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Supplemental information

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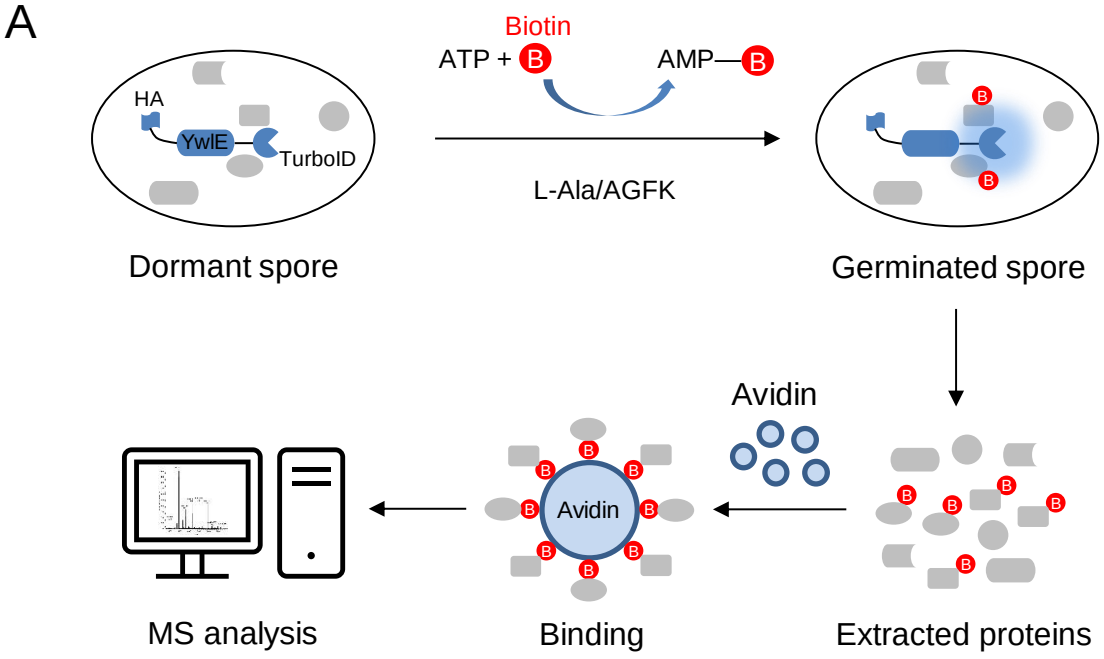
Supplementary Figures S1-S9 and Table S1

Figure S1


A
Biotin
ATP +
AMP
HA
YwlE
TurboID
L-Ala/AGFK
Dormant spore
Germinated spore
Avidin
Avidin
MS analysis
Binding
Extracted proteins

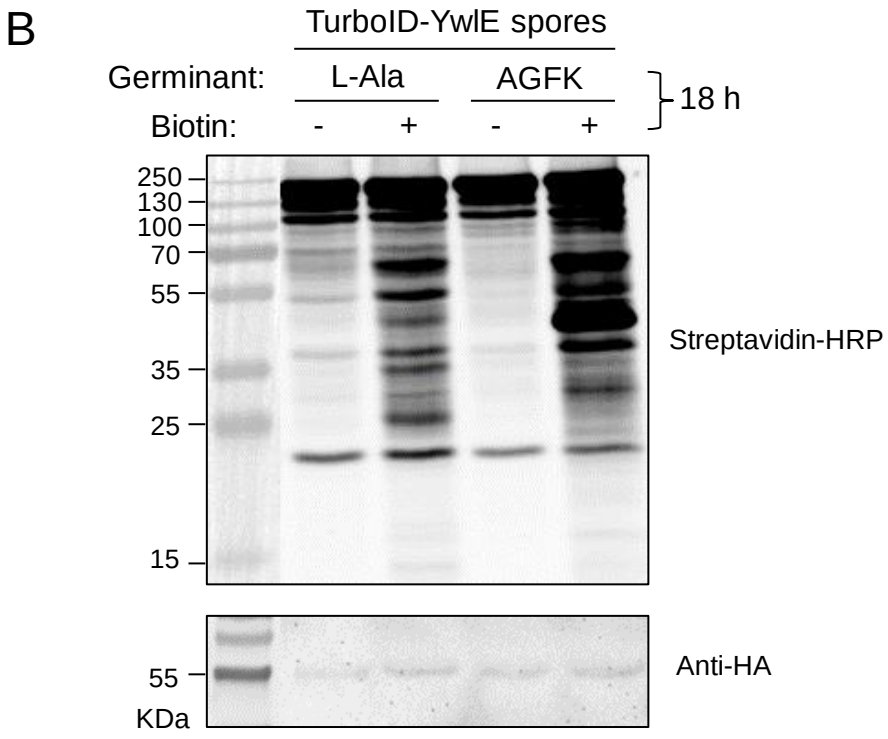

B
TurboID-YwlE spores
Germinant:
L-Ala
AGFK
18 h
Biotin:
-
+
-
+
250
130
100
70
55
35
25
15
Streptavidin-HRP
55
KDa
Anti-HA

Figure S2

Protein	Description	DPA release
Translation		
Tig	Trigger factor	Normal (Sinai et al., 2015)
RpmEB	Accessory ribosomal protein	Normal
YpfD	Similar to ribosomal protein S1	Normal
Metabolism		
LysC	Aspartokinase II	Normal
RocR	Arginine utilization regulatory protein	Deficiency
GmuR	HTH-type transcriptional regulator	Normal
SpeD	S-adenosylmethionine decarboxylase	Normal
Cellular processes		
YdiF	Uncharacterized ABC transporter	Normal
RbsA	Ribose ABC transporter	Normal
Zur	Zinc-specific metallo-regulatory protein	Normal
PrkC	Serine/threonine-protein kinase	Normal
Sporulation		
YkoU	DNA repair polymerase/ligase	Normal
CdaS	Cyclic di-AMP synthase	Normal
YabS	Unknown function	Normal
YkoN	Unknown function	Normal
YabR	Unknown function	Normal
Unknown		
YvaK	Unknown function	Normal
YbfQ	Unknown function	Normal
YtpQ	Unknown function	Normal
YkoG	Unknown function	Normal
YhaH	Unknown function	Normal
YdhI	Unknown function	Normal
YugI	Unknown function	Normal

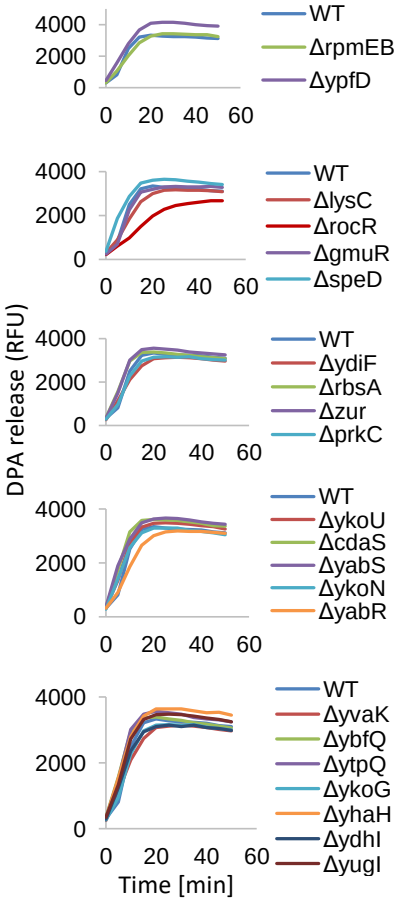


Figure S3

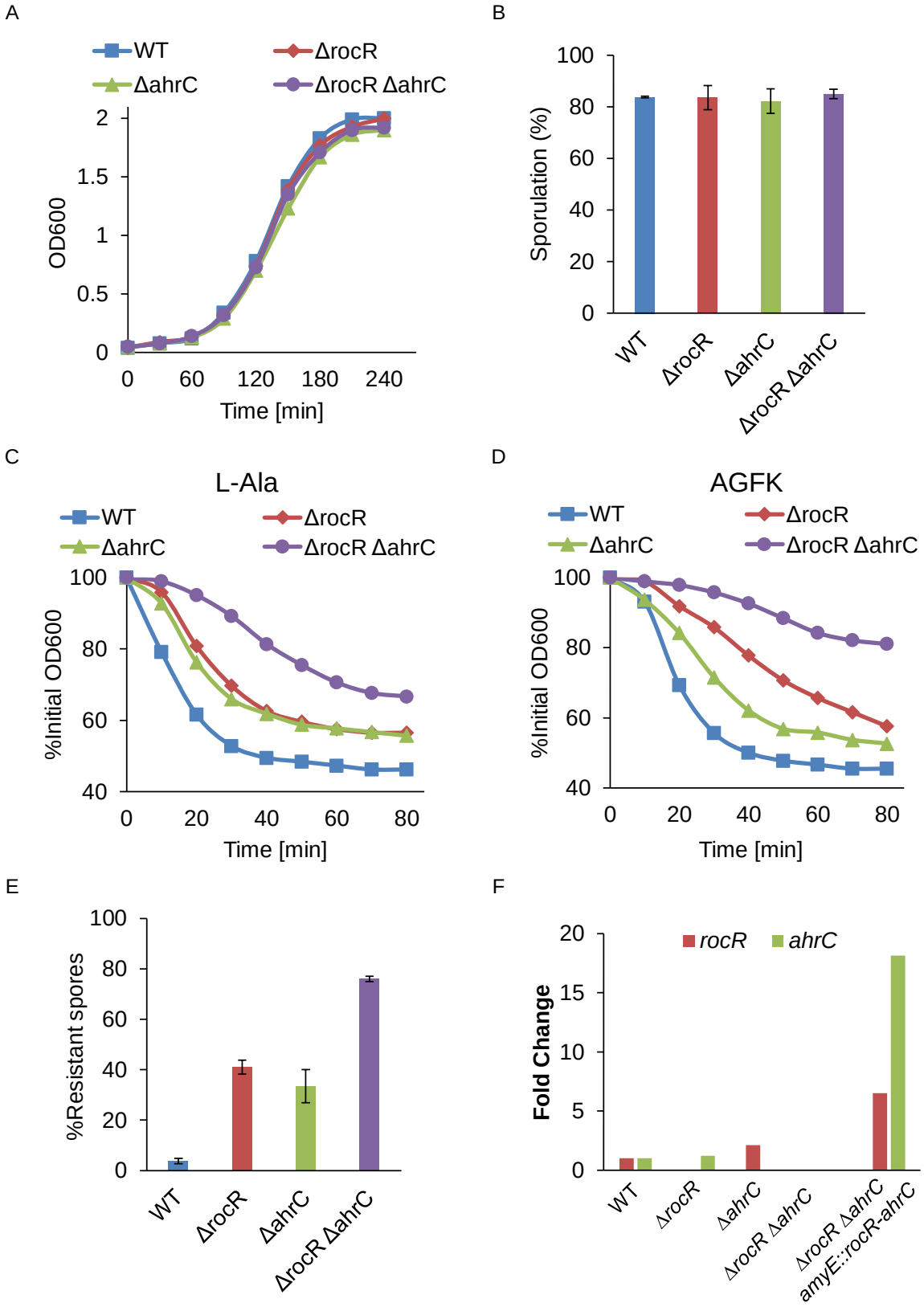

A
WT
ΔrocR
ΔahrC
ΔrocR ΔahrC
OD600
Time [min]
B
Sporulation (%)
WT
ΔrocR
ΔahrC
ΔrocR ΔahrC
C
L-Ala
%Initial OD600
Time [min]
D
AGFK
%Initial OD600
Time [min]
E
%Resistant spores
F
rocR
ahrC
Fold Change
ΔrocR ΔahrC amyE::rocR-ahrC

Figure S4

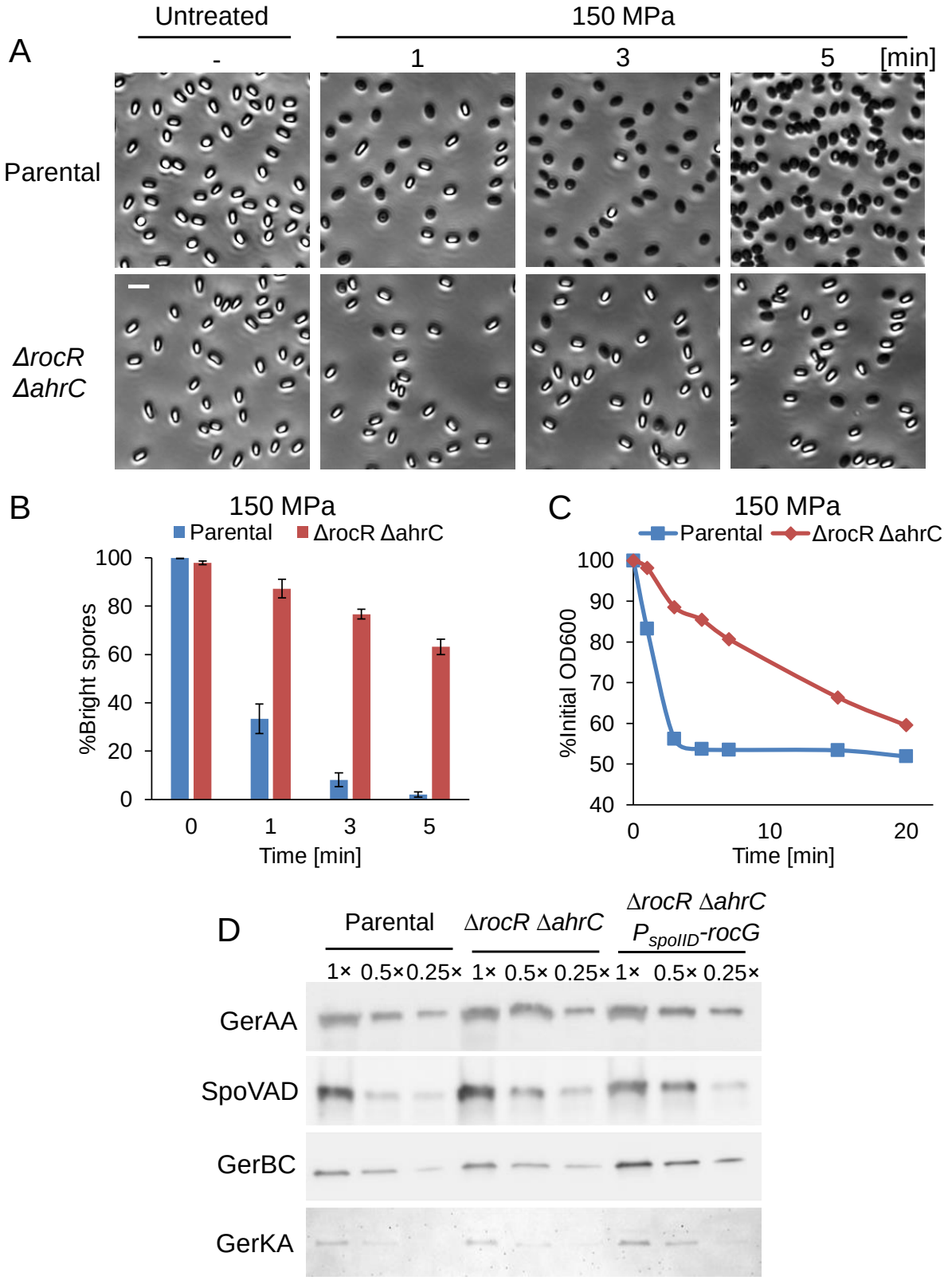

A
Untreated
150 MPa
-
1
3
5
[min]
Parental
ΔrocR
ΔahrC
B
150 MPa
Parental
ΔrocR ΔahrC
%Bright spores
100
80
60
40
20
0
0
1
3
5
Time [min]
C
150 MPa
Parental
ΔrocR ΔahrC
%Initial OD600
100
90
80
70
60
50
40
0
10
20
Time [min]
D
Parental
ΔrocR ΔahrC
ΔrocR ΔahrC
PspoIID-rocG
1× 0.5× 0.25× 1× 0.5× 0.25× 1× 0.5× 0.25×
GerAA
SpoVAD
GerBC
GerKA

Figure S5

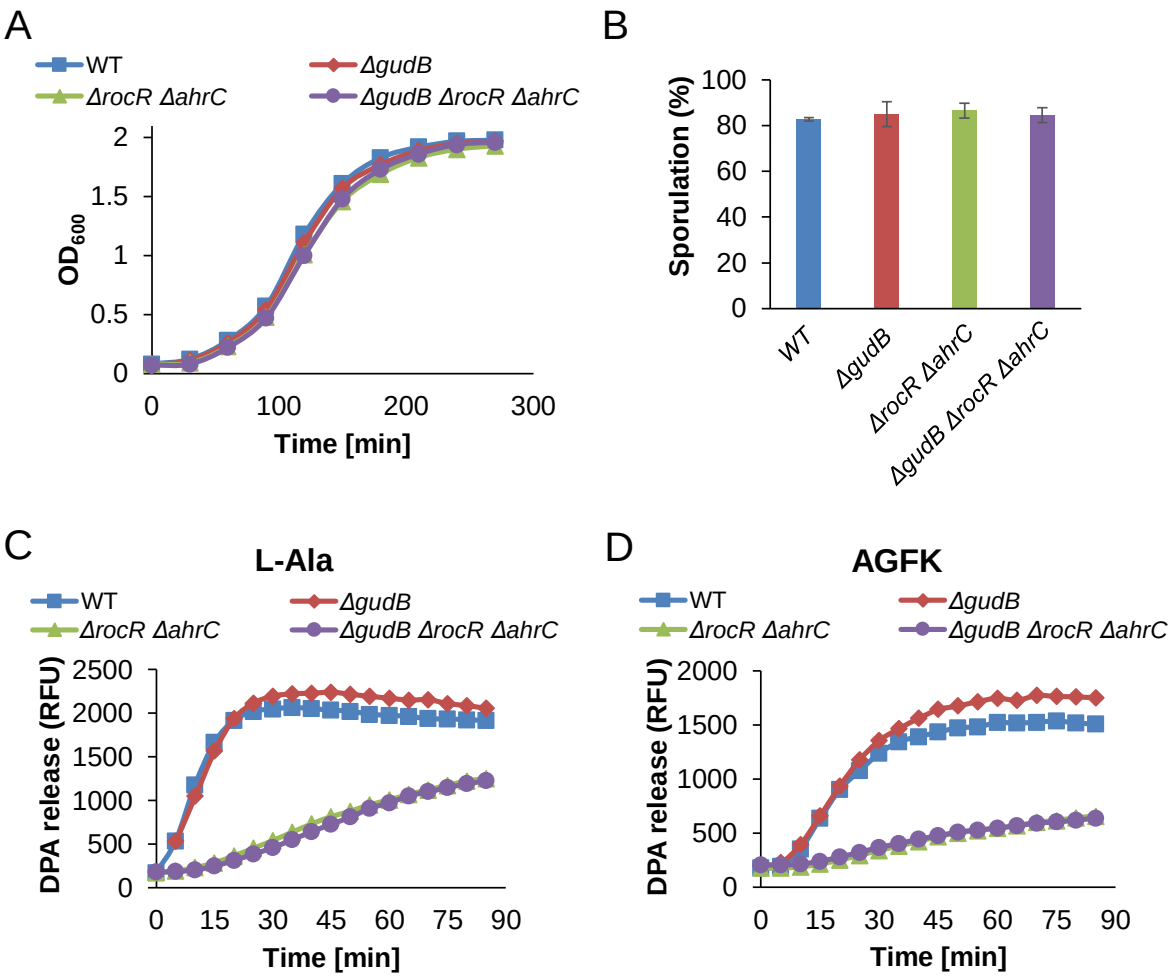

A
WT
ΔgudB
ΔrocR ΔahrC
ΔgudB ΔrocR ΔahrC
OD600
Time [min]
B
Sporulation (%)
WT
ΔgudB
ΔrocR ΔahrC
ΔgudB ΔrocR ΔahrC
C
L-Ala
DPA release (RFU)
Time [min]
D
AGFK
DPA release (RFU)
Time [min]

Figure S6

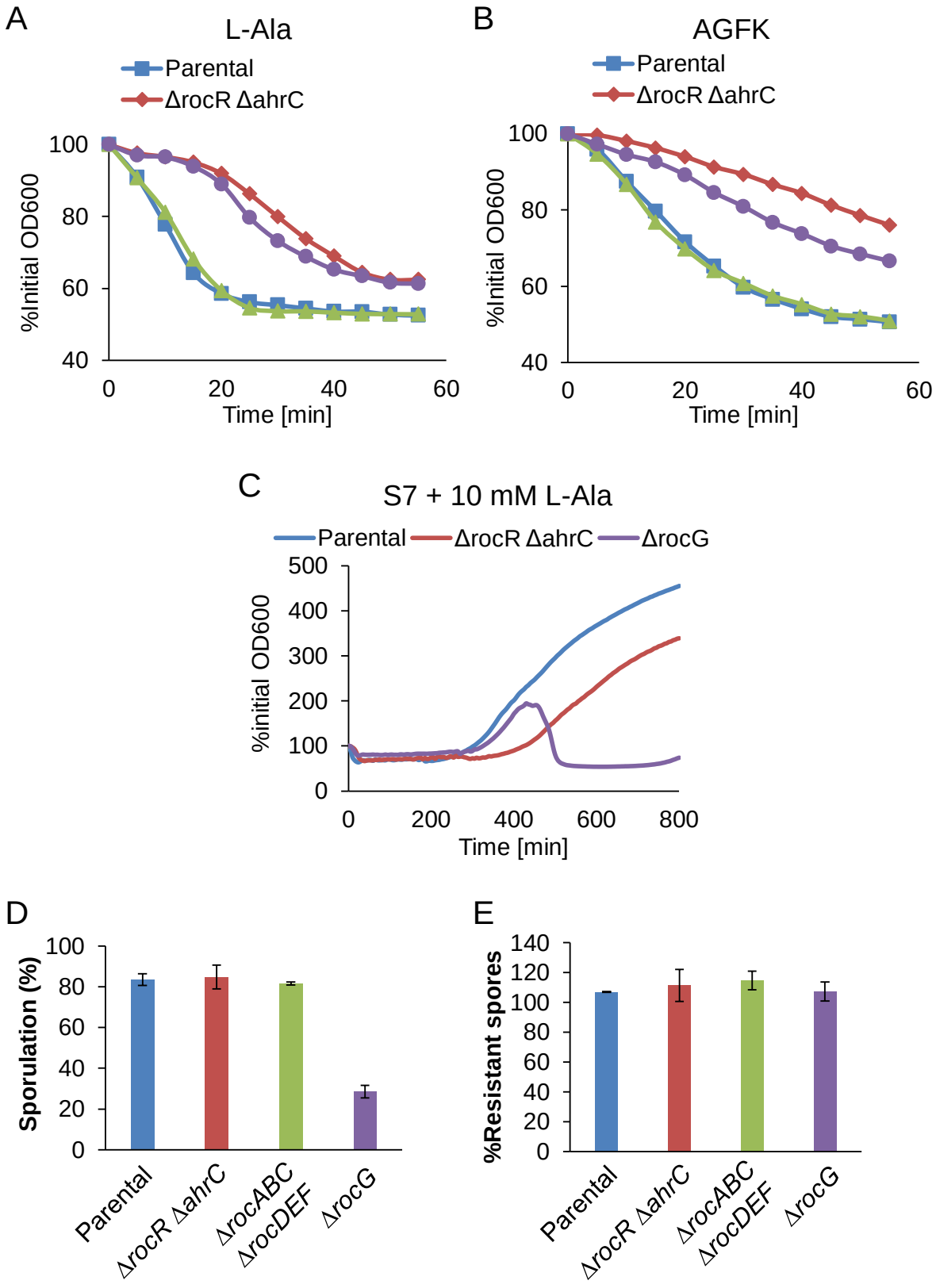

A
L-Ala
Parental
ΔrocR ΔahrC
%Initial OD600
100
80
60
40
0
20
40
60
Time [min]
B
AGFK
Parental
ΔrocR ΔahrC
%Initial OD600
100
80
60
40
0
20
40
60
Time [min]
C
S7 + 10 mM L-Ala
Parental
ΔrocR ΔahrC
ΔrocG
%initial OD600
500
400
300
200
100
0
0
200
400
600
800
Time [min]
D
Sporulation (%)
100
80
60
40
20
0
Parental
ΔrocR ΔahrC
ΔrocABC ΔrocDEF
ΔrocG
E
%Resistant spores
140
120
100
80
60
40
20
0
Parental
ΔrocR ΔahrC
ΔrocABC ΔrocDEF
ΔrocG

Figure S7

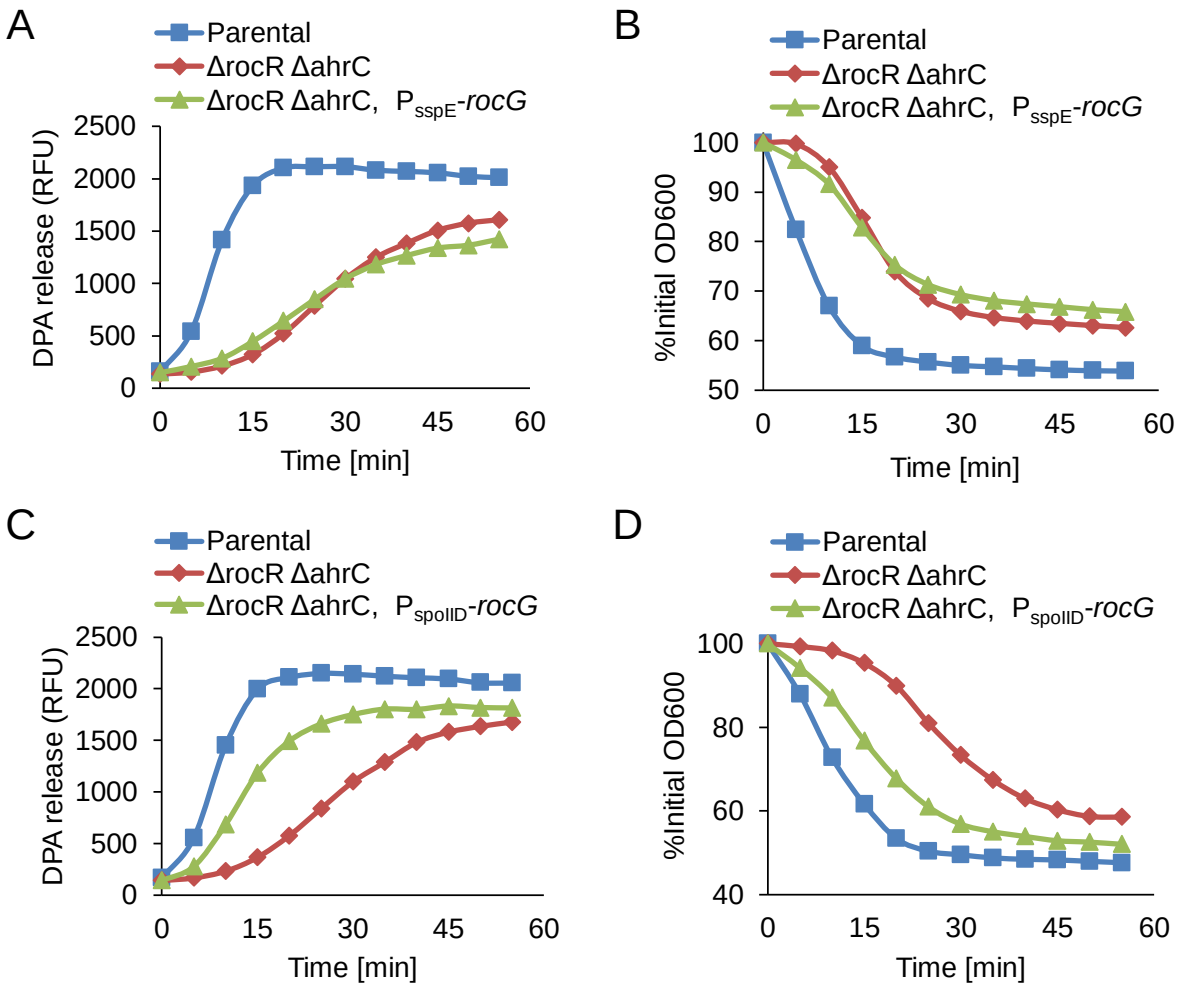

A
Parental
ΔrocR ΔahrC
ΔrocR ΔahrC, P$_{sspE}$-*rocG*
DPA release (RFU)
2500
2000
1500
1000
500
0
0
15
30
45
60
Time [min]
B
Parental
ΔrocR ΔahrC
ΔrocR ΔahrC, P$_{sspE}$-*rocG*
%Initial OD600
100
90
80
70
60
50
0
15
30
45
60
Time [min]
C
Parental
ΔrocR ΔahrC
ΔrocR ΔahrC, P$_{spoIID}$-*rocG*
DPA release (RFU)
2500
2000
1500
1000
500
0
0
15
30
45
60
Time [min]
D
Parental
ΔrocR ΔahrC
ΔrocR ΔahrC, P$_{spoIID}$-*rocG*
%Initial OD600
100
80
60
40
0
15
30
45
60
Time [min]

Figure S8

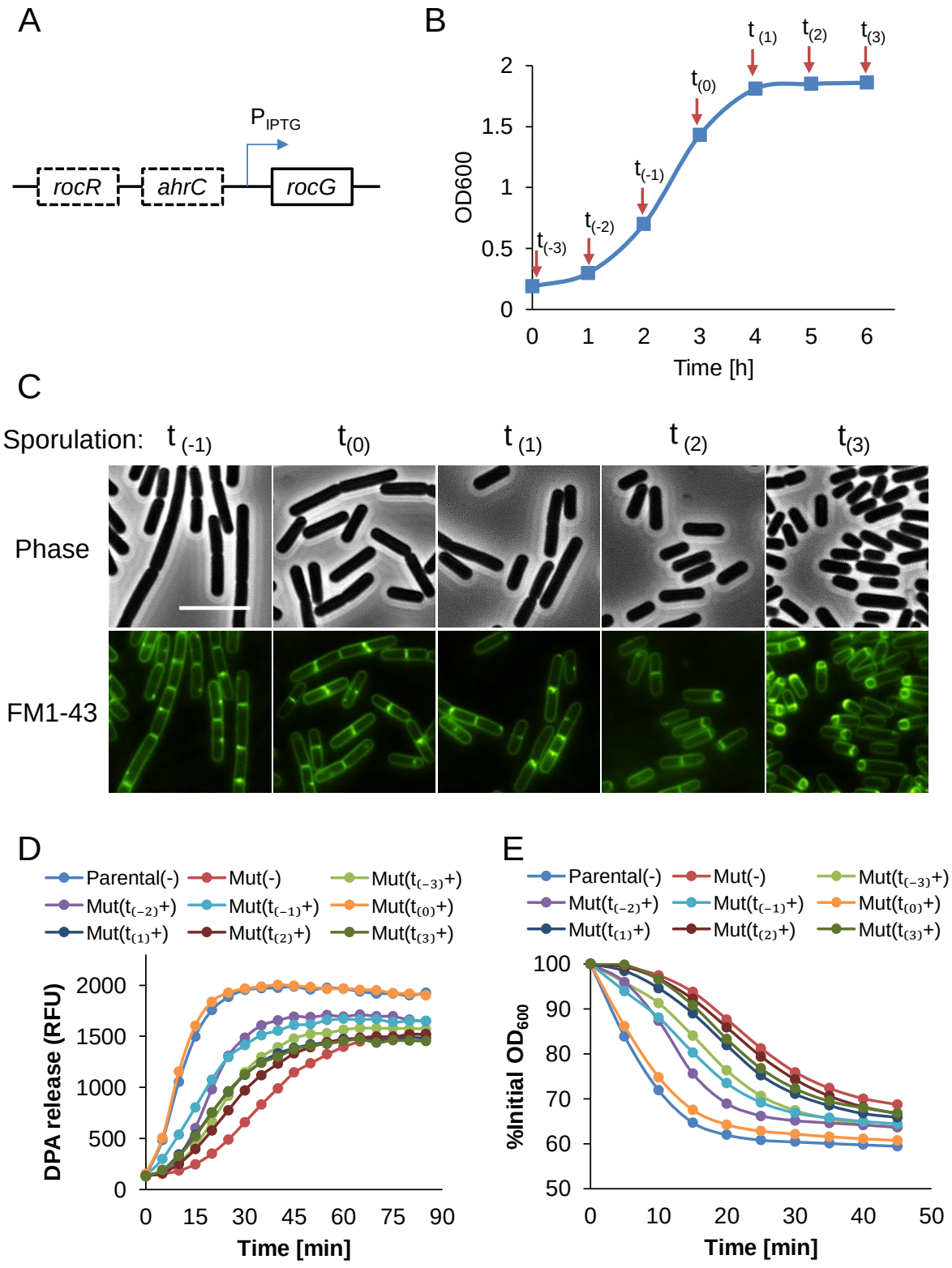
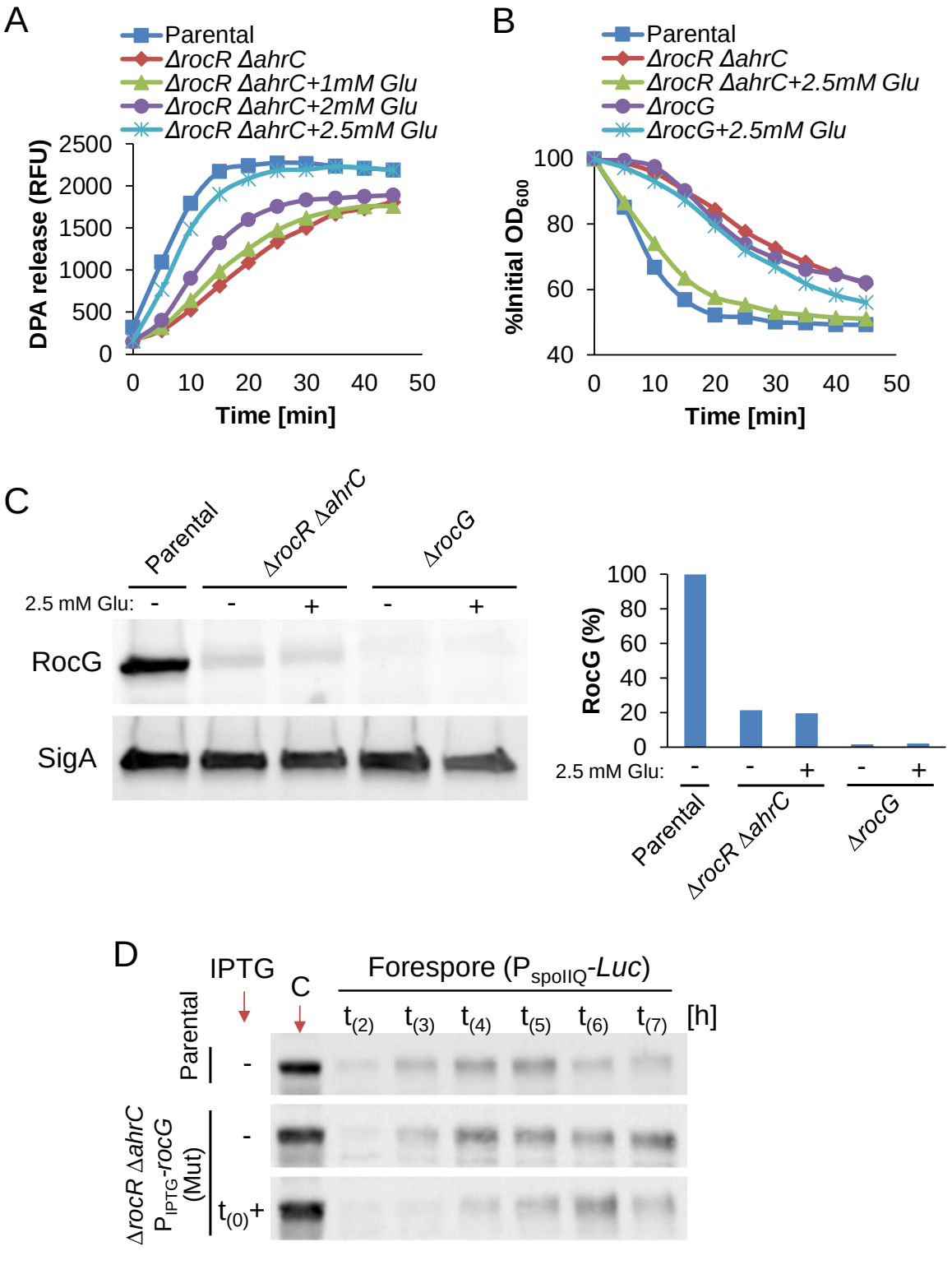

A
P IPTG
rocR
ahrC
rocG
B
OD600
Time [h]
t (1)
t(2)
t(3)
t(0)
t(-1)
t(-2)
t(-3)
C
Sporulation: t (-1) t(0) t (1) t (2) t(3)
Phase
FM1-43
D
Parental(-)
Mut(-)
Mut(t(-3)+)
Mut(t(-2)+)
Mut(t(-1)+)
Mut(t(0)+)
Mut(t(1)+)
Mut(t(2)+)
Mut(t(3)+)
DPA release (RFU)
Time [min]
E
%Initial OD600
Time [min]

Figure S9


A
Parental
ΔrocR ΔahrC
ΔrocR ΔahrC+1mM Glu
ΔrocR ΔahrC+2mM Glu
ΔrocR ΔahrC+2.5mM Glu
DPA release (RFU)
2500
2000
1500
1000
500
0
0 10 20 30 40 50
Time [min]
B
Parental
ΔrocR ΔahrC
ΔrocR ΔahrC+2.5mM Glu
ΔrocG
ΔrocG+2.5mM Glu
%Initial OD600
100
80
60
40
0 10 20 30 40 50
Time [min]
C
Parental
ΔrocR ΔahrC
ΔrocG
2.5 mM Glu: - - + - +
RocG
SigA
RocG (%)
100
80
60
40
20
0
2.5 mM Glu: - - + - +
Parental
ΔrocR ΔahrC
ΔrocG
D
IPTG
C
Forespore (PspoIIQ-Luc)
t(2) t(3) t(4) t(5) t(6) t(7) [h]
Parental
-
ΔrocR ΔahrC PIPTG-rocG (Mut)
-
t(0)+

Supplementary Figure Legends

Figure S1. TurboID-based proximity labelling of YwlE interactome, related to Figure 1.

(A) Schematic description of the main steps in defining TurboID-based proximity labelling of YwlE interactome. Spores harboring HA-labeled YwlE fused to TurboID were induced to germinate in the presence of Biotin and ATP. TurboID uses ATP and biotin to generate biotin-5'-AMP, a reactive intermediate that can covalently label the proximal proteins. The germinated spores were disrupted for protein extraction, and the biotinylated proteins were separated and enriched using Avidin beads and identified by mass spectrometry.

(B) Spores of LR14 (*turboID-ywlE-*$_{2\times HA}$) strain were germinated with L-Ala or AGFK in PBS × 1 supplemented with biotin and ATP. Proteins extracted from the germinated spores were analyzed by Western blot using anti-biotin (streptavidin-HRP) (upper panel) or anti-HA (lower panel) antibodies. Samples without biotin addition were processed as a control.

Figure S2. Screening for mutants defective in germination, related to Figure 1.

Shown is a table summarizing the germination capacity of potential YwlE-interacting factors. Spores of the indicated mutant strains were induced to germinate with L-Ala (10 mM), and germination was followed by monitoring the relative fluorescence units (RFU) of Tb^{3+}-DPA. Shown is a representative experiment out of three independent biological repeats. Similar results were obtained when germination was triggered by AGFK.

Figure S3. RocR and ArhC are required for spore germination, related to Figure 1.

(A) PY79 (WT), LR31 (Δ*rocR*), LR30 (Δ*ahrC*), LR32 (Δ*rocR* Δ*ahrC*) strain cultures were grown in LB

medium, and OD_{600} was measured at the indicated time points. Shown is a representative out of three independent biological repeats.

(B) PY79 (WT), LR31 (Δ*rocR*), LR30 (Δ*ahrC*), LR32 (Δ*rocR* Δ*ahrC*) strains were induced to sporulate in DSM for 24 hrs. Next, cultures were subjected to heat treatment (80ºC, 30 min), serial decimal dilutions were plated on LB, and colonies were counted after 24 hrs of incubation at 37ºC. Percentage of sporulation was calculated by the number of colonies after heat treatment/number of colonies before heat treatment. Shown are average values and SD obtained from three independent biological repeats.

(C-D) Spores of PY79 (WT), LR31 (Δ*rocR*), LR30 (Δ*ahrC*), LR32 (Δ*rocR* Δ*ahrC*) strains were incubated with L-Ala (10 mM) (C) or AGFK (D) to trigger germination, and OD_{600} was measured at the indicated time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Shown is a representative experiment out of three independent biological repeats.

(E) Spores of PY79 (WT), LR31 (Δ*rocR*), LR30 (Δ*ahrC*), LR32 (Δ*rocR* Δ*ahrC*) strains were purified, incubated with L-Ala (10 mM) for 10 min, and heat treated at 80ºC for 30 min. The percentage of non-germinating heat resistant spores was determined by the number of colonies after heat treatment/number of colonies before heat treatment.

(F) PY79 (WT), LR31 (Δ*rocR*), LR30 (Δ*ahrC*), LR32 (Δ*rocR* Δ*ahrC*) and LR205 (Δ*rocR* Δ*ahrC, amyE::rocR-ahrC*) strains were induced to sporulate in DSM medium. At t_0 of sporulation, cells of each strain were collected, RNA was extracted, and the mRNA levels of *rocR* and *ahrC* were determined by quantitative RT-PCR. The results are presented as the fold expression change of the investigated genes relative to that of PY79 (WT).

Figure S4. Δ*rocR* Δ*ahrC* mutant spores exhibit germination defect when exposed to high pressure, related to Figure 2 and Figure 3.

(A) Spores of LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*) strains, lacking *gudB*, were induced to germinate by high pressure treatment at 150 MPa for various times, and spores were collected by centrifugation and examined by phase contrast microscopy. Shown are phase contrast images captured at the indicated time points from a representative experiment out of three independent biological repeats. Scale bar, 1 μm.

(B) Quantification of the experiment describe in (A). Data are presented as percentages of the initial number of the phase bright spores. Shown are average values and SD obtained from three independent biological repeats (n ≥ 300 for each strain).

(C) Spores of LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*) strains, lacking *gudB*, were induced to germinate by high pressure treatment at 150 MPa for various times, and spore suspensions were tested for OD_{600} decrease. Shown is a representative experiment out of three independent biological repeats.

(D) Equal amounts of protein extracts from spores of LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*) and LR133 (Δ*rocR* Δ*ahrC,* P_{spoIID}-*rocG*)) strains, lacking *gudB*, were subjected to SDS-PAGE. Western blot analysis using antibodies against GerAA, GerBC, GerKA, and SpoVAD was conducted. Dilutions of the different samples were loaded on the same gel for comparison.

Figure S5. Sporulation and germination are unaffected by *gudB* deletion, related to Figure 2.

(A) PY79 (WT), LR33 (Δ*gudB*), LR32 (Δ*rocR* Δ*ahrC*), LR38 (Δ*gudB* Δ*rocR* Δ*ahrC*) strains were grown in LB medium, and OD_{600} was measured at the indicated time points. Shown is a representative out of three independent biological repeats.

(B) PY79 (WT), LR33 (Δ*gudB*), LR32 (Δ*rocR* Δ*ahrC*), LR38 (Δ*gudB* Δ*rocR* Δ*ahrC*) strains were induced to sporulate in DSM for 24 hrs. Next, cultures were subjected to heat treatment (80ºC, 30 min), serial decimal dilutions were plated on LB, and colonies were counted after 24 hrs of incubation at 37ºC. Percentage of sporulation was calculated by the number of colonies after heat treatment/number of colonies before heat treatment. Presented are average values and SD obtained from three independent biological repeats.

(C-D) Spores of PY79 (WT), LR33 (Δ*gudB*), LR32 (Δ*rocR* Δ*ahrC*), LR38 (Δ*gudB* Δ*rocR* Δ*ahrC*) strains were incubated with L-Ala (10 mM) (C) or AGFK (D) to trigger germination, and DPA release was followed by monitoring the relative fluorescence units (RFU) of Tb^{3+}-DPA. Shown is a representative experiment out of three independent biological repeats.

Figure S6. RocG affects sporulation and spore revival, related to Figure 2.

(A-B) Spores of LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*), LR203 (Δ*rocABC* Δ*rocDEF*), LR137 (Δ*rocG*) strains, lacking *gudB*, were incubated with L-Ala (10 mM) (A) or AGFK (B) to trigger germination, and OD_{600} was measured at the indicated time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Shown is a representative experiment out of three independent biological repeats.

(C) Spores of LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*), LR137 (Δ*rocG*) strains were incubated in S7 minimal medium supplemented with 10 mM L-Ala and OD_{600} was measured at 5 min intervals. Decreasing OD_{600} signifies spore germination, and increasing OD_{600} indicates spore outgrowth. Data are presented as percentage of the initial OD_{600} of spore suspension. Shown is a representative experiment out of three independent biological repeats.

(D) LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*), LR203 (Δ*rocABC* Δ*rocDEF*), LR137 (Δ*rocG*) strains, lacking *gudB*,

were induced to sporulate in DSM for 24 hrs. Next, cultures were subjected to heat treatment (80ºC, 30 min), serial decimal dilutions were plated on LB, and colonies were counted after 24 hrs incubation at 37ºC. Percentage of sporulation was calculated by the number of colonies after heat treatment/number of colonies before heat treatment. Presented are the average sporulation percentage from three independent biological repeats.

(E) Purified spores of LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*), LR203 (Δ*rocABC* Δ*rocDEF*), LR137 (Δ*rocG*) strains, lacking *gudB*, were heat treated at 80ºC for 30 min. The percentage of heat resistant spores was determined by the number of colonies after heat treatment/number of colonies before heat treatment.

Figure S7. RocG expression at early stages of sporulation could partially rescue the Δ*rocR* Δ*ahrC* germination deficiency, related to Figure 3.

(A-B) Spores of LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*), LR134 (Δ*rocR* Δ*ahrC,* P_{sspE}-*rocG*) strains, lacking *gudB*, were incubated with L-Ala (10 mM), and the germination was followed by monitoring the relative fluorescence units (RFU) of Tb^{3+}-DPA (A), and by OD_{600} decrease (B). Shown is a representative experiment out of three independent biological repeats.

(C-D) Spores of LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*), LR133 (Δ*rocR* Δ*ahrC,* P_{spoIID}-*rocG*) strains, lacking *gudB*, were incubated with L-Ala (10 mM), and the germination was followed by monitoring the relative fluorescence units (RFU) of Tb^{3+}-DPA (A), and by OD_{600} decrease (B). Shown is a representative experiment out of three independent biological repeats.

Figure S8. **RocG induction at onset of sporulation could fully rescue the Δ*rocR* Δ*ahrC* germination deficiency, related to Figure 4.**

(A) Schematic of the genotype of stain LR127 (Δ*rocR* Δ*ahrC, P_{IPTG}-rocG*) (lacking *gudB*).

(B-C) LR127 (Δ*rocR* Δ*ahrC, P_{IPTG}-rocG*) strain, lacking *gudB*, was incubated in DSM medium and OD_{600} was measured during growth at the indicated time points (B). At each time point, cells were collected for microscopic examination by phase contrast (upper panels) and FM 1-43 membrane staining (lower panels) (C). Shown is a representative out of three independent biological repeats.

(D-E) LR33 (Parental) and LR127 (Δ*rocR* Δ*ahrC,* P_{IPTG}-*rocG*) (Mut) strains, lacking *gudB*, were incubated in DSM medium. IPTG was added to LR127 cultures at the indicated sporulation time points to induce *rocG* expression, and the corresponding spores were purified. The spores were then incubated with L-Ala (10 mM), and the germination was followed by monitoring the relative fluorescence units (RFU) of Tb^{3+}-DPA (D), and by OD_{600} decrease (E). Shown is a representative experiment out of three independent biological repeats.

Figure S9. The effect of glutamate supplementation during sporulation on Δ*rocR* Δ*ahrC* germination, related to Figure 5 and Figure 6.

(A) LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*) strains, lacking *gudB*, were grown in DSM medium with or without glutamate at the indicated concentrations. The corresponding spores were purified, and germination was followed by monitoring the relative fluorescence units (RFU) of Tb^{3+}-DPA. Shown is a representative experiment out of three independent biological repeats.

(B) LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*), LR137 (Δ*rocG*) strains, lacking *gudB*, were grown in DSM sporulation medium with or without 2.5 mM glutamate. After 20 hrs of incubation, spores were

purified, induced to germinate by L-Ala (10 mM) (t=0), and germination was followed by monitoring OD_{600} decrease. Shown is a representative experiment out of three independent biological repeats.

(C) LR33 (Parental), LR38 (Δ*rocR* Δ*ahrC*), LR137 (Δ*rocG*) strains, lacking *gudB*, were incubated in DSM medium supplemented with 2.5 mM glutamate. At t_0 of sporulation, cells were collected and disrupted for protein extraction. Equal amounts of protein extracts were subjected to Western blot analysis (left panel) with antibodies against RocG or SigA, which serves as a loading control. Quantification of the RocG signal was shown as percentage of the parental signal (right panel).

(D) LR209 [Parenal (P_{spoIIQ}-*luc*)], LR227 [Mut (P_{spoIIQ}-*luc*, P_{IPTG}-*rocG*, Δ*rocR* Δ*ahrC*)] strains were incubated in DSM medium to induce sporulation. At t_0 of sporulation, IPTG was added to induce *rocG* expression, and samples were collected and disrupted for protein extraction at indicated sporulation time points. Equal amounts of protein extracts were subjected to Western blot analysis with antibody against Luciferase. C-indicates a loading control, derived from LR209 strain at t_5.

Table S1. Primers used in this study

Primer name	Primer sequence
P_{IPTG}-*rocG*-P1	5’-AATTGTGAGCGGATAACAATTAAGCTTAAAGGTGGTGAACTACT ATCCAATGAAGAAAGGAATTGGC-3’
P_{IPTG}-*rocG*-P2	5’-ATGCGGCTAGCTGTCGACTATCATTAGACCCATCCGCGGAAA-3’
P_{spoIID}-P1	5’-CAACTGGTAATGGTAGCGACCGGCGCTCAGGAATACGTAAATGACAAATAAA GTTTCTGTCC-3’
P_{spoIID}-P2	5’-GCCAATTCCTTTCTTCATTGGATATTCAGCTGCCTCCTGCT-3’
P_{sspE}-P1	5’-CCAACTGGTAATGGTAGCGACCGGCGCTCAGCGGGACGCATGGTCGAAATT AAAGAC-3’
P_{sspE}-P2	5’-GCCAATTCCTTTCTTCATTGGATTGTTATCACCTCCACGGTCATTAGAATGTG-3’
rocG-P1	5’-ATCCAATGAAGAAAGGAATTGGC-3’
rocG-P2	5’-GTCAAACATGAGAATTCGATAAGCTTCTAGTCATTAGACCCATCCGCGGAAA-3’
P_{rocR}-*rocR*-P1	5’-CCAACTGGTAATGGTAGCGACCGGCGCTCAGATTTGAATTCCCCCTTGTTT TCTG-3’
P_{rocR}-*rocR*-P2	5’-GATATGCAAAAAAAACTGAACAGGGG-3’
P_{ahrC}-P1	5’-CCCCTGTTCAGTTTTTTTTGCATATCAACGATCCTAATTTGAAGCGG-3’
P_{ahrC}-P2	5’-TTGTACATGAATGCAGAAAAAAGGAATAAGCGGATCAACTCACTTTCA-3’
ahrC-P1	5’-TATTCCTTTT TTCTGCATTC ATGTACAA-3’
ahrC-P2	5’-GTCAAACATGAGAATTCGATAAGCTTCTAGTTACAGCAGTTCAAGGAGCCT-3’
P_{spoIIG}-P1	5’-CCAACTGGTAATGGTAGCGACCGGCGCTCAGGTGGAAAAAAAGCTGCCGT-3’
P_{spoIIG}-P2	5’-CAGTGAAAAGTTCTTCTCCTTTACTCATATCTGACTCCTTTCTTTCTTGCC-3’
rocR-KO-P1	5’-ACATATCCGG AACAATGCCG-3’
rocR-KO-P2	5’-CTGAGCGAGGGAGCAGAATGTATGAACCTCCCTCAATTATTTTC-3’
rocR-KO-P3	5’-GTTGACCAGTGCTCCCTGTCCGGAGCAGGAAGCCTG-3’
rocR-KO-P4	5’-GGGCGATCCCAGTAGATTCA-3’
gudB-KO-P1	5’-GCGGCATATC TGATCAGCAA-3’
gudB-KO-P2	5’-CTGAGCGAGGGAGCAGAATTGAGTTAACCTCCTAGAATCTTCTG-3’

gudB-KO-P3	5’-GTTGACCAGTGCTCCCTGGTTGATGATTTGCATA AAAATAAAAAATCTCC-3’
gudB-KO-P4	5’-TGTTTCCCGCAGCAATAACA-3’
rocABC-KO-P1	5’-ATTCTCCGGGCTTTATTACAGG-3’
rocABC-KO-P2	5’-CTGAGCGAGGGAGCAGAAATGTAGTCCCCCTCGTGTTAT-3’
rocABC-KO-P3	5’-GTTGACCAGTGCTCCCTGAAAAAGCTCTCCGGGAGG-3’
rocABC-KO-P4	5’-ATGATAAATAAGCCCGCAGC-3’
rocDEF-KO-P1	5’-TGAAGGCTGAGATTCAGCGA-3’
rocDEF-KO-P2	5’-CTGAGCGAGGGAGCAGAAATTTGAATTCCCCCTTGTTTTCTG-3’
rocDEF-KO-P3	5’-GTTGACCAGTGCTCCCTGTAAGAAAACCCCCGCACC-3’
rocDEF-KO-P4	5’-AGCAGTCAAAACATTCAGAGAAGA-3’
rocR-RT-L	5’-TTATGACTGGCCGGGAAATA-3’
rocR-RT-R	5’-TTGATAGGGCAGATGGGAAG-3’
ahrC-RT-L	5’-GATGGACGCATTTGTGAAAA-3’
ahrC-RT-R	5’-CCATCATTTCATCCCAGTCC-3’
rocG-RT-L	5’-TTTGAATGGGTGCAAAACAA-3’
rocG-RT-R	5’-TTTCTGATGCCCGTCATGTA-3’
yoxA-RT-L	5'-CACAAGCAGCTGGATGATGT-3'
yoxA-RT-R	5'-ACCCATGTGTAAGGCTCAGG-3'

All primers were designed during this study, and synthesized by Integrated DNA Technologies (IDT).