Supplemental Methods

Construction of plasmid pJOE8739.1

The plasmid pJOE8739.1 is derived from pIC20HE (Altenbuchner et al., 1992). First, a PmlI site was introduced into pIC20HE behind the bla gene by amplification of the plasmid with primers s6549 (5´-cagcttgatgtaaacttggtctgagtt) / s6550 (5´-cccccgtagaaaaagatcaag) and religation of the fragment to pJOE6767.1. The copy number of this plasmid was reduced by insertion of the rop gene, amplified from plasmid pJOE5751.1 (Hoffmann et al., 2012) by primers s7797 (5´-gttaaacccgtagaaaaagatcaag) and s7803 (5´-aaaaagcatgctgctgacctga) and insertion between the PmlI/SphI site to pJOE7445.1.

The spectinomycin resistance gene from pJOE6743.1 was amplified with the primers s9681 (5´-gctgctagaaataatcaaat) / s9682 (5´-atatatgtaaatatactttcatgctgctgctgac) and inserted between the PmlI/NdeI sites of pJOE7445.1 to pJOE8714.2. The manP gene from pJOE6743.1 was isolated as NdeI fragment inserted into the NdeI site of pJOE8714.1 to give pJOE8731.1. The ccdB gene (van Melderen, 2002) was amplified from the F´plasmid of E. coli JM109 total DNA with the primers s6895 (5´-gttaagacgagagagagact) / s6905 (5´-gaagctttatataatactttcatgctgctgctgac) and inserted behind the rhamnose promoter in pJOE5751.1, cut with AflII/HindIII (pJOE6946.1). The rhaP-ccdB cassette was excised from pJOE6946.1 with MluI/HindIII and inserted blunt-ended between the SmaI sites of pJOE4786.1 (Altenbuchner et al., 1992) to give pJOE6958.4. Finally, a SphI/HindIII fragment from pJOE6958.4 was inserted between the SphI/HindIII sites in pJOE8731.1 to give pJOE8739.1. The DNA sequence of pJOE8731.1 has been deposited in GenBank, KY200664.

Microscopy and determination of the cytoplasmic volume of B. subtilis cells

B. subtilis strains were grown in triplicate in LB medium supplemented with 0.5% glucose to an OD600 of 0.5. An aliquot of each culture was stained with the membrane specific dye Nile Red (200 ng/ml, Thermo Fisher Scientific, Eugene, OR, USA) and mounted on an agarose-covered glass slide (1% w/v). Samples were observed using an Axio Imager.M2 (Carl Zeiss Microscopy GmbH, Germany) equipped with an AxioCam MRm, an EC-Plan-NEOFLUAR 100/1.3 Oil Ph3 objective and a 63 HE filter for dye
visualization. Images were taken randomly from each slide. Based on the fluorescence signal of stained membranes, the lengths and widths of 200 individual cells per replicate were measured using ZEN 2012 software (Carl Zeiss Microscopy GmbH, Germany). The cytoplasmic volume was calculated per cell, depending on the cell division status (single, dividing or chained cells). For chained cells we assumed a cylinder, for dividing cells a cylinder plus one hemisphere, and for single cells a cylinder plus two hemispheres. Finally, the median cytoplasmic volume was calculated from the values of 600 cells per strain.

**Quantification of metabolites**

5 ml of each culture were immediately cooled down in liquid nitrogen, and then harvested via filtration sampling as described previously (Kohlstedt et al., 2014). The bacteria were removed from the filters by adding 4 ml extraction solution (cooled 60% ethanol). Cell disruption was performed by a freeze/thaw cycle as described previously (Kohlstedt et al., 2014). Centrifugation was carried out for 10 min at 4°C and 8,000 rpm to remove the filter and the cell fragments. After combining the supernatants of the first and second extraction, the samples were dried under nitrogen stream.

The dry pellet or alternatively 20 µl of medium were extracted with 400 µl of extraction buffer (methanol/chloroform/water 32.25:12.5:6.25 [v/v/v]). 200 µl of 0.05 mg/ml allo-inositol in water was added to induce phase separation and to introduce allo-inositol as the internal standard. The polar fraction was evaporated under N₂-stream and derivatized with 15 µl methoxyamine hydrochloride and 30 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as previously described (Bellaire et al., 2014) to transform the metabolites into their methoxyimino (MEOX) and trimethylsilyl (TMS) derivatives. The samples were analysed on a Agilent 5973 Network mass selective detector connected to an Agilent 6890 gas chromatograph equipped with a capillary HP5-MS column (30 m x 0.25 mm; 0.25 µm coating thickness; J&W Scientific, Agilent). Helium was used as carrier gas (1 ml/min). The inlet temperature was set to 230°C and the temperature gradient applied was 50°C for 2 min, 50 – 330°C at 5 K/min 330°C for 2 min. Electron energy of 70 eV, an ion source temperature of 230°C, and a transfer line temperature of 330°C was used. Spectra were recorded in
the range of 71-600 da/e. For absolute quantification, all compounds were quantified using a calibration curve created with pure substances based on a total of twelve measurements for each substance.

Sample preparation for proteome analysis

Frozen cell pellets were resuspended in 1 ml UT buffer (8 M urea and 2 M thiourea) and added to 500 µl of unwashed glass beads (160 µm, Sigma Life sciences). Cell disruption was performed as previously described by Kohlstedt et al, 2014. For the spectral library generation, protein samples (for details, see Supplemental Table S5) were pre-fractionated using SDS-PAGE. Briefly, 20 µg of protein from each sample was denatured with loading buffer for 5 min at 95°C and separation was carried by NuPAGE® Bis-Tris Gels 4-12% (Life Technologies Corporation, Carlsbad, California). Separation, staining and destaining were carried out according to the manufacturer’s guidelines. Each lane was sliced into 5 equal pieces which were processed independently for the subsequent MS analysis as previously described by Kohlstedt et al, 2014.

Protein samples without pre-fractionation (details in Supplemental Table S5) were subjected to in-solution trypsin digestion. The in-solution trypsin digestion and peptide desalting were performed as described (Samal et al, 2015). 1 µg of the purified peptide mixture was used for subsequent LC-MS/MS analysis.

For data-dependent acquisition experiments, iRT peptides (Biognosys, Schlieren, Switzerland) were spiked into the sample, and for the data-independent acquisition experiments, HRM calibration peptides (Biognosys) were added to the samples prior to analysis according to manufacturers’ instructions.

Mass spectrometric data-dependent-acquisition (DDA) analyses using a Q Exactive™ instrument

MS analyses were performed with an on-line coupled UltiMate 3000RS-LC system (Thermo Fisher Scientific, Idstein, Germany) connected to a Q Exactive™ Orbitrap-MS or a Q Exactive Plus™ Orbitrap-MS (Thermo Fisher Scientific Inc.). For LC-separation, peptides were enriched on an Acclaim
PepMap 100, 100 µm × 2 cm, nanoViper C18, 5 µM, 100 Å pre-column (Thermo Fisher Scientific Inc.) and separated using an Accucore 150-C18 with a column length of 25 cm (150 Å, 2.6 µm, Thermo Fisher Scientific Inc.) at 40°C. For separation, a 145 min gradient was used with a solvent mixture of buffer A (5% acetonitrile (ACN) in water with 0.1% acetic acid) and increasing percentages of buffer B (ACN with 0.1% acetic acid): 2% for 10 min, 2–25% for 120 min, 25–40% for 5 min, 40–90% for 2 min, and 90% for 5 min. Peptides were eluted with a flow rate of 300 nl/min for the 25 cm column and 200 nl/min for the 50 cm column. Full-scan MS was carried out using a mass range of m/z 300 to 1,650. Data were acquired in a data-dependent strategy in profile mode with a resolution of 70,000 for MS at m/z 400 and 17,500 for MS/MS and a positive polarity. The method used allowed sequential isolation of the top ten most intense ions for fragmentation using high-energy collisional dissociation (HCD) with dynamic exclusion for 30 s and disabled early expiration. An intensity threshold of 8.3e4 was applied with an isolation width of 3 m/z, normalized collision energy of 27.5 eV, and a starting mass of m/z 100. The charge state screening and monoisotopic precursor selection rejected +1 and +7, +8, and >+8 charged ions.

**Mass spectrometric data-independent-acquisition (DIA) analyses using a Q Exactive™ instrument**

LC-MS/MS analysis was performed on an UltiMate 3000 RSLC (Dionex/Thermo Fisher Scientific, Idstein, Germany) coupled to a QExactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The peptide digest was enriched on a 2 cm x 100 µm Acclaim PepMap 100 trap column (100 Å pore size, 5 µm C18 particles, Thermo Fisher Scientific) and separated on a 50 cm x 75 µm Accucore 150-C18 analytical column (150 Å pore size, 2.6 µm particles, Thermo Fisher Scientific) with a flow rate of 200 nl/min or a 25 cm x 75 µm Accucore 150-C18 analytical column (150 Å pore size, 2.6 µm particles, Thermo Fisher Scientific) with a flow rate of 300 nl/min at a constant temperature of 40°C. Reversed-phase chromatography was performed with a binary buffer system consisting of 0.1% acetic acid, 5% ACN in water (buffer A) and 100% ACN in 0.1% acetic acid (buffer B). The peptides were separated by applying a linear gradient from 2% to 25% buffer B over a time of 120
min and 180 min for the 25 cm and the 50 cm analytical column, respectively. Eluting peptides were ionized using the chip-based TriVersa NanoMate ion source (Advion Biosciences, Norwich, UK). For the data-independent mode the previously published parameters (Bruderer et al, 2015) were used.

**Building the *B. subtilis* ion library for DIA data analysis**

Four spectral libraries were generated from various search engines and systematically combined to generate a comprehensive spectral library for *B. subtilis* that contains 40,076 peptides corresponding to 3,093 unique proteins. All the spectral libraries were generated using the same 93 DDA acquisitions (for details, see Appendix Table S3) that were performed on Q Exactive™ Orbitrap-MS or a Q Exactive Plus™ Orbitrap-MS (Thermo Fisher Scientific Inc.).

Common search parameters for all the search engines include semi-tryptic search specificity, methionine oxidation as variable modification, carbamidomethylation of cysteine as a static modification and 2 missed cleavages were allowed. The protein sequence database was obtained from UniProt/Swiss-Prot (http://www.uniprot.org/) for *B. subtilis* (November 2015) and additional new features derived from a recent transcriptome analysis were added to the database (Nicolas et al, 2012). In addition, iRT peptides sequences were also added to the final database.

The spectral libraries 1 and 2 were generated by converting the vendor MS raw data files to mzML or mzXML format using ProteoWizard (v3.0.7364) (Chambers et al, 2012). MS/MS spectra were searched with Comet (2014.02 rev. 2) (Eng et al, 2013) against the above mentioned database including common contaminants listed in the common repository of adventitious proteins (cRAP) as well as decoy reverse sequences. The parent mass error was set to ± 30 ppm and a fragment mass error of 0.01 Da was used. Peptides were allowed to be semi-tryptic with up to two internal cleavage sites. The search parameters included a fixed modification of +57.021464 for carbamidomethylated cysteines and a variable modification of +15.9949 for oxidized methionines. The search results were processed with the Trans-Proteomic Pipeline (TPP, version v4.8.0 PHILAE) (Deutsch, 2010) including PeptideProphet and iProphet (Deutsch, 2010; Keller et al, 2002; Keller et al, 2005; Nesvizhskii et al, 2003; Shteynberg et al, 2011). Peptide spectrum matches (PSM) issuing from the database search
were analyzed with PeptideProphet using accurate mass binning model and the non-parametric model to assign each PSM a probability of being correct. iProphet was used to process the PeptideProphet results further providing a more accurate and conservative representation of the identified peptides resulting in an iProphet probability (Shteynberg et al, 2011). To resolve chimeric spectra, reSpect (Shteynberg et al, 2015) was used with a minimum probability of spectra to respect of 0.5 and a m/z tolerance of 0.1 Da. The resulting mzML files were searched and processed as described above.

All the resulting Comet-searched iProphet files were converted to blib files using ProteoWizard (v3.0.7364) (Chambers et al, 2012) (iProphet cut-off score = 0.9). The resulting peptide error ranged from 0.0023 to 0.0054 with a median error over all files of 0.0035. The non-redundant blib files were used to generate a library using R version 3.2.2 and the bioconductor package specL (1.5.2) (Panse et al, 2015) [parameter: max MZ error: 0.01 Da; TopN: 10; fragment ion m/z range: 300 - 2000; fragment ion type: b,y]. The final library was filtered for 6-10 transitions and fully-tryptic peptides.

Spectral libraries 3 and 4 were generated in Spectronaut 8.0 (Biognosys) using a Q value cutoff of 0.001 and minimum of 6 and a maximum of 10 fragment ions. Spectral library 3 was generated from the MaxQuant search results (Bruderer et al, 2015). Spectral library 4 was generated from the search results of Proteome Discoverer 1.4 using a combination of search engines that include Mascot, Sequest HT and MS Amanda (Dorfer et al, 2014).

To merge the libraries, all the peptide precursors from spectral library 1 were retained and the unique peptide precursors belonging to spectral library 2 were added to the spectral library 1. Furthermore to the resultant spectral library, unique precursors from the spectral library 3 were added. Finally the unique precursors from the spectral library 4 were added to the previously combined spectral library. The sequential addition of the spectral libraries was performed via an in-house R-script.
**DIA data analysis**

The data analysis was performed using Spectronaut™ (Spectronaut v8.0.9600.8.23526 academic). Raw files from DIA acquisition were converted to HTRMS™ format by “Raw to HTRMS converter” supplied with the Spectronaut™. HTRMS format files were analyzed using Spectronaut™ with the settings of iRT correction factor set to 1; interference correction set to TRUE. The number of fragment ions was defined in the spectral library (at least 6 and up to 10), and all were required for identification and quantification. All results were filtered by a Q value of 0.001. All other settings were set to default. Protein intensity was calculated by summing the peptide peak areas (sum of fragment ion peak areas as calculated by Spectronaut) of each protein from the Spectronaut default output file. Shared peptide sequences were excluded from the analysis. Peptides sequences that were wrongly assigned to the deleted genes in either of the mutants (PG10 and PS38) were discarded from the entire analysis. The resulting protein intensity values were then converted to percentage abundance (alpha values) for the corresponding sample and log transformed. The resultant protein alpha values were used for statistical comparison.

Further statistical analyses were conducted in the GeneData Analyst software package (GeneData, Basel, Switzerland) and R. The statistical comparison of sample groups includes four biological replicates comparing mutants (PG10 and PS38) versus the wild type strain (Δ6). Proteins that were identified in at least three of the 4 biological replicates were considered for T-test. Proteins were considered as significantly regulated between both groups after T-test with a statistical cut off of (q value = 0.05 after Benjamini-Hochberg correction) with a mean fold change of at least 2. Multi-omic maps were generated in Inkscape software (v0.91, [www.Inkscape.org](http://www.Inkscape.org)) using XML annotations. Pathway visualization for the Multi-omic maps was generated in R using SVGMapping package (v1.43, author: Jean-Christophe AUDE, Repository: CRAN).

**Transcriptome analysis**

RNA was isolated as described previously (Eymann et al, 2002; Nicolas et al, 2012). The quality of the RNA preparations was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara,
CA, USA) according to the manufacturer’s instructions. 5 µg of total RNA were subjected to tiling array analysis. Synthesis and fluorescence labeling of cDNA followed a strand-specific method using the FairPlay III Microarray Labeling Kit (Agilent Technologies) and actinomycin D (Calbiochem) (Mäder & Nicolas, 2012). 200 ng of Cy3-labeled cDNA were hybridized to a tiling array (containing 383,238 probes covering both strands of the *B. subtilis* 168 genome with a 22 nucleotides tiling step) following Agilent’s hybridization, washing and scanning protocol (One-Color Microarray-based Gene Expression Analysis, version 5.5). Data were extracted and processed using the Feature Extraction software (version 10.5).

An aggregated expression value per gene was computed as the median intensity of probes lying entirely within the corresponding genomic region (Nicolas *et al.*, 2012). Gene-level intensities were scaled based on the intensity values of ten different in-vitro synthesized transcripts contained in the One Color RNA Spike-In kit (Agilent Technologies). Further analysis was performed with the Genedata Analyst software (Genedata AG, Basel, Switzerland). Genes entirely present in the reference strain Δ6 and the respective deletion strain were included in a t-test analysis (with four biological replicates per group) to identify significantly differentially expressed genes defined as those with an FDR adjusted P-value less than 0.05 and a mean ratio of ≤ 0.5 or ≥ 2.

For the calculation of total mRNA intensities, log2 intensities of all annotated genes entirely present in the corresponding strain were displayed in a density plot for each array. The x-value of the first low point was calculated and all intensities above this threshold were added up. Mean summed intensity and standard deviation of four biological replicates were computed.

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