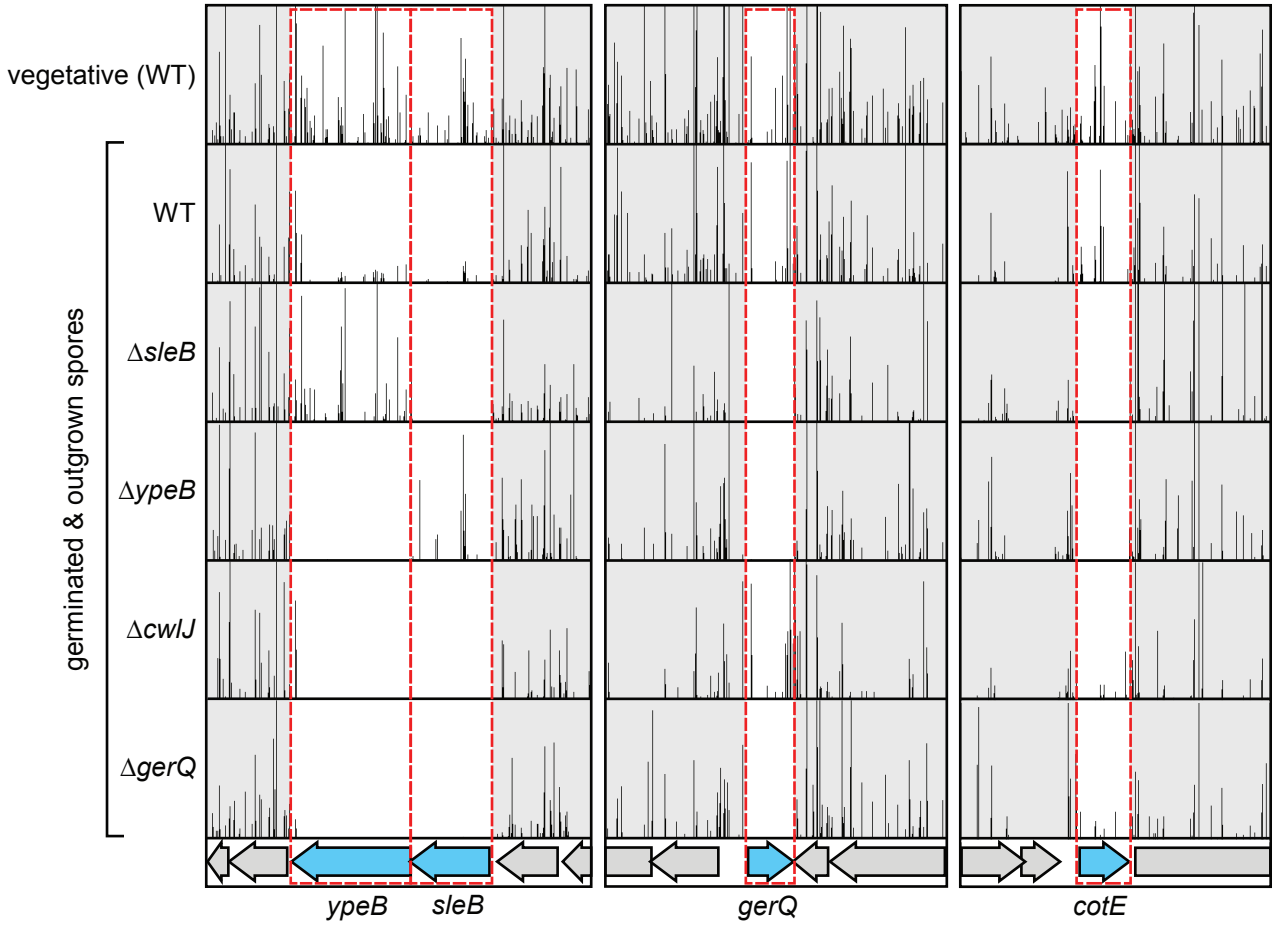
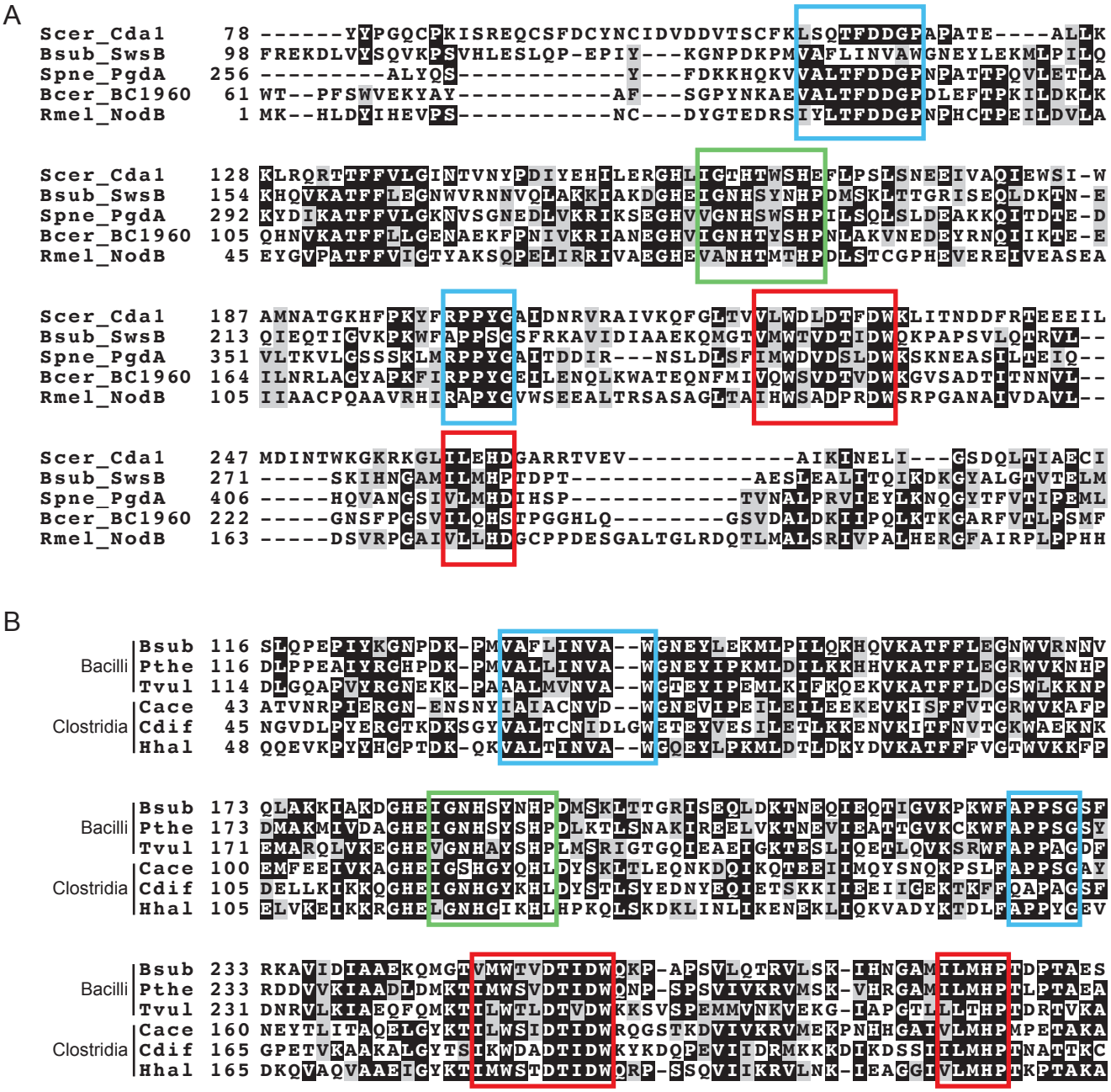


Supplemental Figure 1: Transposon insertion profiles of germinated and outgrown mutant spores. Transposon libraries generated in strains of the indicated genotype were sporulated by nutrient exhaustion for 30 hours. Cultures were heat treated and then plated on LB agar. Colonies from germinated and outgrown spores from each library were separately pooled and transposon junctions were deep sequenced and mapped to a reference genome. At the onset of starvation, a sample of wild-type (WT) cells was collected ("vegetative") and transposon insertions mapped. Shown are regions containing the *ypeB*, *sleB*, *cotE*, and *gerQ* genes. The height of each vertical line corresponds to the number of transposon junctions mapped to that locus, with the height of each box set to 250 reads.



Supplemental Figure 2: Multiple protein alignment of selected CE4 family enzymes. Protein sequences in the CE4 family were obtained from the Carbohydrate Active Enzymes (CAZy) database (http://www.cazy.org) and subjected to multiple sequence alignment (36). Blue boxes highlight residues that act as or position the catalytic base in polysaccharide deacetylases from other species (41, 42). Green box indicates zinc-binding domain. Red boxes indicate residues that act as or position the catalytic acid. (A) Multiple protein alignment of broadly conserved polysaccharide deacetylases. Labels indicate species followed by gene name, with the species listed as follows: Scer – *Saccharomyces cerevisiae*; Bsub – *Bacillus subtilis*; Spne – *Streptococcus pneumoniae*; Bcer – *Bacillus cereus*; Rmel – *Rhizobium meliloti*. (B) Multiple protein alignment of more-narrowly conserved SwsB-like proteins. Colored boxes are in the same positions as in (A). Labels indicate species, listed as follows: Bsub – *Bacillus subtilis* (SwsB); Pthe – *Parageobacillus thermoglucosidasius* (WP_06550991.1); Tvul – *Thermoactinomyces vulgaris* (WP_037996499); Cace – *Clostridium aceticum* (WP_044825898.1); Cdif – *Clostridioides difficile* (WP_003438319.1); Hhal – *Halobacteroides halobius* (WP_015326413).

Figure S3
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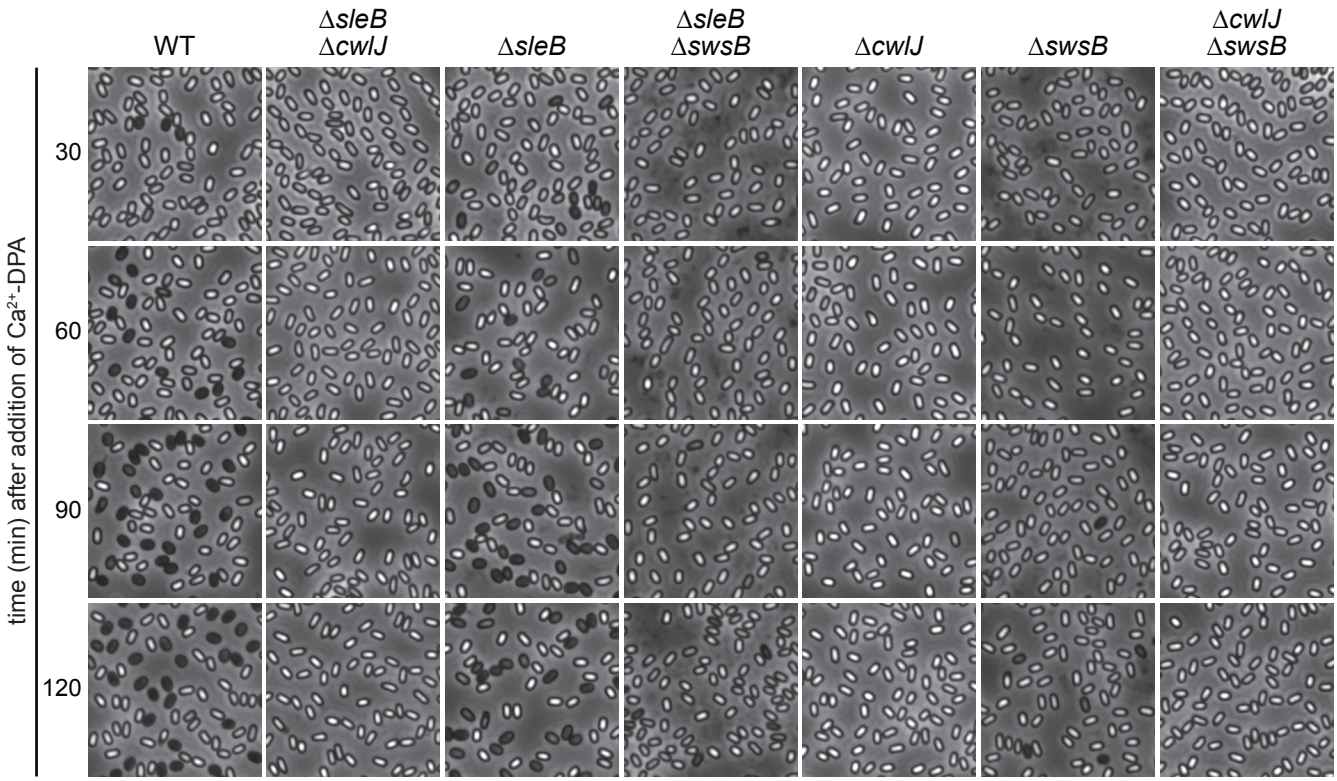
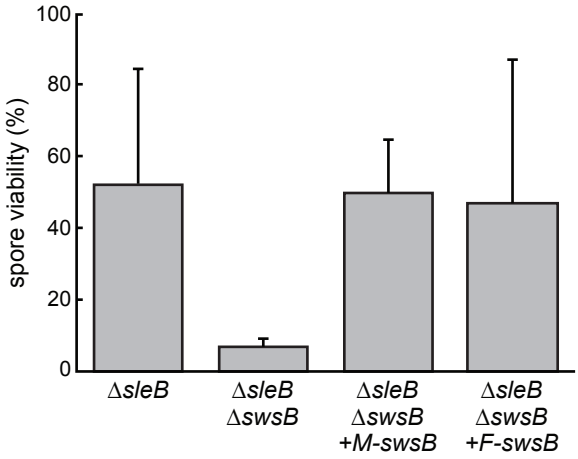
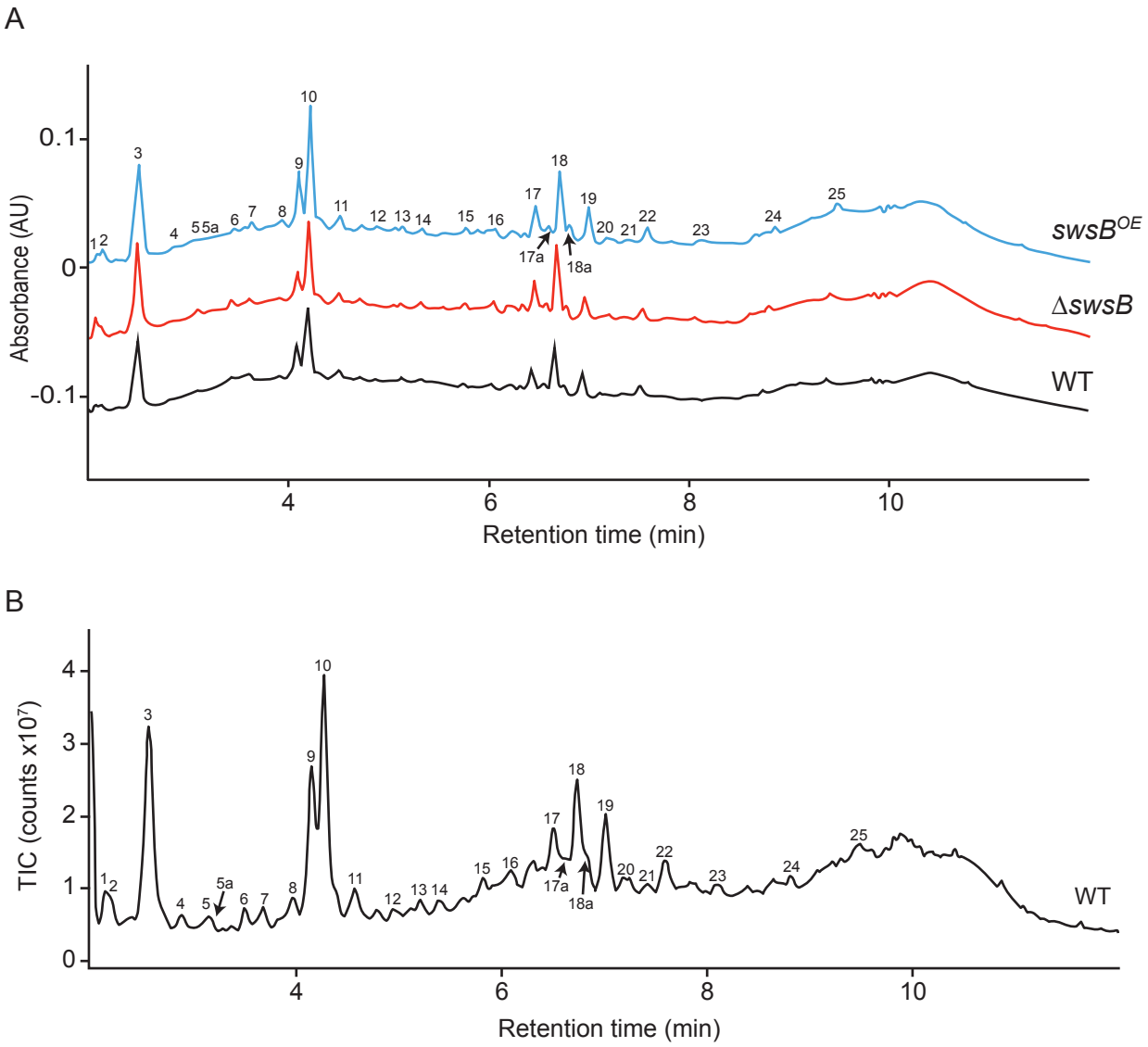


Supplemental Figure 3: SwsB is required for Ca^{2+}-DPA-mediated spore germination. Representative phase-contrast micrographs of spores germinating in Ca^{2+}-DPA. Spores of the indicated genotype were purified by density gradient centrifugation and then resuspended in freshly prepared 60mM Ca^{2+}-DPA followed by incubation at 22 °C with agitation. Aliquots were removed every 30 minutes for imaging. Experiments were performed in biological triplicate, with representative images shown. For each sample, >1000 spores were scored at the 120-minute timepoint.



Supplemental Figure 4: Complementation of Δ*swsB* Δ*sleB* with *swsB* alleles containing strong forespore- and mother-cell-specific promoters. The Δ*swsB* Δ*sleB* double mutant was transformed at an ectopic locus (*ycgO*) with *swsB* under control of the strong mother cell promoter *PspoIID* (*M-swsB*) or the strong forespore promoter *PsspB* (*F-swsB*). Cells were then sporulated by nutrient exhaustion, heat-treated, and plated on LB agar to assess heat-resistant colony forming units. Wild-type spore viability (~ 3.8x108 CFU/ml) was set to 100%. Error bars indicate standard deviation, n = 3.



Supplemental Figure 5: Major muropeptide species from spore peptidoglycan identified by UPLC-QTOF analysis. (A) Figure 6 enlarged to show numbered peaks with identified masses. (B) Total ion chromatogram of the wild-type sample. Numbers correspond to the same peaks in (A). All experiments were performed in triplicate, with representative chromatograms shown.

Supplemental Figure 6 (following page): Proposed structures of identified muropeptides. Hypothetical chemical structures were drawn based on the digestion pattern of mutanolysin and the molecular weights of the major species found in Supplemental Table 1. LD (3-3) and DD (4-3) peptide crosslinks are ambiguous and could be interchanged. Site of deacetylation on muropeptide 5 was determined by MS-MS fragmentation analysis (see Supplemental Figure 7). Site of deacetylation on muropeptide 16 was assumed at the same location.

Figure S6
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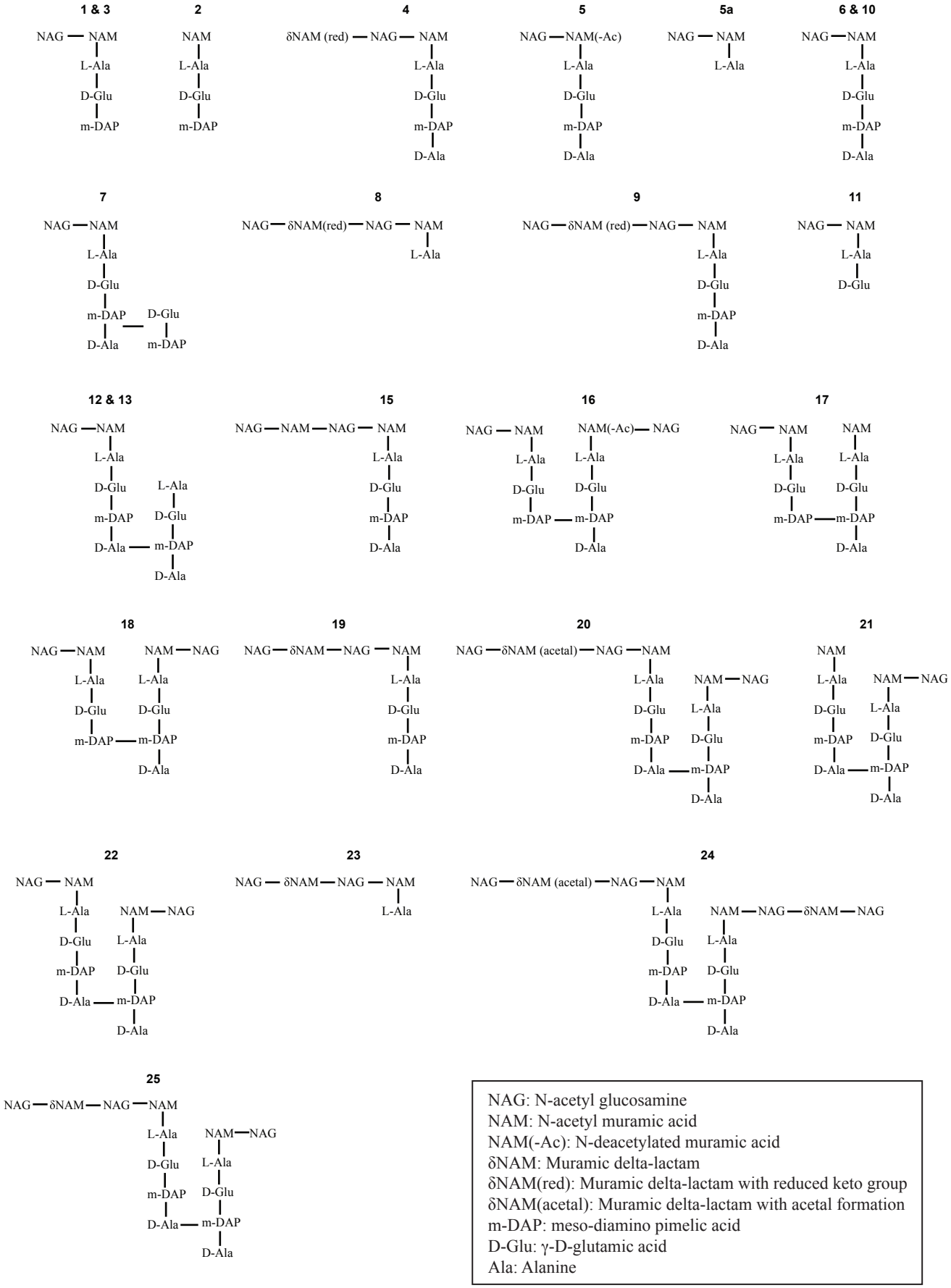
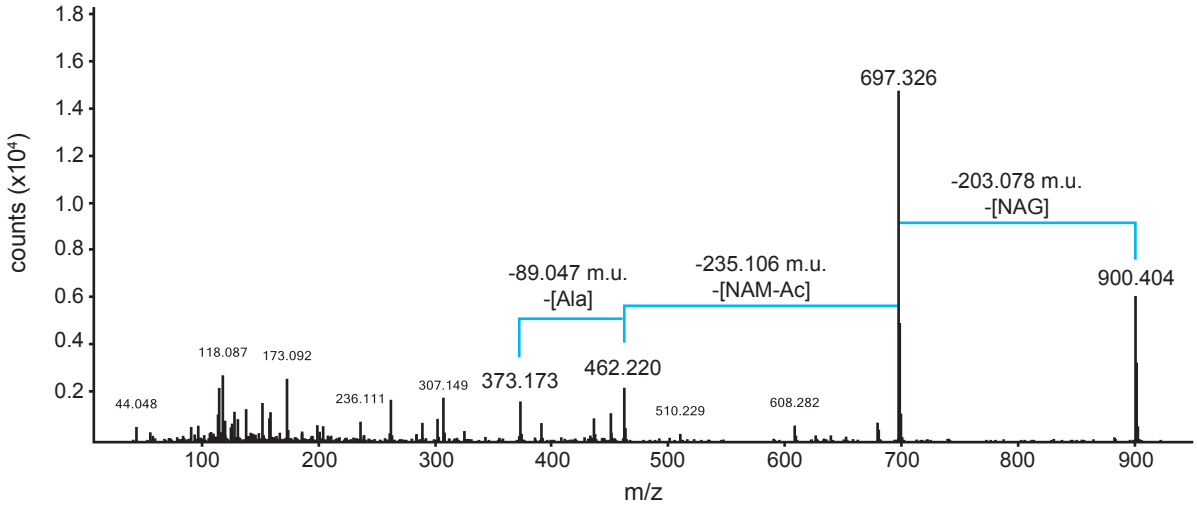
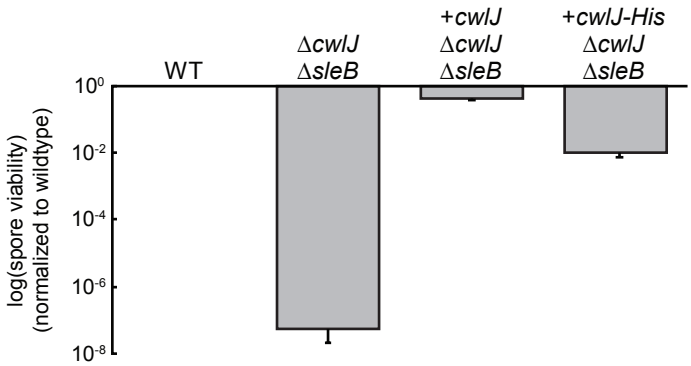


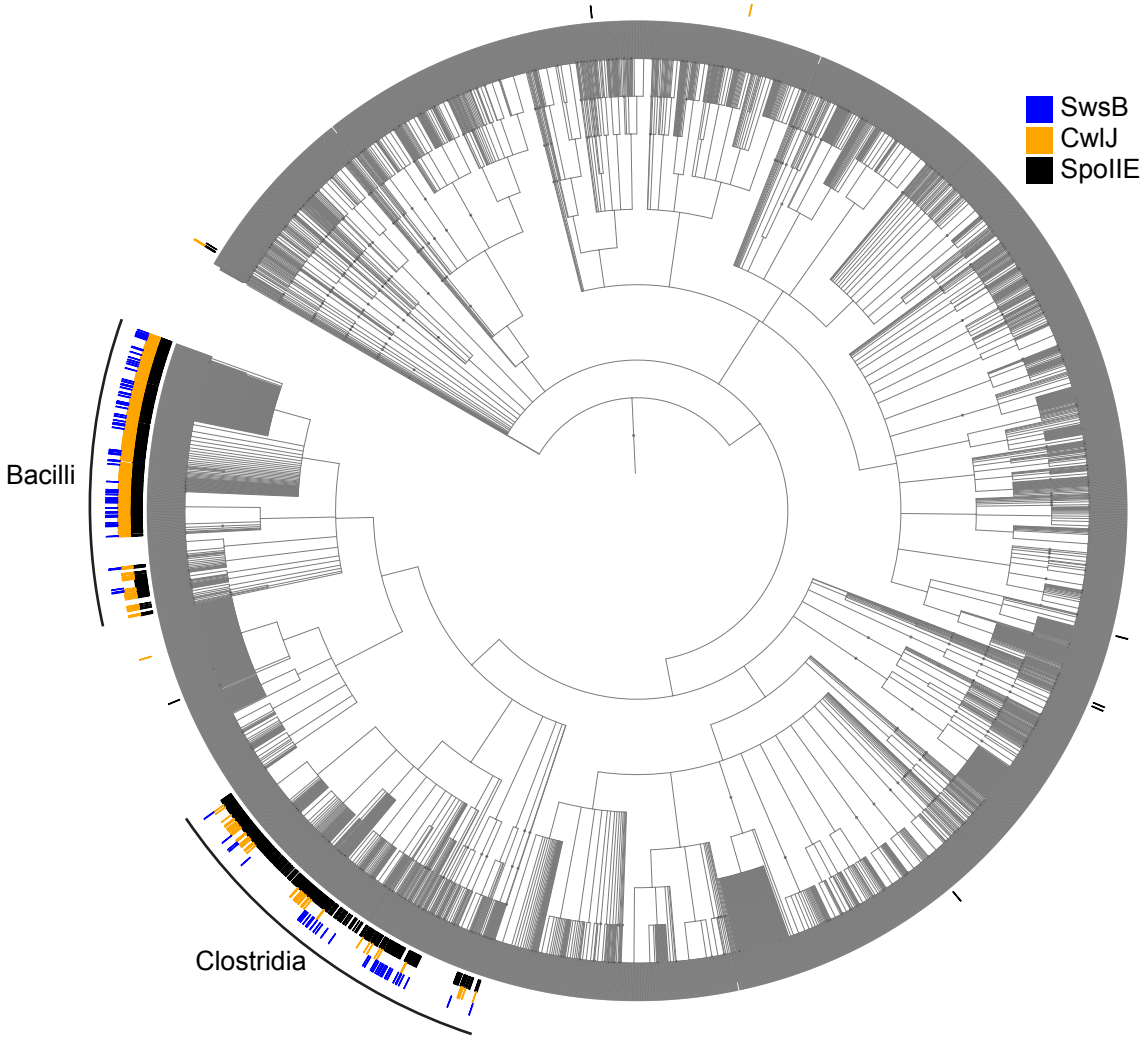
NAG: N-acetyl glucosamine
NAM: N-acetyl muramic acid
NAM(-Ac): N-deacetylated muramic acid
δNAM: Muramic delta-lactam
δNAM(red): Muramic delta-lactam with reduced keto group
δNAM(acetal): Muramic delta-lactam with acetal formation
m-DAP: meso-diamino pimelic acid
D-Glu: γ-D-glutamic acid
Ala: Alanine



Supplemental Figure 7: Mass fragmentation pattern of deacetylated monomer. Muropeptide 5 was subjected to MS-MS analysis to determine location of deacetylation. Mass spectrum after fragmentation is shown. m.u. – mass units.



Supplemental Figure 8: Complementation of Δ*sleB* Δ*cwlJ* with ectopically expressed cwlJ alleles. The Δ*sleB* Δ*cwlJ* double mutant was transformed at an ectopic locus (*amyE*) with either *cwlJ* or a tagged version of CwlJ containing a C-terminal hexahistidine tag (*cwlJ-His*). Cells of the indicated genotype were sporulated by nutrient exhaustion, heat-treated, and plated on LB agar to assess heat-resistant colony forming units. The data are presented with a logarithmic y-axis for clarity, with wild-type spore viability (~ 3.8x108 CFU/ml) set to 1. Error bars indicate standard deviation, n =3.



Supplemental Figure 9: Phylogenetic tree of SwsB- CwlJ- and SpoIIE orthoglogs. Protein sequences of CwlJ, SwsB, and SpoIIE (to serve as a marker for spore-forming organisms) were subjected to homology searches using BLAST against the NCBI Reference Sequence protein database. E-value cutoff for all proteins was 1x10-8. CwlJ hits were further selected to be at least 35% identical to B. subtilis CwlJ so as to differentiate them from SleB, which shares some homology with CwlJ. SwsB hits were also subjected to subsequent filtering to differentiate from multiple other CE4 family deacetylases with high homology. SwsB homologs were defined as BLAST hits that retained zinc-binding (H192 and H188 in *B. subtilis*) and catalytic-acid residues (D256 and H282 in *B. subtilis*) but lacked catalytic-base residues (N137 and A226 in *B. subtilis* SwsB; D and R, respectively, in non-SwsB deacetylases). These regions were defined by performing multiple alignments of protein sequences in the CE4 family from the CAZy database (see Supplemental Figure 2). Hits were then plotted on a Newick tree built from the Reference Prokaryotic Representative Genomes library available at NCBI and visualized using iTOL (64, 65). Full interactive tree is available at http://itol.embl.de/shared/jamon. Note that not all organisms with hits are represented on this tree. A complete list of hits for CwlJ and SwsB is available in Dataset S1.

Table S1: List of muropeptides identified by UPLC-QTOF analysis

Peak	$[M+H]^+$	Proposed muropeptide composition								
		NAG	NAM	NAM(-Ac)	δ-NAM	δ-NAM (Reduced)	δ-NAM (acetal)	Ala	Glu	Dap
1	871.3797	1	1	0	0	0	0	1	1	1
2	668.2983	0	1	0	0	0	0	1	1	1
3	871.3798	1	1	0	0	0	0	1	1	1
4	1143.5076	1	1	0	0	1	0	2	1	1
5	900.4025	1	0	1	0	0	0	2	1	1
5a	570.2516	1	1	0	0	0	0	1	0	0
6	942.4146	1	1	0	0	0	0	2	1	1
7	1243.5612	1	1	0	0	0	0	2	2	2
8	974.4388	2	1	0	0	1	0	1	0	0
9	1346.5937	2	1	0	0	1	0	2	1	1
10	942.4142	1	1	0	0	0	0	2	1	1
11	699.2949	1	1	0	0	0	0	1	1	0
12	1385.6221	1	1	0	0	0	0	4	2	2
13	1385.6377	1	1	0	0	0	0	4	2	2
14	1801.6968	-	-	-	-	-	-	-	-	-
15	1420.5998	2	2	0	0	0	0	2	1	1
16	1752.7580	2	1	1*	0	0	0	3	2	2
17	1591.6933	1	2	0	0	0	0	3	2	2
17a	1874.7662	-	-	-	-	-	-	-	-	-
18	1794.7730	2	2	0	0	0	0	3	2	2
18a	1386.5952	-	-	-	-	-	-	-	-	-
19	1360.5706	2	1	0	1	0	0	2	1	1
20	2301.9964	3	2	0	0	0	1	4	2	2
21	1662.7328	1	2	0	0	0	0	4	2	2
22	1865.8013	2	2	0	0	0	0	4	2	2
23	988.4106	2	1	0	1	0	0	1	0	0
24	2720.1401	4	2	0	1	0	1	4	2	2
25	2283.9676	3	2	0	1	0	0	4	2	2

* site of deacetylation was assumed at NAM as per NAM deacetylation in muropeptide 5.

TABLE S2: *Bacillus subtilis* strains used in this study

Strain	Genotype	Source	Figure
168	*trpC2*	Zeigler *et al*., 2008 (54)	1-4, 6, 7, S1, S3, S5, S8
BAM476	Δ*sleB::lox72*	This study	1, S1
BAM478	Δ*ypeB::lox72*	This study	1, S1
BAM477	Δ*cwlJ::lox72*	This study	1, S1
BAM837	Δ*gerQ::lox72*	This study	1, S1
BDR3488	Δ*swsB::lox72*	This study	2-7, S3, S4, S5
BDR3486	Δ*cwlJ::lox72*	This study	2, 3, 4, 7, S3
BDR3487	Δ*sleB::lox72*	This study	2-5, 7, S1, S3, S4
BDR3196	Δ*sleB::lox72* Δ*cwlJ::lox72*	This study	2, 3, 4, 7, S3, S8
BDR3497	Δ*swsB::lox72* Δ*cwlJ::lox72*	This study	2, 3, 4, 7, S3
BDR3498	Δ*swsB::lox72* Δ*sleB::lox72*	This study	2-5, 7, S3
BJA018a	Δ*cotE::cat*	This study	2
BJA023a	Δ*cotE::cat* Δ*cwlJ::erm*	This study	2
BJA024a	Δ*cotE::cat* Δ*sleB::erm*	This study	2
BJA020a	Δ*safA::tet*	This study	2
BJA027a	Δ*safA::tet* Δ*cwlJ::erm*	This study	2
BJA028a	Δ*safA::tet* Δ*sleB::erm*	This study	2
BJA019a	Δ*gerQ::tet*	This study	2
BJA025a	Δ*gerQ::tet* Δ*cwlJ::erm*	This study	2
BJA026a	Δ*gerQ::tet* Δ*sleB::erm*	This study	2
BDR3513	Δ*swsB::lox72* Δ*sleB::lox72 amyE::swsB-His*	This study	5
BJA047a	Δ*swsB::lox72* Δ*sleB::lox72 amyE::swsB (H282A)-His (spec)*	This study	5
BJA048a	Δ*swsB::lox72* Δ*sleB::lox72 amyE::swsB (D256A)-His (spec)*	This study	5
BJA049a	Δ*swsB::lox72* Δ*sleB::lox72 amyE::swsB (D256N)-His (spec)*	This study	5
BJA073a	Δ*swsB::lox72* Δ*sleB::lox72 amyE::PspoIID-swsB (spec)*	This study	S4
BJA075a	Δ*swsB::lox72* Δ*sleB::lox72 amyE::PsspB-swsB (spec)*	This study	S4
BJA093a	*amyE::PspoIID-swsB (spec) ycgO::PsspB-swsB (erm)*	This study	6, S5
BJA087a	*amyE::cwlJ-His (spec)*	This study	7
BJA088a	Δ*swsB::lox72 amy::cwlJ-His (spec)*	This study	7
BJA083a	Δ*sleB::lox72* Δ*cwlJ::lox72 amyE::cwlJ-His (spec)*	This study	S8
BJA102a	Δ*sleB::lox72* Δ*cwlJ::lox72 amyE::cwlJ (spec)*	This study	S8
BJA094a	Δ*cotE::cat amyE::cwlJ-His (spec)*	This study	7
BJA095a	Δ*gerQ::tet amyE::cwlJ-His (spec)*	This study	7
BJA096a	Δ*safA::tet amyE::cwlJ-His (spec)*	This study	7
BJA105a	Δ*swsB::lox72 amyE::swsB (spec) ycgO::cwlJ-His (cat)*	This study	7
BJA106a	Δ*swsB amyE::swsB-His (spec) ycgO::cwlJ-His (cat)*	This study	7
BDR3178	Δ*gerAB::lox72* Δ*spoVFA::erm*	Ramirez-Guadiana *et al.,* 2017 (66)	7
BDR3970	Δ*sleB::lox72* Δ*spoVA::tet*	Ramirez-Guadiana *et al.,* 2017 (66)	7

TABLE S3: Plasmids used in this study

Plasmid	Description	Source
pFR12	*amyE::swsB (spec)*	This study
pFR13	*amyE::swsB-GSG-His6 (spec)*	This study
pJA003	*amyE::swsBH282A)-GSG-His6 (spec)*	This study
pJA004	*amyE::swsB(D256A)-GSG-His6 (spec)*	This study
pJA005	*amyE::swsB(D256N)-GSG-His6 (spec)*	This study
pJA016	*amyE::PspoIID-swsB (spec)*	This study
pJA018	*amyE::PsspB-swsB (spec)*	This study
pJA022	*amyE::cwlJ-GSG-His6 (spec)*	This study
pJA030	*ycgO::PsspB-swsB (erm)*	This study
pJA035	*amyE::cwlJ (spec)*	This study
pJA038	*ycgO::cwlJ-GSG-His (cat)*	This study

TABLE S4 Oligonucleotides used in this study

Oligonucleotide	Sequence
oFR25	gccGGATCCGGTGCACATTTCAGAGCTTGC
oFR26	gccGAATTCATCGCAACAGAACGGACTGTC
oFR28	gccGAATTCTTAGTGATGGTGATGGTGATGTCCTGAGCCCTTCAATAGTCTTGTTTCATC
oJA020	ACATAATGGTGCCATGATTTTAATGGCTCCGACTGACCCTACGGCGGAAAGTC
oJA042	GACTTTCCGCCGTAGGGTCAGTCGGAGCCATTAAAATCATGGCACCATTATGT
oJA021	AGTCATGTGGACAGTTGATACAATCGCTTGGCAAAAGCCGGCTCCGTCTGTAC
oJA043	GTACAGACGGAGCCGGCTTTTGCCAAGCGATTGTATCAACTGTCCACATGACT
oJA022	AGTCATGTGGACAGTTGATACAATCAATTGGCAAAAGCCGGCTCCGTCTGTAC
oJA044	GTACAGACGGAGCCGGCTTTTGCCAATTGATTGTATCAACTGTCCACATGACT
oJA058	ctagagtcgaatcccgAAGCTTACATAAGGAGGAACTACTATGTACAAAAAATTTGTACC
oJA059	TGGTAGCGACCGGCGCTCAGGATCCTTACTTCAATAGTCTTGTTTCATCC
oJA062	aaaaggagattttacacaagctTacataaggaggaactactATGTACAAAAAATTTGTAC
oJA063	aaaaggagattttacacaagctTacataaggaggaactactATGAATCACTTCTATGTGT
oJA068	AGCGACCGGCGCTCAGGATCCTGTGAATCCAGGAATTGAGG
oJA069	CGCCAGGGCTGCAGGAATTCTTAGTGATGGTGATGGTGATGTCCTGAGCCAAATGTGTTATATACATTTTC
oJA093	CGCCATTCGCCAGGGCTGCAGGAATTCTGTGAATCCAGGAATTGAGG
oJA094	AGCGACCGGCGCTCAGGATCCCTAAAATGTGTTATATACATTTTC

Supplemental Methods

Plasmid construction

pFR12 [*amyE*::P_{swsB}-*swsB (spec)*] was generated in a two-way ligation with a *Bam*HI-*Eco*RI PCR product containing P_{swsB}-*swsB* (oligonucleotide primers oFR25 + oFR26 using genomic DNA from *B. subtilis* 168 as template) and pLD30 cut with *Bam*HI-*Eco*RI. pLD30 [*amyE*::spec] is an ectopic integration vector for double crossover integrations at the *amyE* locus (laboratory stock).

pFR13 [*amyE*::P_{swsB}-*swsB*-GSG-His_6 *(spec)*] was generated in a two-way ligation with a *Bam*HI-*Eco*RI PCR product containing P_{swsB}-*swsB*-GSG-His_6 (oligonucleotide primers oFR25 + oFR28 using genomic DNA from *B. subtilis* 168 as template) and pLD30 cut with *Bam*HI-*Eco*RI.

pJA003 [*amyE:: *P_{swsB}-*swsB(H292A)*-GSG-His_6 *(spec)*] was generated by site-directed mutagenesis of pFR13 using oligonucleotide primers oJA020 and oJA042.

pJA004 [*amyE:: *P_{swsB}-*swsB(D256A)*-GSG-His_6 *(spec)*] was generated by site-directed mutagenesis of pFR13 using oligonucleotide primers oJA021 and oJA043.

pJA005 [*amyE:: *P_{swsB}-*swsB(D256N)*-GSG-His_6 *(spec)*] was generated by site-directed mutagenesis of pFR13 using oligonucleotide primers oJA022 and oJA044.

pJA016 [*amyE::*P_{spoIID}-*swsB (spec)*] was generated by isothermal assembly of a PCR product containing *swsB* (oligonucleotide primers oJA058 + oJA059 using genomic DNA from *B. subtilis 168* as template) and pKM012 [*amyE-PspoIID-yfp (spec)*] that had been linearized with HindIII and BamHI followed by gel purification to isolate the backbone from the *yfp* gene. pKM012 is a modified version of pLD30 with a YFP reporter under control of the SpoIID promoter designed for double-crossover integration at the *amyE* locus.

pJA018 [*amyE::*P_{sspB}-*swsB (spec)*] was generated by isothermal assembly of a PCR product containing *swsB* (oligonucleotide primers oJA062 + oJA063 using genomic DNA from *B. subtilis 168* as template) and pNC153 [*amyE-*P_{sspB}-*yfp*] that had been linearized with HindIII and BamHI followed by gel purification to isolate the backbone from the *yfp* gene. pNC153 is a modified version of pLD30 with a YFP reporter under control of the SspB promoter designed for double-crossover integration at the *amyE* locus

pJA022 [*amyE::*P_{cwlJ}-*cwlJ*-GSG-His_6 *(spec)*] was generated by isothermal assembly of a PCR product containing P_{cwlJ}-*cwlJ*-GSG-His_6 (oligonucleotide primers oJA068 + oJA069 using genomic DNA from *B. subtilis 168* as a template) and pLD030 linearized with BamHI and EcoRI.

pJA030 [*ycgO::*P_{sspB}-*swsB (erm)*] was created by double ligation of the EcoRI-BamHI fragment (P_{sspB}-*swsB*) from pJA018 into pER118. pER118 [*ycgO::erm*] is an ectopic integration vector for double crossover integrations at the *ycgO* locus (laboratory stock).

pJA035 [*amyE::*P_{cwlJ}-*cwlJ (spec)*] was created by isothermal assembly of a PCR product containing P_{cwlJ}-*cwlJ* (oligonucleotide primers oJA093 + oJA094 using genomic DNA from *B. subtilis 168* as template) into pLD30 linearized with BamHI and EcoRI.

pJA038 [*ycgO::*P_{cwlJ}-*cwlJ*-GSG-His_6 *(cat)*] was created by double ligation of the EcoRI-BamHI fragment (P_{cwlJ}-*cwlJ*-GSG- His_6) from pJA022 into pCB042. pCB042 [*ycgO::cat*] is an ectopic integration vector for double crossover integrations at the *ycgO* locus (laboratory stock).

Supplemental References

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