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Supplementary Information for

Arginine dephosphorylation propels spore germination in bacteria

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Supplementary Text

Supplemental materials and methods

Spore purification

For ribosome purification and *in vitro* transcription experiments, spores were purified by water washing as described by Nicholson and Setlow (1). Briefly, 500 ml of 48 h DSM culture was centrifuged and washed 3 times using 200 ml of DDW. The pellet was resuspended in 100 ml of DDW and kept in 4°C with constant rotation. At the next few days the suspension was repeated washing once a day and resuspended in DDW (3 washes a day). Eventually, after at least 7 days, the pellet contained spores, surrounded by a brown layer of cell debris. This layer became very viscous and tight when swirled and therefore easy to remove. The remaining pellet included almost exclusively free spores, as evaluated by phase contrast microscopy.

For other experiments, 3-step Histodenz gradient was used to purify spores. In brief, a 12 ml 24 h DSM culture was washed in DDW and resuspended in 1 ml of 20% Histodenz solution for 30 minutes on ice. This spore suspension was then placed on top of a two-layer gradient, made up from 2 ml 40% histodenz on top of 6 ml 50% histodenz. After centrifugation (90 min, 4,000 RPM, 23°C), a pellet was observed at the bottom of the tube. The pellet contains >99% pure spore population, as evaluated by phase contrast microscopy.

Allelic Replacement ("pop in pop out" technique)

Allelic replacement was carried out as described previously (2, 3). Briefly, to replace the WT allele of a given gene, a PCR product containing ~500 bp upstream and ~500 bp downstream of the target gene was amplified using chromosomal DNA purified from PY79 as the template. The PCR product was digested, and cloned into multiple cloning site of pMINImad2, which carries a temperature sensitive origin of replication and an erythromycin (*mls*) resistance cassette. Site directed mutagenesis was conducted on the resulting construct using appropriate primer pair and the Quickchange II kit (Stratagene). The plasmid bearing the point mutation was introduced into PY79 by single crossover integration by transformation at the restrictive temperature for plasmid replication (37ºC),

using *mls* resistance as a selective marker. To evict the plasmid, the strain was incubated in 3 ml LB at a permissive temperature for plasmid replication (23ºC) for 14 h, diluted 30 fold into fresh LB, and incubated at 23ºC for additional 8 h. Cells were then serially diluted and plated on LB agar at 37ºC. Individual colonies were patched on both LB and LB containing *mls* plates, to identify *mls* sensitive colonies that had evicted the plasmid was purified and screened by PCR to determine which isolate had retained the mutant allele. Finally, allelic replacement was verified by DNA sequencing.

Quantitative phosphoproteomic analysis

Purified spores were suspended 50 mM Tris–HCl (pH 7.4) supplemented with 0.05 % SDS, Halt Protease and phosphatase inhibitors (Pierce), and lysed using Fastprep (FastPrep (MP) 6.5, 60 sec, ×3). Following cell lysis, the supernatant was additionally centrifuged at 15,000 rpm for 30 min. The protein suspension was precipitated using acetone. Protein precipitates were resuspended in a denaturation buffer containing 6 M urea and 2 M thiourea in 10 mM Tris (pH 8.0). Protein concentration was measured using standard Bradford assay (Bio-Rad). In total, 6 mg of proteins were reduced with 1 mM dithiotreitol for 1 h and subsequently alkylated with 5.5 mM iodoacetamide for 1 h in the dark at room temperature (RT). Proteins were predigested with endoproteinase Lys-C (1:100 w/w) for 3 h, then diluted with 4 volumes of water and supplemented with trypsin (1:100 w/w) for overnight digestion at RT. Digested protein sample was diluted 1:1 in 50 mM heptafluorobutyric acid (HFBA), pH 8 and desalted by solid-phase extraction using Sep-Pak Vac 100 mg C18 column (Waters). For that, column was activated with methanol, washed with 70% acetonitrile and equilibrated with 25 mM HFBA, pH 8. After loading the sample, the column was washed with water and peptides were eluted with a titanium dioxide (TiO_2) loading solution (300 mg/ml lactic acid, 12% v/v acetic acid, 0.2% v/v HFBA, pH 4 with NH_4OH). Phosphopepetide enrichment was performed with TiO_2 spheres (5 μm, 300 Å, ZirChrom) at pH 4 as described previously (4). Peptides were incubated with TiO_2 beads in 1:10 peptide to bead ratio for 10 min for 7 consecutive rounds. Phosphopeptides were eluted in two steps with 1.25% v/v NH_4OH and 5% v/v NH_4OH in 60% v/v acetonitrile. The pH was adjusted to 7 with formic acid and samples were concentrated by vacuum centrifugation.

Peptides from phosphopeptide enrichment fractions were separated by EASY-nLC 1200 system (Thermo Scientific) coupled to Q Exactive mass spectrometer (Thermo Scientific) through a nanoelectrospray ion source (Thermo Scientific). Chromatographic separation was performed on a 20 cm long, 75 μm inner diameter analytical column packed in-house with reversed-phase ReproSil-Pur C18-AQ 1.9 μm particles (Dr. Maisch GmbH) at 40°C. Peptides were loaded onto the column at a flow rate of 700 nL/min with solvent A (0.1% v/v formic acid) under maximum back-pressure of 850 bar. Peptides were eluted using 46 min segmented analytical gradient of 10–50% solvent B (80% v/v acetonitrile, 0.1% v/v formic acid) at a constant flow rate of 200 nL/min. Peptides were ionized by nanoelectrospray ionization at 2.3 kV and the capillary temperature of 275°C. The mass spectrometer was operated in a data-dependent mode, switching between one MS and subsequent MS/MS scans of 7 (Top7 method) most abundant peaks selected with an isolation window of 1.4 m/z. MS spectra were acquired in a mass range from 300–1650 m/z with a maximum injection time (IT) of 25 ms and a resolution of 60,000 (defined at m/z 200). The higher energy collisional dissociation (HCD) MS/MS spectra were recorded with the maximum IT of 220 ms, resolution of 60,000 and a normalized collision energy of 27%. The masses of sequenced precursor ions were dynamically excluded for fragmentation for 30 sec. Ions with single, unassigned or 6 and higher charge states were excluded from fragmentation.

Raw MS data was processed using a MaxQuant software suite (version 1.5.2.8) (5). Derived peak list was searched using Andromeda search engine integrated in MaxQuant (6) against a reference *B. subtilis* proteome (taxonomy ID 1423) obtained from UniProt (4,207 protein entries, released in October 2015), and a file containing 245 common laboratory contaminants implemented in MaxQuant. During the first search, peptide mass tolerance was set to 20 ppm and in the main search to 4.5 ppm. The minimum required peptide length was set to 7 amino acids. Trypsin was set to cleave at lysine and arginine C-terminus with the maximum of three missed cleavages. Carbamidomethylation of cysteines was set as a fixed modification and methionine oxidation, protein N-terminal acetylation and Ser/Thr/Tyr/Arg (STYR) phosphorylation were defined as variable modifications. Neutral loss was defined for STR and diagnostic peak for Y phosphorylation. Peptide, protein and modification site identifications were

filtered using a target-decoy approach at FDR set to 0.01 (7). Phosphorylation sites in Phospho(STYR)Sites.txt file were filtered for Andromeda score of ≥50 and localization probability of ≥0.75 to obtain high confidence data. Best identified MS/MS spectra of all 18 peptides phosphorylated on arginine were manually inspected and provided in Table S1.

Ribosome purification

Ribosome purification was carried out by chromatography method as described previously (8), in which cysteine-Sulfolink resin was employed for covalent immobilization of ribosome. Sulfolink resin is a cross-linked agarose resin (CL-6B) with 18-atom alkyl chains carrying a reactive iodoacetyl group at their terminus. All of the alkyl chains were reacted with cysteine to produce the resin. In brief, 2 ml of 50% slurry of Sulfolink coupling gel were placed in 15 ml tubes and centrifuged (5 min, 4000 RPM), and then the storage buffer was carefully decanted. The gel was washed (x3) with 3 ml sulfolink coupling buffer (50mM Tris, 5mM EDTA-Na, pH 8.5). Next, 3 ml of 50 mM L-cysteine in coupling buffer was added to the gel and the slurry was gently shaken for 1 h at room temperature. Next, residual cysteine was removed by centrifugation (5 min, 4000 RPM). 2 ml binding buffer (10 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 60 mM NH_4Cl) was added to the cysteine-Sulfolink resin gel and this gel was packed in Polyprep columns (Bio-Rad) and pre-equilibrated. Cell extracts from vegetative cells, dormant and germinating spores were loaded onto the column, and allowed to flow through by gravity at 4°C. The ribosome in the cell extracts were bound to the cysteine-Sulfolink resin in the column. The columns were washed with 5x1 ml portions of the same buffer to remove the nonspecific binding to cysteine-Sulfolink resin and then eluted with 5x1 ml portions of the same buffer supplemented with 0.5 M NH_4Cl to obtain purified ribosome, and 0.5 ml fractions were collected.

BONCAT spore germination experiments

Cultures of 10 ml of dormant or germinating spores were centrifuged and washed with PBS x 1. Pellets were resuspended in PBS x 1 supplemented with protease inhibitors (Thermo, 78439), lysed using Fastprep [FastPrep (MP) 6.5, 60 seconds, x3], and

centrifuged (5 min, 14,000 RPM). Supernatants containing a mixed population of AHA-labeled newly synthesized proteins and unlabeled pre-existing proteins were collected. The enrichment for newly synthesized proteins was performed using BONCAT as previously described (9, 10). For tagging of AHA-labeled proteins, samples were incubated overnight at 4°C with triazole ligand (0.25 mM, Sigma), alkyne-bearing biotin-flag tag (0.063 mM, Genscript) and CuBr (2 mM in DMSO, Sigma).

Quantitative Real time PCR (qRT-PCR)

Spore cultures (10 ml) were washed (x3) with PBS×1 and RNA was extracted using FastRNA Pro Blue kit (MP Biomedicals) as described previously (11). Extracted RNA (2 μg) was treated with RQ1 DNase (Promega) and subjected to cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer protocol. qRT-PCR reactions were conducted using SYBR-green mix (Bio Rad), and fluorescence detection was performed using CFX Connect Real-Time PCR Detection System (Bio-Rad), according to manufacturer instructions. qRT-PCR primers (Table S3) were designed using Primer3 software (v.0.4.0, available on-line). *yoxA* gene was used to normalize expression data, as its expression was unchanged throughout germination as indicated by qRT-PCR analysis. The relative gene expression levels were calculated from threshold cycle (C_T) values using the $2^{-\Delta\Delta CT}$ method (12). Each assay was performed in duplicates with at least two RNA templates prepared from independent biological repeats.

Western blot analysis

Proteins were extracted from vegetative cells, dormant or germinating spores as described in "**Quantitative phosphoproteomic analysis"**. Extracts were incubated at 100ºC for 10 min with Laemmli sample buffer. Proteins were separated by SDS-PAGE 12.5% and electroblotted onto a polyvinylidene 48 difluoride (PVDF) transfer membrane (Immobilon-P; Millipore). For Immunoblot analysis of GFP or mCherry fusion proteins, membranes were blocked for 1 hr at room temperature (0.05% Tween-20, 5% skim milk in TBSx1). Blots were then incubated for 1.5 h at room temperature with polyclonal rabbit anti-GFP or anti-mCherry antibodies (1:10,000 in 0.05% Tween-20, 5% skim milk in TBSx1). Next, membranes were incubated for 1 h at room temperature with peroxidase

conjugated goat anti-rabbit secondary antibody (Bio-Rad) (1:10,000 in 0.05% Tween-20, 5% skim milk in). EZ-ECL kit (Biological Industries, Beit Haemek, Israel) was used for final detection.

Determination of the levels of spore germination proteins

Expression levels of germination receptor subunits (GerAA, GerAC, GerBC and GerKA) and SpoVAD were detected by Western blot analyses using rabbit antibodies against these proteins and a secondary antibody, as described previously (13). Briefly, 125 ODs of spores were decoated at 70°C for 2 hrs with decoating solution (0.1 M DTT, 0.1 M NaCl, 0.1 M NaOH, 1% SDS), followed by 10 water washes. The decoated spores were lysed with 1 mg lysozyme, 1 mM PMSF, 1µg RNase, 1 µg Dnase I, and 20 µg of $MgCl_2$ in 0.5 ml TEP buffer (50mM Tris-HCl pH 7.4, 5 mM EDTA) at 37°C for 5 min, and then incubated on ice for 20 min. The lysed spores were then disrupted using Fastprep (MP) (6.5, 60 seconds, x3), and 100 µL of the lysate was added to 100 µl Laemmli sample buffer containing 55 mM DTT (425 µL BioRad 161-0737 plus 25 µL 1 M DTT) and incubated at 23°C for one hour. Western blot analysis was carried out as described above, using rabbit antibodies against these proteins and a secondary antibody.

***In vitro* transcription**

Lysates were extracted from vegetative cells, dormant and germinated spores as described above for ribosome purification. Samples from vegetative cells, dormant spores and germinating spores were collected by centrifugation, washed with transcription buffer supplemented with protease inhibitors (Thermo, 78439), and lysed using Fastprep (FastPrep (MP) 6.5, 60 sec, ×3). Following cell lysis, the supernatant was additionally centrifuged at 15,000 rpm for 15 min to obtain extracts. The extracts were dialyzed for 2 h against 200 volumes of dialyzed buffer (0.01 M Tris-HCl (pH 8.2), 0.014 M magnesium chloride, 0.06 M KCI, 0.001 M dithiothreitol, 20% glycerol, and 2 mM pefablock sc). These dialyzed extracts were stored at -80°C until usage.

In vitro transcription reactions were performed in a final volume of 25 µL that contained 15 µL of extract, 1× transcription buffer (12 mM Tris-HCl at pH 7.9, 5 mM $MgCl_2$, 80 mM KCl, 0.5 mM DTT, 20 mM creatine phosphate), NTPs (0.5 mM ATP, 0.5

mM CTP, 0.05 mM GTP, 0.5 mM UTP), 10 μCi of [α-32P]-UTP (3000 Ci/mmol; Perkin Elmer), and 0.25–0.5 μg of PCR products carrying ~500 bp upstream and ~ the first 400 bp of the desired gene (*recA*), providing an additional external template. After 40 min of incubation at 37°C, reaction mixtures were diluted 1:1 with H_2O, passed through a G-50 column, diluted to 250 μL with 1× digestion buffer (20 mM Tris-HCl at pH 7.9, 250 mM sodium acetate, 1 mM EDTA, 0.25% SDS), and digested with 120 μg/mL Proteinase K for 30 min at 37°C. Reaction products were recovered following 25:24:1 phenol:chloroform:isoamylalcohol extraction by ethanol precipitation. Labeled RNAs were analyzed in 8% polyacrylamide gels, and bands were visualized by autoradiography.

Northern Blot of mRNA

Northern blot was carried out as described previously (14, 15). In brief, total RNA (20~30 μg) was denatured for 10 min at 70°C in formamide loading buffer, separated on an agarose gel (1.4% in MOPS buffer), and transferred to Zeta-Probe GT membranes (Bio-Rad Laboratories) by capillary blotting overnight. Membranes were then UV auto-crosslinked, and hybridized overnight at 42ºC with the corresponding biotinylated probes (~30 nM) (Table S3) in ULTRAhyb Hybridization Buffer (ThermoFisher). Membranes were then further incubated with a streptavidin-conjugated Horseradish Peroxidase (HRP) (1:3000 in 0.5% SDS, 0.1% I-Block reagent in PBSx1, abcam, Israel). The blot was developed using EZ-ECL kit (Biological Industries, Beit Haemek, Israel) according to the manufacturer's instructions.

Plasmid Construction

pMINImad2 (3)

pBZ100 (*tig erm amp oriBsTs*), constructed by amplifying genomic region containing ~500 bp upstream and ~500 bp downstream of the *tig-R45* site with primer pair *tig*-BamHI-U/*tig*-SalI-L. The PCR product was digested with BamHI and SalI, and cloned into the BamHI and SalI sites of pMINImad2, which carries a temperature sensitive origin of replication and an erythromycin (*mls*) resistance cassette.

pBZ98 (*tig-R45A erm amp oriBsTs*), to change the codon encoding R45 to a codon encoding Ala, site directed mutagenesis was conducted on pBZ100 using primer pair *tig*-R45A-F/*tig*-R45A-R and the Quickchange II kit (Stratagene). Plasmid was sequenced to verify the presence of the mutation.

pBZ97 (*tig-R45D erm amp oriBsTs*), to change the codon encoding R45 to a codon encoding Asp, site directed mutagenesis was conducted on pBZ100 using primer pair *tig*-R45D-F/*tig*-R45D-R and the Quickchange II kit (Stratagene). Plasmid was sequenced to verify the presence of the mutation.

pBZ200 **(***sigA erm amp oriBsTs***),** constructed by amplifying genomic region containing ~500 bp upstream and ~500 bp downstream of the *sigA-R365* site with primer pair *sigA*-BamHI-U/*sigA*-SalI-L. The PCR product was digested with BamHI and SalI, and cloned into the BamHI and SalI sites of pMINImad2.

pBZ90 (*sigA-R365A erm amp oriBsTs*), to change the codon encoding R45 to a codon encoding Ala, site directed mutagenesis was conducted on pBZ200 using primer pair *sigA*-R365A-F/*sigA*-R365A-R and the Quickchange II kit (Stratagene). Plasmid was sequenced to verify the presence of the mutation.

pBZ91 (*sigA-R365D erm amp oriBsTs*), to change the codon encoding R45 to a codon encoding Asp, site directed mutagenesis was conducted on pBZ200 using primer pair *sigA*-R365D-F/*sigA*-R365D-R and the Quickchange II kit (Stratagene). Plasmid was sequenced to verify the presence of the mutation.

pBZ141 (*tig-R45E erm amp oriBsTs*), to change the codon encoding R45 to a codon encoding Glu, site directed mutagenesis was conducted on pBZ100 using primer pair *tig*-R45E-F/*tig*-R45E-R and the Quickchange II kit (Stratagene). Plasmid was sequenced to verify the presence of the mutation.

pBZ143 (*sigA-R365E erm amp oriBsTs*), to change the codon encoding R45 to a codon encoding Glu, site directed mutagenesis was conducted on pBZ200 using primer pair *sigA*-R365E-F/*sigA*-R365E-R and the Quickchange II kit (Stratagene). Plasmid was sequenced to verify the presence of the mutation.

pAR5 (*rpsB-mCherry-kan*) (16)

pAR68 (*pupG-gfp-spc*) laboratory stock

pDB27 (*rplA-dronpa-spc*) (17)

pLS146 (*rpmE-dronpa-spc*), containing the 3’ region of *rpmE* fused to *dronpa*, was constructed by amplifying the 3’ region of *rpmE* gene by PCR using primers 3019 and 3020, which replaced the stop codon with a XhoI site. The PCR-amplified DNA was digested with EcoRI and XhoI and was cloned into the EcoRI and XhoI sites of pDB27 (*spc*), which contains the *dronpa* coding sequence.

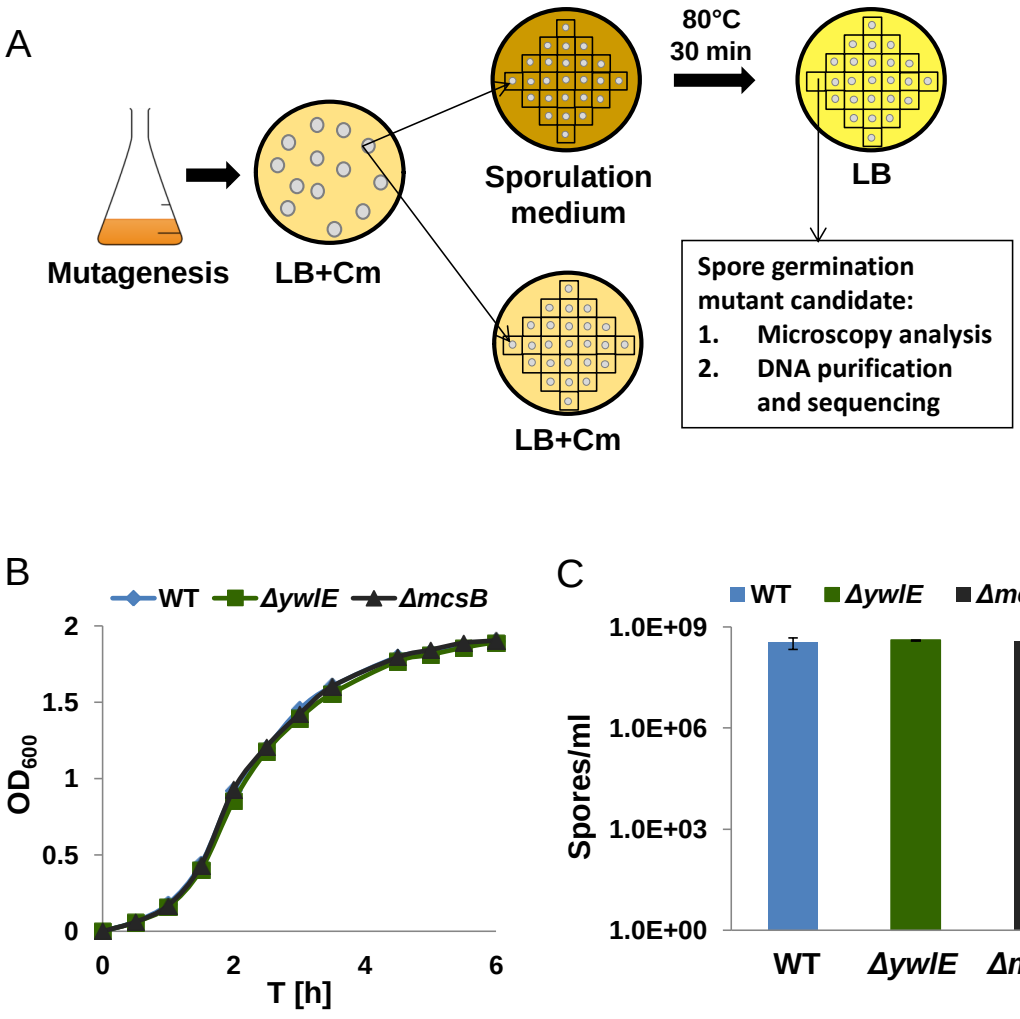


Fig. S1: Screening for mutants perturbed in spore germination

(*A*) A schematic description of transposon mutagenesis strategy, screening for mutants deficient in germination. DS8274 strain was grown in LB medium overnight, and cells plated on LB+chloramphenicol (LB+cm) plates at 48°C to allow transposition and the formation of mutant colonies. Each colony was streaked onto LB+cm plate (source plate) and DSM plate (sporulation plate), and the plates were incubated at 30°C. After 6 days DSM plates were incubated at 80°C for 30 min to kill the non-sporulated cells. Heated DSM colonies were then replicated into fresh LB plates to allow revival. Candidates that could not revive on LB plates were further examined using light microscopy to exclude

sporulation mutants, and those that sporulated well were considered as revival mutants. Candidates were recovered from the source LB+Cm plate for DNA purification and sequencing.

(*B*) PY79 (WT), BZ16 (Δ*ywlE*), and BZ129 (Δ*mcsB*) strain cultures were diluted to OD_{600}=0.05 in LB medium, incubated at 37°C and OD_{600} was measured during growth at the indicated time points. Shown is a representative out of 3 independent biological repeats.

(*C*) PY79 (WT), BZ16 (Δ*ywlE*), and BZ129 (Δ*mcsB*) strains were induced to sporulate in DSM for 24 h. Next, cultures were subjected to heat kill treatment (80°C, 30 min), serial decimal dilutions were plated on LB, and colonies were scored after 24 h. Presented are the averaged numbers and SD of spores/ml from 3 independent biological repeats.

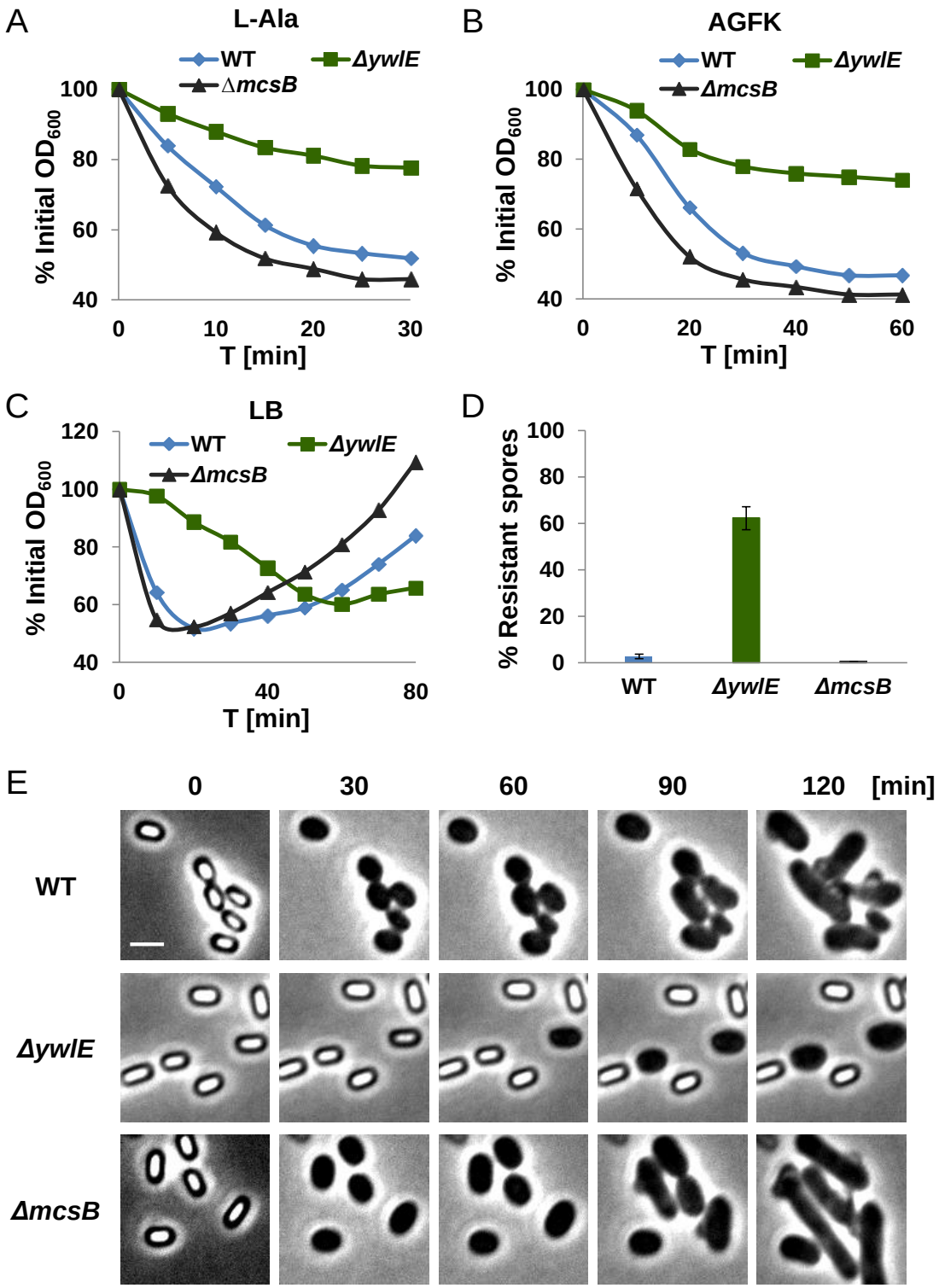


Fig. S2: Spore germination is facilitated by arginine dephosphorylation

(*A*-*C*) Spores of PY79 (WT), BZ16 (Δ*ywlE)* and BZ129 (Δ*mcsB)* strains were incubated with L-Ala (10 mM) (*A*), AGFK (*B*), or LB (*C*) and OD_{600} was measured at the indicated time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Germination assays were carried out in triplicates, and representative data are presented. Decreasing OD_{600} signifies spore germination.

(*D*) Spores of PY79 (WT), BZ16 (Δ*ywlE)* and BZ129 (Δ*mcsB)* strains were supplemented with L-Ala for 10 min, and incubated at 80°C 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.

(*E*) Spores of PY79 (WT), BZ16 (Δ*ywlE)* and BZ129 (Δ*mcsB)* strains were incubated on LB agar and monitored by time lapse microscopy. Shown are phase contrast images from a representative experiment out of 3 independent biological repeats. Scale bar represents 1 μm.

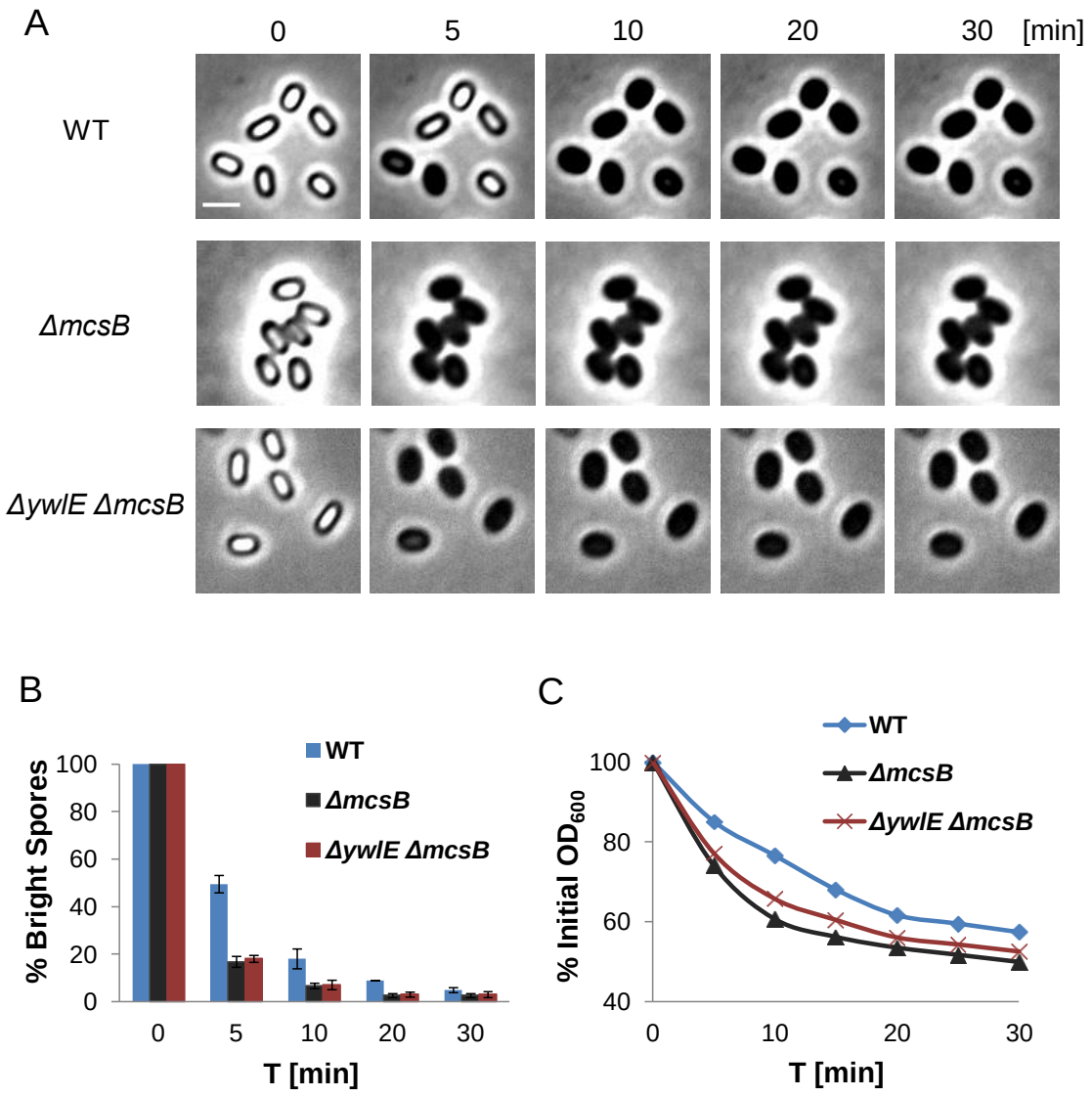


Fig. S3: McsB is the major kinase for the arginine phosphorylation sites detected in the Δ*ywlE* strain during germination

(*A*) Spores of PY79 (WT), BZ129 (*ΔmcsB*) and BZ130 (*ΔywlE ΔmcsB*) were incubated on agarose supplemented with L-Ala (10 mM) and monitored by time lapse microscopy. Shown are phase contrast images from a representative experiment out of 3 independent biological repeats. Scale bar represents 1 μm.

(*B*) Spores of PY79 (WT), BZ129 (*ΔmcsB*) and BZ130 (*ΔywlE ΔmcsB*) strains were incubated on agarose supplemented with L-Ala (10 mM) and monitored by time lapse microscopy. Data are presented as percentage of the initial number of the phase bright spores. Shown are average values and SD obtained from 3 independent biological repeats (n≥300 for each strain).

(*C*) Spores of PY79 (WT), BZ129 (Δ*mcsB*) and BZ130 (*ΔywlE ΔmcsB*) strains were incubated with L-Ala (10 mM) and OD_{600} was measured at the indicated time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Germination assays were carried out in triplicates, and representative data are presented. Decreasing OD_{600} signifies spore germination.

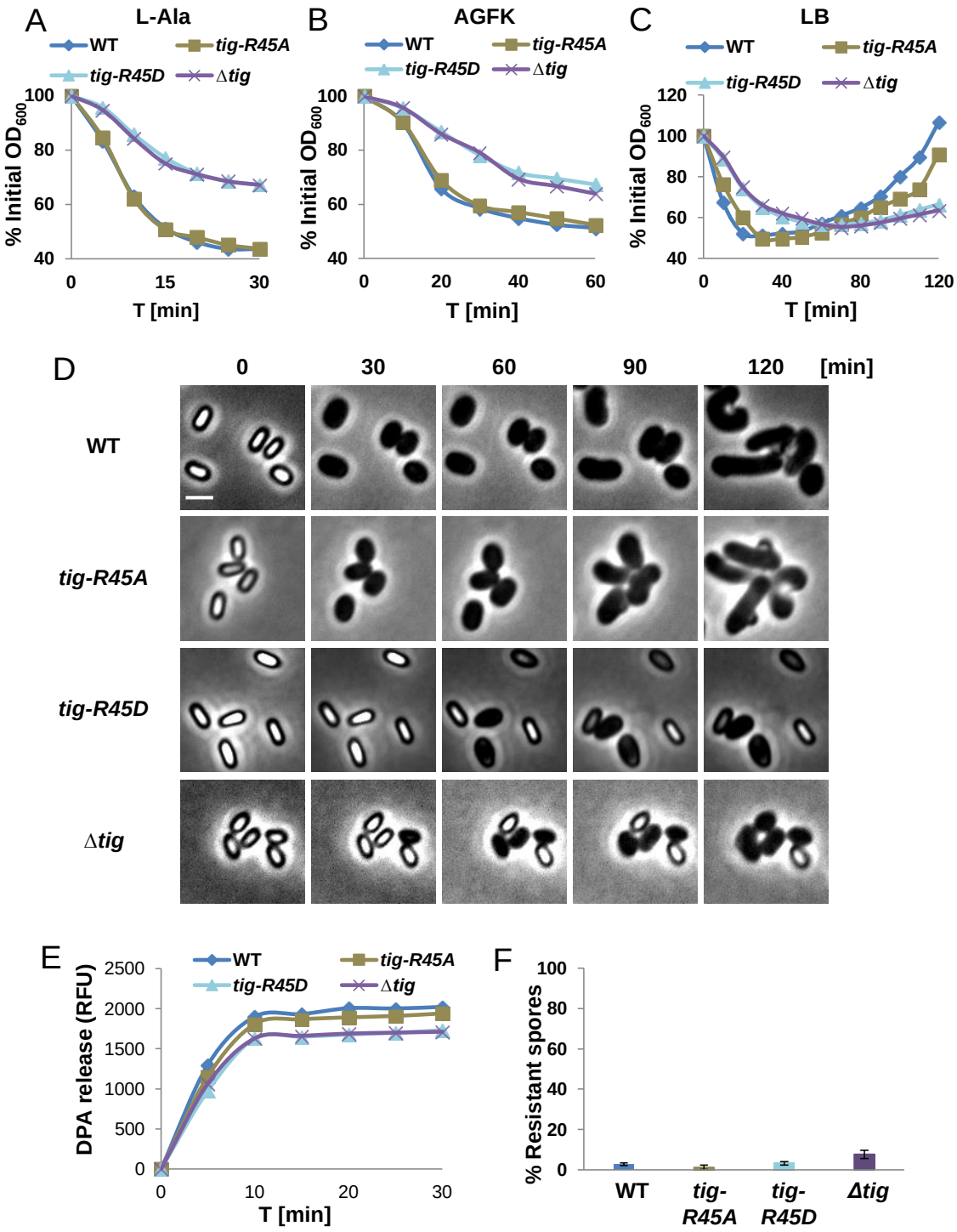


Fig. S4: Dephosphorylation of Tig drives spore germination

(*A-C*) Spores of PY79 (WT), BZ98 (*tig-R45A*), BZ97 (*tig-R45D)* and LS38 (Δ*tig*) strains were incubated with L-Ala (10 mM) (*A*), AGFK (*B*), or LB (*C*) and OD_{600} was measured

at the indicated time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Germination assays were carried out in triplicates, and representative data are presented.

(*D*) Spores of PY79 (WT), BZ98 (*tig-R45A*), BZ97 (*tig-R45D)* and LS38 (Δ*tig*) strains were incubated on LB agar and monitored by time lapse microscopy. Shown are phase contrast images from a representative experiment out of 3 independent biological repeats. Scale bar represents 1 μm.

(*E*) Spores of PY79 (WT), BZ98 (*tig-R45A*), BZ97 (*tig-R45D)* and LS38 (Δ*tig*) strains were incubated with L-Ala to trigger germination. DPA release to the medium was determined by Tb-DPA assay. Presented are relative fluorescence units (RFU) measured at 545 nm with excitation at 270 nm. Shown is a representative experiment out of 3 independent biological repeats.

(*F*) Spores of PY79 (WT), BZ98 (*tig-R45A*), BZ97 (*tig-R45D)* and LS38 (Δ*tig*) strains were supplemented with L-Ala for 10 min and then incubated at 80°C 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.

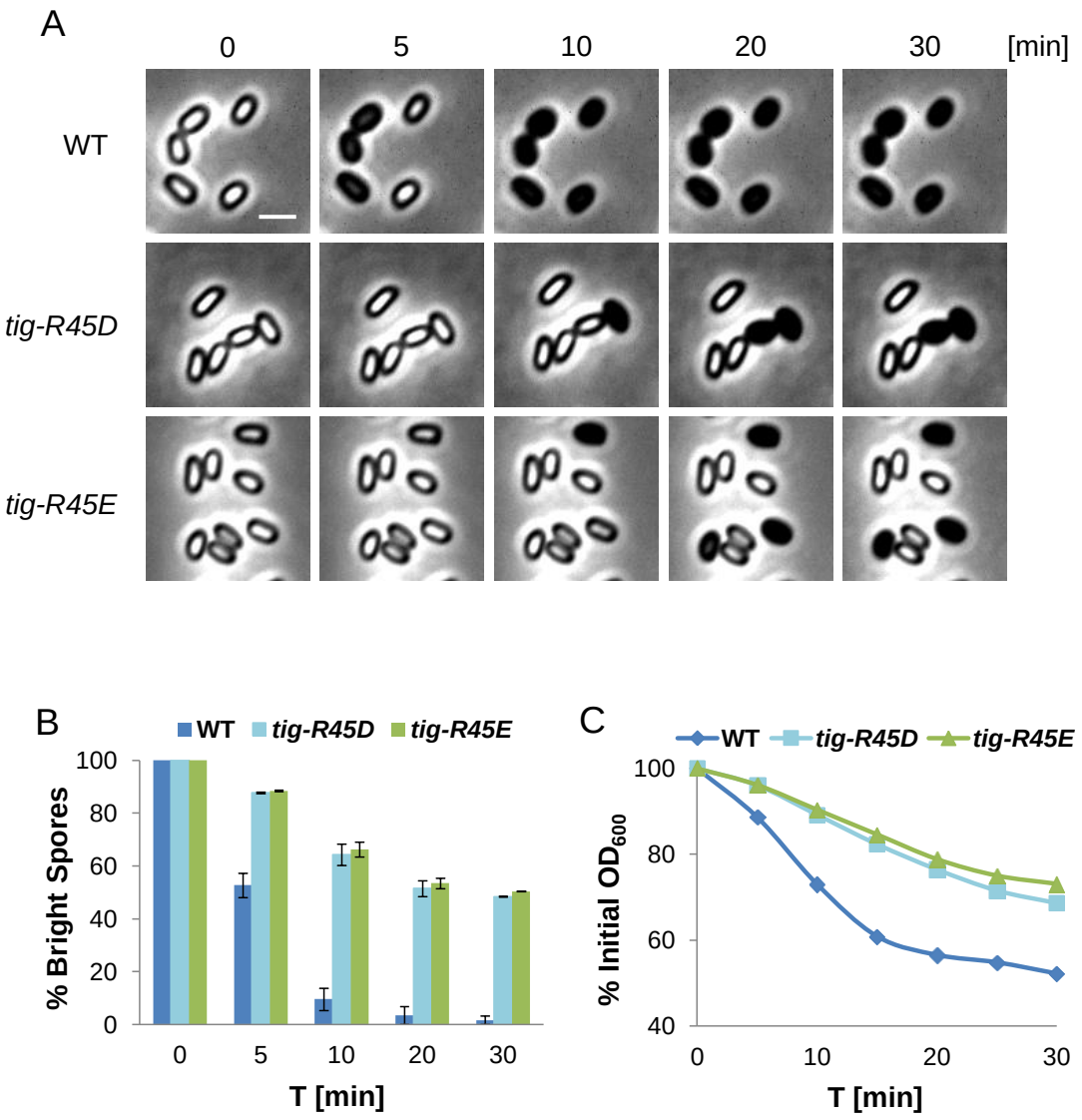
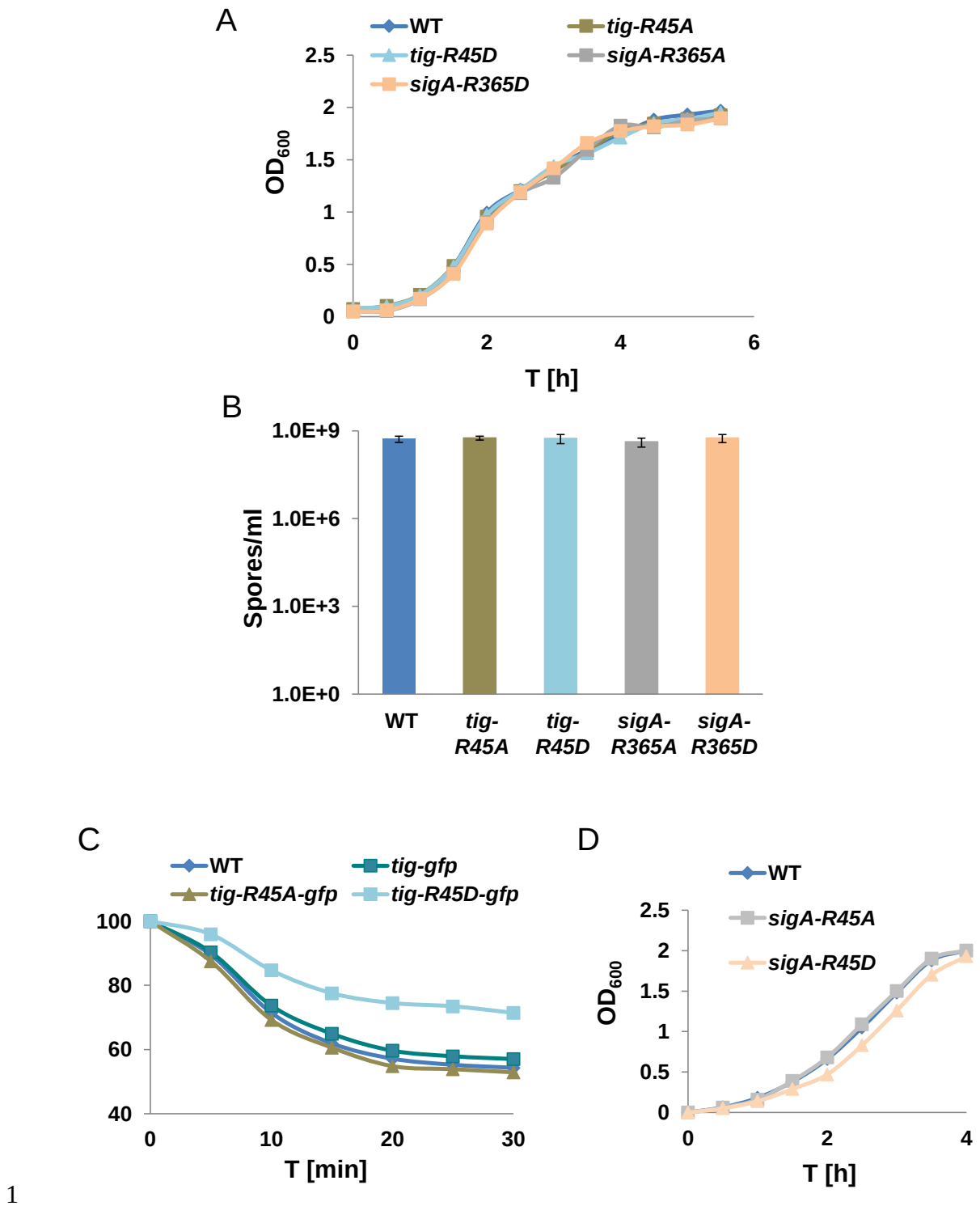


Fig. S5: *tig-R45E* mutant spores shows germination defects similar to that of spores harboring *tig-R45D*

(*A*) Spores of PY79 (WT), BZ97 (*tig-R45D*) and BZ141 (*tig-R45E*) were incubated on agarose supplemented with L-Ala (10 mM) and monitored by time lapse microscopy. Shown are phase contrast images from a representative experiment out of 3 independent biological repeats. Scale bar represents 1 μm.

(*B*) Spores of PY79 (WT), BZ97 (*tig-R45D*) and BZ141 (*tig-R45E*) strains were incubated on agarose supplemented with L-Ala (10 mM) and monitored by time lapse microscopy. Data are presented as percentage of the initial number of the phase bright spores. Shown are average values and SD obtained from 3 independent biological repeats ($n \geq 300$ for each strain).

(*C*) Spores of PY79 (WT), BZ97 (*tig-R45D*) and BZ141 (*tig-R45E*) strains were incubated with L-Ala (10 mM) and OD_{600} was measured at the indicated time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Germination assays were carried out in triplicates, and representative data are presented. Decreasing OD_{600} signifies spore germination.



1

2 **Fig. S6: characterization of *tig* and *sigA* mutants**

(*A*) PY79 (WT), BZ98 (*tig-R45A*), BZ97 (*tig-R45D*), BZ90 (*sigA-R365A*) and BZ91 (*sigA-R365D*) strain cultures were diluted to OD_{600}=0.05 in LB medium, incubated at 37°C and OD_{600} was measured during growth at the indicated time points.

(*B*) PY79 (WT), BZ98 (*tig-R45A*), BZ97 (*tig-R45D*), BZ90 (*sigA-R365A*) and BZ91 (*sigA-R365D*) strains were induced to sporulate in DSM for 24 h. Next, cultures were subjected to heat kill treatment (80°C, 30 min), serial decimal dilutions were plated on LB and colonies were scored after 24 h. Presented are the averaged numbers and SD of spores/ml from 3 independent biological repeats.

(*C*) Spores of PY79 (WT), LS50 (*tig-gfp*), BZ108 (*tig-R45D-gfp*) and BZ109 (*tig-R45A-gfp*) strains were incubated with L-Ala (10 mM) and OD_{600} was measured at the indicated time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Decreasing OD_{600} signifies spore germination.

(*D*) PY79 (WT), BZ90 (*sigA-R365A*) and BZ91 (*sigA-R365D*) strain cultures were diluted to OD_{600}=0.05 in S7 medium, incubated at 37°C and OD_{600} was measured during growth at the indicated time points.

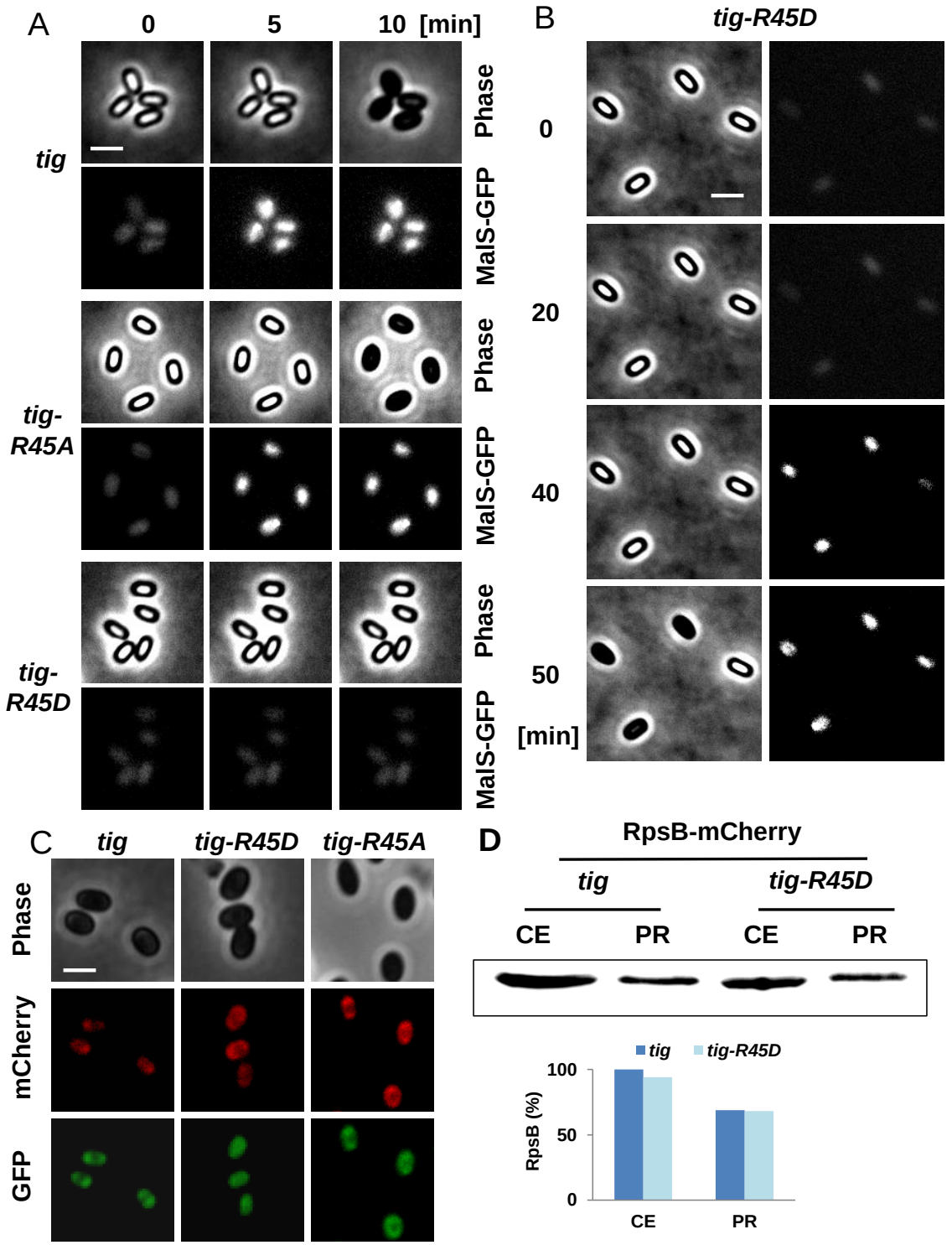


Fig. S7: The impact of Tig phosphorylation state on protein synthesis during spore germination

(*A-B*) Spores of AR71 (WT, *malS-gfp*), BZ107 (*tig-R45A, malS-gfp*) and BZ106 (*tig-R45D, malS-gfp)* strains were incubated on agar with L-Ala and followed by time lapse microscopy. Shown are phase contrast and fluorescence from MalS-GFP images taken at the indicated time points. For each strain, images were scaled to the same intensity range. A representative experiment out of three independent biological repeats is shown. Scale bars represent 1 μm.

(*C*) Spores of BZ118 (*tig-gfp, rpsB-mCherry*), BZ120 (*tig-R45A-gfp, rpsB-mCherry*) and BZ119 (*tig-R45D-gfp, rpsB-mCherry*) strains were incubated with 10 mM L-Ala and observed by microscopy at indicated time points. Shown is phase contrast (Phase), fluorescence from mCherry (red) and from GFP (green) images. For BZ118 and BZ120, the images were taken at 10 min after germination induction. For BZ119, the images were taken at 60 min after germination induction. Shown are images from a representative experiment out of 3 independent biological repeats. Scale bar represents 1 μm.

(*D*) Intact ribosomes were purified from the cell extracts (CE) of the vegetative cells of BZ118 (*tig-gfp, rpsB-mCherry*) and BZ119 (*tig-R45D-gfp, rpsB-mCherry*) strains. Shown is a Western blot analysis carried out using antibody against mCherry to detect the levels of RpsB in cell extracts (CE) and in purified ribosomes (PR) (Upper panel). Signal from mCherry fusion proteins was quantified by MetaMorph software (version 7.7, Molecular Devices) (Lower panel). The signal from RpsB-mCherry in CE from BZ118 (*tig-gfp*) was considered as 100%. Shown is a representative experiment out of two biological repeats.

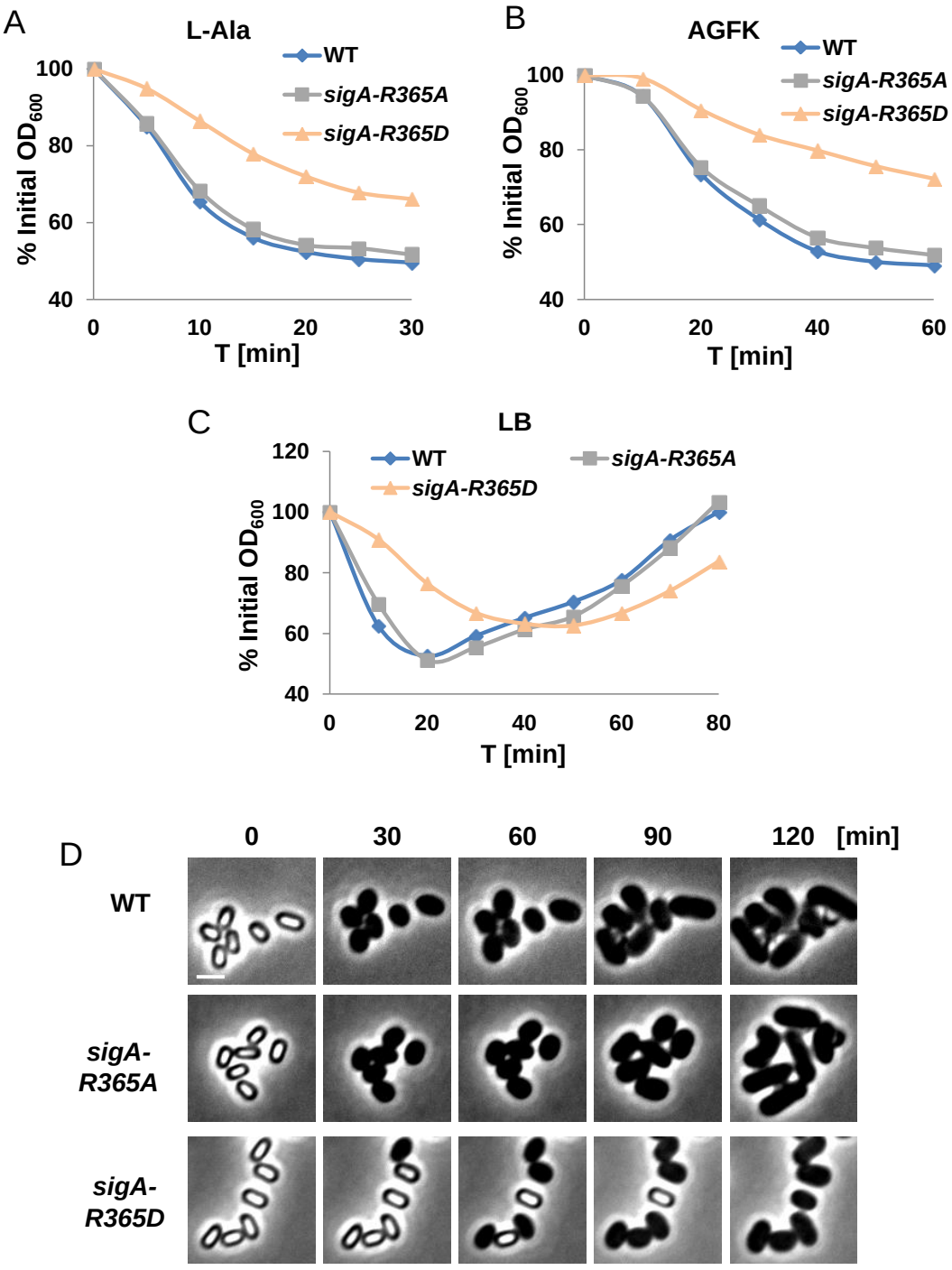


Fig. S8: Dephosphorylation of SigA contributes to spore germination

(*A-C*) Spores of PY79 (WT), BZ90 (*sigA-R365A*) and BZ91 (*sigA-R365D*) strains were incubated with L-Ala (*A*), AGFK (*B*) or LB (*C*) and OD_{600} was measured at the indicated

time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Germination assays were carried out in triplicates, and representative data are presented. (*D*) Spores of PY79 (WT), BZ90 (*sigA-R365A*) and BZ91 (*sigA-R365D*) strains were incubated on LB agar and monitored by time lapse microscopy. Shown are phase contrast images from a representative experiment out of 3 independent biological repeats. Scale bar represents 1 μm.

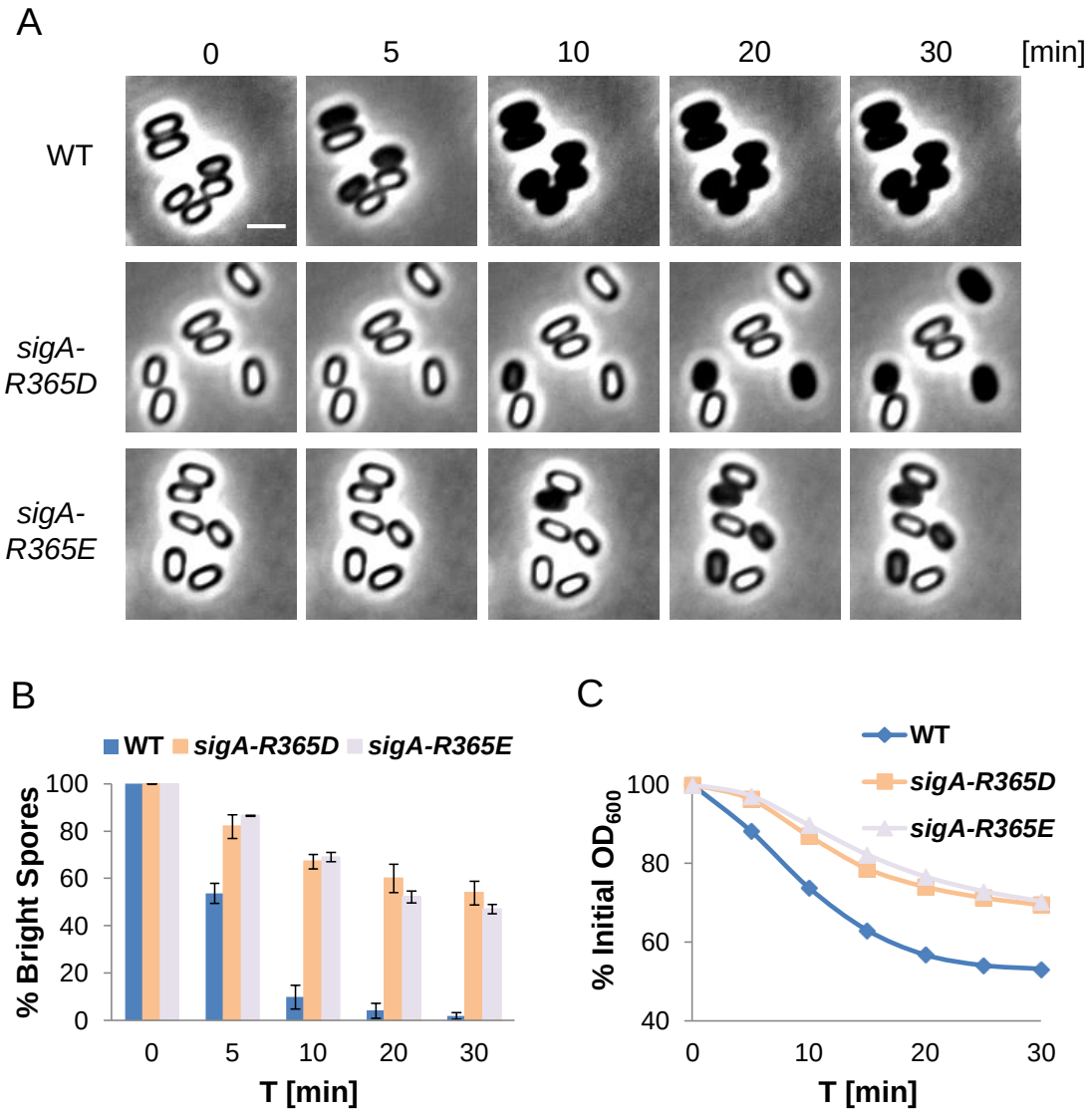


Fig. S9: *sigA-R365E* mutant spores show germination defect similar to that of spores harboring *sigA-R365D*

(*A*) Spores of PY79 (WT), BZ91 (*sigA-R365D*) and BZ143 (*sigA-R365E*) were incubated on agarose supplemented with L-Ala (10 mM) and monitored by time lapse microscopy. Shown are phase contrast images from a representative experiment out of 3 independent biological repeats. Scale bar represents 1 μm.

(*B*) Spores of PY79 (WT), BZ91 (*sigA-R365D*) and BZ143 (*sigA-R365E*) strains were incubated on agarose supplemented with L-Ala (10 mM) and monitored by time lapse microscopy. Data are presented as percentage of the initial number of the phase bright spores. Shown are average values and SD obtained from 3 independent biological repeats ($n \geq 300$ for each strain).

(*C*) Spores of PY79 (WT), BZ91 (*sigA-R365D*) and BZ143 (*sigA-R365E*) strains were incubated with L-Ala (10 mM) and OD_{600} was measured at the indicated time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Germination assays were carried out in triplicates, and representative data are presented. Decreasing OD_{600} signifies spore germination.

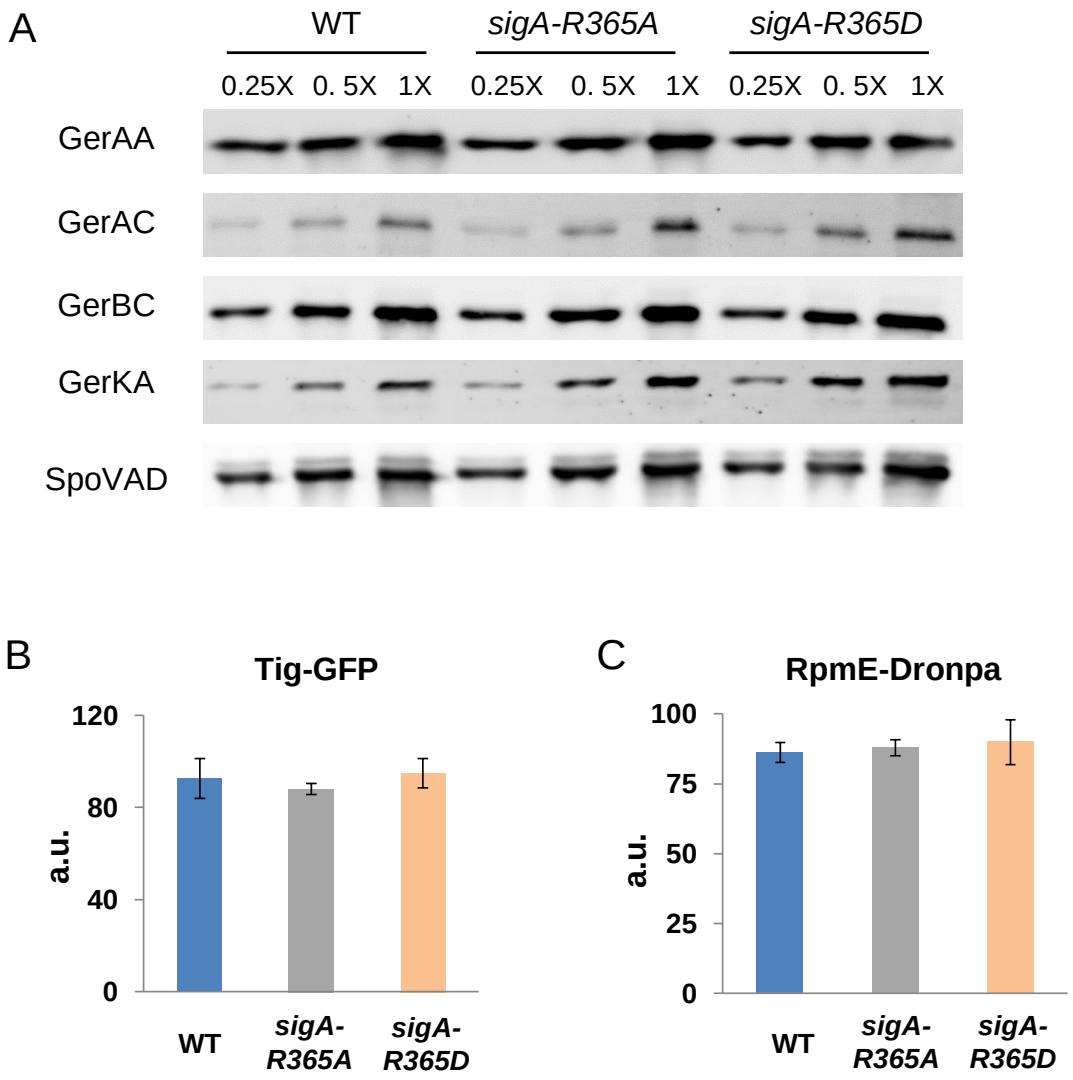


Fig. S10: Determination of the levels of spore germination proteins

(*A*) Equal amounts of protein extracts from spores of PY79 (WT), BZ90 (*sigA-R365A*) and BZ91 (*sigA-R365D*) strains were subjected to SDS-PAGE. Western blot analysis using antibodies against GerAA, GerAC, GerBC, GerKA, and SpoVAD was conducted. Dilutions of the different samples were loaded on the same gel for comparison.

(*B*) Dormant spores of LS50 (WT, *tig-gfp*), BZ127 (*sigA-R365A*, *tig-gfp*), and BZ128 (*sigA-R365D, tig-gfp*) were observed by fluorescence microscopy. Shown are average

values and SD of the fluorescence signal in arbitrary units (a.u.) obtained from 3 independent biological repeats (n≥300 for each strain).

(*C*) Dormant spores of LS108 (WT, *rpmE-dronpa*), BZ131 (*sigA-R365A, rpmE-dronpa*), and BZ132 (*sigA-R365D*, *rpmE-dronpa*) were processed as in (*B*).

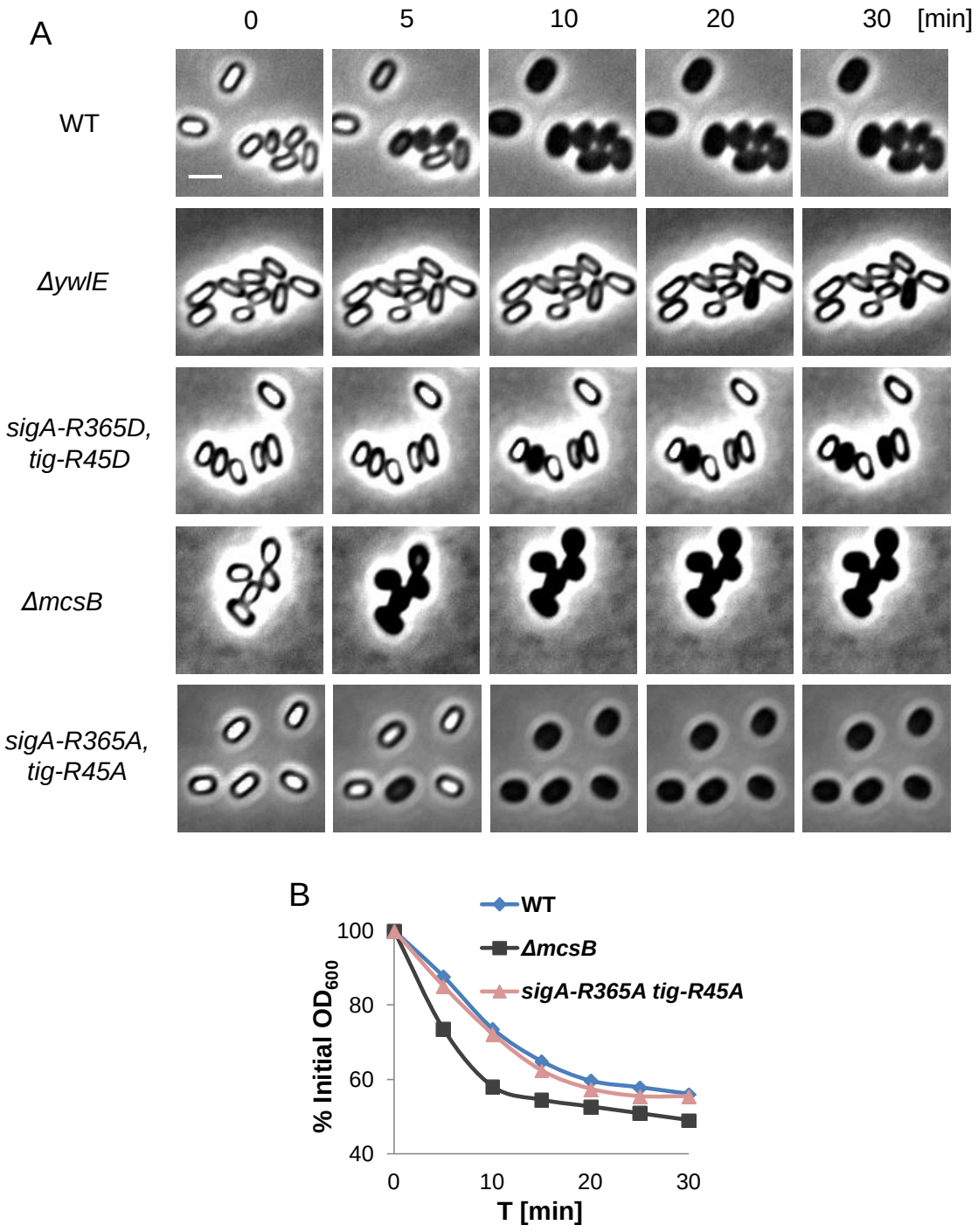


Fig. S11: Combining *sigA* and *tig* phosphomimetic mutations failed to recapitulate the germination phenotypes of Δ*ywlE* and Δ*mcsB* spores

(*A*) Spores of PY79 (WT), BZ16 (Δ*ywlE*), BZ104 (*tig-R45D*, *sigA-R365D*), BZ129 (Δ*mcsB*) and BZ139 (*tig-R45A*, *sigA-R365A*) strains were incubated on agarose

supplemented with L-Ala (10 mM) and monitored by time lapse microscopy. Shown are phase contrast images from a representative experiment out of 3 independent biological repeats. Scale bar represents 1 μm.

(*B*) Spores of PY79 (WT), BZ129 (Δ*mcsB*) and BZ139 (*tig-R45A*, *sigA-R365A*) strains were incubated with L-Ala (10 mM) and OD_{600} was measured at the indicated time points. Data are presented as percentage of the initial OD_{600} of spore suspension. Germination assays were carried out in triplicates, and representative data are presented. Decreasing OD_{600} signifies spore germination.

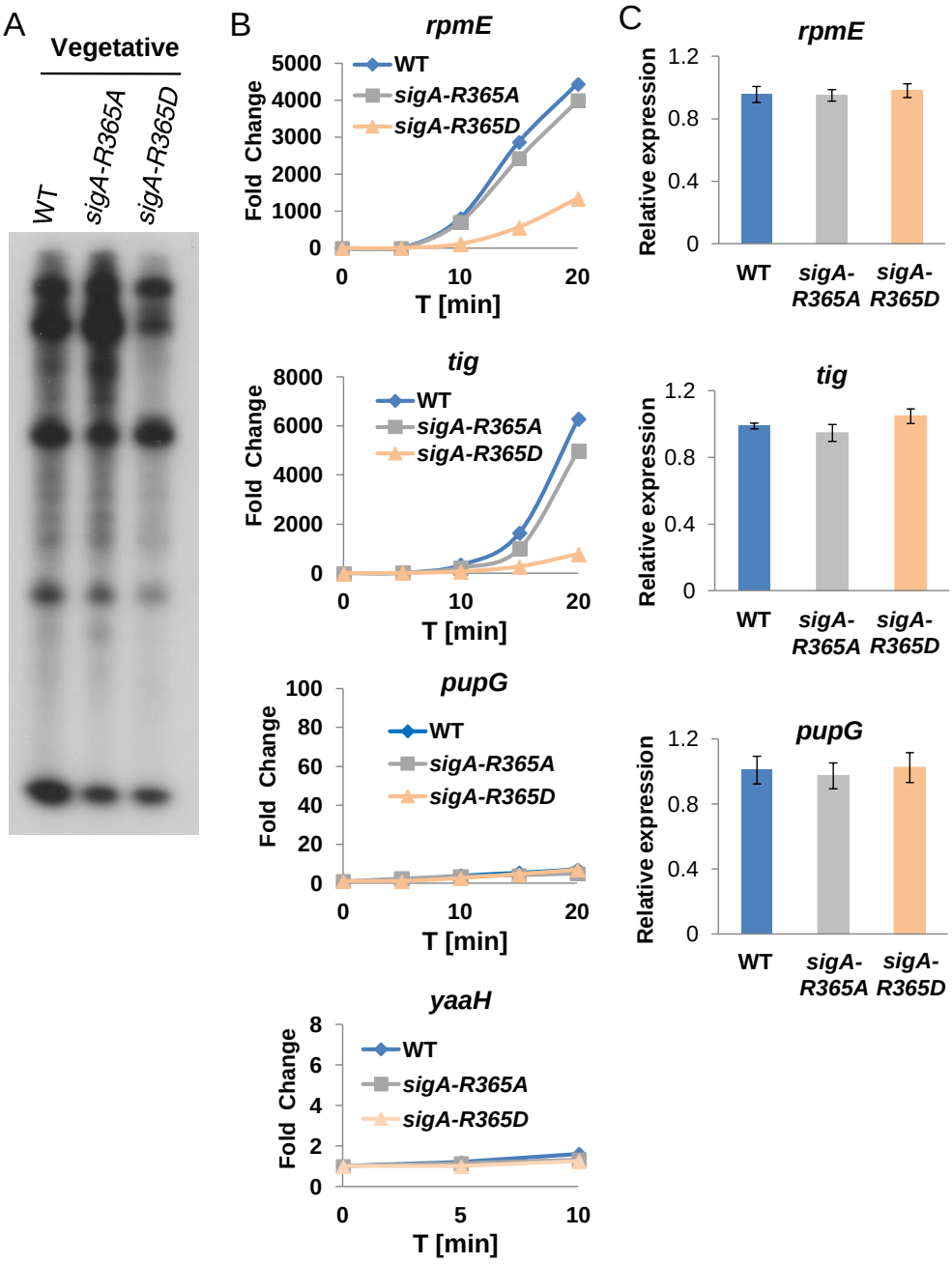


Fig. S12: Measuring transcription during spore revival

(*A*) *In vitro* transcription reaction was carried out in whole extracts from vegetative cells of PY79 (WT), BZ90 (*sigA-R365A*) and BZ91 (*sigA-R365D*) strains, in transcription buffer supplemented with NTPs (ATP, CTP, GTP, UTP, [α-32P]-UTP). After 40 min of incubation at 37°C the reaction was stopped, RNA was purified and radioactively labeled

RNAs were analyzed in 8% polyacrylamide gels, and bands were visualized by autoradiography. The analysis was carried out in two biological repeats, and a representative experiment is presented.

(*B*) Spores of PY79 (WT), BZ90 (*sigA-R365A*) and BZ91 (*sigA-R365D*) strains were incubated in LB or L-Ala to trigger germination, collected by centrifugation at indicated time and their RNA were extracted. The mRNA levels of selective genes, *tig*, *rpmE*, *pupG*, and *yaaH* were determined by quantitative RT-PCR. The result is presented as the fold change of target gene expression after germination relative to that before germination. The Assays were carried out in triplicates, and representative data are presented.

(*C*) RNA were extracted from PY79 (WT), BZ90 (*sigA-R365A*) and BZ91 (*sigA-R365D*) vegetative cells grown to the mid logarithmic phase. The mRNA levels of selective genes, *tig*, *rpmE*, and *pupG* were determined by quantitative RT-PCR. Transcript levels are relative to that of WT strain. Each bar represents an average value and s.d. of three independent experiments.

Supplementary Tables

Table S1: Phospho-sites detected in dormant spores

Shown is the phosphoproteome of BZ16 (Δ*ywlE*) dormant spores. Table S1b contains only phosphorylation sites on R. Table S1c contains all detected phosphorylation sites on STYR.

Table S2: *B. subtilis* strains used in this study

Strain	Genotype	Comments
PY79	Wild type	(18)
LS5	*metE::mls*	Laboratory stock
BZ16	*ywlE::mls*	The ORF of *ywlE* was replaced by *mls* gene using a long-flanking-homology PCR with primers *ywlE*-KO-P1- P4 (Table S3)
LS26	*rpmE::kan*	Laboratory stock
LS38	*tig::kan*	Laboratory stock
LS50	*tig-gfp-spc*	Laboratory stock
AR68	*pupG-gfp-spc*	Laboratory stock
AR71	*malS-gfp-spc*	Laboratory stock
LS76	*pupG::kan*	Laboratory stock
BZ90	*sigA-R365A*	*sigA-R365A* allele was constructed by allele replacement technique, transforming PY79 with plasmid pBZ90.
BZ91	*sigA-R365D*	*sigA-R365D* allele was constructed by allele replacement technique, transforming PY79 with plasmid pBZ91.
BZ97	*tig-R45D*	*tig-R45D* allele was constructed by allele replacement technique, transforming PY79 with plasmid pBZ97.
BZ98	*tig-R45A*	*tig-R45A* allele was constructed by allele replacement technique, transforming PY79 with plasmid pBZ98.
BZ102	*tig-R45D, metE::mls*	*tig-R45D, metE::mls* allele was constructed by transformation of BZ97 with genomic DNA from LS5.
BZ103	*tig-R45A,*	*tig-R45A, metE::mls* allele was constructed by

	metE::mls	transformation of BZ98 with genomic DNA from LS5.
BZ104	*tig-R45D, sigA-R365D*	*tig-R45D, sigA-R365D* allele was constructed by allele replacement technique, transforming BZ97 with plasmid pBZ91.
BZ106	*tig-R45D, malS-gfp-spc*	BZ97 was transformed with pAR71 (*malS-gfp-spc*)
BZ107	*tig-R45A, malS-gfp-spc*	BZ98 was transformed with pAR71 (*malS-gfp-spc*)
BZ108	*tig-R45D-gfp-spc*	BZ97 was transformed with pLS50(*tig-gfp-spc*)
BZ109	*tig-R45A-gfp-spc*	BZ98 was transformed with pLS50(*tig-gfp-spc*)
BZ118	*tig-gfp-spc, rpsB-mcherry-kan*	LS50 was transformed with pAR5 (*rpsB-gfp-mcherry-kan*)
BZ119	*tig-R45D-gfp-spc, rpsB-mcherry-kan*	BZ108 was transformed with pAR5 (*rpsB-gfp-mcherry-kan*)
BZ120	*tig-R45A -gfp-spc, rpsB-mcherry-kan*	BZ109 was transformed with pAR5 (*rpsB-gfp-mcherry-kan*)
BZ127	*sigA-R365D, tig-gfp-spc*	BZ91 was transformed with pLS50 (*tig-gfp-spc*)
BZ128	*sigA-R365A, tig-gfp-spc*	BZ90 was transformed with pLS50 (*tig-gfp-spc*)
BZ129	*mcsB::tet,* P_{ctsR}*-clpC*	The ORF of *mcsB* was replaced by *tet* gene using a long-flanking-homology PCR with primers *mcsB*-KO-P1- P6 (Table S3)
BZ130	*ywlE::mls, mcsB::tet,* P_{ctsR}*-clpC*	BZ 129 was transformed with genomic DNA of BZ16
BZ131	*sigA-R365A, rpmE-dronpa-spc*	BZ90 was transformed with pLS146 (*rpmE-dronpa-spc*)
BZ132	*sigA-R365D,*	BZ91 was transformed with pLS146 (*rpmE-*

	rpmE-dronpa-spc	*dronpa-spc*)
BZ139	*tig-R45A, sigA-R365A*	*tig-R45A,sigA-R365A* allele was constructed by allele replacement technique, transforming BZ98 with plasmid pBZ90.
BZ141	*tig-R45E*	*tig-R45E* allele was constructed by allele replacement technique, transforming PY79 with plasmid pBZ141.
BZ143	*sigA-R365E*	*sigA-R365E* allele was constructed by allele replacement technique, transforming PY79 with plasmid pBZ143.
BZ144	*tig-R45A, pupG-gfp-spc*	BZ98 was transformed with pAR68 (*pupG-gfp-spc*)
BZ145	*tig-R45D, pupG-gfp-spc*	BZ97 was transformed with pAR68 (*pupG-gfp-spc*)
DS8274	*pEP25* *TnLacJump* *cat amp mls* *mariner-Himar1* *ori(TS)Bs*	A gift from Daniel B. Kearns (Indiana University)

Long-flanking-homology PCR replacement strategy was based on (19) and the resultant PCR product was used to transform PY79. For some of the constructs, Gibson Assembly kit (New England Biolabs) was utilized to assemble the PCR products.

Table S3: Primers used in this study

Primer name	Primer sequence
ywlE-KO-P1	5'-ATTCAAGAATATCAACAGGGTGGAAGACGG-3'
ywlE-KO-P2	5'-CTGAGCGAGGGAGCAGAAGAAACGGCGACTTCGTTTCTTTTATTGGTA-3'
ywlE-KO-P3	5'-GTTGACCAGTGCTCCCTGAATCTGCAAACAGGTCATTTGCAAATCCTG-3'
ywlE-KO-P4	5'-CTGAATGGTTTCAACAAGAACAGGAGACTT-3'
mcsB-KO-P1	5'-CGAGTGTTAGATCAAAATGGCAAGG-3'
mcsB-KO-P2	5'-CTGAGCGAGGGAGCAGAATACTCCTGTTCCTCCTCACTATCTG-3'
mcsB-KO-P3	5'-GTTGACCAGTGCTCCCTGGAGCTGGGTTTTTTGTATTTTGGTTTATTGG-3'
mcsB-KO-P4	5'-CTGTAAATCTTCCAAACATCATCACTCAACCCCCTCCTTTACTGA-3'
mcsB-KO-P5	5'-TCAGTAAAGGAGGGGGTTGAGTGATGATGTTTGGAAGATTTACAGAACGAGC -3'
mcsB -KO-P6	5'-CATCAAGAGTCGTTGCACCAATACATTG-3'
tig-BamHI-U	5'-TAGGGATCCCATTTGTACGTTTTATATCCGTTCCAGCCG -3'
tig-SalI-L	5'-TAGGTCGACGTACTCCTCAGGGAATGTCACTTCAACATC-3'
tig-R45A-F	5'-CAAGTTTCAATTCCTGGATTCGCTAAAGGTAAAATTCCTCGCGG -3'
tig-R45A-R	5'-CCGCGAGGAATTTTACCTTTAGCGAATCCAGGAATTGAAACTTG-3'
tig-R45D-F	5'-CAAGTTTCAATTCCTGGATTCGATAAAGGTAAAATTCCTCGCGG-3'
tig-R45D-R	5'- CCGCGAGGAATTTTACCTTTATCGAATCCAGGAATTGAAACTTG-3'
sigA-BamHI-U	5'-TAGGGATCCGATCCATGAAGGAAACATGGGCCTGATG-3'
sigA-SalI-L	5'-TAGGTCGACCTTTTCAGCAGATTTGCTTTCTCCTCCG-3'

sigA-R365A-F	5'- ACTAAGACATCCTAGCAGAAGTAAAGCTTTGAAAGATTT CCTTGAATAAGA -3'
sigA-R365A-R	5'-TCTTATTCAAGGAAATCTTTCAAAGCTTTACTTCTGCTAG GATGTCTTAGT -3'
sigA-R365D-F	5'- AAACTAAGACATCCTAGCAGAAGTAAAGATTTGAAAGAT TTCCTTGAATAAGATG -3'
sigA-R365D-R	5'- CATCTTATTCAAGGAAATCTTTCAAATCTTTACTTCTGCT AGGATGTCTTAGTTT -3'
tig-R45E-F	5'- AGTTTCAATTCCTGGATTCGAAAAAGGTAAAATTCCTCGCG -3'
tig-R45E-R	5'- CGCGAGGAATTTTACCTTTTTCGAATCCAGGAATTGAAACT -3'
sigA-R365E-F	5'-GGAAACTAAGACATCCTAGCAGAAGTAAAGAATTGAAAGA TTTCCTTGAATAAGATG -3
sigA-R365E-R	5'- CATCTTATTCAAGGAAATCTTTCAATTCTTTACTTCTGCTA GGATGTCTTAGTTTCC -3'
3019	5'-TGGATCGAATTCCAAAAAAGCAACAGTTAAATGC GCTTG-3'
3020	5'-TGGATCCTCGAGCTTAAGACCGTATTTTTTGTTAA AGCG-3'
yoxA-RT-U	5'-CACAAGCAGCTGGATGATGT-3'
yoxA-RT-L	5'-ACCCATGTGTAAGGCTCAGG-3'
tig-RT-U	5'-TAGTTGGCCTTGAAGCAGGT-3'
tig-RT-L	5'-CGAATTCATCGTCAAGCTCA-3'
rpmE-RT-U	5'-GCAACAGTTAAATGCGCTTG-3'
rpmE-RT-L	5'-TTTGTTAAAGCGATCAACACG-3'
pupG-RT-U	5'-GGCTGAAAAGATTGCGAAAG-3'
pupG-RT-L	5'-ATGCAGGAAATGCCAAGAAC-3'

yaaH-RT-U	5'-GGATCCTTTTGAACGACGAA-3'
yaaH-RT-L	5'-TCGATGGAAAAGATCCCTTG-3'
recA-Vitro-U	5'-GAAACAGAGGCCGTTATCTTAGAG-3'
recA-Vitro-L	5'-TTATTAGCTTGGTACAGTAGAAATTCTTGTATCT-3'
Primers used for Northern blot	
rpmE-bio-P1	Biotin- CCGCAAGCGCATTTAACTGTTGC-3'
rpmE-bio-P2	Biotin- GGGTGGCATTCAGAGCAAATCTCAACGCGTACC -3'
rpmE-bio-P3	Biotin- GCGATCAACACGACCATCAGCAGAAGCG -3'
pupG-bio-P1	Biotin- CCGGCATGCCCTTCAACAGTAGATACCGGG -3'
pupG-bio-P2	Biotin- CTGGCGCCGAAATCTGCTTCGTTTGGCCCG -3'
pupG-bio-P3	Biotin- GCAGGAAATGCCAAGAACCCGCATTCCCGC -3'

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

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