ΔspoVAD::lox72 yhdG::PVA-spoVAD(E152A)(spec)*	This work	S18
bYG974	*ΔgerA::cat ΔspoVAD::lox72 yhdG::PVA-spoVAD(Y153A)(spec)*	This work	S18
bYG975	*ΔgerA::cat ΔspoVAD::lox72 yhdG::PVA-spoVAD(E152A Y153A)(spec)*	This work	S18
bYG991	*spoVAC(D79A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	S17
bYG1016	*spoVAC(L123A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	S17
bYG935	*spoVAC(D79A L123A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	6, S17
bYG992	*spoVAC(D79A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(E152A)(spec)*	This work	S17
bYG993	*spoVAC(D79A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(Y153A)(spec)*	This work	S17
bYG1011	*spoVAC(L123A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(E152A)(spec)*	This work	6, S17
bYG1012	*spoVAC(L123A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(Y153A)(spec)*	This work	6, S17
bYG828	*ΔsleB::lox72 ΔspoVAD::lox72 yhdG::PVA-spoVAD(E152A Y153A)-gfp(spec)*	This work	6
bYG947	*ΔsleB::lox72 spoVAC(D79A L123A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec)*	This work	6
bYG1017	*ΔsleB::lox72 spoVAC(L123A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(E152A)-gfp(spec)*	This work	6
bYG670	*ΔsleB::lox72 ΔspoVAD::lox72 yhdG::PVA-spoVAD-gfp(spec) yvbJ::PsspB-mScarlett(kan)*	This work	S7
bYG671	*ΔsleB::lox72 ΔspoVA::tet yhdG::PVA-spoVAD-gfp(spec) yvbJ::PsspB-mScarlett(kan)*	This work	S7
bDR3146	*ΔspmA::erm*	(Koo et al. 2017)	S14
bDR3209	*ΔgerAB::lox72 ΔspoVV::lox72*	(Ramírez-Guadiana et al. 2017)	S18
bYG1155	*ΔgerA::cat spoVAC(D79A L123A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(spec)*	This work	S18, S19
bYG1153	*ΔgerA::cat spoVAC(L123A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(E152A)(spec)*	This work	S18, S19
bYG1154	*ΔgerA::cat spoVAC(L123A)-ΔspoVAD::lox72 yhdG::PVA-spoVAD(Y153A)(spec)*	This work	S18, S19

Supplemental Table 2. Plasmids used in this study.

Plasmid	Genotype	Source
pLA73	*MCS-proC His-SUMO-FLAG-MCS (amp)*	(Taguchi et al. 2019)
pETDuet-1	COLA replicon (*kan)*	Novagen
pAM174	arabinose-inducible Ulp1[L403-K621] protease *(cat)*	(Meeske et al. 2016)
pCB179	*yhdG::PVA(spec) (amp)*	This work
pYG01	*yhdG::PVA-spoVAA(spec) (amp)*	This work
pYG21	*yhdG::PVA-optRBS-spoVAB(spec) (amp)*	This work
pYG24	*yhdG::PVA-optRBS-spoVAC(spec) (amp)*	This work
pYG04	*yhdG::PVA-spoVAD(spec) (amp)*	This work
pYG22	*yhdG::PVA-optRBS-spoVAEb(spec) (amp)*	This work
pYG06	*yhdG::PVA-spoVAEa(spec) (amp)*	This work
pYG07	*yhdG::PVA-spoVAF(spec) (amp)*	This work
pCB175	*yhdG::PVA-spoVAC-spoVAD-spoVAEb(spec) (amp)*	This work
pYG30	*yhdG::PVA-optRBS-spoVAA-His8(spec) (amp)*	This work
pYG25	*yhdG::PVA-optRBS-His8-spoVAC(spec) (amp)*	This work
pYG11	*yhdG::PVA-spoVAD-His8(spec) (amp)*	This work
pYG27	*yhdG::PVA-optRBS-spoVAEb-(GGS)3-His8(spec) (amp)*	This work
pYG13	*yhdG::PVA-spoVAEa-His8(spec) (amp)*	This work
pYG14	*yhdG::PVA-spoVAF-His8(spec) (amp)*	This work
pYG48	*ycgO::PVA-optRBS-spoVAC(cat) (amp)*	This work
pYG47	*ycgO::PVA-optRBS-spoVAEb(cat) (amp)*	This work
pYG50	*yhdG::PVA-optRBS-gfp-(GGS)3-spoVAC(spec) (amp)*	This work
pYG49	*yhdG::PVA-optRBS-spoVAEb-(GGS)3-gfp(spec) (amp)*	This work
pYG103	*yhdG::PVA-spoVAD-gfp(spec) (amp)*	This work
pYG75	*ycgO::PVA-optRBS-spoVAC(K11E)(cat) (amp)*	This work
pYG100	*ycgO::PVA-optRBS-spoVAC(N7A K11E)(cat) (amp)*	This work
pYG101	*ycgO::PVA-optRBS-spoVAC(N7A K11E Q16A)(cat) (amp)*	This work
pYG102	*ycgO::PVA-optRBS-spoVAC(N7A K11E Q16A Y15A)(cat) (amp)*	This work
pYG121	*ycgO::PVA-optRBS-spoVAC(Y8A)(cat) (amp)*	This work
pYG123	*ycgO::PVA-optRBS-spoVAC(C36A)(cat) (amp)*	This work
pYG124	*ycgO::PVA-optRBS-spoVAC(Q40A)(cat) (amp)*	This work
pYG127	*ycgO::PVA-optRBS-spoVAC(N98A)(cat) (amp)*	This work
pYG158	*yhdG::PVA-spoVAD(I242A)(spec) (amp)*	This work
pYG160	*yhdG::PVA-spoVAD(I242A D245A)(spec) (amp)*	This work
pYG176	*yhdG::PVA-spoVAD(I242A D245A T160A)(spec) (amp)*	This work
pYG169	*yhdG::PVA-spoVAD(I242A D245A T160A D200A)(spec) (amp)*	This work
pYG180	*yhdG::PVA-spoVAD(I242A D245A T160A)-gfp(spec) (amp)*	This work
pYG172	*yhdG::PVA-spoVAD(I242A D245A T160A D200A)-gfp(spec) (amp)*	This work
pYG250	*yhdG::PVA-spoVAD(E152A)(spec) (amp)*	This work
pYG251	*yhdG::PVA-spoVAD(Y153A)(spec) (amp)*	This work
pYG252	*yhdG::PVA-spoVAD(E152A Y153A)(spec) (amp)*	This work
pYG256	*yhdG::PVA-spoVAD(E152A)-gfp(spec) (amp)*	This work
pYG258	*yhdG::PVA-spoVAD(E152A Y153A)-gfp(spec) (amp)*	This work
pYG286	*ycgO::PVA-optRBS-spoVAC(D79A)(cat) (amp)*	This work
pYG290	*ycgO::PVA-optRBS-spoVAC(D79A L123A)(cat) (amp)*	This work
pYG227	*yvbJ::PsspB-optRBS-mScarlett(kan) (amp)*	This work
pYG82-1	*spoVAEb1-proC (amp)*	This work
pYG82	*spoVAEb1-proC His-SUMO-FLAG-spoVAC1 (amp)*	This work
pYG239	*spoVAEb1-proC His-SUMO-spoVAC1(No FLAG) (amp)*	This work
pYG241-1	*spoVAD1-His8 (kan)*	This work
pYG241	*His8-spoVAB-1 spoVAD1-His8 (kan)*	This work

Supplemental Table 3. Oligonucleotides used in this study.

oligos	sequence	use
oCB106	cggACTAGttaaggaggtttcaagatg	pCB175
oCB107	ggcCTCGAGattatcctttcggtttgaa	pCB175
oCB110	CGCAAGCTTtcggtggttatatatgta	pCB179
oCB55	GCCGAATTCGccagacacagtccgagg	pCB179, pYG01
oFR5	TGAATGGTTTCTTTATTAGGC	bYG793
oFR6	CTGAGCGAGGGAGCAGAACAATGAGGTCACCTCTTATC	bYG793
oFR7	GTTGACCAGTGCTCCCTGTAGCAGCCGCCTAATTCAC	bYG793
oFR8	GTTTCGCCTCAGGGTATATG	bYG793
oJM028	TTCTGCTCCCTCGCTCA	bYG793
oJM029	CAGGGAGCACTGGTCAAC	bYG793
oYG09	ACATACtacatatataaccaccgaGATGTCATAGGAGGAGAAGAAAATG	pYG11
oYG109	GCGATTTTCGTTCGTGAATACATGT	pYG49, pYG103, pYG172, pYG180, pYG258, pYG256
oYG110	ctggcggaagcggaggatccAAGGGAGAAGAGTTGTTTACGGGT	pYG49, pYG103, pYG172, pYG180, pYG258, pYG256
oYG111	GTATTCACGAACGAAAATCGCTTaggtgctACTAGTAGAACCACCGCCT	pYG49, pYG103, pYG172, pYG180, pYG258, pYG256
oYG112	CATagtagttCCTCCTTAtgtAAGCTTtcggtg	pYG50
oYG113	ctggcggaagcggaggatccACAAACATAAAAGAAAATTACAAATCAAAAGTG	pYG50
oYG114	GCTTacaTAAGGAGGaactactATGAAGGGAGAAGAGTTGTTTACGGGT	pYG50
oYG115	ggatcctccgcttccgccagagcctccTTTATACAATTCGTCCATACCGTGCGT	pYG50
oYG13	ACATACtacatatataaccaccgaTTCAAACCGAAAGGATAATGCCGAC	pYG13
oYG15	ACATACtacatatataaccaccgaTTTATTTTAGAAAGGAGCGGGTATC	pYG14
oYG167	CATATGTATATCTCCTTCTTATACTTAACTAATATACTAAGATGGGGAATTG	pYG241-1, pYG241
oYG176	CATAAAAGAAAATTACAAATCAGAAGTGAAAACATATCAGCCTAAG	pYG75
oYG177	CTTAGGCTGATATGTTTTCACTTCTGATTTGTAATTTTCTTTTATG	pYG75
oYG19	tcggtggttatatatgtaGTATGTGGTTCGA	pYG11, pYG13, pYG14
oYG190	CATCCATGgtatatctccttcttaaagttaaac	pYG82-1, pYG82, pYG239
oYG191	ggaggctctggcggaagcggaggatccA	pYG82-1, pYG82, pYG239
oYG193	CTCGAgtctggtaaagaaaccgctgctgcga	pYG82, pYG239
oYG194	ctttaagaaggagatatacCATGGATGGATTTTATATATGCATTTCTTGTAGGT	pYG82-1, pYG82, pYG239
oYG195	ccgcttccgccagagcctccTCCTTTCGGTTTACATATAAGTGCT	pYG82-1, pYG82, pYG239
oYG196	GCggcggaagcggaggatccACAAGTCAGAAATTGAAGGATGATTAC	pYG82
oYG197	ggtttctttaccagacTCGAGTTAAGACATAAAAATTTTAAAGGTATACCT	pYG82, pYG239
oYG199	GGATCCGAATTCGAGCTCG	pYG241
oYG20	CACcatcatCACCACcatcatCACTAGGCGATTTTCGTTCGTGAATACATGT	pYG11, pYG13, pYG14
oYG200	CACcatcatCACCACcatcatCACTAGCTCGAGTCTG	pYG241-1, pYG241
oYG202	CCGAGCTCGAATTCGGATCCCTACTTTACGAAATAAATCCAGTGAAAC	pYG241

oYG203	AGTTAAGTATAAGAAGGAGATATACATatgAGGTTGACCGGAAAACAAACGT	pYG241-1, pYG241
oYG204	tgatgGTGGTGatgatgGTGCTCTCCTTTAACTCTCTCAAATACGACA	pYG241-1, pYG241
oYG21	tgatgGTGGTGatgatgGTGACGATCATGCAGATCCTTTATGGT	pYG30
oYG24	tgatgGTGGTGatgatgGTGAGATGCACCTCCTGCACGCTCA	pYG11
oYG26	tgatgGTGGTGatgatgGTGGCTGGATTCCGATAAACCCGA	pYG13
oYG269	ctATGACAAACATAAAAGAAGCTTACAAATCAGAAGTGAAAAC	pYG100
oYG27	tgatgGTGGTGatgatgGTGTGAATTGGTAGGCTGCCTTAAGCGA	pYG14
oYG270	GTTTTCACTTCTGATTTGTAAGCTTCTTTTATGTTTGTCATag	pYG100
oYG271	CAAATCAGAAGTGAAAACATATGCGCCTAAGCCGCCTTACGTCTG	pYG101
oYG272	CAGACGTAAGGCGGCTTAGGCGCATATGTTTTCACTTCTGATTTG	pYG101
oYG273	CTTACAAATCAGAAGTGAAAACAGCTGCGCCTAAGCCGCCTTACGTCTG	pYG102
oYG274	CAGACGTAAGGCGGCTTAGGCGCAGCTGTTTTCACTTCTGATTTGTAAG	pYG102
oYG275	ggatcctccgcttccgccagagcctccAGATGCACCTCCTGCACGCTCA	pYG103, pYG172, pYG180, pYG258, pYG256
oYG276	ggatcctccgcttccgccGCTAGCTCCGGATCcCCCAGGGCCT	pYG82
oYG28	cacggCTCGAGCTAACGATCATGCAGATCCTTTATG	pYG01
oYG292	GAATTCGACATCAAGAGCGGGAAGGGAGATTTG	pYG227
oYG293	CTCGAGatGCTAGCatGGATCCcagc	pYG227
oYG30	cacggCTCGAGTTATGAATGGTCAATAAAGTACAG	pYG21
oYG32	ctggcCTCGAGCTATGACATCAGTTTCTCAAAAGCA	pYG24, pYG25, pYG48
oYG322	ACtacatatataaccaccgaAAGCTTGATGTCATAGGAGGAGAAGAAAATG	pYG04
oYG323	AGGGGGATCCatGCTAGCatCTCGAGGACAAAAGCCAAAAGGTAGTCCAT	pYG04
oYG325	actATGACAAACATAAAAGAAAATGCCAAATCAAAAGTGAAAACATATCAG	pYG121
oYG326	CTGATATGTTTTCACTTTTGATTTGGCATTTTCTTTTATGTTTGTCATagt	pYG121
oYG329	CTTTTTAGTGGGCGGACTGATTGCTGCAATCGGGCAAGGTCTGCA	pYG123, bYG651, bYG663
oYG330	TGCAGACCTTGCCCGATTGCAGCAATCAGTCCGCCCACTAAAAAG	pYG123, bYG651, bYG663
oYG331	GACTGATTTGTGCAATCGGGGCAGGTCTGCAAAATTTTTATATCCA	pYG124, bYG652, bYG664
oYG332	TGGATATAAAAATTTTGCAGACCTGCCCCGATTGCACAAATCAGTC	pYG124, bYG652, bYG664
oYG337	GTACCTGTCACGGGTTTTGCCGCCAGTATGGCAAGTGCGGCTC	pYG127, bYG653, bYG665
oYG338	GAGCCGCACTTGCCATACTGGCGGCAAAACCCGTGACAGGTAC	pYG127, bYG653, bYG665
oYG34	cacggCTCGAGTTATCCTTTCGGTTTGAAAATAACA	pYG22, pYG47
oYG348	CTGTTAATTTCATTTTCTTCTCCTCCTATGACATCAGTTTCTCAAAAGCA	bYG514, bYG515, bYG424, bYG481, bYG935, bYG947
oYG349	CTTGAAACCTCCTTATGAATGGTCAATAAAGT	bYG514, bYG515, bYG424, bYG481, bYG935, bYG947
oYG35	cagcgACTAGTTTTATTTTAGAAAGGAGCGGGTATC	pYG07
oYG350	GAGGAGAAGAAAATGAAATTAACAGGAAAGCA	bYG514, bYG515, bYG424, bYG481, bYG935, bYG947
oYG36	cacggCTCGAGTTATGAATTGGTAGGCTGCCTTAAG	pYG07
oYG37	cagcgACTAGTTTCAAACCGAAAGGATAATGCCGAC	pYG06

oYG38	cagccCTCGAGTTAGCTGGATTCCGATAAACCCGA	pYG06, bYG514, bYG515, bYG651, bYG652, bYG653, bYG424, bYG481, bYG663, bYG664, bYG665, bYG991, bYG1016, bYG935, bYG947, bYG1017
oYG392	CATTCATAAGGAGGTTTCAAGATGACAAACATAAAAGAAAATGCCAAATC	bYG514, bYG515, bYG424, bYG481, bYG935, bYG947
oYG399	TGTCAGGCGTCGGTTCTCCAGCCGTAAAAGACATTTTAAAAGAAGATGG	pYG158
oYG400	CCATCTTCTTTTAAAATGTCTTTTACGGCTGGAGAACCGACGCCTGACA	pYG158
oYG403	TGTCAGGCGTCGGTTCTCCAGCCGTAAAAGCCATTTTAAAAGAAGATGGATATC	oYG160
oYG404	GATATCCATCTTCTTTTAAAATGGCTTTTACGGCTGGAGAACCGACGCCTGACA	oYG160
oYG405	ACGGAGGCCAAAAACCGGACGCTGCTACCTCCACTGTAACCGGAAG	pYG176
oYG406	CTTCCGGTTACAGTGGAGGTAGCAGCGTCCGGTTTTTGGCCTCCGT	pYG176
oYG407	GATTTAGGAATTACAGATCCTTTTGCTATGGGATCGGCTATGGCTC	pYG169
oYG408	GAGCCATAGCCGATCCCATAGCAAAAGGATCTGTAATTCCTAAATC	pYG169
oYG45	CACcatcatCACCACcatcatCACACAAACATAAAAGAAAATTACAAATC	pYG25
oYG522	TCCCGCTCTTGATGTCGAATTCctcaagatttaccacacaattctc	pYG227
oYG523	GGATCCatGCTAGCatCTCGAGGGATCCTTacttatacagttcatccatac	pYG227
oYG55	cagcgAAGCTTacaTAAGGAGGaactactATGATCGTTAGTGTATTGTTCATCA	pYG21
oYG56	cagcgAAGCTTacaTAAGGAGGaactactATGGACTACCTTTTGGCTTTTGTC	pYG22, pYG27, pYG47
oYG563	ACCACCAATCTGTTCTCTGTGAGCCT	pYG239
oYG564	GCTCACAGAGAACAGATTGGTGGTACAAGTCAGAAATTGAAGGATGATTAC	pYG239
oYG566	gatgGTGGTGatgatgGTGCATCCATGGTATATCTCCTTATTAAAGTTAAAC	pYG241
oYG567	ATGCACcatcatCACCACcatcatCACCCGATGATTGAGTCTGGATTTG	pYG241
oYG583	ACAGTTTCGCTATCCGACAGCATACGGAGGCCAAAAACCGGA	pYG250
oYG584	TCCGGTTTTTGGCCTCCGTATGCTGTCGGATAGCGAAACTGT	pYG250
oYG585	CAGTTTCGCTATCCGACAGAAGCCGGAGGCCAAAAACCGGACA	pYG251
oYG586	TGTCCGGTTTTTGGCCTCCGGCTTCTGTCGGATAGCGAAACTG	pYG251
oYG587	GACAGTTTCGCTATCCGACAGCAGCCGGAGGCCAAAAACCGGACA	pYG252
oYG588	TGTCCGGTTTTTGGCCTCCGGCTGCTGTCGGATAGCGAAACTGTC	pYG252
oYG61	cagcgAAGCTTacaTAAGGAGGaactactATGACAAACATAAAAGAAAATTACA	pYG24, pYG48
oYG62	gcgAAGCTTacaTAAGGAGGaactactATGCACcatcatCACCACcatcatCACACAAAC	pYG25
oYG627	ACAGGGTTTGGAATCTATGCCAGAATCGGACAATTCGCAGG	pYG286, bYG991
oYG628	CCTGCGAATTGTCCGATTCTGGCATAGATTCCAAACCCTGT	pYG286, bYG991
oYG633	GAGTAGCGACAAATATGTTTAAAGCGGCAGGAAATGTTATTGTTTTC	pYG290, bYG1016, bYG1017
oYG634	GAAAACAATAACATTTCCTGCCGCTTTAAACATATTTGTCGCTACTC	pYG290, bYG1016, bYG1017
oYG64	ggatcctccgcttccgccagagcctccTCCTTTCGGTTTGAAAATAACAGCT	pYG27, pYG49
oYG65	cacggCTCGAGTTAGTGatgatgGTGGTGatgatgGTGggatcctccgcttccgccagag	pYG27
oYG72	cagcgAAGCTTacaTAAGGAGGaactactATGGAACGACGAATATTTATCCGGCT	pYG30

oYG73	ctggcCTCGAGCTAGTGatgatgGTGGTGatgatgGTGACGATCATG	pYG30
oYG77	cggcctgtatggccGAATTCCCAGACACAGTCCGAGGTGGCTGA	bYG514, bYG515, bYG651, bYG652, bYG653, bYG424, bYG481, bYG663, bYG664, bYG665, bYG991, bYG1016, bYG935, bYG947, bYG1017

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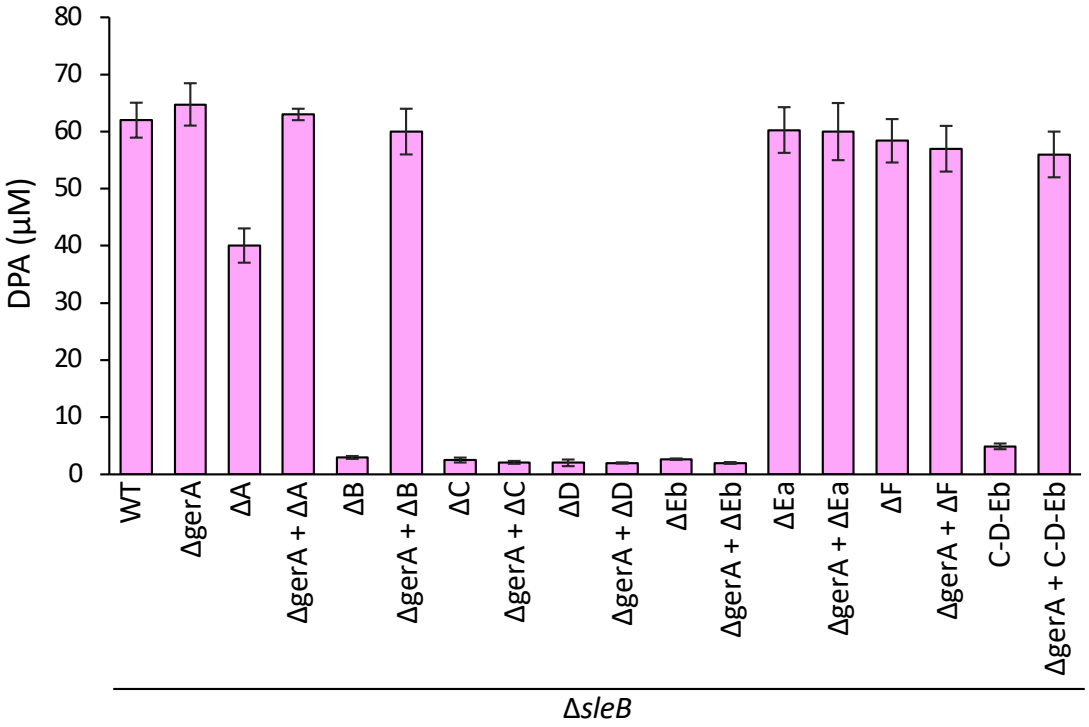
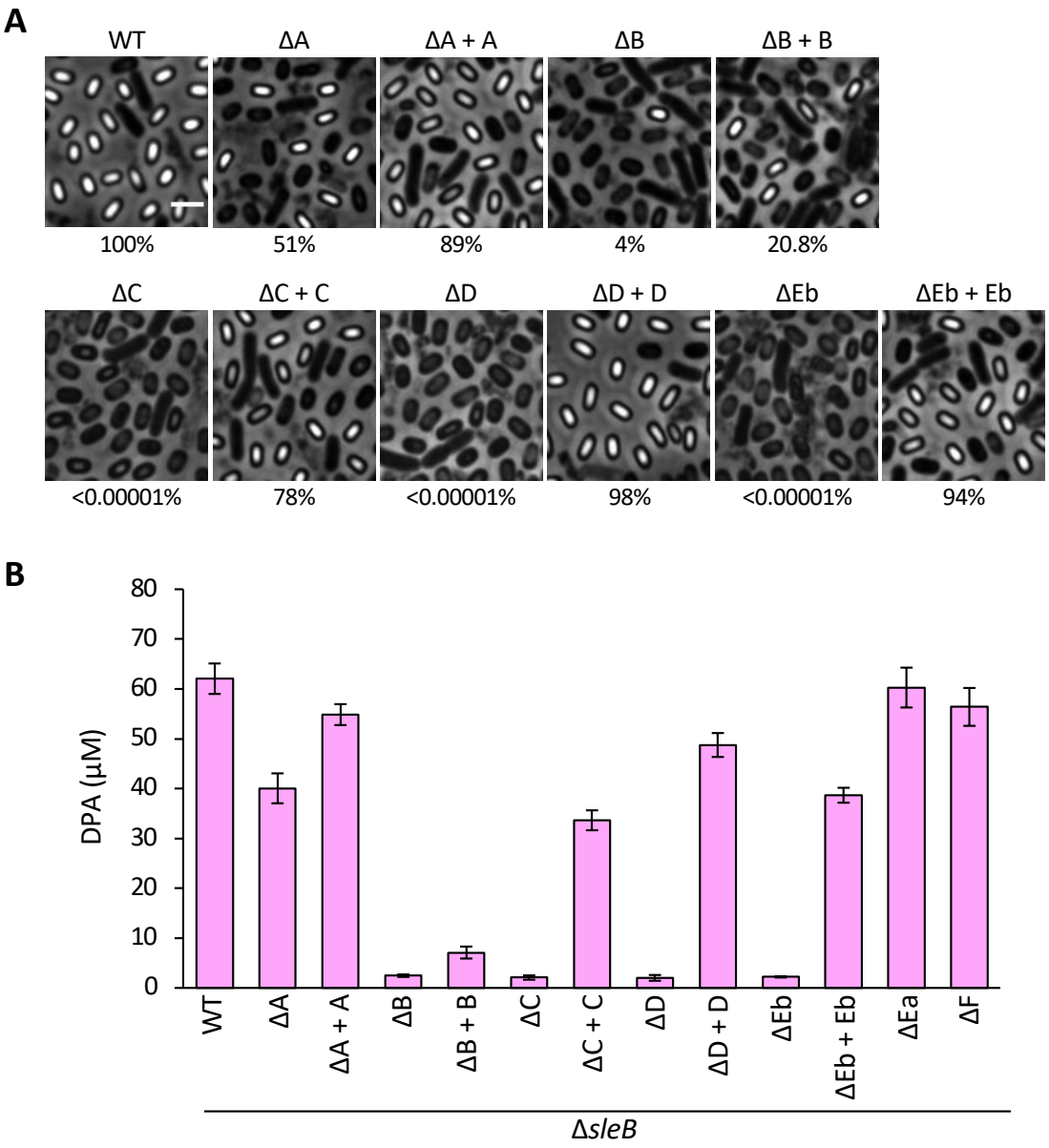
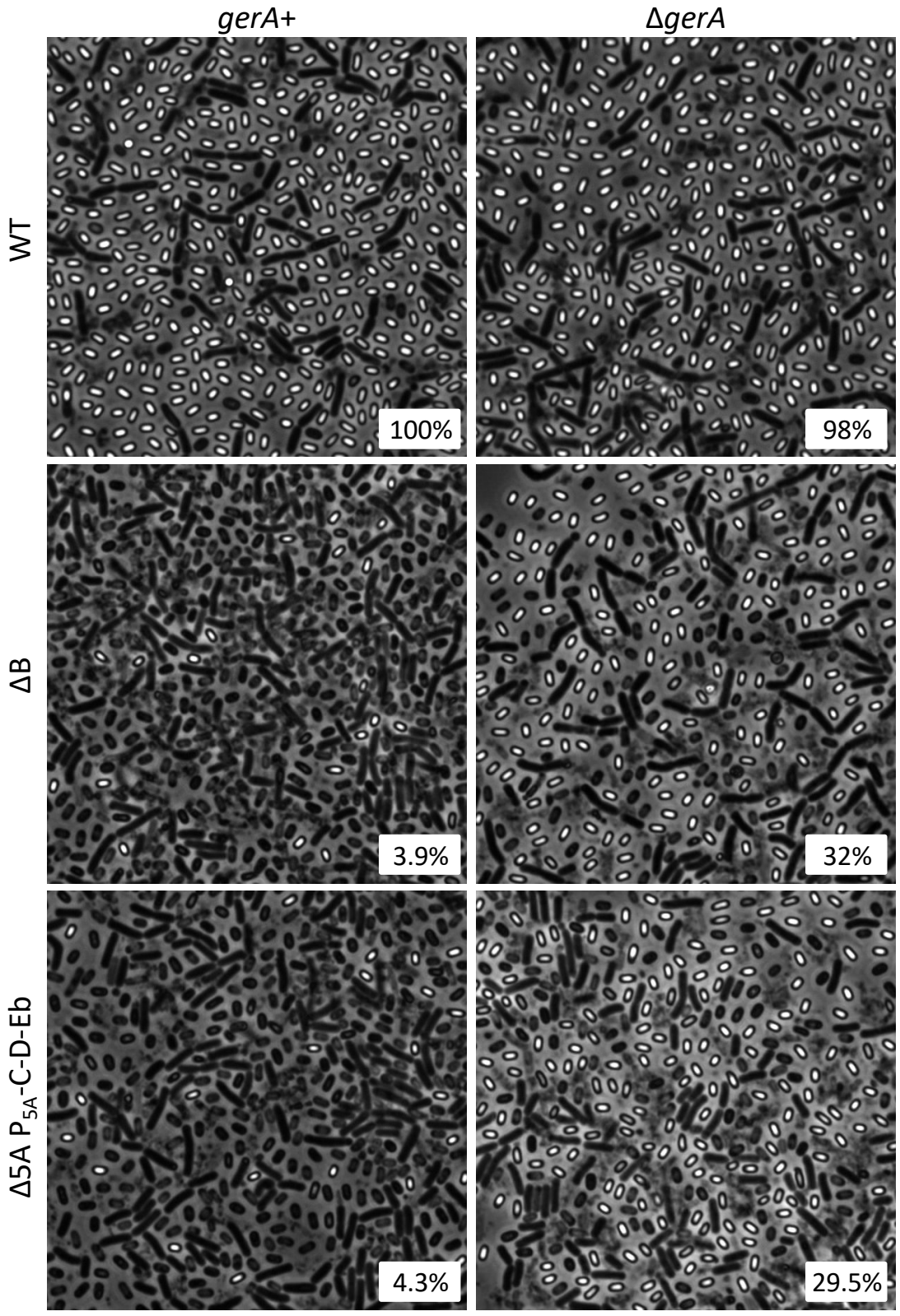


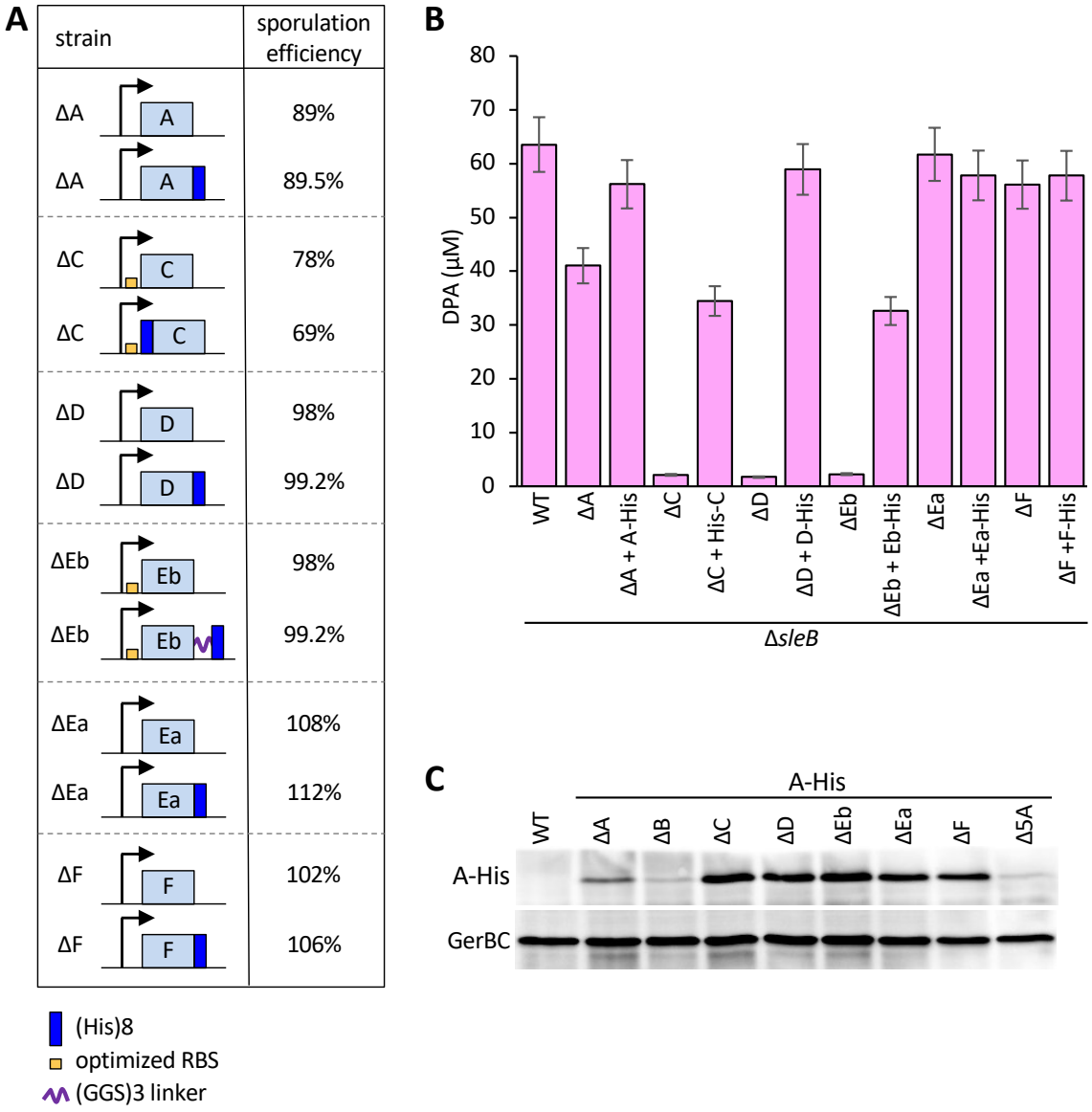
Supplemental Figure S1. Comparison of DPA accumulation in 5A mutants with and without the GerA germination receptor. Bar graph showing DPA levels in spores from the indicated strains. Total spores were isolated with lysozyme followed by SDS, normalized, and boiled to release DPA. DPA in the supernatant was mixed with $TbCl_3$ and detected by fluorimetry and compared to a standard curve.



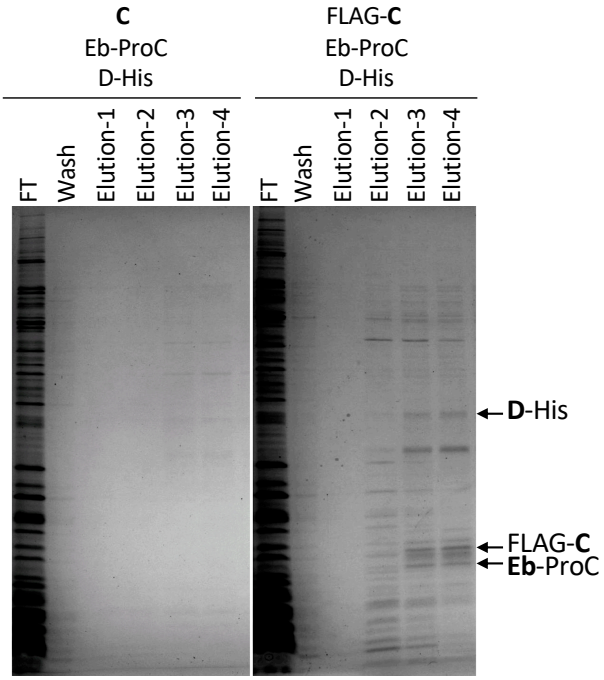
Supplemental Figure S2. Complementation of the 5A mutants. (A) Representative phase-contrast images and sporulation efficiencies below the images of the indicated strains. Scale bar is 2 μm. **(B)** Bar graph showing DPA levels in spores from the indicated strains. Total spores were isolated with lysozyme followed by SDS, normalized, and boiled to release DPA. DPA in the supernatant was mixed with $TbCl_3$ and detected by fluorimetry.



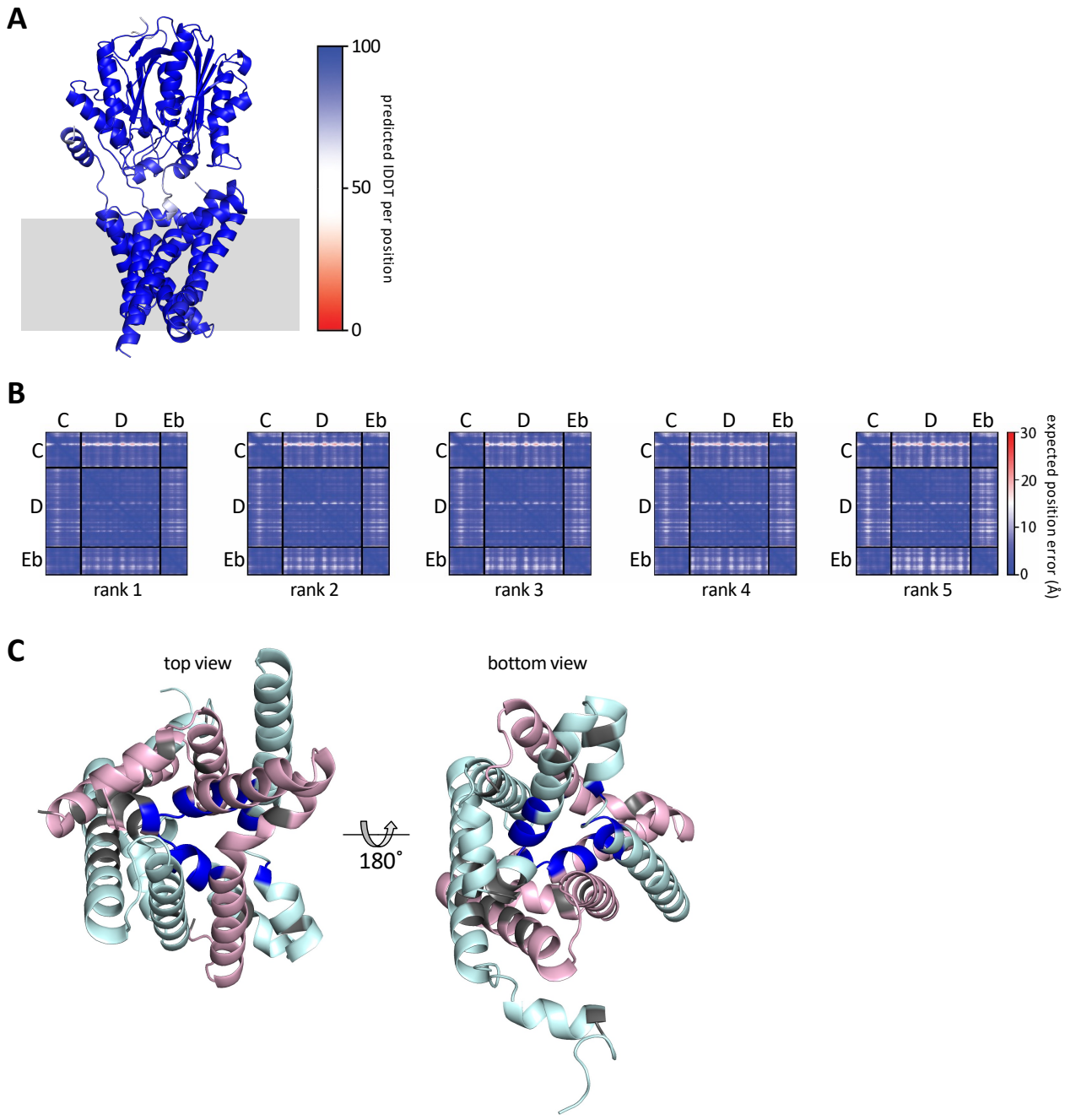
Supplemental Figure S3. A minimal set of 5A proteins required for DPA accumulation. Representative phase-contrast images of sporulated cultures of the indicated strains in the presence and absence of *gerA*. Sporulation efficiencies based on heat resistance (20 min at 80 ˚C) compared to wild-type are shown in the lower right. The two mutants produce a mixture of phase-bright, phase-grey, phase-dark, and lysed spores.



Supplemental Figure S4. His-tag fusions to 5A proteins are functional. (A) Sporulation efficiency of the His fusions to A, C, D, Eb, Ea, and F. Diagrams schematize the design of each fusion. **(B)** Bar graph showing DPA levels in spores from the indicated strains. Total spores were isolated with lysozyme followed by SDS, normalized, and boiled to release DPA. DPA in the supernatant was mixed with $TbCl_3$ and detected by fluorimetry. **(C)** Stability of A-His in spores from strains lacking individual *5A* genes. GerBC controls for loading. All strains harbor Δ*sleB* to prevent spore cortex degradation and enable spore purification for immunoblots.

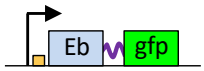


Supplemental Figure S5. C, D, and Eb form a membrane complex. Coomassie-stained gels showing co-purification of FLAG-C, Eb-ProC, and D-His using anti-FLAG resin. The *B. cereus* 5A-1 proteins were co-expressed in *E. coli* and detergent-solubilized membrane preparations were subject to immunopurification with anti-FLAG resin. Flow through (FT), wash, and elutions are shown for side-by-side purifications in which C was expressed with and without a FLAG tag along with Eb-ProC and D-His. The indicated bands were excised and subjected to mass spectrometry to confirm the identities of C, D, and Eb.



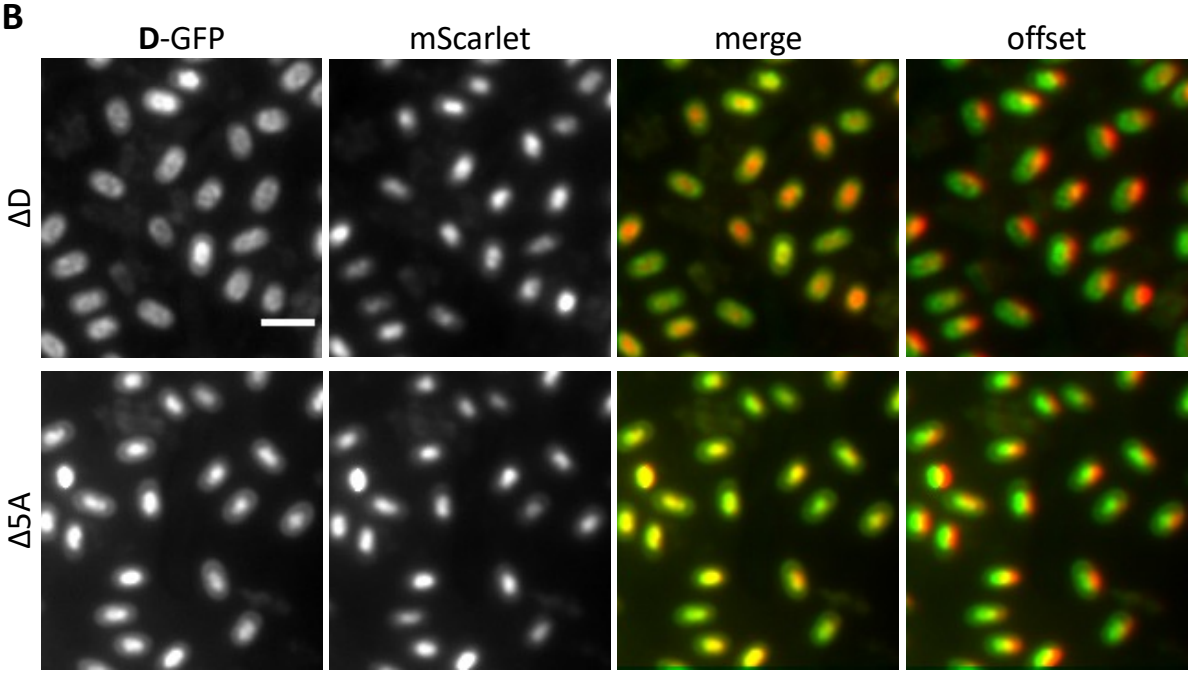
Supplemental Figure S6. Predicted local distance difference tests and alignment error for the AlphaFold-predicted C-D-Eb complex. (A) Predicted local distance difference tests (plDDT) per position mapped onto the predicted C-D-Eb structure. Higher plDDT (blue) corresponds to a more confident prediction. **(B)** Expected position error in Å of all residues against all residues for five top-ranked C-D-Eb models. Low error (blue) corresponds to well-defined relative domain positions. **(C)** Many of the conserved residues in C and Eb line the AlphaFold-predicted membrane channel. Conserved residues in C (cyan) and Eb (pink) that line the channel are highlighted in dark blue. Other conserved residues are shown in grey. Top view is from the integument layer looking in. Bottom view is from the spore core looking out.

A

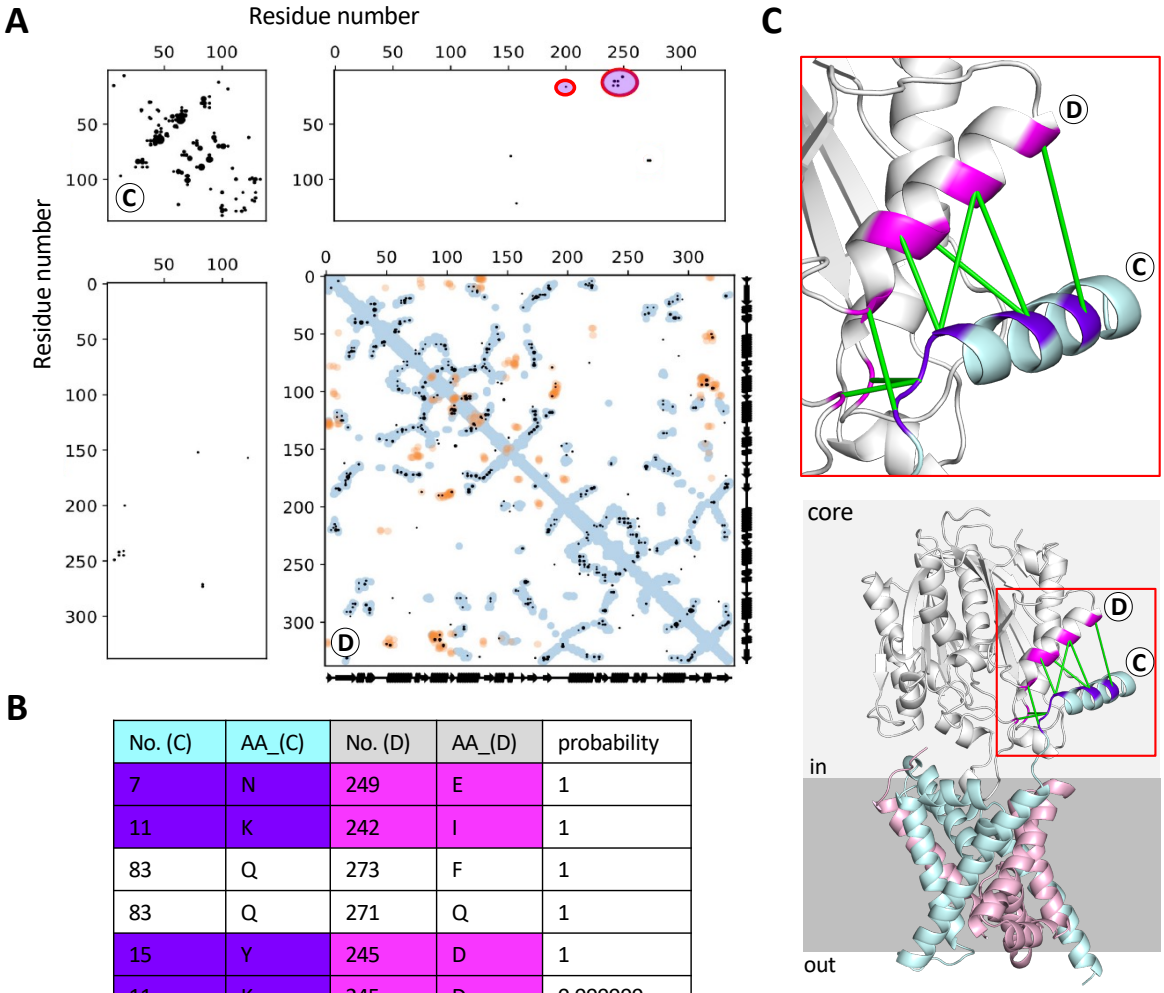
strain	sporulation efficiency
ΔC	<0.00001%
ΔC 	28.2%
ΔD	<0.00001%
ΔD 	95.3%
ΔEb	<0.00001%
ΔEb 	15.9%

optimized RBS
$(GGS)_3$ linker

B

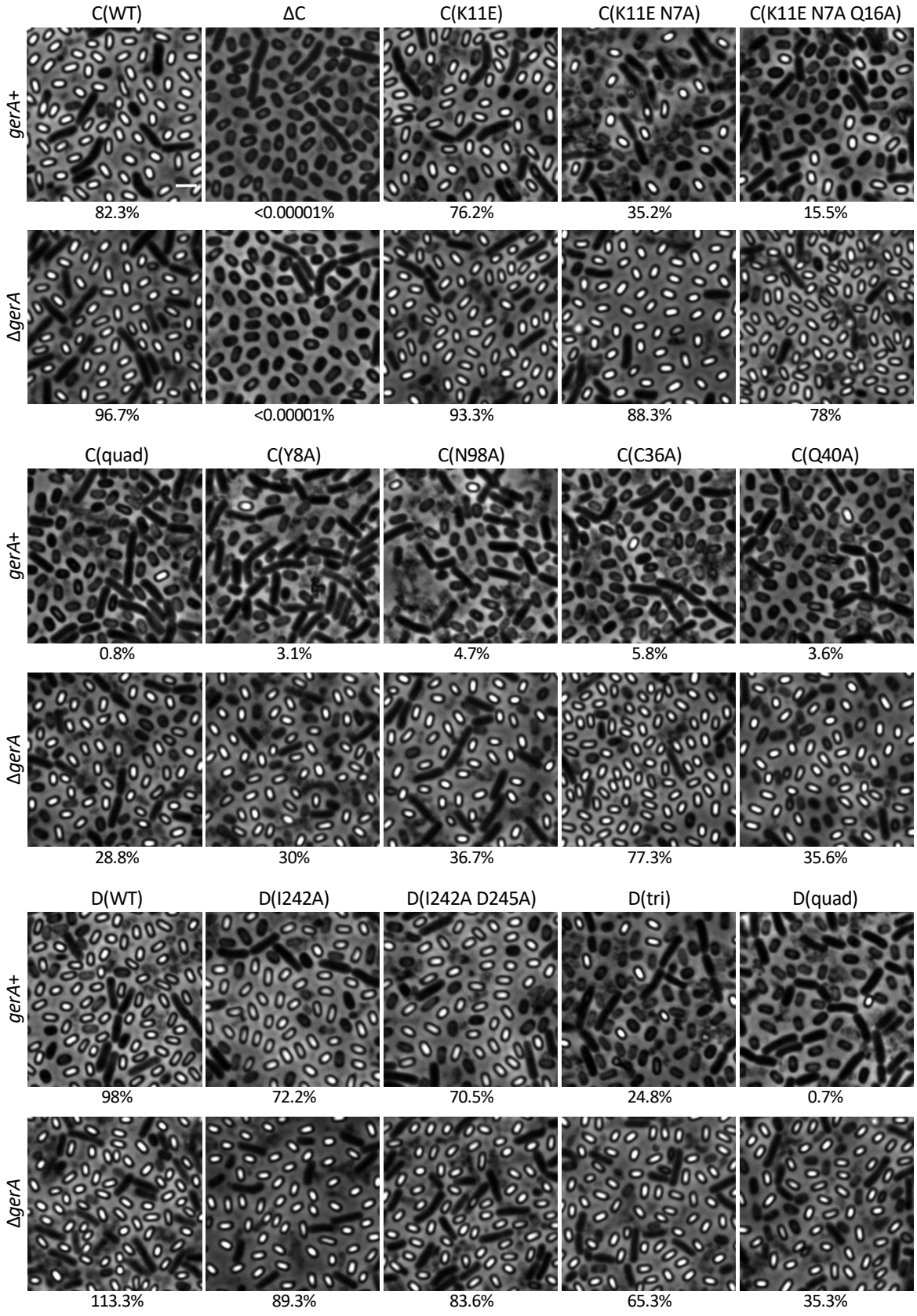


Supplemental Figure S7. GFP fusions to C, D, and Eb are functional. (A) Sporulation efficiency of the GFP fusions to C, D and Eb. Diagrams schematize the design of each fusion. **(B)** Representative images of D-GFP and cytoplasmic mScarlet in spores in the presence and absence of the *5A* locus. mScarlet fluorescence co-localizes with D-GFP when the *5A* operon is deleted. Both strains harbor Δ*sleB* to maintain the protective cortex layer. Scale bar is 2 µm.

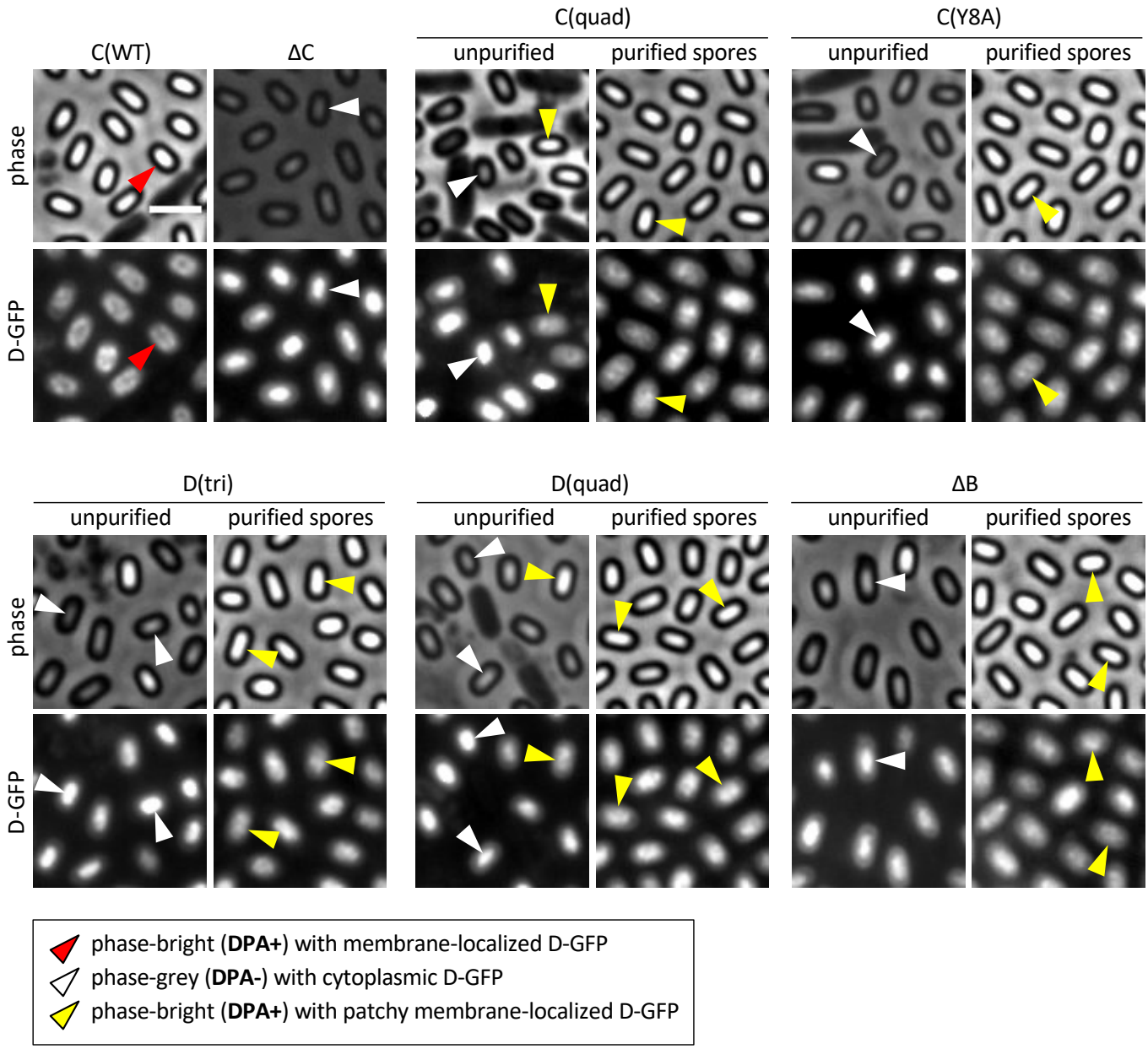


No. (C)	AA_(C)	No. (D)	AA_(D)	probability
7	N	249	E	1
11	K	242	I	1
83	Q	273	F	1
83	Q	271	Q	1
15	Y	245	D	1
11	K	245	D	0.999999
79	D	152	E	0.999993
16	Q	200	D	0.999852
122	K	157	K	0.999702
15	Y	241	P	0.999557
17	P	238	V	0.999537
16	Q	160	T	0.998599

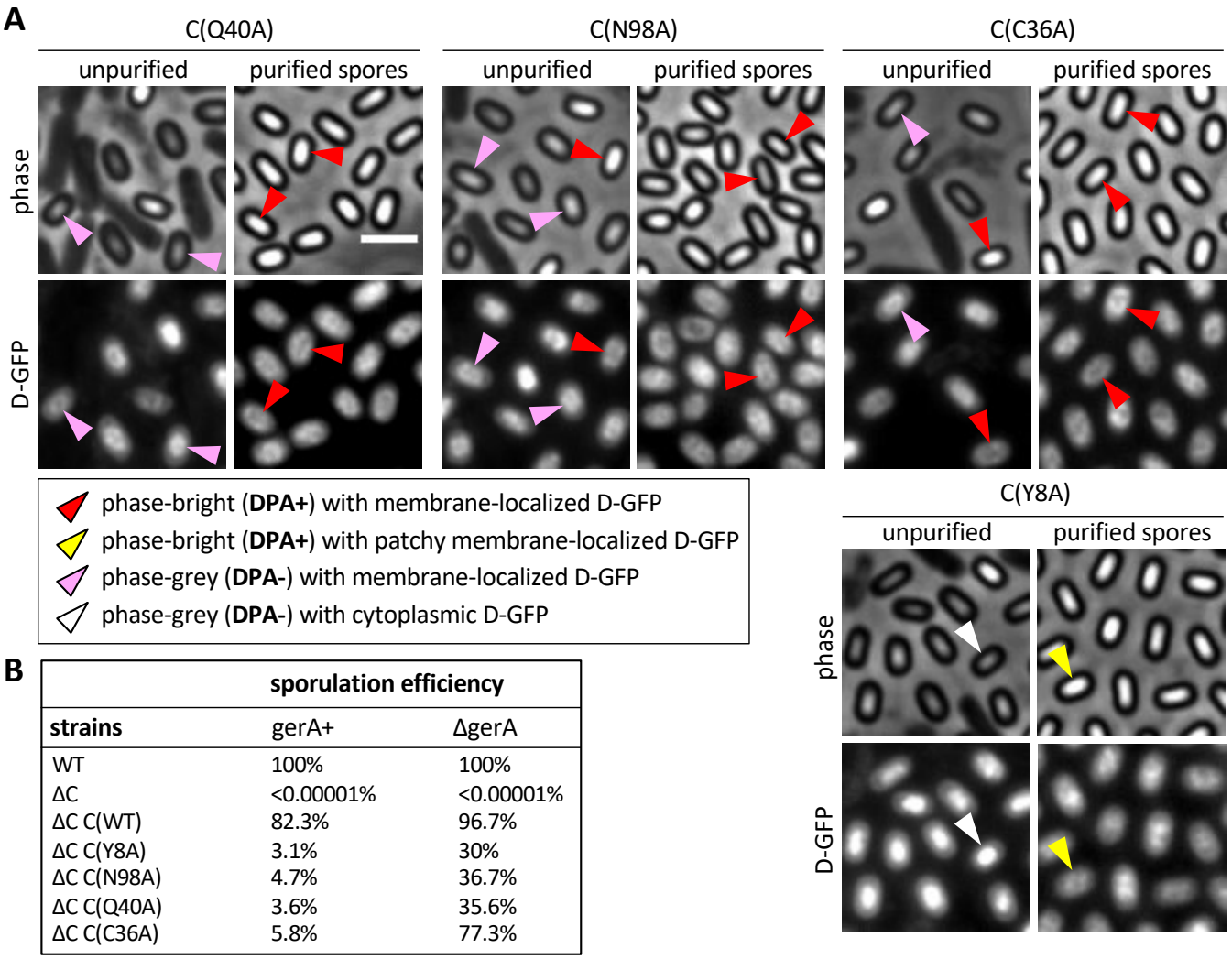
Supplemental Figure S8. Evolutionary co-variation analysis of C and D. (A) Evolutionarily coupled (EC) residue pairs in C, D and between C and D are plotted as black circles. Relevant EC residue pairs between C and D are highlighted (purple with red ovals). Residue pairs that are ≤5 Å apart in the D structure (PDB ID: 3LM6) are shown as blue circles. Orange circles show residue pairs in adjacent protomers in the crystal. **(B)** Table of EC residues pairs between C and D with probabilities >0.998 **(C)** AlphaFold model of the C-D-Eb complex. Green lines connect all the EC residue pairs between the N-terminus of C (dark purple) and D (magenta).



Supplemental Figure S9. Suppression of premature germination and sporulation efficiency defects in the C and D point mutants by Δ*gerA*. Representative phase-contrast images and sporulation efficiencies of the indicated strains in the presence (*gerA+*) and absence (Δ*gerA*) of the GerA germinant receptor. The suppression of sporulation efficiency and premature germination in the absence of GerA argues that the mutants are impaired in DPA accumulation. Scale bar is 2 μm.

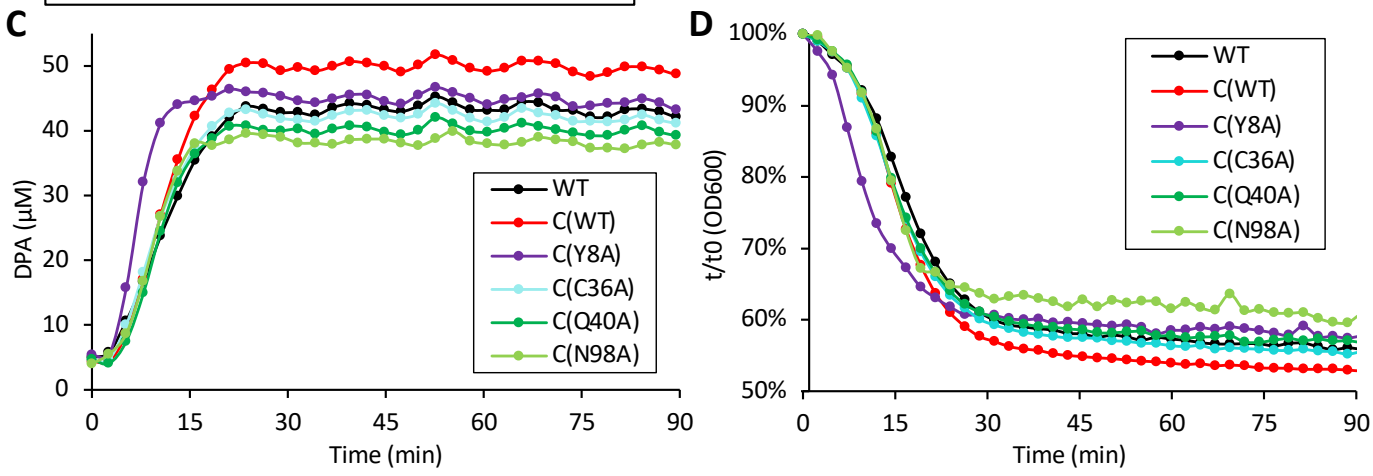


Supplemental Figure S10. D-GFP localization in spores with mutations in *B*, *C*, or *D*. Representative phase-contrast and fluorescence images of the indicated strains. Total unpurified spores and Histodenz-purified phase-bright spores are shown. Wild-type phase-bright spores have membrane-localized D-GFP (red carets). Mutant phase-grey spores, a hallmark of DPA deficiency, have cytoplasmic D-GFP (white carets). Phase-bright mutant spores have patchy membrane-localized D-GFP (yellow carets). All strains in this figure harbor Δ*sleB* to maintain the protective cortex layer. Scale bar is 2 µm.

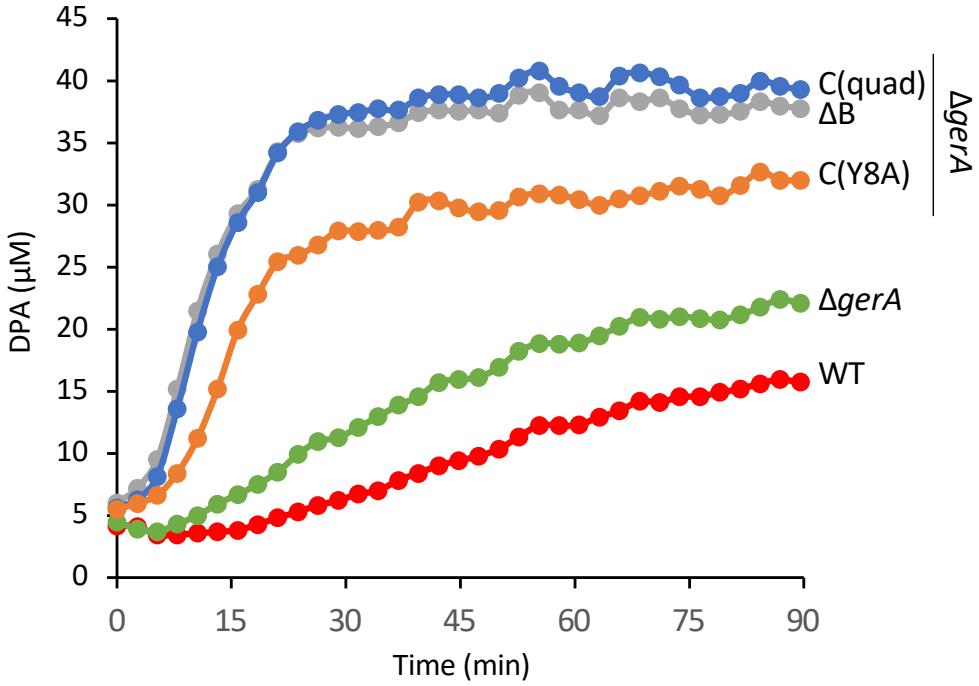


B

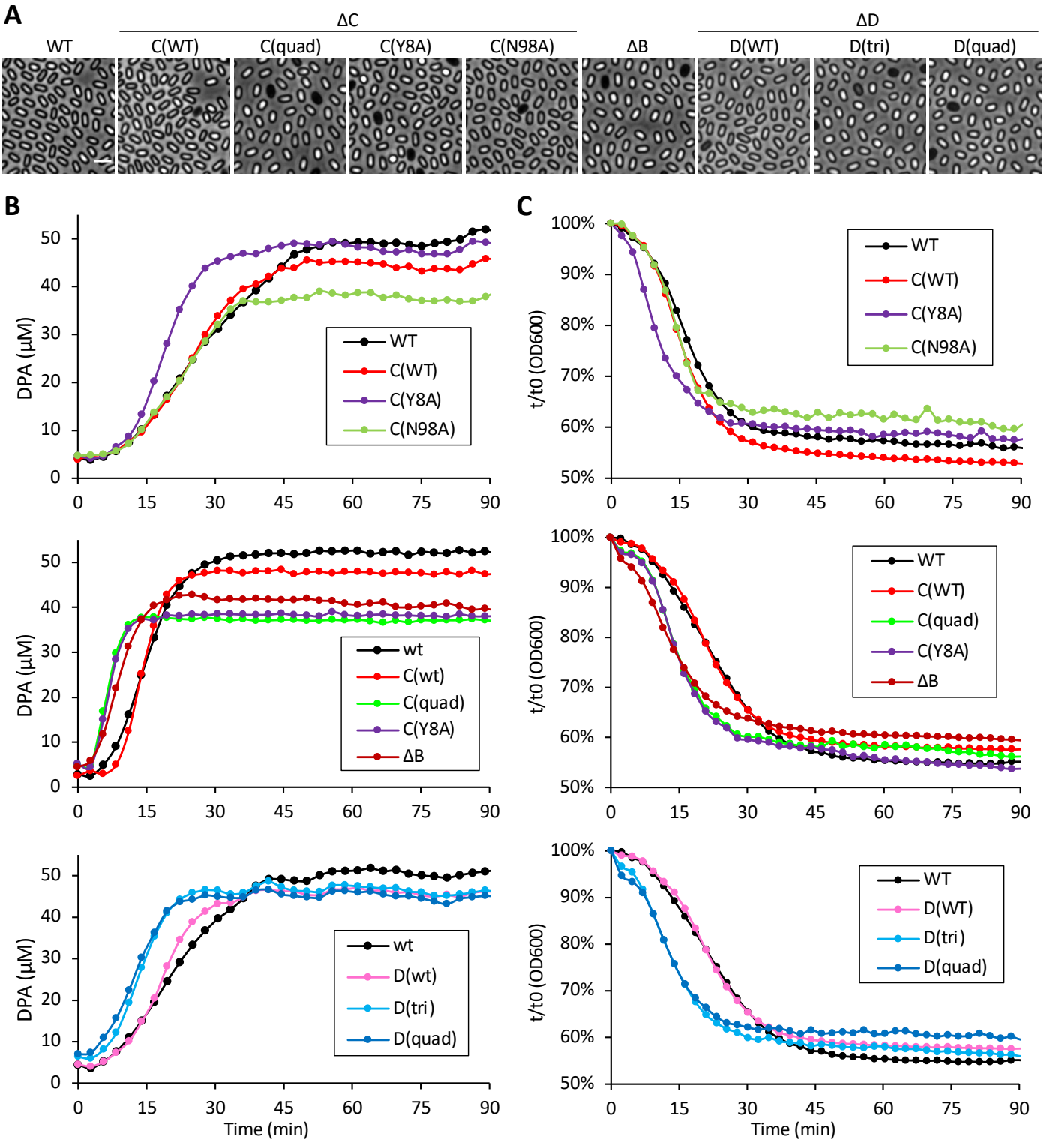
	sporulation efficiency	
strains	gerA+	ΔgerA
WT	100%	100%
ΔC	<0.00001%	<0.00001%
ΔC C(WT)	82.3%	96.7%
ΔC C(Y8A)	3.1%	30%
ΔC C(N98A)	4.7%	36.7%
ΔC C(Q40A)	3.6%	35.6%
ΔC C(C36A)	5.8%	77.3%



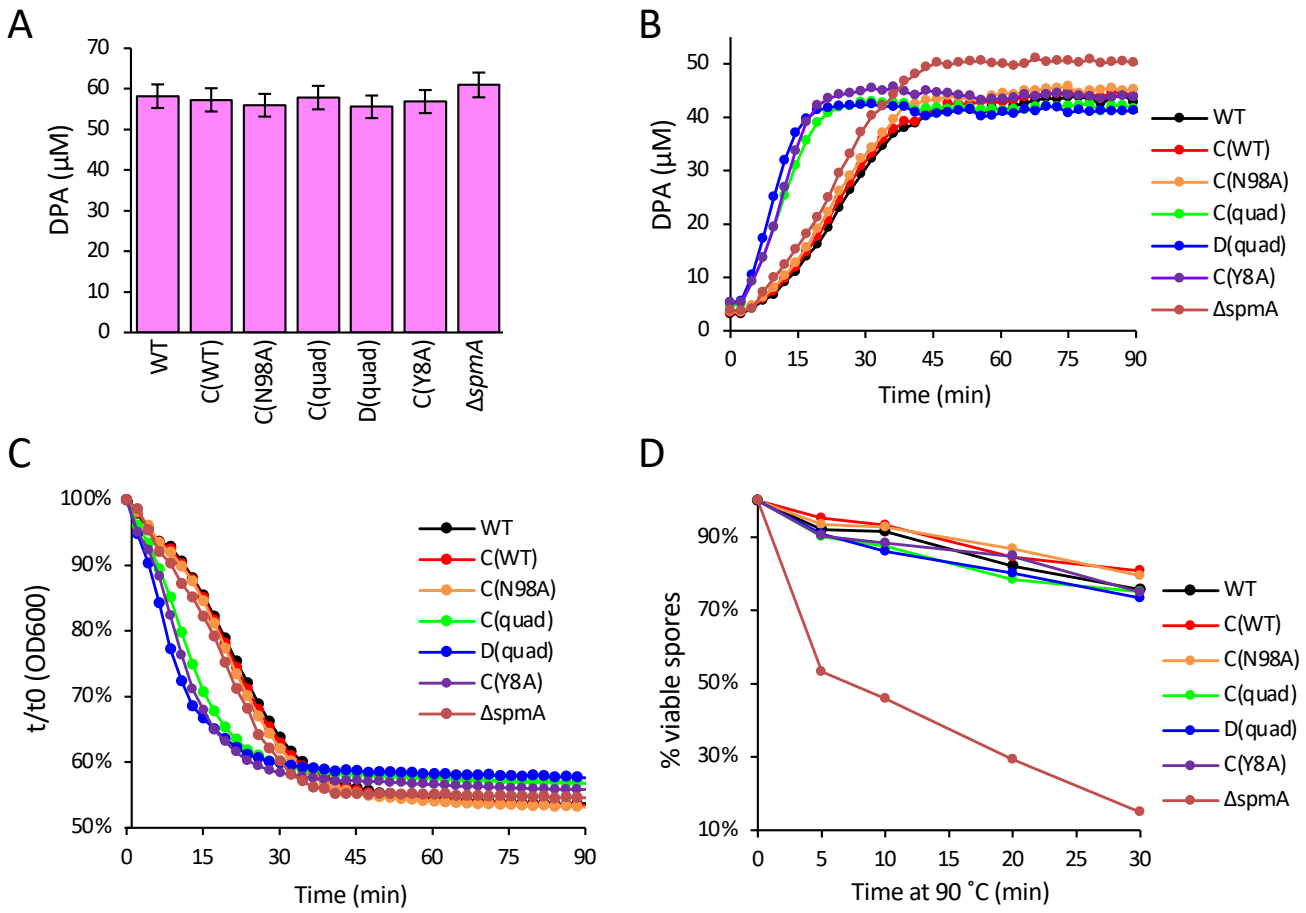
Supplemental Figure S11. Point mutations in C with similar sporulation defects have different D-GFP localization patterns that correlate with the timing of germination. (A) Representative phase-contrast and fluorescence images of the indicated strains. Total unpurified spores and Histodenz-purified spores are shown. Phase-bright (red carets) and phase-grey (pink carets) spores of C(Q40A), C(N98A), and C(C36A) mutants have membrane-localized D-GFP. Phase-grey C(Y8A) spores (white carets) have cytoplasmic D-GFP and phase-bright C(Y8A) spores (yellow carets) have patchy membrane-localized D-GFP. All strains in (A) harbor Δ*sleB* to maintain the protective cortex layer. Scale bar is 2 μm. **(B)** Sporulation efficiencies of the indicated mutants in the presence and absence of *gerA*. **(C)** Germination in response to 1 mM L-alanine as assayed by release of DPA in the indicated strains. Purified phase-bright spores were induced to germinate with 1 mM L-alanine and DPA release was monitored over time using $TbCl_3$. Spores harboring C(Y8A) initiated DPA release faster than wild-type and the other mutants. **(D)** Germination in response to 1 mM L-alanine as assayed by the reduction in OD600. Spores harboring C(Y8A) initiated a drop in OD600 faster than wild-type and the other mutants.



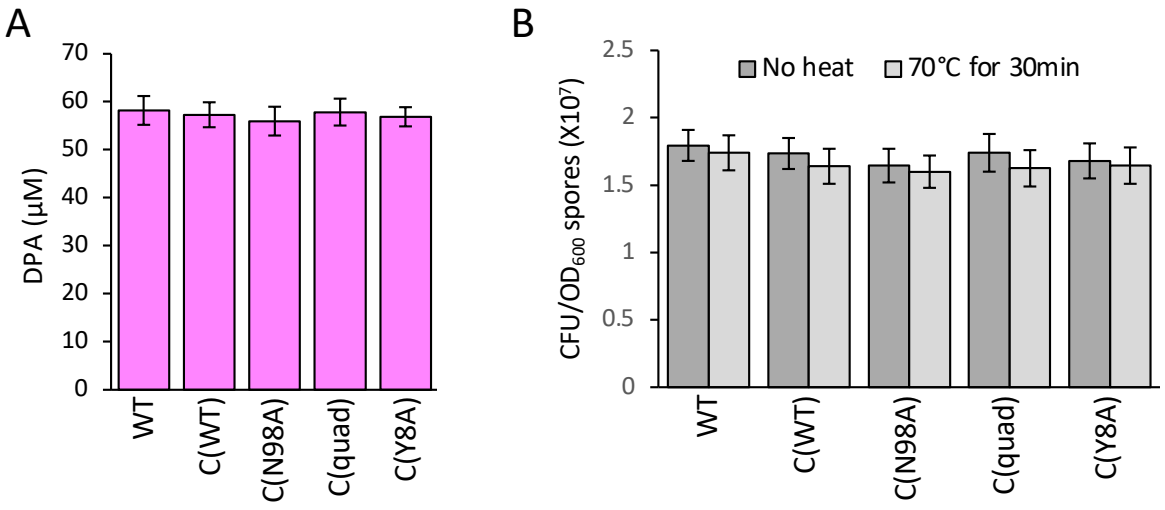
Supplemental Figure S12. Spores harboring mutations that impair the interaction between C and D initiate germination faster in response to AGFK. Spore germination in response to the mixture of 10 mM L-asparagine, 10 mM D-Glucose, 10 mM Fructose, 10 mM KCl (AGFK) as assayed by release of DPA in the indicated strains. Purified phase-bright spores were heat-activated at 70˚C for 30 min and induced to germinate with AGFK. DPA release was monitored over time using $TbCl_3$.



Supplemental Figure S13. Spores harboring mutations that impair the interaction between C and D initiate germination faster in response to L-alanine. (A) Representative phase-contrast images of Histodenz-purified spores from the indicated strains. Scale bar is 2 μm. **(B)** Spore germination in response to 1 mM L-alanine as assayed by release of DPA in the indicated strains. Purified phase-bright spores were induced to germinate with 1 mM L-alanine and DPA release was monitored over time using $TbCl_3$. The germination assays are biological replicates of those shown in Figure 5. **(C)** Spore germination in response to 1 mM L-alanine as assayed by a reduction in OD600 in the indicated strains. Purified phase-bright spores were induced to germinate with 1 mM L-alanine and OD600 was monitored over time. Data were plotted as the percent reduction in OD600 relative to time 0.

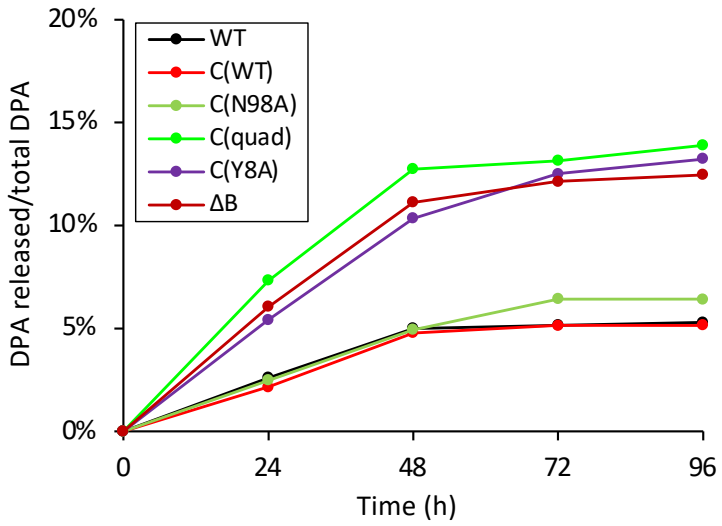


Supplemental Figure S14. Comparison of spores lacking *spmA* to C and D mutants spores analyzed in this study. (A) Bar graph showing DPA levels in spores of the indicated strains. Phase-bright spores were isolated using a Histodenz step-gradient, normalized to an OD600 of 1, and boiled for 30 min to release DPA. The supernatant was mixed with $TbCl_3$ and DPA quantified by fluorimetry and compared to a standard curve. **(B)** Spore germination in response to 1 mM L-alanine as assayed by release of DPA in the indicated strains. Purified phase-bright spores were induced to germinate with 1 mM L-alanine and DPA release was monitored over time using $TbCl_3$. **(C)** Spore germination in response to 1 mM L-alanine as assayed by a reduction in OD600 in the indicated strains. Purified phase-bright spores were induced to germinate with 1 mM L-alanine and OD600 was monitored over time. Data were plotted as the percent reduction in OD600 relative to time 0. As reported previously (Popham et al. 1995), Δ*spmA* spores initiate germination slightly faster than wild-type. This increase has been attributed to incomplete core dehydration. The C and D mutant spores with impaired interaction reproducibly initiated germination even more quickly. **(D)** Analysis of spore heat resistance. Purified spores were incubated at 90 ˚C for the indicated times and then serially diluted and plated on LB agar. CFU were enumerated after overnight incubation at 37 ˚C and the percentage of viable spores was determined. As reported previously, Δ*spmA* spores are less heat resistant due to incomplete core dehydration (Popham et al. 1995). By contrast, all other mutants tested had heat resistance comparable to wild-type.



Supplemental Figure S15. Purified phase-bright spores have similar Colony Forming Units (CFUs) and similar levels of DPA. (A) Bar graph showing DPA levels in spores of the indicated strains. Phase-bright spores were isolated using a Histodenz step-gradient, normalized to an OD600 of 1, and boiled for 30 min to release DPA. The supernatant was mixed with $TbCl_3$ and the DPA quantified by fluorimetry and compared to a standard curve. **(B)** Colony forming units per OD600 of purified spores used in (A). Bar graph shows CFUs from spores with or without heat-activation at 70 ˚C for 30 min. The CFU per OD600 were similar allowing direct comparison of DPA levels between wild-type and the mutants.

A

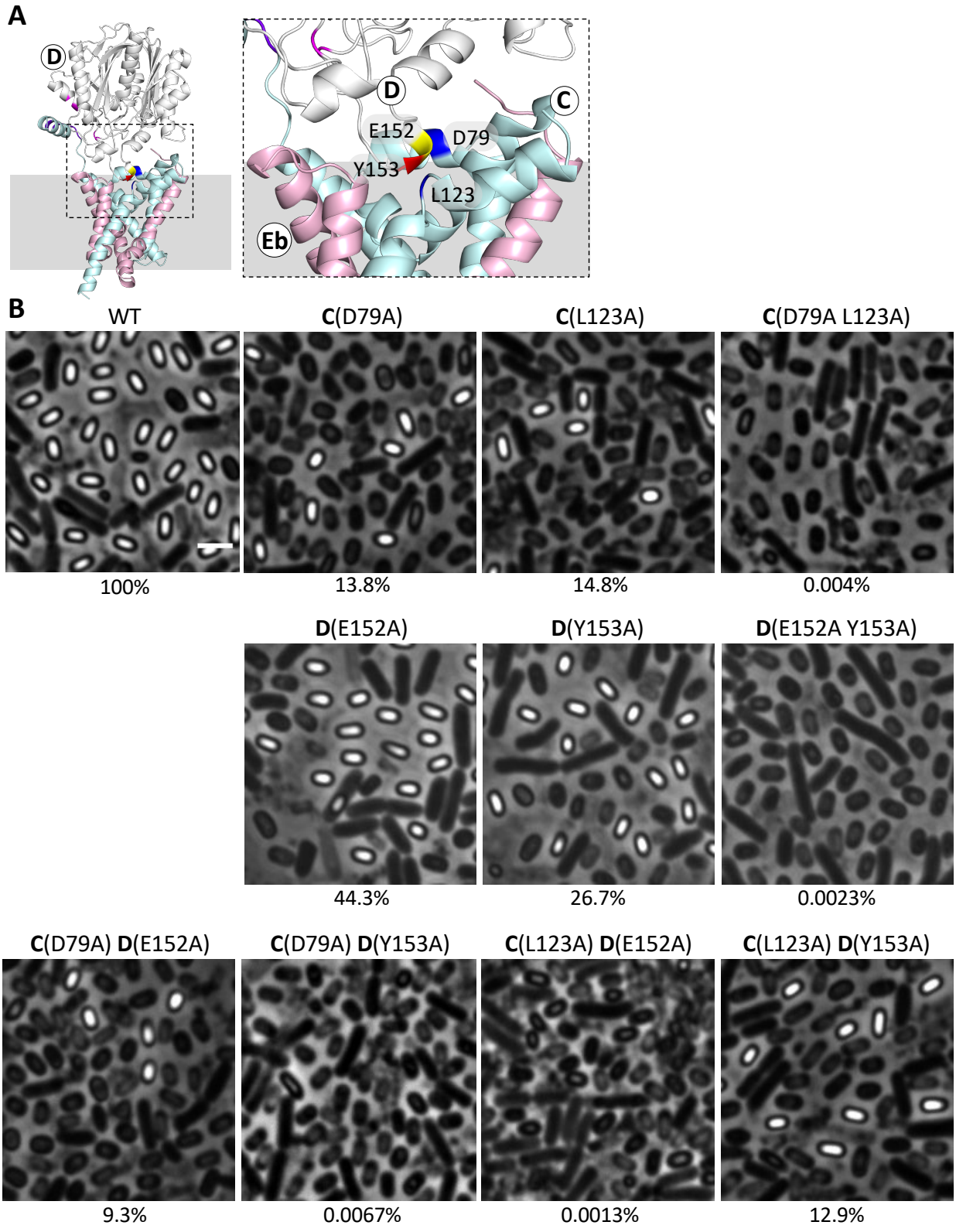


B

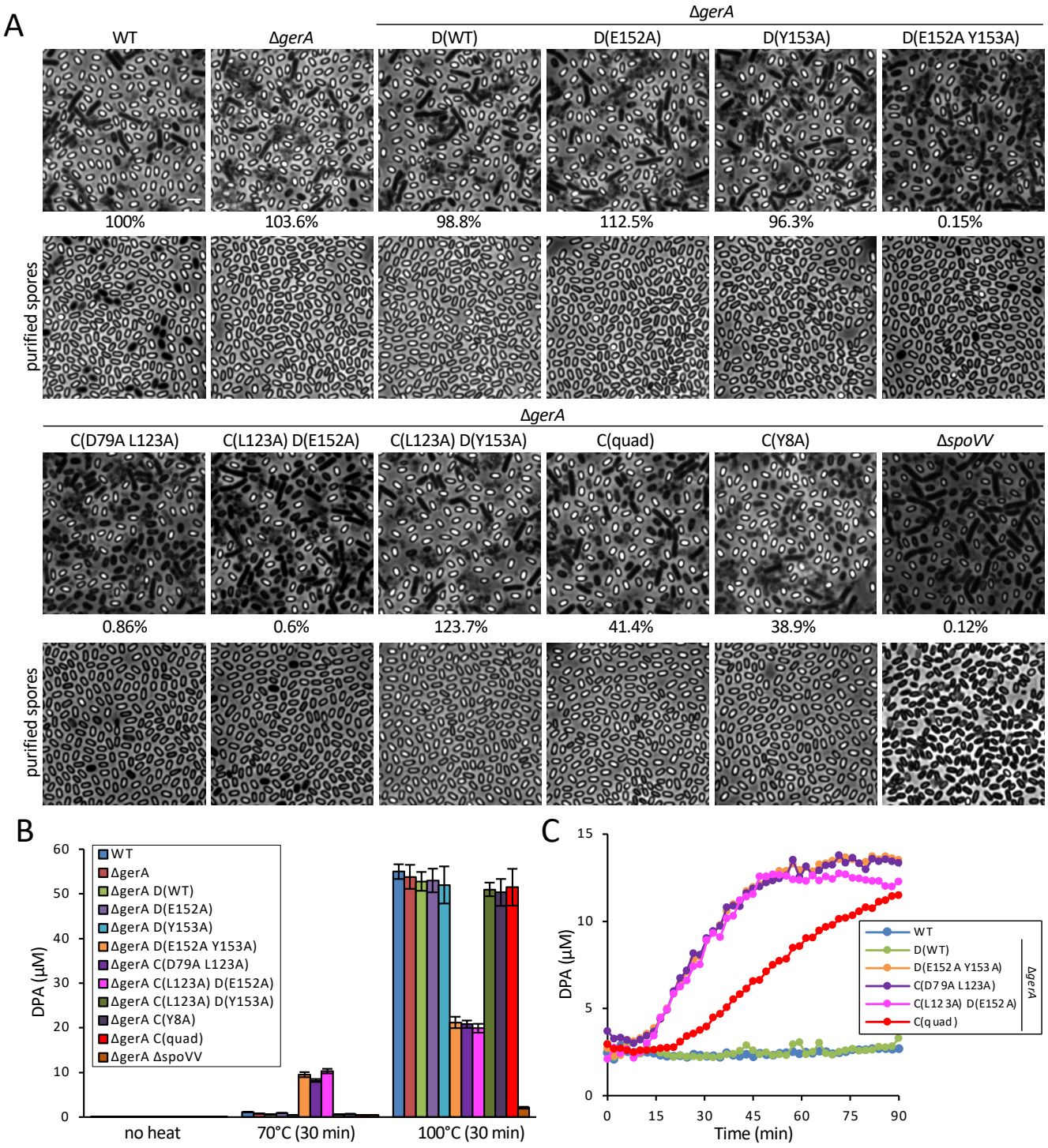
% heat-resistant CFUs relative to 0h

strains	0h	24h	48h	72h	96h
WT	100%	95.3%	98.2%	87.1%	85.2%
C(WT)	100%	98.9%	89.9%	82.7%	84.8%
C(N98A)	100%	95.5%	97.3%	87.8%	83.6%
C(quad)	100%	91.5%	86.2%	84.3%	81.7%
C(Y8A)	100%	89.1%	85.2%	83.5%	82.3%
ΔB	100%	96%	82.3%	84.8%	82.8%

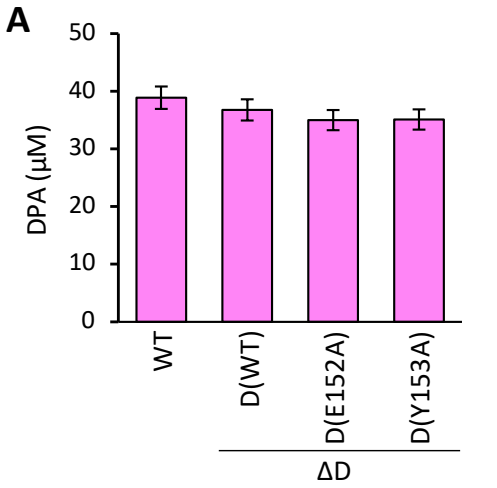
Supplemental Figure S16. Analysis of DPA leakage out of dormant spores. (A) DPA present in the buffer of purified phase-bright spores incubated at 37˚C over a 4-day time course. Data were plotted as the percent DPA released compared to total DPA. **(B)** Heat-resistant colony forming units (CFUs) from the same spores and timepoints used in (A). The reduction in heat-resistant CFU is similar to wild-type in all strains tested.

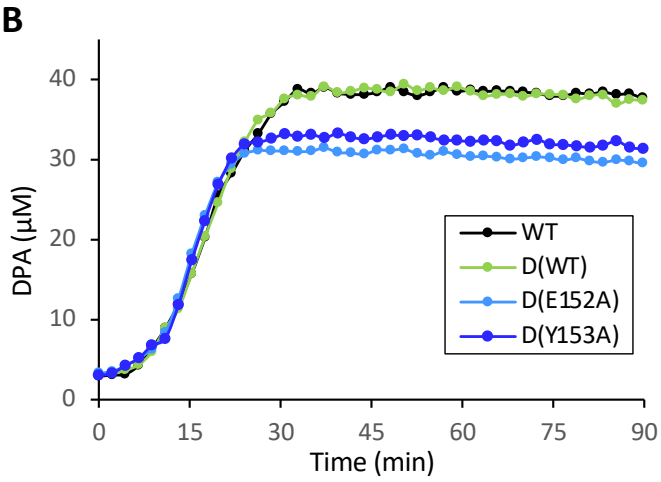


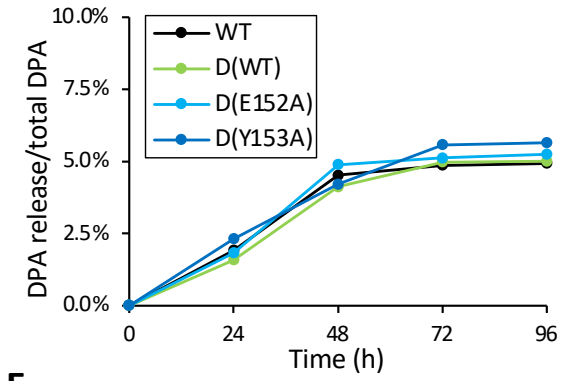
Supplemental Figure S17. Mutational analysis of the residues in C and D that form the plug. (A) AlphaFold model of the C-D-Eb complex with E152 (yellow) and Y153 (red) in D and D79 and L123 (blue) in C highlighted. **(B)** Representative phase-contrast images and sporulation efficiencies (below the images) of the indicated strains. Amino acid substitutions predicted to disrupt one of the two interactions in the plug have similar sporulation defects and degree of premature germination. Substitutions predicted to disrupt both interactions are synergistic. Scale bar is 2 µm.



Supplemental Figure S18. Characterization of C and D mutant spores with defects in the predicted plug. (A) Representative phase-contrast images of sporulated cultures and Histodenz-purified spores from the indicated strains. Sporulation efficiency as assayed by heat-resistant (20 min at 80 ˚C) CFU from the same sporulated cultures are indicated below the images. The purified C and D mutant spores that disrupt both interactions in the putative plug are less bright by phase-contrast microscopy. Δ*spoVV* Δ*gerA* spores that lack DPA are included for comparison and were purified with Lysozyme and SDS. Scale bar indicated 2 μm. **(B)** Bar graph showing total DPA and DPA retention in spores of the indicated strains. Purified spores were incubated for 30 min at 100 ˚C to release total DPA, at 70 ˚C, a condition used to heat-activate spores, or without heat. DPA in the supernatant was quantified using $TbCl_3$ and fluorimetry. C and D mutants that disrupt both interactions in the putative plug contained less total DPA and were impaired in DPA retention at 70˚C. **(C)** Spore germination in response to the mixture of 10 mM L-asparagine, 10 mM D-Glucose, 10 mM Fructose, 10 mM KCl (AGFK) as assayed by release of DPA in the indicated strains. Purified spores were not heat-activated prior to addition of AGFK. C and D mutants that disrupt both interactions in the putative plug released DPA after a modest delay. The C(quad) mutant released DPA after a delay. Wild-type spores did not respond. For comparison, Figure S11 shows germination of heat-activated spores in response to AGFK.



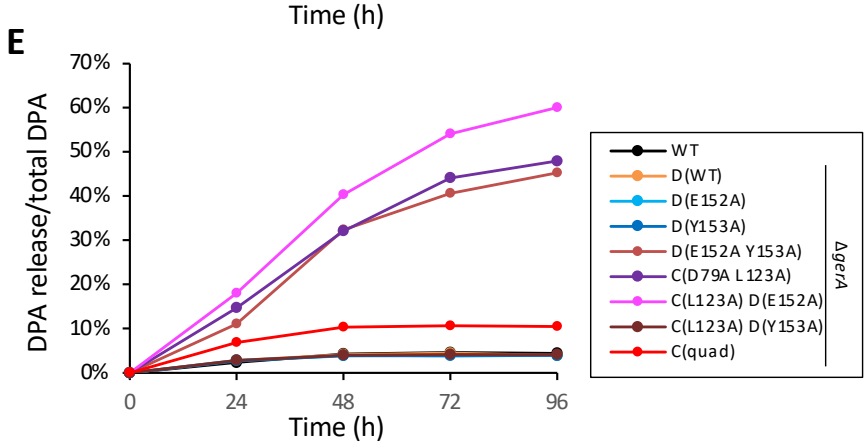




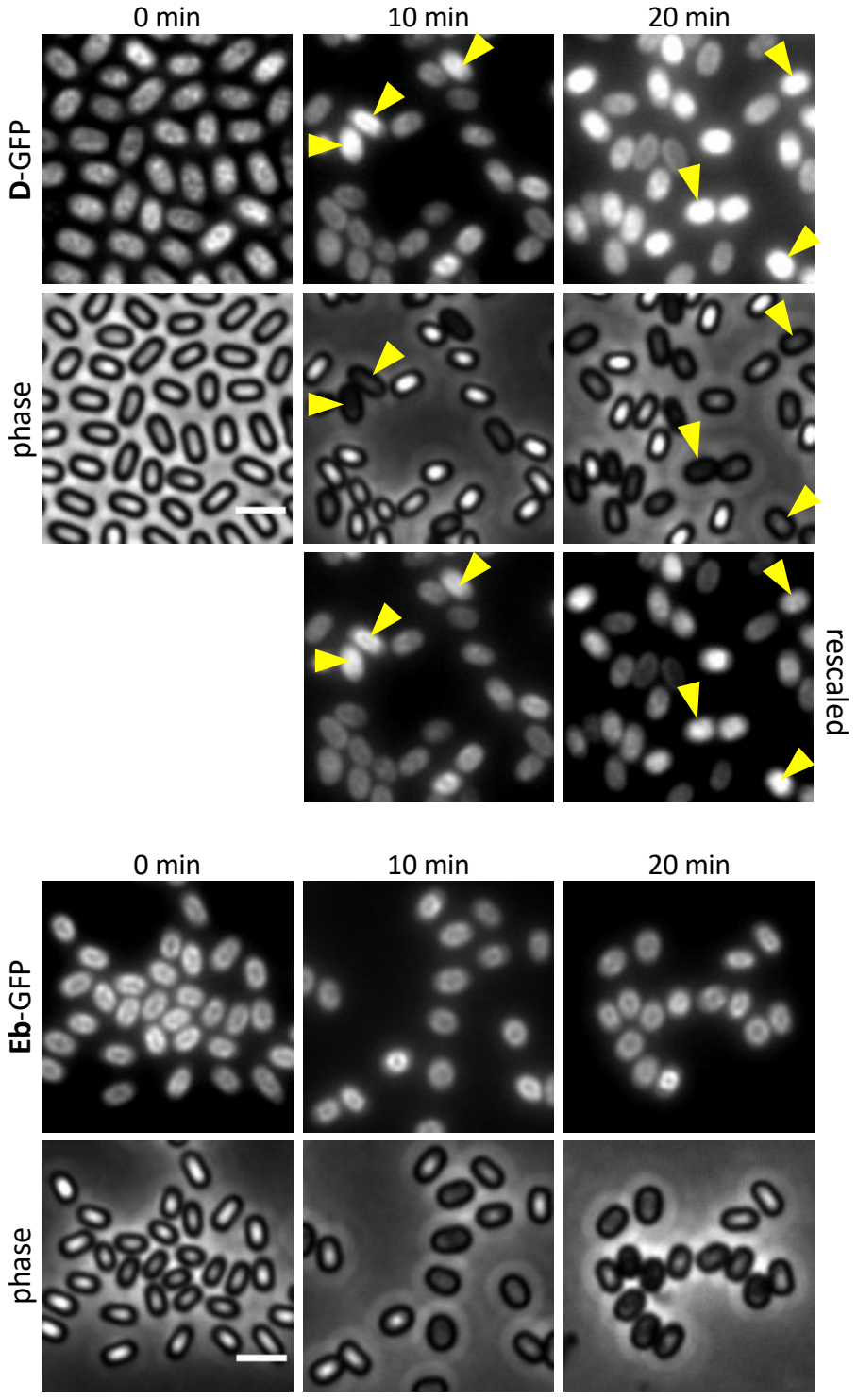
D

% heat-resistant CFUs relative to 0h

strains	0h	24h	48h	72h	96h
WT	100%	92.6%	87.5%	85.4%	84.2%
D(WT)	100%	97.2%	92.7%	86.3%	82.7%
D(E152A)	100%	94.3%	89.8%	87.7%	85.5%
D(Y153A)	100%	91.8%	85.2%	86.2%	83.8%



Supplemental Figure S19. Analysis of DPA leakage out of dormant spores. (A) Bar graph of DPA levels in histodenz-purified spores of the indicated strains. **(B)** Spore germination in response to 1 mM L-alanine as assayed by release of DPA in the indicated strains. Purified phase-bright spores were induced to germinate with 1 mM L-alanine and DPA release was monitored over time using $TbCl_3$. **(C)** DPA present in the buffer of purified phase-bright spores of the indicated strains at 37˚C over 4 days. Data were plotted as the percent released compared to total DPA in the input spores. **(D)** Heat-resistant colony forming units from the same spores used in (C). The reduction in heat-resistant CFUs is similar to wild-type in all strains tested. **(E)** DPA present in the buffer of the indicated purified phase-bright spores incubated at 37˚C over 4 days. The double mutant spores that disrupt both interactions in the putative plug released >50% of their DPA over the 4-day time course.



Supplemental Figure S20. D-GFP becomes cytoplasmic during germination. Representative phase-contrast and fluorescence images of D-GFP and Eb-GFP in purified spores before and 10 and 20 minutes after exposure to 1 mM L-alanine. Examples of germinated phase-dark spores with cytoplasmic D-GFP are highlighted (yellow carets). Rescaled images of D-GFP are included to assess its localization in germinated spores. Scale bar is 2 µm.