SUPPLEMENTAL DATA

A highly coordinated cell wall degradation machine governs spore morphogenesis in *Bacillus subtilis*

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Plasmid Construction

The plasmids used in this study are listed in Table S2.

pDR198 [*his$_6$-IIP*] was generated in a two-way ligation with an *Nhe*I-*Bam*HI PCR product containing the extracellular domain of SpoIIP (oligonucleotide primers oDR386 and oDR387 and PY79 genomic DNA as template) and pRsetA (Invitrogen) cut with *Nhe*I and *Bam*HI.

pDR199 [*his$_6$-IID*] was generated in a two-way ligation with an *Nhe*I-*Xho*I PCR fragment encoding the extracellular domain of SpoIID (oligonucleotide primers oDR379 and oDR380 and PY79 genomic DNA as template) and pRsetA cut with *Nhe*I and *Xho*I.

pCM187 [*his$_6$-IIP^{H189R}*] was generated by site-directed mutagenesis using oligonucleotide primers oCM160 and oCM161 and plasmid pDR198 as template.

pCM188 [*his$_6$-IIP^{H278R}*] was generated by site-directed mutagenesis using oligonucleotide primers oCM162 and oCM163 and plasmid pDR198 as template.

pCM189 [*his$_6$-IIP^{D280G}*] was generated by site-directed mutagenesis using oligonucleotide primers oCM164 and oCM165 and plasmid pDR198 as template.

pKM339 [*amyE::spoIID*] was generated in a two-way ligation with an *Eco*RI-*Bam*HI PCR fragment containing the *spoIID* gene (oligonucleotide primers oDR65 and oDR728 and PY79 genomic DNA as template) and pDL30 [amyE::spec] (Garsin et al. 1998) cut with *Eco*RI and *Bam*HI.

pKM341 [*his$_6$-IID^{E88A}*] was generated by site-directed mutagenesis using oligonucleotide primers oDR734 and plasmid pDR199 as template.

pKM342 [*his$_6$-IID^{R106A}*] was generated by site-directed mutagenesis using oligonucleotide primers oDR738 and plasmid pDR199 as template.

pKM343 [*his$_6$-IID^{T188A}*] was generated by site-directed mutagenesis using oligonucleotide primers oDR740 and plasmid pDR199 as template.

pKM344 [*his$_6$-IID^{H297A}*] was generated by site-directed mutagenesis using oligonucleotide primers oDR744 and plasmid pDR199 as template.

pKM345 [*his$_6$-IID^{Y323A,Y324A}*] was generated by site-directed mutagenesis using oligonucleotide primers oDR746 and plasmid pDR199 as template.

pKM346 [*amyE::spoIID^{E78A}*] was generated by site-directed mutagenesis using oligonucleotide primers oDR732 and plasmid pKM339 as template.

pKM347 [*amyE::spoIID^{Y80A}*] was generated by site-directed mutagenesis using oligonucleotide primers oDR733 and plasmid pKM339 as template.

pKM348 [*amyE::spoIID*^{E88A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR734 and plasmid pKM339 as template.

pKM349 [*amyE::spoIID*^{E96A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR735 and plasmid pKM339 as template.

pKM350 [*amyE::spoIID*^{K99A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR736 and plasmid pKM339 as template.

pKM351 [*amyE::spoIID*^{Q101A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR737 and plasmid pKM339 as template.

pKM352 [*amyE::spoIID*^{R106A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR738 and plasmid pKM339 as template.

pKM353 [*amyE::spoIID*^{T164A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR739 and plasmid pKM339 as template.

pKM354 [*amyE::spoIID*^{T188A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR740 and plasmid pKM339 as template.

pKM355 [*amyE::spoIID*^{Y201A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR741 and plasmid pKM339 as template.

pKM356 [*amyE::spoIID*^{Y269A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR742 and plasmid pKM339 as template.

pKM357 [*amyE::spoIID*^{S276A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR743 and plasmid pKM339 as template.

pKM358 [*amyE::spoIID*^{H297A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR744 and plasmid pKM339 as template.

pKM359 [*amyE::spoIID*^{Q303A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR745 and plasmid pKM339 as template.

pKM360 [*amyE::spoIID*^{Y323A,Y324A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR746 and plasmid pKM339 as template.

pKM361 [*amyE::spoIID*^{Y171A}*)*] was generated by site-directed mutagenesis using oligonucleotide primers oDR747 and plasmid pKM339 as template.

pKM362 [*amyE::spoIID*^{Y323A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR749 and plasmid pKM339 as template.

pKM363 [*amyE::spoIID*^{Y324A}] was generated by site-directed mutagenesis using oligonucleotide primers oDR750 and plasmid pKM339 as template.

pTD68 [P_{T7}::*his*$_6$-*sumo*] was generated in a two-way ligation with an *Xba*I-*Xho*I PCR product containing the his$_6$-SUMO domain (oligonucleotide primers oTB178 and oTB684 and pTB146 (Bendezu et al. 2009)as template) and pET21a (Novagen) cut with *Xba*I and *Xho*I.

pTU204 [P_{T7}*::his6-sumo-sltY*] was generated in a two-way ligation with an *Sac*I-*Hind*III PCR product containing the extracellular domain of SltY (28-645aa) (oligonucleotide primers oTB773 and oTB774 and *E. coli* MG1655 genomic DNA as template) and pTD68 cut with *Sac*I and *Hind*III.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Cell wall degradation activity of AmiD using Remazol brilliant blue coupled to *E. coli* PG. Reactions contained 4 μM of each protein. Activity was normalized to a reaction using Lysozyme.

Figure S2. Cell wall degradation activities of IID and IIP using Remazol brilliant blue coupled to *B. subtilis* PG. Reactions contained 12.5 μM of each protein. Activity was normalized to a reaction using the muramidase Mutanolysin.

Figure S3. IIP has endopeptidase activity on crosslinked muropeptides. RP-HPLC elution profiles of the soluble products after treatment of unlabeled *E. coli* PG with indicated enzymes followed by reduction with sodium borohydride as described in the Experimental Procedures. (**A**) Incubation with Mutanolysin. (**B**) Incubation with Mutanolysin followed by heat-inactivation and then treatment with IIP. The muropeptide and crosslinked muropeptide products are shown schematically above the elution peaks. The lack of tetrapeptide products in this reaction indicates that IIP is unable to act as an amidase on the small muropeptide products.

Figure S4. IID has does not cleave intact PG. RP-HPLC elution profiles of the soluble products after treatment of unlabeled *E. coli* PG with indicated enzymes in 1xPBS followed by reduction with sodium borohydride. (**A**) Untreated PG. (**B**) PG incubated with IID. (**C**) PG incubated with AmiD. Under these reaction conditions, AmiD inefficiently cleaves crosslinked muropeptides (**D**) PG incubated with IID followed by heat-inactivation and treatment with AmiD. (**E**) PG incubated

with Mutanolysin. (**F**) PG incubated with IID followed by heat-inactivation and treatment with Mutanolysin.

Figure S5. IID cell wall cleavage activity depends on IIP activity. RP-HPLC elution profiles of the soluble products after treatment of unlabeled *E. coli* PG with indicated enzymes followed by reduction with sodium borohydride. (**A**) Untreated PG. (**B**) PG incubated with IID. (**C**) PG incubated with IID and IIP^{H189R}.

Figures S6. Heating is sufficient to inactivate AmiD and IIP. Cell wall degrading activities of AmiD and IIP with and without incubation at 95°C for 15 min using the RBB dye-release assay. All reactions contained 4 μM protein. The dye-coupled soluble products released by AmiD and IIP were normalized to the release by 4 μM Lysozyme (Lys).

Figure S7. IID cleaves naked glycan strands. RP-HPLC elution profile of the soluble products after treatment of unlabeled PG with IIP (4 μM) followed by heat-inactivation and treatment with IID (4 μM). The tetrapeptide and anhydro-disaccharide products are shown schematically above the elution peaks.

Figure S8. Amino acid sequence alignment of the extracellular domains of IID homologs from representative phyla. Bacteria include *B. subtilis*, *Geobacillus sp. WCH70*, *Bacillus cereus BDRD-ST26*, *Bacillus mycoides Rock1-4*, *Bacillus clausii KSM-K16*, *Eubacterium dolichum DSM 3991*, *Mollicutes bacterium D7*, *Clostridium beijerinckii NCIMB 8052*, *Symbiobacterium thermophilum IAM 14863, Thermosinus carboxydivorans Nor1, Syntrophomonas wolfei*, *Alkaliphilus metalliredigens QYMF, Bacteroides capillosus ATCC 29799, Heliobacterium modesticaldum Ice1, Anabaena variabilis ATCC 29413, Microcystis aeruginosa NIES-843, Synechococcus sp. PCC 7335, Myxococcus xanthus, Geobacter sp. FRC-32, Dictyoglomus thermophilum H-6-12, Leptospira biflexa*, *Bdellovibrio bacteriovorus HD100*, *Blautia hydrogenotrophica DSM 10507*, *Halothermothrix orenii H 168*. Conserved amino acids (black boxes) and similar residues (grey boxes) are highlighted. Amino acid substitutions that were tested are indicated above the sequence. Black circles indicate a strong block in engulfment and a >1000-fold defect in sporulation efficiency. Grey circles indicate aberrant engulfment as assessed by fluorescence microscopy.

White circles indicate alanine substitutions with no significant affect on engulfment or sporulation efficiency.

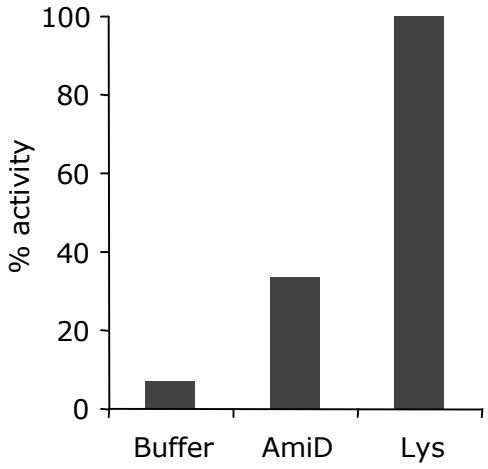
Figure S9. Morphological defects in the IID mutants. At hour 2 of sporulation, the indicated strains were analyzed by fluorescence microscopy using the membrane dye TMA-DPH.

Figure S10. Amino acid sequence alignment of the extracellular domains of *B. subtilis* IID and *B. subtilis* LytB. Conserved amino acids (black boxes) and similar residues (grey boxes) are highlighted. Amino acid substitutions in IID that were tested are indicated above the sequence. Black circles indicate a strong block in engulfment, a >1000-fold defect in sporulation efficiency, and a loss in lytic transglycosylase activity. Grey circles indicate aberrant engulfment as assessed by fluorescence microscopy. White circles indicate alanine substitutions with no significant affect on engulfment or sporulation efficiency.

REFERENCES

Bendezu, F.O., C.A. Hale, T.G. Bernhardt, and P.A. de Boer. 2009. RodZ (YfgA) is required for proper assembly of the MreB actin cytoskeleton and cell shape in E. coli. *Embo J* **28**: 193-204.

Garsin, D.A., D.M. Paskowitz, L. Duncan, and R. Losick. 1998. Evidence for common sites of contact between the antisigma factor SpoIIAB and its partners SpoIIAA and the developmental transcription factor sigmaF in Bacillus subtilis. *J Mol Biol* **284**: 557-68.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60
40
20
0
% activity
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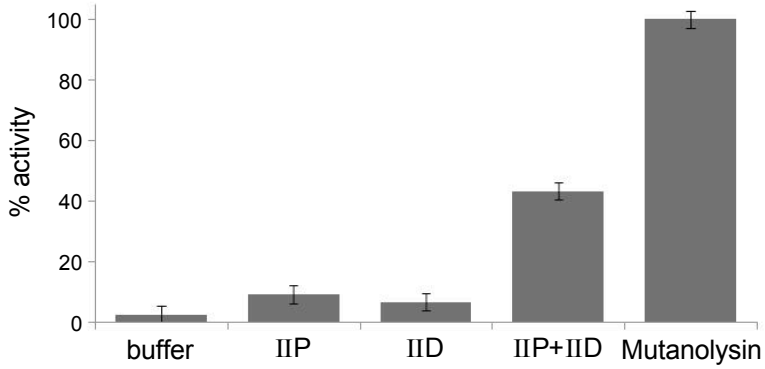
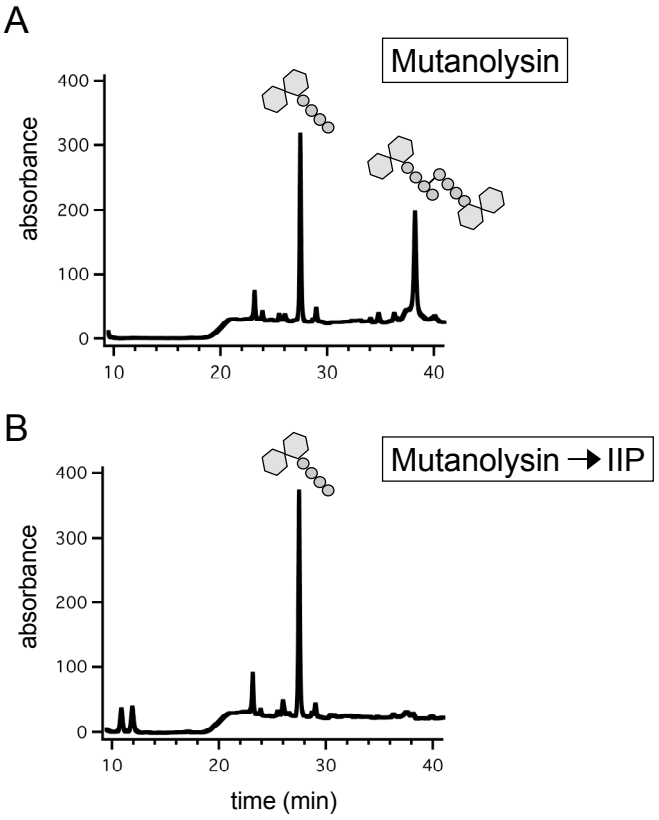
AmiD
Lys

Morlot_FigS2

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Mutanolysin
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10
20
30
40
B
Mutanolysin → IIP
absorbance
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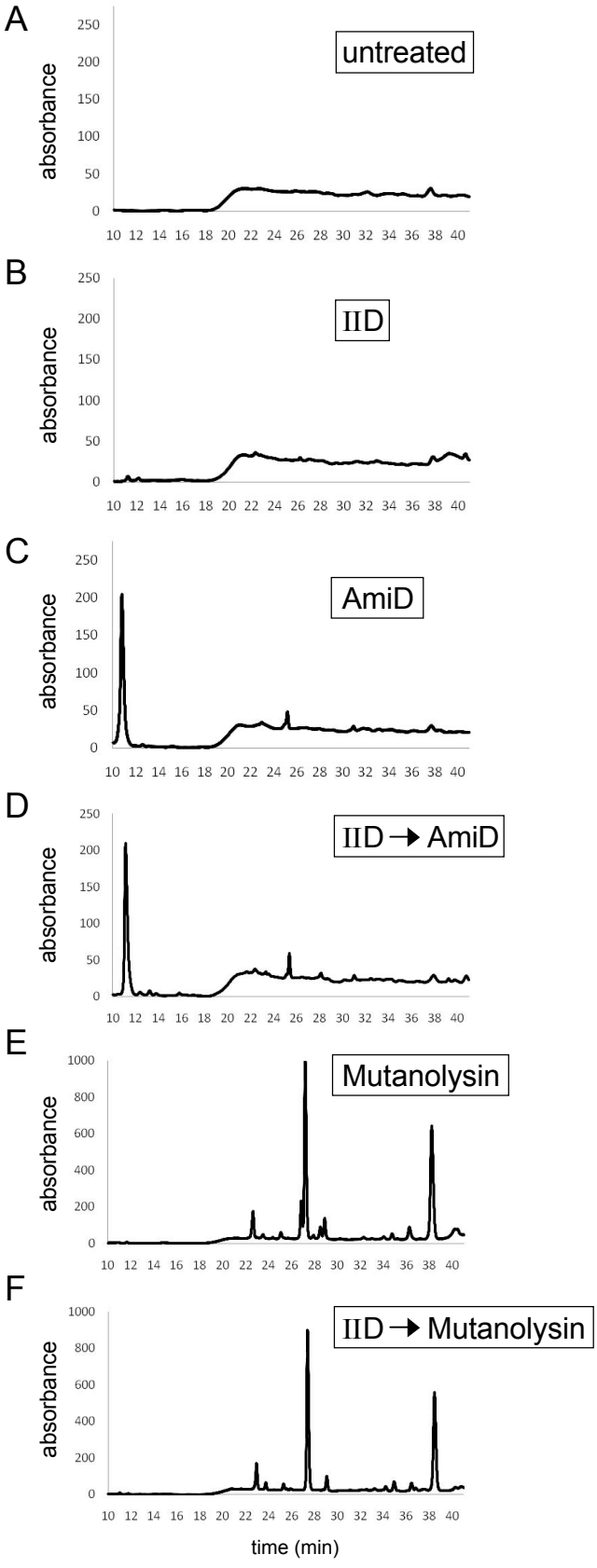
20
30
40
time (min)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B
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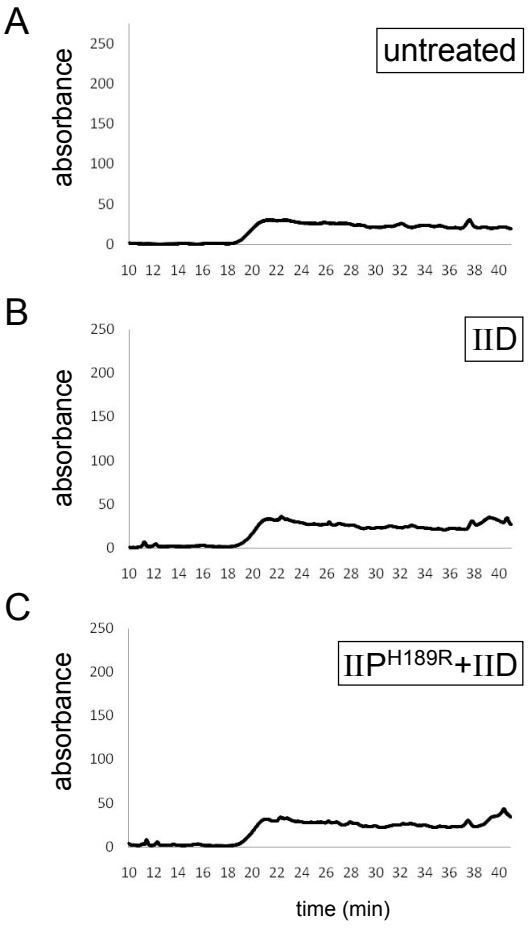
C
AmiD
D
IID → AmiD
E
Mutanolysin
F
IID → Mutanolysin
absorbance
time (min)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50
0
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B
IID
absorbance
250
200
150
100
50
0
10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
C
IIP^H189R+IID
absorbance
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200
150
100
50
0
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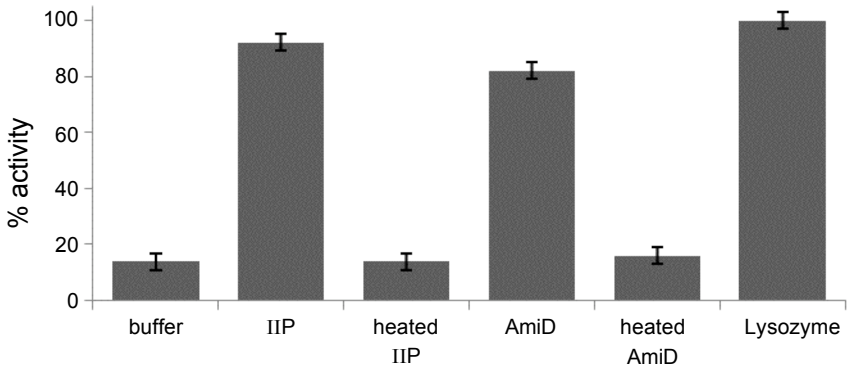
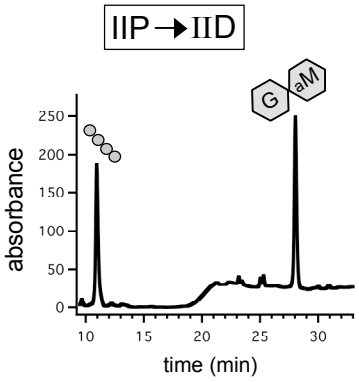
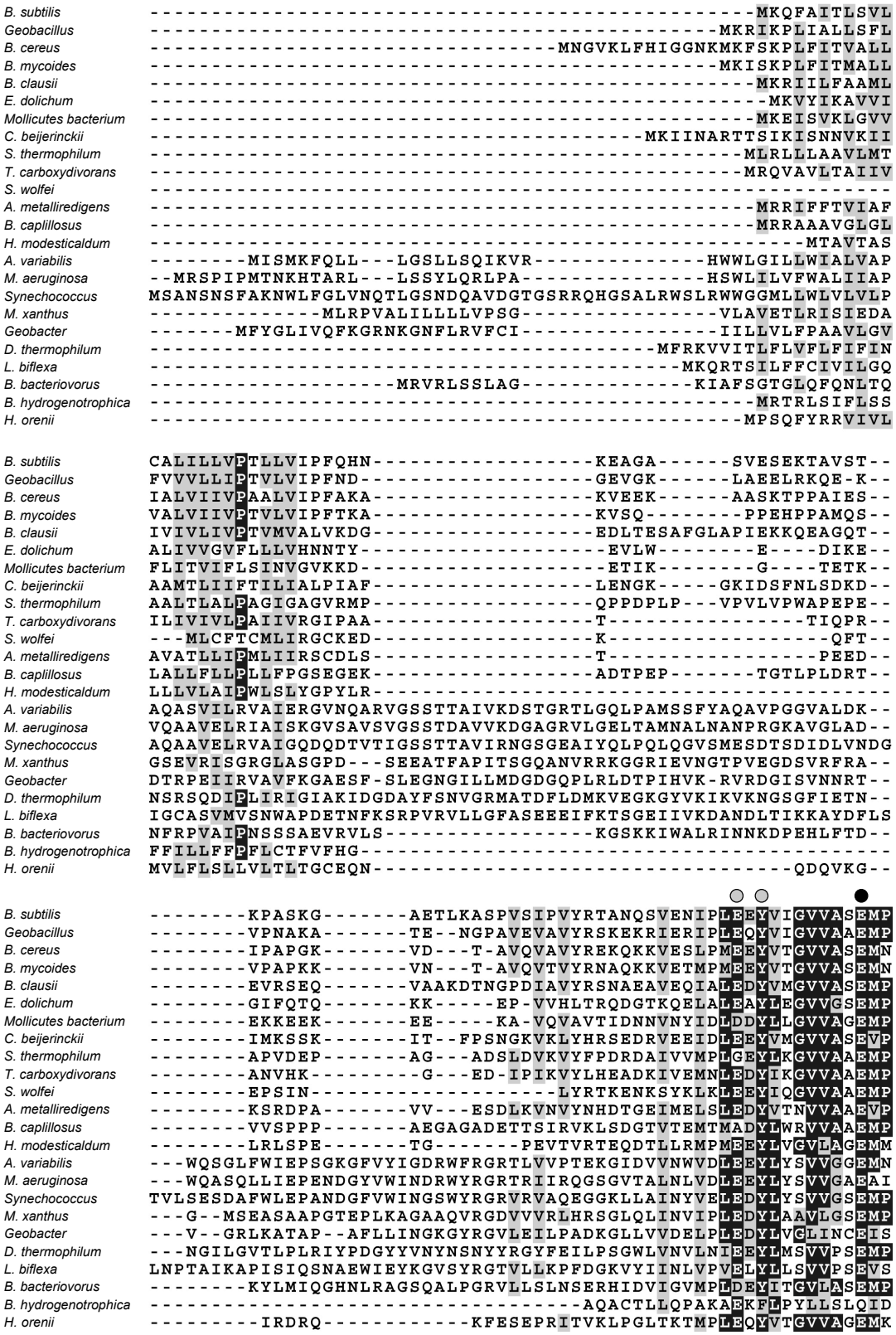
time (min)

Morlot_FigS6




IIP→IID
G
aM
absorbance
250
200
150
100
50
0
10
15
20
25
30
time (min)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Geobacillus
B. cereus
B. mycoides
B. clausii
E. dolichum
Mollicutes bacterium
C. beijerinckii
S. thermophilum
T. carboxydivorans
S. wolfei
A. metalliredigens
B. caplillosus
H. modesticaldum
A. variabilis
M. aeruginosa
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B. bacteriovorus
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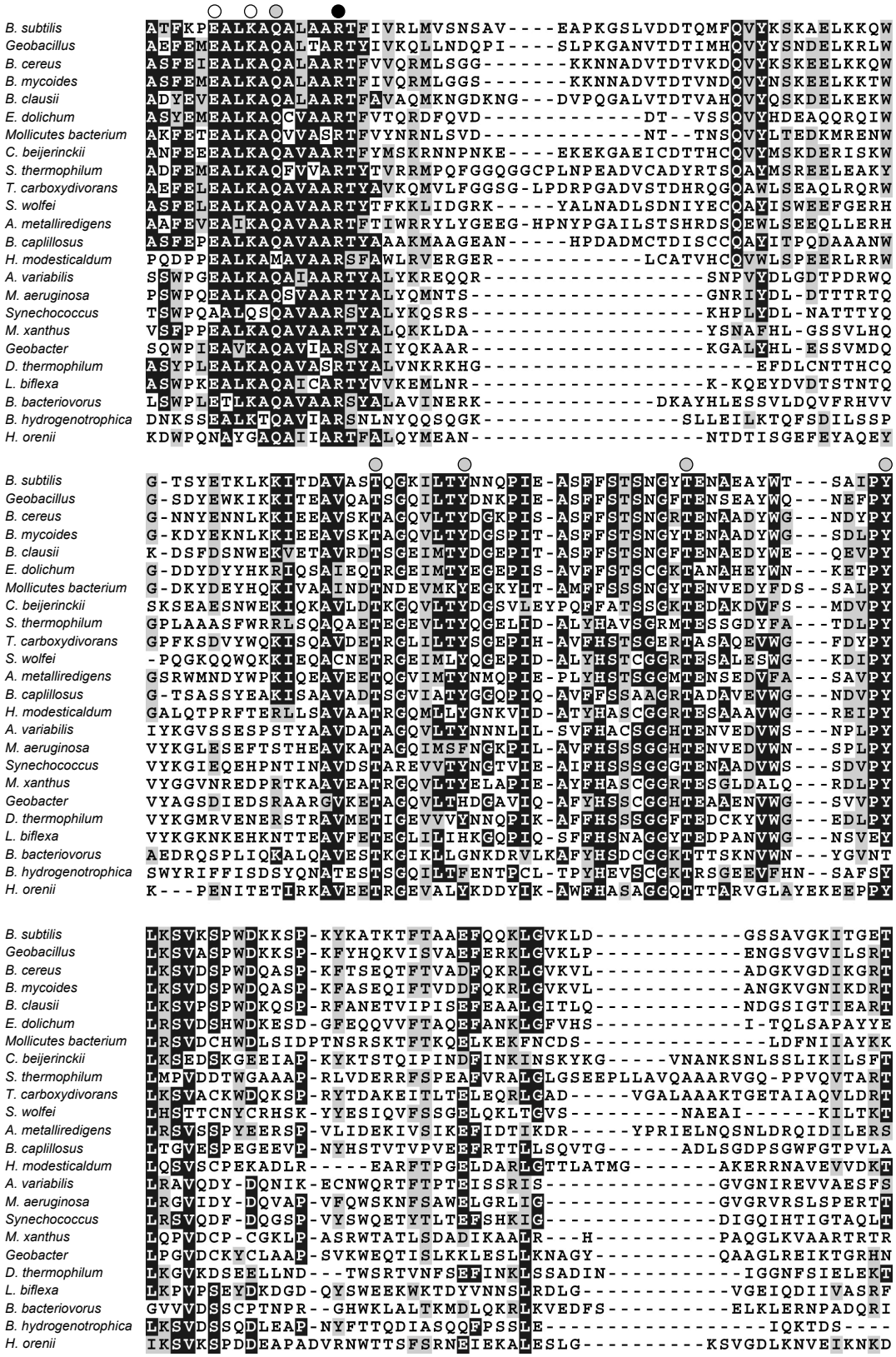
H. orenii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Geobacillus
B. cereus
B. mycoides
B. clausii
E. dolichum
Mollicutes bacterium
C. beijerinckii
S. thermophilum
T. carboxydivorans
S. wolfei
A. metalliredigens
B. caplillosus
H. modesticaldum
A. variabilis
M. aeruginosa
Synechococcus
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Geobacter
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B. bacteriovorus
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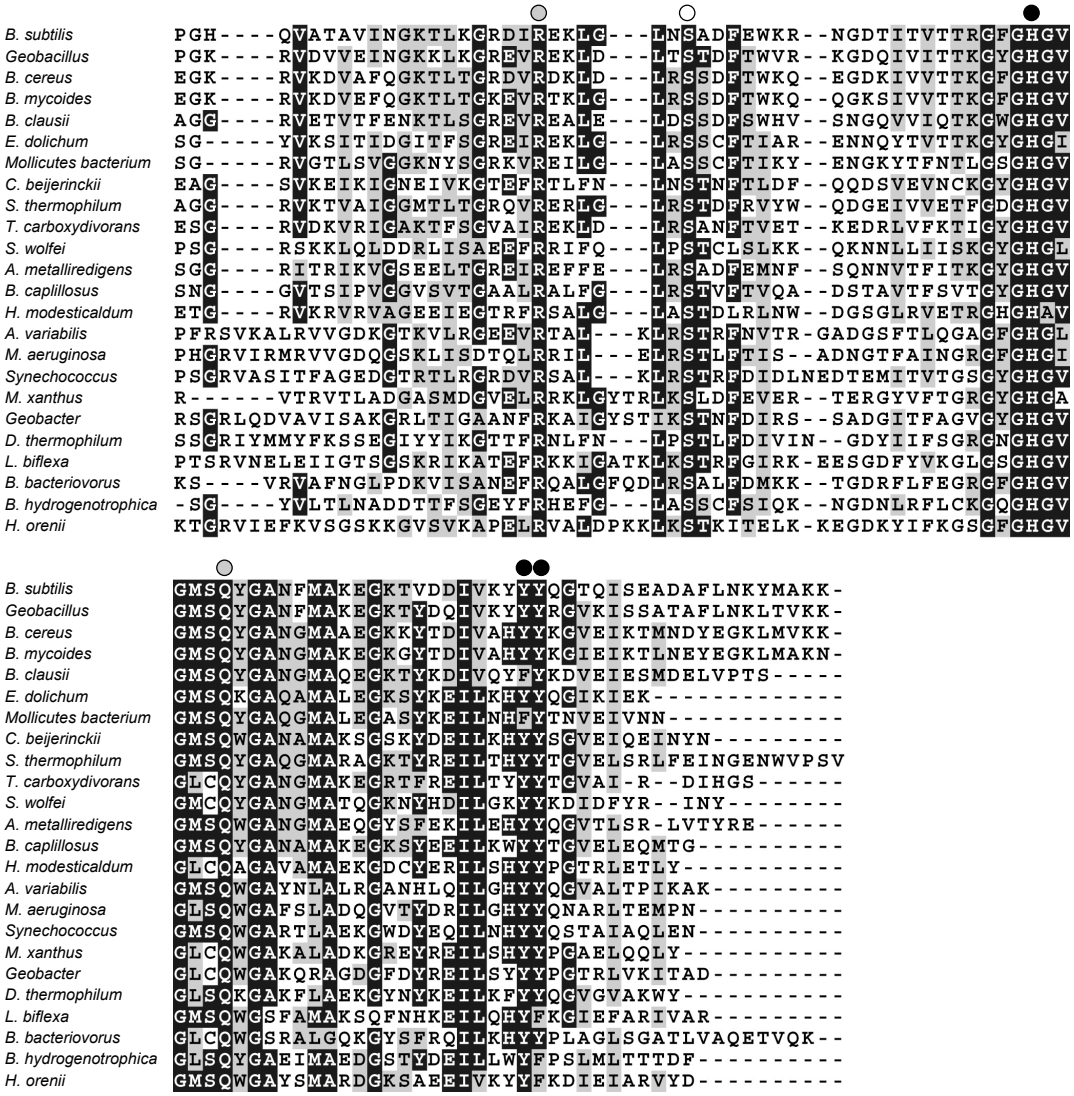
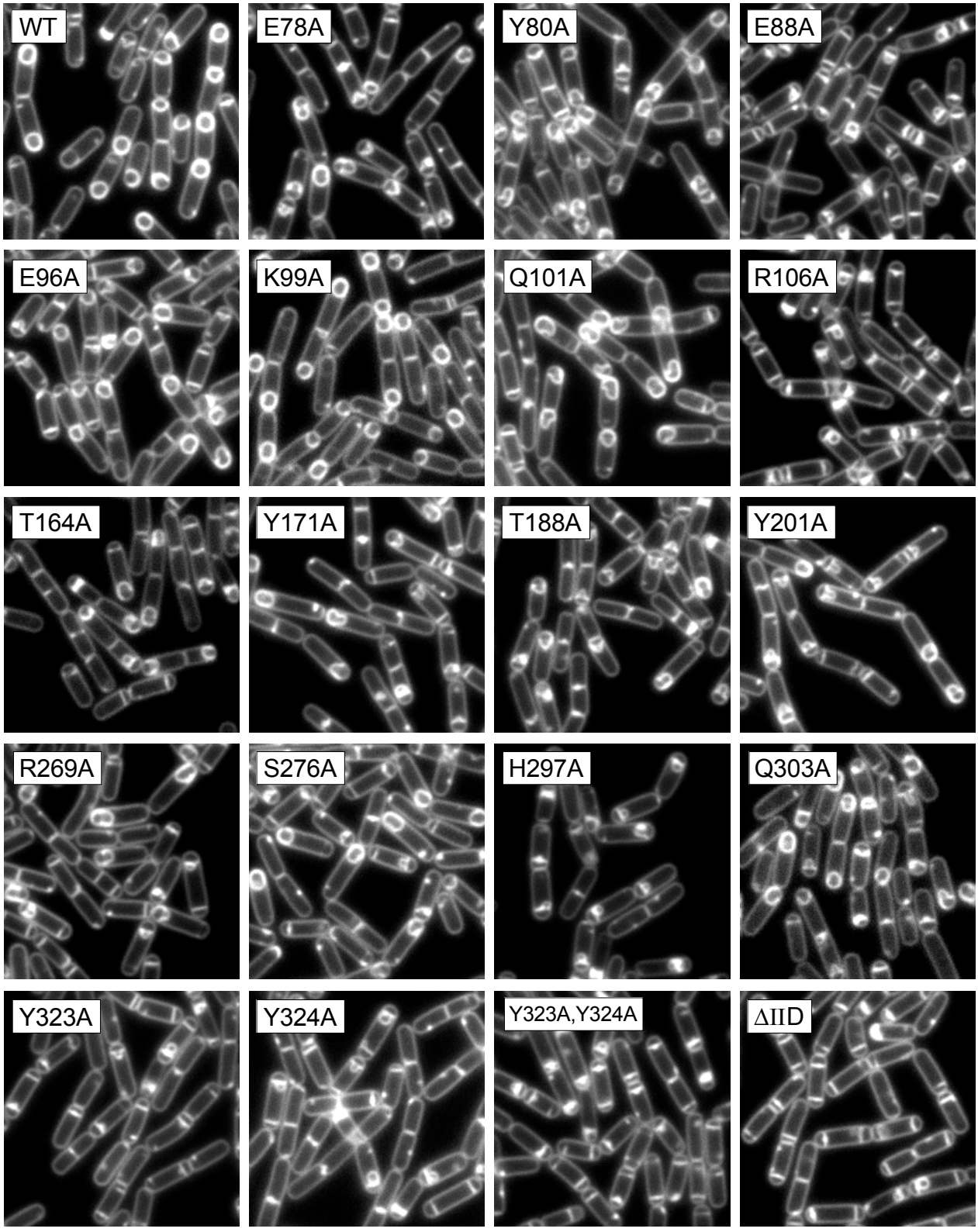
H. orenii

Morlot_FigS8

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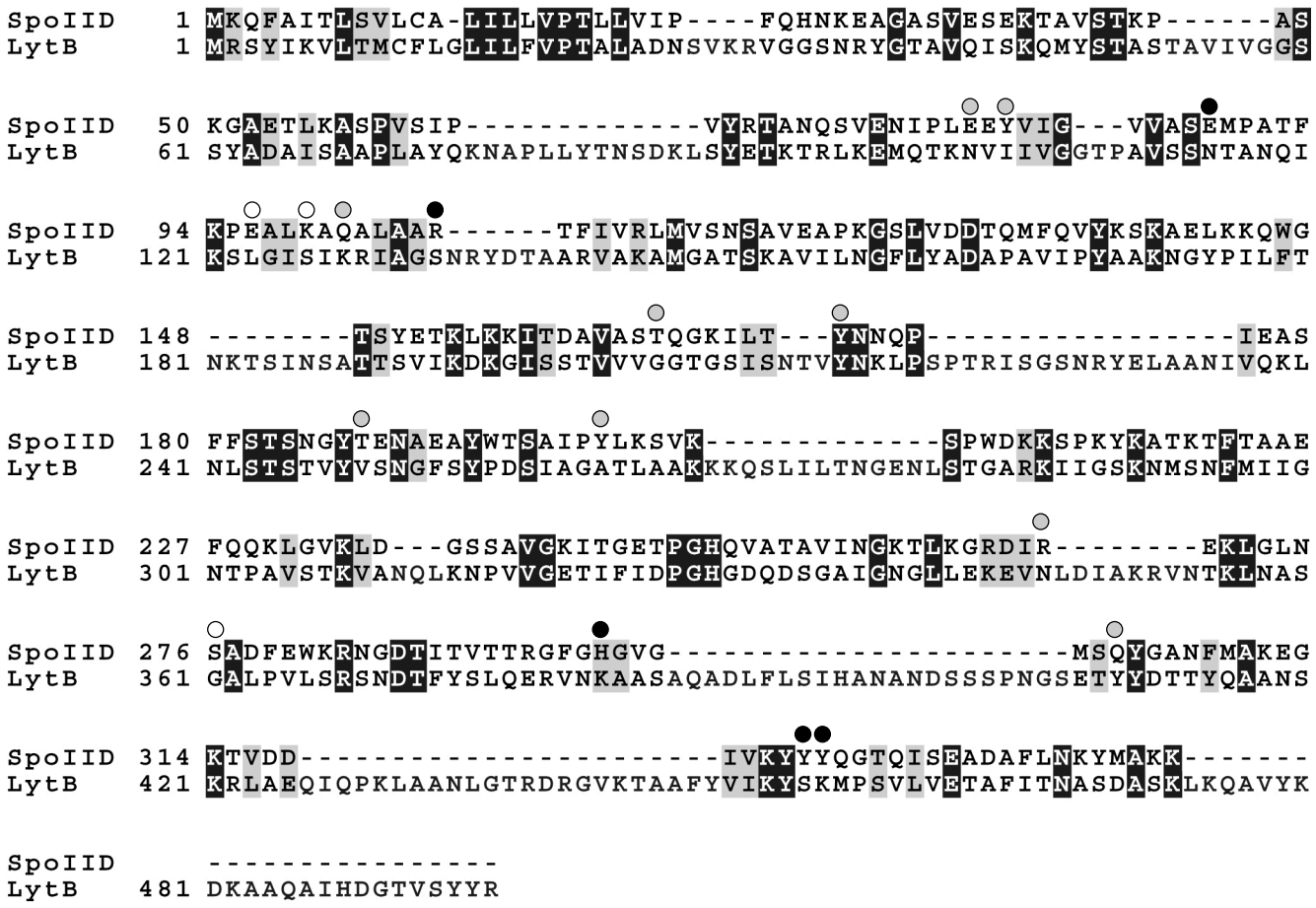
ΔIID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SpoIID 50 KGAETLKASPVSIP-------------VYRTANQSVENIPLEEYVIG---VVASEMPATF
LytB 61 SYADAISAAPLAYQKNAPLLYTNSDKLSYETKTRLKEMQTKNVIIVGGTPAVSSNTANQI
SpoIID 94 KPEALKAQALAAR------TFIVRLMVSNSAVEAPKGSLVDDTQMFQVYKSKAELKKQWG
LytB 121 KSLGISIKRIAGSNRYDTAARVAKAMGATSKAVILNGFLYADAPAVIPYAAKNGYPILFT
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LytB 241 NLSTSTVYVSNGFSYPDSIAGATLAAKKKQSLILTNGENLSTGARKIIGSKNMSNFMIIG
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SpoIID 276 SADFEWKRNGDTITVTTRGFGHGVG----------------------MSQYGANFMAKEG
LytB 361 GALPVLSRSNDTFYSLQERVNKAASAQADLFLSIHANANDSSSPNGSETYYDTTYQAANS
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SpoIID ----------------
LytB 481 DKAAQAIHDGTVSYYR

Table S1 *Strains used in this study*

Strain	Genotype	Source
PY79	prototrophic wild-type strain	Youngman *et al.*, 1983
RL324	*spoIID::cat*	Eichenberger et al., 2002
BKM1824	*spoIID::cat, amyE::spoIID (spec)*	This work
BKM1832	*spoIID::cat, amyE::spoIID^{E78A} (spec)*	This work
BKM1833	*spoIID::cat, amyE::spoIID^{Y80A} (spec)*	This work
BKM1834	*spoIID::cat, amyE::spoIID^{E88A} (spec)*	This work
BKM1835	*spoIID::cat, amyE::spoIID^{E96A} (spec)*	This work
BKM1836	*spoIID::cat, amyE::spoIID^{K99A} (spec)*	This work
BKM1837	*spoIID::cat, amyE::spoIID^{Q101A} (spec)*	This work
BKM1838	*spoIID::cat, amyE::spoIID^{R106A} (spec)*	This work
BKM1839	*spoIID::cat, amyE::spoIID^{T164A} (spec)*	This work
BKM1840	*spoIID::cat, amyE::spoIID^{T188A} (spec)*	This work
BKM1841	*spoIID::cat, amyE::spoIID^{Y201A} (spec)*	This work
BKM1842	*spoIID::cat, amyE::spoIID^{R269A} (spec)*	This work
BKM1843	*spoIID::cat, amyE::spoIID^{S276A} (spec)*	This work
BKM1844	*spoIID::cat, amyE::spoIID^{H297A} (spec)*	This work
BKM1845	*spoIID::cat, amyE::spoIID^{Q303A} (spec)*	This work
BKM1846	*spoIID::cat, amyE::spoIID^{Y323A,Y324A} (spec)*	This work
BKM1847	*spoIID::cat, amyE::spoIID^{Y171A} (spec)*	This work
BKM1848	*spoIID::cat, amyE::spoIID^{Y323A} (spec)*	This work
BKM1849	*spoIID::cat, amyE::spoIID^{Y324A} (spec)*	This work

Table S2 *Plasmids used in this study*

plasmid	description	source
pDR198	*his$_6$-spoIIP*	This work
pDR199	*his$_6$-spoIID*	Doan & Rudner, 2007
pCM187	*his$_6$-spoIIP^{H189R}*	This work
pCM188	*his$_6$-spoIIP^{H278R}*	This work
pCM189	*his$_6$-spoIIP^{D280G}*	This work
pKM341	*his$_6$-spoIID^{E88A}*	This work
pKM342	*his$_6$-spoIID^{R106A}*	This work
pKM343	*his$_6$-spoIID^{T188A}*	This work
pKM344	*his$_6$-spoIID^{H297A}*	This work
pKM345	*his$_6$-spoIID$^{Y323A, Y324A}$*	This work
pKM339	*amyE::spoIID (spec)*	This work
pKM346	*amyE::spoIID^{E78A} (spec)*	This work
pKM347	*amyE::spoIID^{Y80A} (spec)*	This work
pKM348	*amyE::spoIID^{E88A} (spec)*	This work
pKM349	*amyE::spoIID^{E96A} (spec)*	This work
pKM350	*amyE::spoIID^{K99A} (spec)*	This work
pKM351	*amyE::spoIID^{Q101A} (spec)*	This work
pKM352	*amyE::spoIID^{R106A} (spec)*	This work
pKM353	*amyE::spoIID^{T164A} (spec)*	This work
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pKM355	*amyE::spoIID^{Y201A} (spec)*	This work
pKM356	*amyE::spoIID^{R269A} (spec)*	This work
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pKM359	*amyE::spoIID^{Q303A} (spec)*	This work
pKM360	*amyE::spoIID$^{Y323A, Y324A}$ (spec)*	This work
pKM361	*amyE::spoIID^{Y171A} (spec)*	This work
pKM362	*amyE::spoIID^{Y323A} (spec)*	This work
pKM363	*amyE::spoIID^{Y324A} (spec)*	This work
pTD68	*his$_6$-sumo*	Bendezu et al., 2009
pTU204	*his$_6$-sumo-sltY*	This work
pET28a-AmiD	*his$_6$-AmiD*	Uehara & Park, 2007

Table S3. *Oligonucleotide primers used in this study*

primer	sequence
oDR379	gccGCTAGCcataataaggaagcgggggcc
oDR380	cggCTCGAGctacttttttcgccatatatttattc
oDR386	cgcGAATTCgatacgggtattcggtttcgg
oDR387	cgcGGATCCgccgtgctgaatcgtttcac
oCM160	gtgtttatctatcacacgcGcaatacggaatcatatctc
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oCM162	caatatatcattgacatccGcagagactctcggcgc
oCM163	gcgccgagagtctctgCggatgtcaatgatatattg
oCM164	cattgacatccacagagGctctcggcgcaaaaaagac
oCM165	gtctttttgcgccgagagCctctgtggatgtcaatg
oDR065	ggcGAATTCgccgctctgggcgcagac
oDR728	cgcGGATCCgacaaatgtggatgactttacc
oDR732	cgtagaaaacattccgcttgCagagtatgtgattggagtcg
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oDR738	gcccaggcgcttgccgccGCaacatttattgtcagactgatgg
oDR739	cacagatgcggtagccagtGcgcaaggcaaaatcttaacg
oDR740	ctccacaagcaacggctacGcagagaatgcagaagcttattgg
oDR741	ttggacaagtgctatcccaGCtttaaaaagcgtcaaaagcccatg
oDR742	cgctgaaaggaagagacataGCtgaaaagttgggtctcaactc
oDR743	cgtgaaaagttgggtctcaacGccgccgattttgaatggaag
oDR744	cacgacgagaggatttggcGCAggtgtggggatgagccaatac
oDR745	cacggtgtggggatgagcGCatacggagcgaattttatggc
oDR746	cggttgatgacattgtaaagtacGCtGCccaaggcacacaaatttctg
oDR747	cgcaaggcaaaatcttaacgGCcaacaaccagccgattgaag
oDR749	cggttgatgacattgtaaagtacGCttaccaaggcacacaaatttctg
oDR750	cggttgatgacattgtaaagtactatGCccaaggcacacaaatttctg
oTB178	gactctcttccgggcgctatc
oTB684	gtcaGTCGACAAGCTTattaCTCGAGgagctcGGATCCaccaatctgttctctgtgag
oTB773	gtcaGAGCTCgactcactggatgagcagcgtag
oTB774	gtcaAAGCTTaacgtgcggatcagtaacgacg

capital letters indicate recognition sites for restriction endonucleases or mutations introduced by site-directed mutagenesis.