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´-cacgtgatgagtaaacttggtctgacagtt)/ s6550 (5´-ccccgtagaaaagatcaaaggat) 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´-gtttaaaccccgtagaaaagatcaaag) and s7803 (5´-aaaaaagcatgctggcattgaccctga) and insertion between the PmlI/SphI site to pJOE7445.1. The spectinomycin resistance gene from pJOE6743.1 was amplified with the primers s9681 (5´-gctgctagaagaaatcgaat)/s9682 (5´-atatatgtttaaacgtgtttccaccatttttttcaa) 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´-gcttaagacgagaacagggact)/s6905 (5´-gaagcttattaatttatattccccagaacat) 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 OD_{600} 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 N_2-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[TM] instrument

MS analyses were performed with an on-line coupled UltiMate 3000RSLC system (Thermo Fisher Scientific, Idstein, Germany) connected to a Q Exactive[TM] Orbitrap-MS or a Q Exactive Plus[TM] 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 $8.3e^4$ 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[TM] 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) 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.

References

Altenbuchner J, Viell P, Pelletier I (1992) Positive selection vectors based on palindromic DNA sequences. *Methods Enzymol* **216:** 457-466

Bellaire A, Ischebeck T, Staedler Y, Weinhaeuser I, Mair A, Parameswaran S, Ito T, Schönenberger J, Weckwerth W (2014) Metabolism and development – integration of micro computed

tomography data and metabolite profiling reveals metabolic reprogramming from floral initiation to silique development. *New Phytol* **202:** 322-335

Bruderer R, Bernhardt OM, Gandhi T, Miladinovic SM, Cheng LY, Messner S, Ehrenberger T, Zanotelli V, Butscheid Y, Escher C, et al. (2015) Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acetaminophen-treated three-dimensional liver microtissues. *Mol Cell Proteomics* **14:** 1400-1410.

Chambers MC, MacLean B, Burke R, Amodei D, Ruderman DL, Neumann S, Gatto L, Fischer B, Pratt B, Egertson J et al. (2012) A cross-platform toolkit for mass spectrometry and proteomics. *Nat Biotechnol* **30:** 918–920.

Deutsch EW, Shteynberg D, Lam H, Sun Z, Eng JK, Carapito C, von Haller PD, Tasman N, Mendoza L, Farrah T et al. (2010) Trans-Proteomic Pipeline supports and improves analysis of electron transfer dissociation data sets. *Proteomics* **10:** 1190-1195.

Dorfer V, Pichler P, Stranzl T, Stadlmann J, Taus T, Winkler S, Mechtler K (2014) MS Amanda, a universal identification algorithm optimized for high accuracy tandem mass spectra. *J Prot Res* **13:** 3679–3684.

Eng JK, Jahan TA, Hoopmann MR (2013) Comet: an open-source MS/MS sequence database search tool. *Proteomics* **13:** 22–24.

Eymann C, Homuth G, Scharf C, Hecker M (2002) *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *J Bacteriol* **184:** 2500-2520.

Hoffmann J, Bóna-Lovász J, Beuttler H, Altenbuchner J (2012) *In vivo* and *in vitro* studies on the carotenoid cleavage oxygenases from *Sphingopyxis alaskensis* RB2256 and *Plesiocystis pacifica* SIR-1 revealed their substrate specificities and non-retinal-forming cleavage activities. *FEBS J* **279:** 3911–3924

Keller A, Nesvizhskii AI, Kolker E, Aebersold R (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* **74:** 5383–5392.

Keller A, Eng J, Zhang N, Li XJ, Aebersold R (2005) A uniform proteomics MS/MS analysis platform utilizing open XML file formats. *Mol Syst Biol* **1:** 2005.0017.

Kohlstedt M, Sappa PK, Meyer H, Maaß S, Zaprasis A, Hoffmann T, Becker J, Steil L, Hecker M, van Dijl JM et al. (2014) Adaptation of *Bacillus subtilis* carbon core metabolism to simultaneous nutrient limitation and osmotic challenge: a multi-omics perspective. *Env Microbiol* **16:** 1898-1917

Mäder U, Nicolas P (2012) Array-based approaches to bacterial transcriptome analysis. *Meth Microbiol* **39:** 151-182.

Nesvizhskii AI, Keller A, Kolker E, Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem* **75:** 4646–4658.

Nicolas P, Mäder U, Dervyn E, Rochat T, Leduc A, Pigeonneau N, Bidnenko E, Marchadier E, Hoebeke M, Aymerich S et al. (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science* **335:** 1103-1106.

Panse C, Trachsel C, Grossmann J, Schlapbach R (2015) specL - an R/Bioconductor package to prepare peptide spectrum matches for use in targeted proteomics. *Bioinformatics* **31:** 2228–2231.

Samal R, Ameling S, Dhople V, Sappa PK, Wenzel K, Völker U, Felix SB, Hammer E, Konemann S (2015) Brain derived neurotrophic factor contributes to the cardiogenic potential of adult resident progenitor cells in failing murine heart. *PloS One* **10:** e0120360

Shteynberg D, Deutsch EW, Lam H, Eng JK, Sun Z, Tasman N, Mendoza L, Moritz RL, Aebersold R, Nesvizhskii AI (2011) iProphet: multi-level integrative analysis of shotgun proteomic data improves peptide and protein identification rates and error estimates. *Mol Cell Proteomics* **10:** M111.007690.

Shteynberg D, Mendoza L, Hoopmann MR, Sun Z, Schmidt F, Deutsch EW, Moritz RL (2015) reSpect: software for identification of high and low abundance ion species in chimeric tandem mass spectra. *J Am Soc Mass Spectrom* **26:** 1837-1847.