Bacillus pumilus Reveals a Remarkably High Resistance to Hydrogen Peroxide Provoked Oxidative Stress

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Abstract

Bacillus pumilus is characterized by a higher oxidative stress resistance than other comparable industrially relevant Bacilli such as B. subtilis or B. licheniformis. In this study the response of B. pumilus to oxidative stress was investigated during a treatment with high concentrations of hydrogen peroxide at the proteome, transcriptome and metabolome level. Genes/proteins belonging to regulons, which are known to have important functions in the oxidative stress response of other organisms, were found to be upregulated, such as the Fur, Spx, SOS or CtsR regulon. Strikingly, parts of the fundamental PerR regulon responding to peroxide stress in B. subtilis are not encoded in the B. pumilus genome. Thus, B. pumilus misses the catalase KatA, the DNA-protection protein MrgA or the alkyl hydroperoxide reductase AhpCF. Data of this study suggests that the catalase KatX2 takes over the function of the missing KatA in the oxidative stress response of B. pumilus. The genome-wide expression analysis revealed an induction of bacillithiol (Cys-GlcN-malate, BSH) relevant genes. An analysis of the intracellular metabolites detected high intracellular levels of this protective metabolite, which indicates the importance of bacillithiol in the peroxide stress resistance of B. pumilus.

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Introduction

Bacillus pumilus is a Gram-positive, rod-shaped and endospore-forming bacterium closely related to the industrially relevant bacteria Bacillus subtilis and Bacillus licheniformis. B. pumilus represents a potential alternative host for the industrial production of enzymes. For the evaluation and optimization of fermentation processes with this organism a comprehensive knowledge on its physiology and stress adaptation is required.

During fermentation processes a variety of stresses (e.g. salt, heat and oxidative stress) can impair the fitness of the production host and the quality of the fermentation product [1–3]. B. pumilus strains are highly resistant against UV radiation and hydrogen peroxide, which may explain the finding of viable spores of B. pumilus in hostile environments such as the interior of the Sonoran desert basalt and spacecrafts [4,5]. This natural potential and oxidative stress (ROS) such as superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) are successive one-electron-reduction products of molecular oxygen and therefore occur in all aerobically living organisms [3,6,7]. Increased ROS production that exceeds the cell defense capacity leads to oxidative stress in the cell and to the oxidation of nucleic acids, proteins and lipids [2,3,8–10].

In B. subtilis, the cellular defense against oxidative stress is ensured by the detoxification of harmful agents, protection of macromolecules and the repair or removal of damaged molecules. The oxidative stress response of this organism is regulated by specific transcriptional regulators, such as PerR, SigB, LexA/RecA, Spx and OhrR, as previously described in detail [11–13]. The oxidative stress response of B. pumilus differs significantly from the response in B. subtilis, as major oxidative stress genes of B.
2.4 2D-Gel Electrophoresis

Cytosolic protein extracts were loaded onto IPG-strips in the pH-range 4–7 (GE Healthcare Bio-Sciences AB, Finland) using 100 μg protein for labeled samples and 500 μg for preparative gels. 2D-PAGE was performed as described by Buttimer et al. [15]. Autoradiography of radioactively labeled gels was performed as previously described [14]. Preparative gels were stained with Coomassie Brilliant Blue as described by Voigt et al. [16]. Proteins were excised from preparative gels, digested and the peptide solution spotted onto MALDI targets using the Ettan Spot Handling Workstation (GE Healthcare, UK). Identification was performed using MALDI-TOF-MS/MS (Proteome Analyzer 5800 MDS Sciex, USA) and an in-house Bacillus pumilus Jo2 (DSM 14395) database as described by Wolf et al. [17]. Protein quantification was done with the Delta2D proteome software (Decodon, Germany).

2.5 Microarray Experiment

Total RNA of B. pumilus was prepared by the acid phenol method [18] with the modifications described elsewhere [19]. The isolated RNA was treated with DNase (RNase-free DNase Set, Quiagen, Germany) and subsequently concentrated and cleaned (RNA cleanup and concentration Kit, Norgen Biotek, Canada). Quantity of RNA was determined on a microscale spectrophotometer (NanoDrop ND-1000, Peqlab Biotechnologie GmbH, Germany) and RNA integrity was analyzed using a capillary electrophoresis system (Bioanalyzer 2100, Agilent Technologies, USA). Synthesis and purification of fluorescently labeled cDNA was carried out according to Schroeter et al. [20] with minor modifications described below. After the labeling and clean-up step [20], 600 ng of respective Cy3- and Cy5-labeled cDNA were admixed (ad. 44 μl), denatured and mixed with 11 μl pre-warmed blocking agent and 60 μl hybridization buffer (both Gene expression hybridization kit, Agilent Technologies, USA). Hybridization was carried out in a Hybridization chamber (Hycel H2000, Agilent Technologies, USA).

Custom-made B. pumilus Jo2 4×44 K gene expression microarrays were obtained from Agilent Technologies (https://earray.chem.agilent.com/earray/), containing 60-mer Oligonucleotide probes (SurePrint technology, Agilent Technologies). Probe design was performed on the chromosome sequence of B. pumilus Jo2 (Sequence Intellectual Property of Henkel KGaA). In addition to the annotated open reading frames (ORFs), ORFs were predicted using (i) Glimmer 3.0 [21], (ii) ZCURVE [22], (iii) Genemark HMM [23], and (iv) Prodigal [24]. Predicted ORFs were added to the design provided that: (i) they were non-overlapping with existing ORFs; or (ii) they were in the reverse complementary strand of existing ORFs. On the annotated and predicted ORFs, up to 5 probes were designed. Altogether, a total of 41377 probes were designed by means of OligoWiz 2.1.3 [25] using default parameters for prokaryotic long-mers. The arrays were hybridized and washed according to the manufacturer’s instructions (Two-Color Microarray-Based Gene Expression Analysis Protocol, Agilent Technologies, USA), followed by a last wash step with acetonitrile (Carl Roth GmbH+Co. KG, Germany) for 30 sec. Microarrays were scanned using the Agilent scanner Type G2565CA with high resolution upgrade G2539A and the software Scan Control 8.4.1 (Agilent Technologies, USA). Data were extracted from scanned images using Agilent’s Feature Extraction Software (version 10.5.1.1; Agilent Technologies, USA) using default settings. A common reference type of design was employed, and data from three biological replicate hybridizations for each point in time were used for data analysis. Spot signals were normalized using Lowess as described earlier [26]. Next, for each ORF a signal was determined by taking the median signal of...
the up to 5 probes per ORF. Differential regulation was determined from the biological triplicate measurements by false-discovery rate (FDR) from the Cyber-T p-values [27] by means of multiple testing correction [26]. Differential regulation was defined as a 2-fold or higher differential expression with a FDR cut-off value of 0.05 or lower.

2.6 Metabolomic Analysis of Thiols as their Monobromobimane-derivatives

Cells were grown in minimal medium as described above and exponentially grown cells from 10 ml culture medium were harvested before oxidative stress, 10, 30 and 60 min after addition of hydrogen peroxide. The isolation of LMW-thiols for HPLC analysis was performed as described previously [28]. In brief, after centrifugation the cells were washed with 50 mM Tris- HCl (pH 8.0) and resuspended in 50% acetonitrile containing 20 mM Tris- HCl (pH 8.0), 1 mM penicillamine as internal standard and 2 mM monobromobimane (mBBr). Control samples were resuspended without penicillamine and 5 mM N-ethylmaleimide (NEM) was used prior to addition of mBBr. Thiols were extracted at 60°C and directly labeled with mBBr. Labeling reaction was stopped with aqueous methane sulfonic acid in a final concentration of 5 mM. BsmB (monobromobimane-derivative of BSH) standards were synthesized as described previously [7,29]. For detection and quantification of LMW-thiols, ion pairing HPLC was performed as described before [30]. For absolute quantification the ratio peak area thiol/peak area internal standard was used and an eight-point calibration between 10 nM and 2000 nM was generated.

2.7 Prediction of the PerR Consensus Sequence

Prediction of the PerR consensus sequence was done with the PRODORIC® database (http://prodoric.tu-bs.de/vfp/index2.php) release 8.9 [31] using the consensus sequence as described by Fuangthong et al. [32].

Results and Discussion

3.1 Effects of H2O2 on Growth and Cell Morphology

Exponentially growing B. pumilus cells were treated with 2 mM hydrogen peroxide. Thus, the concentration of H2O2 that was used to trigger the stress in this study was about 40-fold higher than those used for comparable analyses with B. subtilis or B. licheniformis [13,20]. The highest peroxide concentrations allowing growth for B. subtilis and B. licheniformis were 4 and 1 mM, respectively (Table S1). B. pumilus is still able to grow with 20 mM hydrogen peroxide. This indicates a striking resistance of B. pumilus against peroxide stress. Compared to unstressed cells, growth was significantly impaired for a short time (approximately 15 min) after the H2O2 treatment (Figure 1). However, after that time, cells continued to grow for about one hour. An electron microscopy analysis indicated that after exposure to H2O2 most of the cells are morphologically intact, but some of the cells exhibited major damage of their envelope (Figure 2D). Furthermore, scanning electron microscopy revealed some atypically long cells (up to approximately 10–20% two hours after H2O2 treatment, Figure 2B, 2E) indicating an impact of hydrogen peroxide on processes involved in cell division.

3.2 Global Expression Profile

All values presented for up- and downregulation of genes or proteins are fold change values. The analysis of the soluble intracellular proteome of B. pumilus revealed 54 significantly upregulated and 111 downregulated proteins 10 min after H2O2 treatment (with a threshold of 2-fold, Table 1, Table S2, Figure 3). For the visualization of the fast and early response on proteome level, a labeling with 35S-methionine was necessary. 30 minutes after initiating the stress, 73 proteins were up- and 39 proteins downregulated (Table 1, Table S2 and S3, Figure 4). Transcriptome analysis revealed an at least 2-fold increased transcription of 181 genes three minutes after treatment with H2O2; 76 of them were more than 3-fold upregulated. Eight minutes after treatment, the transcription of 558 genes appeared at least 2-fold increased (307 genes with an at least 3-fold increased transcription). Three minutes after the stress, 266 genes were transcribed with an at least 3-fold lower rate than under control conditions, for 296 genes this decreased transcription rate was shown eight minutes after treatment. To indicate quality of the transcriptome results, raw data for individual probes for selected genes (which were not found to be induced in the proteome analysis) are presented in Table S4. These data show similar basal values and changes following addition of hydrogen peroxide for all five probes corresponding to a gene.

To compare the physiological changes in H2O2 treated B. pumilus cells with the oxidative stress responses of other organisms, the upregulated genes and proteins were assigned to putative regulons known from related organisms like B. subtilis and B. licheniformis [13,20]. 139 of the upregulated genes and proteins could be assigned to these putative regulons (Table S2). The thus classified genes and proteins identified in this study are summarized and discussed below.

3.3 PerR Regulon

The PerR regulon is known to be highly induced by oxidative stress caused by hydrogen peroxide and parapquat [13]. As shown previously for B. licheniformis, the B. pumilus genome encodes a PerR regulator protein with a high level of identity (93%) to the PerR-protein known from B. subtilis [20]. Transcription of the perR gene was significantly increased immediately after stress (Table 1). This indicates a regulation mechanism of PerR in H2O2 treated B. pumilus cells that is similar to the de-repression model reported for B. subtilis [33].

In our study genes assigned to a putative PerR regulon, including those encoding the regulator proteins Fur and SpxA as well as the zinc-uptake protein ZosA, the heme biosynthesis complex HemABCD21LX and the general stress protein YjpC were significantly induced at transcriptional level (Table 1).

Strikingly, some of the PerR-regulated genes exhibiting the highest induction in B. subtilis cells subjected to hydrogen peroxide, are absent from the genome of the B. pumilus strain used in our study, as well as from a previously published B. pumilus genome [34]. This applies e.g. for the genes encoding the catalase KatA and the DNA-protection protein MrgA. Furthermore, B. pumilus lacks not only the genes aphC and aphF, encoding subunits of the alkyl hydroperoxide reductase, but there are no genes annotated with this function in the genome.

Instead of KatA, a gene annotated as catalase KatX2 (53% sequence similarity to B. subtilis KatX) was significantly induced in B. pumilus cells at transcriptional and translational level (up to 10 and 20-fold, respectively, Table 1). Thereby, KatX2 was one of the proteins with the highest induction rates detected. B. subtilis and B. licheniformis subjected to hydrogen peroxide exhibit a more than 100-fold induction of KatA [13,20]. KatX2 comprises about 0.38% of the cytoplasmic protein present in the gel before addition of hydrogen peroxide. The values for B. subtilis and B. licheniformis are 0.13% in both strains [personal communication C. Scharf, B. Voigt]. After addition of hydrogen peroxide KatX2 comprises about 3.8% of the cytoplasmic protein. This is comparable to the
Figure 1. Growth of *B. pumilus*. Growth of *B. pumilus* under control conditions (filled squares) and stressed with 2 mM H$_2$O$_2$ at OD$_{500}$ nm 0.6 (empty squares).
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Figure 2. Electron microscopy micrographs. Scanning (A,B,E) and transmission (C,D) electron microscopy micrographs of *B. pumilus* cells under control conditions (A,C), 30 min (B,D) and 120 min after treatment with 2 mM H$_2$O$_2$ (E).
doi:10.1371/journal.pone.0085625.g002
| ORF ID | gene | transcriptome | proteome | Regulon in other Bacilli |
|--------|------|--------------|----------|-------------------------|
| BPJ13600 | zinc-transporting ATPase ZosA | zosA | 12.74 | 28.72 | perR |
| BPJ25410 | glutamyRNA reductase HemA | hemA | 3.44 | 3.99 | perR |
| BPJ25390 | phosphobiligen deaminase HemC | hemC | 2.68 | 3.90 | perR |
| BPJ25370 | delta-aminolevulinic acid dehydratase HemB | hemB | 2.52 | 3.72 | perR |
| BPJ25400 | putative cytochrome C biogenesis protein HemX | hemX | 2.86 | 4.25 | perR |
| BPJ25380 | uroporphyrinogen III synthase HemD | hemD2 | 2.68 | 4.23 | perR |
| BPJ25360 | glutamate-1-semialdehyde 2,1-aminomutase HemL | hemL | 2.75 | 3.56 | perR |
| BPJ21690 | Fur family ferri uptake regulation protein Fur | fur | 1.92 | 3.62 | perR |
| BPJ1620 | transcriptional regulator Spx | spxA | 4.14 | 3.31 | perR/spx/sigB |
| BPJ11610 | putative N-acetyltransferase YjbC | yjbC | 2.41 | 4.41 | perR/spx/sigB/sigM/sigW/sigX |
| BPJ09760 | catalase KatX2 | katX2 | 6.96 | 10.69 | 15.18 | 21.09 | sigB/sigF |
| BPJ30810 | putative ABC transporter permease YwjA | ywjA | 1.57 | 4.47 | fur |
| BPJ30830 | putative ABC transporter permease FhuC | fhuC1 | 1.51 | 2.46 | fur |
| BPJ30820 | putative ABC transporter permease FhuG | fhuG1 | 1.53 | 3.20 | fur |
| BPJ08440 | ABC transport system permease | bgi08440 | 4.11 | 7.49 | fur |
| BPJ08430 | putative iron complex transport system substrate binding protein | bgi08430 | 4.54 | 7.43 | fur |
| BPJ08420 | putative HTH-type transcriptional regulator | bgi08420 | 3.58 | 5.59 | fur |
| BPJ08580 | putative nitrreductase YfhC | yfhC | 2.67 | 5.00 | 1.10 | fur |
| BPJ08410 | ferredoxin–NAD reductase 2 | bgi08410 | 3.90 | 3.83 | fur |
| BPJ37570 | AraC family transcriptional regulator/putative FeuA-like substrate-binding domain ybbB | ybbB2 | 4.93 | 12.84 | fur |
| BPJ37580 | iron complex ABC transporter substrate-binding protein FeuA | feuA | 3.21 | 10.04 | fur, btr, citB |
| BPJ37590 | putative bacilliactin esterase YbbA | ybbA | 5.24 | 18.42 | fur/btr/citB |
| BPJ07970 | C56 family peptidase Ykm | ykm | 2.94 | 7.61 | 7.39 | 3.09 | fur/sigB |
| RBPU30260 | FeS cluster assembly protein SufB | sufB | 1.87 | 2.10 | 1.73 | FeS cluster biogenesis |
| RBPU30280 | cysteine desulfurase SufS | sufS | –1.77 | 2.69 | FeS cluster biogenesis |
| RBPU30290 | FeS cluster assembly permease SufD | sufD | 1.73 | FeS cluster biogenesis |
| RBPU30300 | FeS cluster assembly ATPase SufC | sufC | 2.52 | 2.13 | FeS cluster biogenesis |
| BPJ11040 | diaminobutyrate-2-oxoglutarate aminotransferase RhbA | rhbA | –1.11 | 11.10 | siderophore synthesis |
| BPJ11080 | rhizobactin siderophore biosynthesis protein RhbE | rhbE | –1.18 | 5.28 | siderophore synthesis |
| ORF ID | gene                                                                 | transcriptome | proteome | Regulon in other Bacilli     |
|--------|----------------------------------------------------------------------|---------------|----------|-----------------------------|
| BPJ1090 | rhizobactin siderophore biosynthesis protein RhbF                      |               |          | siderophore synthesis       |
| BPJ35800 | iron complex ABC transporter ATP-binding protein FhuC                    | 3.88          | 7.85     | iron uptake                 |
| BPJ35810 | iron complex ABC transporter permease FhuB                                 | 3.32          | 7.15     | iron uptake                 |
| BPJ35770 | putative iron complex ABC transporter permease FhuG                     | 2.31          | 4.39     | iron uptake                 |
| BPJ35780 | putative iron complex ABC transporter substrate-binding protein FhuD            | 2.72          | 5.57     | iron uptake                 |
| BPJ35830 | putative iron transport-associated protein/putative siderophore bpj35830 | 3.65          | 5.84     | iron uptake                 |
| BPJ35840 | putative heme uptake protein IsdC                                      | 4.91          | 7.62     | iron uptake                 |
| BPJ35850 | putative iron transport-associated protein                             | 3.89          | 6.47     | iron uptake                 |
| BPJ28430 | DinB-like domain-containing protein YuaE                                |               |          | spx                         |
| BPJ31980 | thioredoxin-disulfide reductase TrxB                                   | 2.37          | 2.87     | spx                         |
| BPJ29110 | putative NADH-dependent butanol dehydrogenase YugJ                    |               |          | spx                         |
| BPJ19830 | methionine sulfoxide reductase MrA                                    | 1.46          | 2.24     | spx                         |
| BPJ19820 | peptide-methionine sulfoxide reductase MrB                             | 1.48          | 2.27     | spx                         |
| BPJ25870 | thioredoxin TrxA                                                       | 1.40          | 2.58     | spx/cysR/sigB               |
| BPJ35200 | NADPH-dependent nitro/flavin reductase NfrA                             | 2.50          | 2.47     | spx/sigD/spo0A              |
| BPJ24450 | cystathionine gamma-lyase MccB                                         | 6.75          | 10.68    | lexA/SOS                    |
| BPJ17710 | putative cell division suppressor protein YneA                          | 2.24          | 44.25    | lexA/SOS                    |
| BPJ10180 | 3’-5’ exoribonuclease YhaM                                           | 0.71          | 2.81     | lexA/SOS                    |
| BPJ21860 | DNA polymerase 4                                                       | 10.68         |          | lexA/SOS                    |
| BPJ32300 | excinuclease ABC subunit B                                             | 7.22          | 4.29     | lexA/SOS                    |
| BPJ32290 | excinuclease ABC subunit A                                             | 1.49          | 6.75     | lexA/SOS                    |
| BPJ29600 | excinuclease ABC subunit UvrC                                          | 3.69          | 7.65     | lexA/SOS                    |
| BPJ17700 | repressor LexA                                                         | 1.55          | 5.66     | lexA/SOS                    |
| BPJ17730 | DUF896 family protein YnuC                                             | 0.65          | 8.85     | lexA/SOS                    |
| BPJ24600 | phage-like PBSX protein XidA                                            | 3.10          | 17.84    | lexA/SOS                    |
| BPJ17720 | resolvase-like protein YneB                                             | 1.38          | 17.03    | lexA/SOS                    |
| BPJ10160 | putative exonuclease YhaO                                              | 8.76          |          | lexA/SOS                    |
| BPJ16880 | recombinase RecA                                                       | 1.63          | 7.22     | lexA/SOS/comK               |
| BPJ35170 | minor extracellular serine protease Vpr                                 | 1.58          | 2.23     | lexA/SOS/phoP               |
| BPJ21470 | hypothetical protein YpuD                                              | 1.93          | 7.12     | lexA/SOS/sigB/sigM          |
| BPJ10170 | putative ATPase YhaN                                                   | 8.73          |          | lexA/SOS                    |
| BPJ13450 | ATP-dependent Clp protease ATP-binding subunit ClpE                    | 2.78          | 45.41    | ctsR                        |
| BPJ25460 | ATP-dependent protease ATP-binding subunit ClpX                        | 2.67          |          | ctsR                        |
| BPJ00800 | DNA repair protein RadA                                                 | 10.02         |          | ctsR/sigB                   |
| ORF ID  | gene                              | transcriptome  | proteome        | Regulon in other Bacilli |
|---------|-----------------------------------|----------------|-----------------|--------------------------|
|         |                                   | 3 min          | 8 min           | 10 min                   | 30 min                   |
| BPJ00760 | transcriptional regulator CtsR   | ctsR           |                 | 9.40                     | ctsR/sigB                |
| BPJ00770 | transcriptional regulator McsA   | mcsA           |                 | 10.26                    | ctsR/sigB                |
| BPJ11850 | ATP-dependent Clp protease proteolytic subunit ClpP | clpP | 1.79             | 4.26                     | 8.74                     | 1.73                     | ctsR/sigB |
| BPJ00780 | putative ATPguanido phosphotransferase McsB | mcSB | 1.43             | 8.87                     |                          |                          | ctsR/sigB/sigF |
| BPJ00790 | ATP-dependent Clp protease ClpC | clpC           |                 | 6.44                     |                          |                          | ctsR/sigB/sigF |
| BPJ00810 | DNA integrity scanning protein DisA | disA | 5.15             |                          |                          |                          | ctsR/sigB/sigM |
| BPJ15470 | adenylly-sulfate kinase CysC | cysC           |                 | 23.93                    | 1.60                     | cymR                     |
| BPJ15480 | uroporphyrin-3 C-methyltransferase CysG | cysG |                 | 14.9                     | 10.53                    | cymR                     |
| BPJ15460 | sulfate adenyllytransferase Sat | sat             |                 | 1.37                     | 13.07                    | cymR                     |
| BPJ20800 | tryptophan synthase alpha subunit TrpA | trpA | 2.16             | 13.51                    | TRAP                     |
| BPJ20810 | tryptophan synthase beta subunit TrpB | trpB | 1.13             | 13.99                    | TRAP                     |
| BPJ20820 | N-(S')-phosphoribosylanthranilate isomerase TrpF | trpF | 1.59             | 2.09                     | TRAP                     |
| BPJ20830 | indole-3-glycerol-phosphate synthase TrpC | trpC | 2.36             | 1.49                     | 10.53                    | TRAP                     |
| BPJ20840 | anthranilate phosphoribosyltransferase TrpD | trpD | 1.44             | 2.72                     | 1.37                     | 13.07                    | TRAP                     |
| BPJ20850 | anthranilate synthase component 1 | trpE | 2.61             |                          | TRAP                     |
| BPJ12980 | transcriptional regulator OhrR | ohrR           |                 | 2.54                     | ohrR                     |
| BPJ2970 | peroxiredoxin OhrA | ohrA           | 10.66            | 9.88                     | 6.29                     | 1.30                     | ohrR |
| BPJ2990 | peroxiredoxin OhrB | ohrB           | 2.10             |                          |                          |                          | ohrR |
| BPJ19510 | putative bacillithiol biosynthesis deacetylase YojG | yojG | 3.31             |                          | bacillithiol-related     |
| BPJ20020 | DUF1094 family protein YphP | yphP           |                 | 1.78                     | 2.22                     | bacillithiol-related     |
| BPJ21140 | putative thioredoxin reductase YpdA | ypdA | 0.67             | 2.58                     | bacillithiol-related     |
| BPJ22220 | DUF1094 family protein YqiW | yqiW           |                 | 2.58                     | bacillithiol-related     |
| BPJ31300 | glycine betaine/carnitine/choline ABC transporter permease OpuCD | opuCD | 3.03             |                          | glycine betaine transport |
| BPJ31310 | glycine betaine/carnitine/choline ABC transporter substrate-binding protein OpuCC | opuCC | 0.88             | 2.62                     | glycine betaine transport |
| BPJ31320 | glycine betaine/carnitine/choline ABC transporter permease OpuCB | opuCB | 0.92             | 2.74                     | glycine betaine transport |
| BPJ31330 | glycine betaine/carnitine/choline ABC transporter ATP-binding protein OpuCA | opuCA | 0.94             | 2.46                     | glycine betaine transport |
| BPJ29250 | glycine betaine ABC transporter ATP-binding protein OpuAA | opuAA | 2.76             | 10.75                    | glycine betaine transport |
| BPJ29260 | glycine betaine ABC transporter membrane protein | opuAB | 2.41             | 10.19                    | glycine betaine transport |
| BPJ29270 | glycine betaine ABC transporter substrate-binding protein | opuAC | 2.37             | 7.48                     | glycine betaine transport |
| BPJ29290 | Na+/H+ antiporter subunit MrpA | mrpA           |                 | 4.57                     | sodium transport         |
| BPJ29370 | Na+/H+ antiporter subunit MrpB | mrpB           |                 | 4.77                     | sodium transport         |
| BPJ29380 | Na+/H+ antiporter subunit MrpC | mrpC           |                 | 3.60                     | sodium transport         |
| BPJ29390 | Na+/H+ antiporter subunit MrpD | mrpD           |                 | 1.48                     | 3.87                     | sodium transport         |
value of 3.6% for *B. licheniformis* (personal communication B. Voigt) but higher than the value for *B. subtilis* (1.2%, personal communication C. Scharf). These values indicate that in *B. pumilus* there is a higher synthesis of KatX2 already in unstressed cells compared to *B. subtilis* and *B. licheniformis* KatA explaining the lower induction rate. In *B. subtilis*, KatX is the major spore catalase and under control of SigB and SigF [35,36]. We detected a *B. subtilis* PerR consensus sequence [32] containing 2 mismatches about 90 bases in front of the start codon of KatX2 indicating a possible involvement of PerR in its regulation.

### 3.4 Fur Regulon and Fe-metabolism

The PerR-regulated fur gene of *B. pumilus*, shows 95% similarity to the fur gene known from *B. subtilis* and was induced 3.6-fold after stress [32]. The regulator protein Fur of *B. subtilis* controls the expression of genes responsible for iron uptake [37]. Immediately after exposure to H$_2$O$_2$, cytosolic iron concentration is considerably reduced to prevent the formation of OH$^-$ by the Fenton reaction [13]. Upregulation of the Fur-controlled genes may be a reaction of the cells to optimize iron uptake in order to face the resulting iron limitation. Alternatively it might be that Fur is H$_2$O$_2$ sensitive as it is in *E. coli* [38].

Nine genes of a putative Fur regulon showed a significantly increased expression in *B. pumilus* cells after H$_2$O$_2$ treatment, including the ABC transporter system fhuB1C1G1 (Table 1). The *fhu* gene was induced by H$_2$O$_2$ in *B. subtilis* and *B. licheniformis*, too [13,20]. Further Fur regulon member genes known to be induced by H$_2$O$_2$ in *B. subtilis* showing an induction in our study were *ykhN*, *ykaP* (flavodoxins) and the hypothetical protein *ykaQ*. With an about 30-fold higher mRNA level 8 minutes after treatment, these were among the highest upregulated genes in this putative regulon. The putative nitroreductase YihC, also induced in H$_2$O$_2$ stressed *B. subtilis* cells, was the only member of the putative Fur regulon we observed to be upregulated at translational level.

The gene *yvaA*, encoding another ABC transporter of yet unknown function, the peptidase encoding gene *yjkM* and the bacillibactin esterase encoding gene *ybbA* were upregulated, too. These genes are Fur-regulated in *B. subtilis*, but they were not upregulated by H$_2$O$_2$ in this organism [13,39]. In *B. subtilis* and *B. licheniformis*, the siderophore biosynthesis complex encoded by *dhhACEFB* was strongly upregulated by H$_2$O$_2$. In our study, these genes showed no significant changes in their expression level.

Other genes that exhibited higher transcription rates after H$_2$O$_2$ treatment were the iron ABC transporter protein encoding gene *fhuA* and its upstream-located regulator *ybhB* (renamed *btr* in *B. subtilis*) [40]. Unlike *B. subtilis*, the *B. pumilus* genome encodes a second Fhu-related iron uptake system. Our study showed an induction of the genes encoding FhuC2-FhuB2-BPJ35820 as well as *fhuG* and *fhuD* immediately after subjecting the cells to the stress. Two further putative iron transporter systems, *bpj33580-bpj33580-rbpj35850* and *bpj08440-bpj08430-bpj08440*, were induced, too. The proteins encoded by the latter genes showed no significant homology to any protein known from related *Bacillus* species.

Furthermore, the proteomic approach revealed a strong induction of the siderophore synthesis proteins RlhA, RlhB and RlhF, encoded by the *rlhABCDEF*-operon (Table 1). A rather slight induction at the translational level was shown for the iron/sulfur cluster biogenesis proteins SulB, SulS, SulD and SulC as previously shown for *B. licheniformis* [20]. The *sufU* gene was found to be only slightly upregulated at the mRNA level.
3.5 Spx Regulon and Bacillithiol

Another regulator protein assigned to the putative PerR regulon is SpxA, controlling the expression of the Spx regulon in *B. subtilis* [41,42]. This gene exhibited an about 4-fold increased transcription rate in H$_2$O$_2$ stressed *B. pumilus* cells. Some of the genes and proteins attributed to a putative Spx regulon in *B. pumilus*

Figure 3. Cytosolic proteome 10 min after H$_2$O$_2$ treatment. The cytosolic proteome of *B. pumilus* cells 10 min after H$_2$O$_2$ treatment. Cell samples were labeled with L-[35S]-methionine during the exponential growth phase (OD$_{500}$ nm 0.6), and 10 min after H$_2$O$_2$ addition. Proteins were separated in a pH gradient 4 (right) – 7 (left).

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appeared to have rather moderately increased expression rates or were not induced after H$_2$O$_2$ treatment.

In our study we detected six genes of a putative Spx regulon to be induced following H$_2$O$_2$ treatment (Table 1). The proteins encoded by three of them, nitro/flavinreductase NfrA, putative NADPH-dependent butanol dehydrogenase YugJ and thioredoxin-disulfide reductase TrxB, were induced in H$_2$O$_2$ treated cells, too. Upregulation of msrAB (methionine sulfoxide reductase

**Figure 4. Cytosolic proteome 30 min after H$_2$O$_2$ treatment.** The cytosolic proteome of *B. pumilus* cells 30 min after H$_2$O$_2$ treatment. Cell samples were labeled with L-[$^{35}$S]-methionine during the exponential growth phase (OD$_{500}$ nm 0.6), and 30 min after H$_2$O$_2$ addition. Proteins were separated in a pH gradient 4 (right) – 7 (left).

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operon) and trxA (thioredoxin) was detected at transcriptional level only. The proteins TrxA and TrxB are described to act in direct detoxification of hydrogen peroxide [43–45]. Cystathionine gamma-lyase MeCB and DinB-like domain-containing protein YuAE showed an induction only at proteome level.

The Spx-regulated sfp operon, mediating competence and metabolic functions in B. subtilis, is absent in the B. pumilus genome as shown before for B. licheniformis [42,46,47].

We noticed an increased transcription of sfdA and yqiW as well as an induction of the ypbP gene product (Table 1). These genes co-occur with bacillithiol (Cys-GlcN-malate, BSH) synthesis genes [48]. However, only one gene encoding a protein involved in bacillithiol synthesis, yogG was transcribed at a slightly elevated level (Table S2). Bacillithiol is one of the major thiols in B. subtilis and known to be involved in resistance against organic peroxide stress and disulfide stress [7,49,50]. For further investigation, we analyzed the cytosolic metabolome of H2O2 treated B. pumilus cells concerning the concentration of thiol compounds. Our analysis revealed a bacillithiol level of 2.6 nmol per mg cell dry weight already under control conditions. Similar BSH concentrations have been detected in B. subtilis (0.6–2.2 nmol per mg) [7,48,51]. Ten minutes after H2O2 treatment, the cytosolic concentration of bacillithiol increased to 5 nmol per mg cell dry weight (Figure 5).

The increase continued up to a concentration of about 6.2 nmol per mg cell dry weight 60 minutes after stress. Since only one bacillithiol synthesis gene (yphP, renamed bshB2 in B. subtilis) was slightly upregulated, increase of bacillithiol concentration in the cells might be regulated allosterically, for example, by an oxidation of the BSH pool leading to a relief of feedback inhibition. [52,53].

### 3.6 SOS Regulon

H2O2 treatment leads to the formation of OH• by Fenton reaction, which exhibits a high DNA-damaging potential. Lowering the concentration of iron in the cells reduces this threat. As a result, B. subtilis and B. licheniformis cells subjected to oxidative stress caused by H2O2, induced the SOS regulon, regulated by the proteins RecA and LexA, responsible for repair of DNA [13,20,54,55].

The proteomic analysis displayed the induction of two proteins, excinuclease subunit UvB and the recombinase RecA, assigned to a putative SOS regulon in B. pumilus following H2O2 treatment (Table 1). The transcriptomic approach added further 13 upregulated genes belonging to this regulon; among them the excinuclease subunits encoding genes ucrA and ucrC. The operon yneBBynzC, induced by H2O2 and involved in suppression of cell division in B. subtilis, was also strongly induced in our study [13,56]. This might be an explanation for the formation of atypically long cells as described above. Showing an about 44-fold increased transcription rate, yneA belongs to the strongest induced genes observed in our study. Furthermore, the putative DNA double-strand break repair cluster yhaONYM exhibited a significantly higher transcription rate following H2O2 addition [57].

### 3.7 CtsR Regulon

The CtsR regulon, mediating repair and/or degradation of misfolded and damaged proteins, was induced by several oxidative stressors in B. subtilis and B. licheniformis [13,20,58]. In our study, we detected an upregulation of nine genes assigned to a putative CtsR regulon in B. pumilus indicating a significant impact of H2O2 on protein quality (Table 1). The operon ctsR-mcsAB-clpC was transcribed with significantly higher intensity after the addition of H2O2 as well as the genes ctpP, ctpX and ctpC, encoding members of the proteolytic complex. Only ClpP was observed to be induced at the protein level. Furthermore, the DNA repair protein encoding gene radA and the DNA integrity scanning protein encoding gene disA showed higher transcription rates compared to control conditions.

### 3.8 SigB Regulon

Besides the induction of the above described putative regulons more or less directly associated to oxidative stress, H2O2 treated cells exhibited an upregulation of 47 genes known to be under control of the general stress sigma factor SigB in B. subtilis (Table 1).
Another of these putative SigB-dependent genes, encoding the putative universal stress protein NhaX, showed the highest induction rate detected in this study (more than 60-fold). Further strongly upregulated genes are the regulator protein encoding gene ngsR and ydaG (general stress protein), both also detected to be induced in H₂O₂ stressed B. licheniformis cells [20]. The upregulated genes ngsR and ydaG encode proteins with still unknown functions. Six of the upregulated putative SigB-dependent genes could be also detected to be induced in the proteomic approach. The putative general stress protein YsxH is among the strongest induced proteins (about 14-fold). The putative general stress protein YsxH is among the strongest induced proteins (about 14-fold). The putative iron storage/DNA protecting protein Dps, providing peroxide resistance in B. anthracis, was induced in H₂O₂ treated B. pumilus cells, too [62].

3.9 CymR Regulon

The results of our study showed an upregulation of several proteins belonging to a putative CymR regulon. In B. subtilis, it is described to be involved in regulation of the sulfur metabolism [63]. An induction of genes belonging to this regulon has been shown in cells afflicted with oxidative stress caused by paraquat, but not stress caused by H₂O₂ [13]. Our proteome study showed a strong induction of three putatively CymR-regulated proteins. The adenylyl-sulfate kinase (CysC) was with an induction of about 24-fold the strongest induced protein. An upregulation of the sulfate adenylyl-sulfate kinase (CysC) was with an induction of about 24-fold the strongest induced protein. An induction of genes belonging to this regulon has been described to be involved in regulation of the sulfur metabolism [63]. An induction of genes belonging to this regulon has been described to be involved in regulation of the sulfur metabolism [63]. A part of a putative SigB-regulon in B. pumilus detected to be upregulated in our study was the sigB gene itself with its signal cascade genes rsbRSTUVW and rsbX indicating an activation of the putative regulon via the general stress response cascade known from B. subtilis [61].

3.10 Other B. pumilus Upregulated Genes/proteins

The OhdB-regulated peroxiredoxin-encoding gene ohdB is reported to be involved in organic peroxide resistance in B. subtilis [64]. Following H₂O₂ treatment, there was no induction of this gene observed in B. subtilis and B. licheniformis [13,20]. In our study, we observed a strongly induced expression of this gene at transcriptional and translational level indicating an involvement of this peroxiredoxin in the H₂O₂ resistance of B. pumilus (Table 1). The ohdB gene itself with its signal cascade genes rsbRSTUVW and rsbX indicating an activation of the putative regulon via the general stress response cascade known from B. subtilis [61].

H₂O₂ treatment induced some additional regulator genes. One of them is fadR, encoding a regulator protein mediating fatty acid degradation in B. subtilis [65]. Two genes putatively controlled by FadR, efAB - encoding the electron transfer flavoprotein alpha and beta subunit, were also induced (Table S2). Another regulator, AbrB1, controlling the expression of genes induced by transition from exponential to stationary growth in B. subtilis [66], was induced at transcriptional and translational level. Similar results, but with significantly higher induction rates in the proteomic approach, were observed for the AbrB1-regulated peroxiredoxin YkuU and thioldisulfide oxidoreductase YkuV. Furthermore, several putative regulator genes with still unknown targets were observed to be upregulated. Bpj13620, bpj17020 and ydaI showed the highest changes in their expression rates. Genes encoding a sensor kinase and a response regulator forming the two-component system YhcYZ, were significantly induced directly after H₂O₂ treatment. Its function is also unknown.

Several genes and proteins involved in transport processes were detected to be upregulated following H₂O₂ stress (Table 1, S2). H₂O₂ treatment caused an upregulation of the sodium uptake system napAB and the nmpABCDEF cluster. This operon encodes a sodium excretion system that is considered to be the major sodium excretion system in bacteria and acts in pH homeostasis and multiple resistances in B. subtilis [67,68].

Strikingly, transcription of the glycine betaine uptake system consisting of opuCA-AB-BC and opuCA-AB-CC-CD was observed to be significantly induced after treatment, indicating that H₂O₂ impacts osmotic homeostasis in B. pumilus cells [69]. Furthermore, it is worth to mention that H₂O₂ induced expression of a putative TRAP regulon in B. pumilus cells. An upregulation of the tryptophan-synthesis operon tpsABCFDE as well as histidinol-phosphate aminotransferase HisC was observed in our analysis. However, neither addition of tryptophan nor addition of glycine betaine before peroxide treatment brought forth better growth or survival of stressed B. pumilus cells (data not shown).

3.11 Downregulated Genes/proteins

As shown for many other organisms, the adaptation mechanism of B. pumilus cells to oxidative stress includes also a downregulation of vegetative cellular functions. Most of the down-regulated genes encode proteins involved in main metabollic pathways. As shown for B. subtilis and B. licheniformis, expression of the purine and pyrimidine synthesis genes was downregulated as well as genes involved in synthesis of arginine (Table S3) [13,20]. Contrary to B. subtilis and B. licheniformis, a repression of histidine synthesis genes was not observed. Instead, isoleucine and leucine synthesis genes were expressed in lower amounts following H₂O₂ treatment. This repression might due to the iron sparing response described by Gaballa et al. [70]. Repression of enzymes involved in branched chain amino acid synthesis has been found during iron starvation in B. subtilis [37]. Furthermore, we observed a reduced expression of most of the aminoaacyl-tRNA-synthetases, with the exception of tryptophanyl-tRNA-synthetase tpsS, which matched the upregulation of the tryptophan operon.

Strikingly, a stringent response, i.e. a downregulation of ribosomal proteins or elongation factors like fusA, tus or tufA, as described for other organisms (B. subtilis, B. licheniformis, E. coli) could not be detected in B. pumilus [13,20,71].

Conclusion

The combination of proteomics and transcriptomics revealed a specific adaptation of B. pumilus cells caused by the oxidative stress trigger H₂O₂. Although many of the induced genes and proteins could be assigned to well-known oxidative stress regulons like PerR, CtsR and Fur, there are particular mechanisms detectable which seem to be involved in the remarkable oxidative stress resistance of B. pumilus. The concentration of H₂O₂ that was used to trigger the stress in our study was about 40-fold higher than those used for comparable analysis of B. subtilis or B. licheniformis. Our study could enlighten several points at which the peroxide stress response of B. pumilus cells is different from its Gram-positive relatives. It is suggested that the catalase KatA is replaced by the catalase KatX2. Furthermore, our study revealed an induction of genes that are highly correlated to bacillliothiol synthesis indicating an involvement of bacillithiol in the peroxide stress response of B. pumilus. Metabolome analysis demonstrated a basal level of this protective metabolite but also an increase of the cytotoxic Bacillus pumilus Resistance to Hydrogen Peroxide
bacillithiol concentration during peroxide stress. Furthermore, a considerable set of $\text{H}_2\text{O}_2$ induced unique proteins with so far unknown function could be identified in this study. These proteins are worth to address in follow up studies to elucidate their specific functions in the control of organosulfur metabolism in Bacillus pumilus.

Supporting Information

Table S1 Determination of minimal inhibition concentration of hydrogen peroxide. (XLSX)

Table S2 Genes and proteins that are upregulated after addition of hydrogen peroxide. (XLSX)

Table S3 Genes and proteins that are downregulated after addition of hydrogen peroxide. (XLSX)

Table S4 Individual signal on the array for five probes for selected genes. (XLSX)

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Author Contributions

Conceived and designed the experiments: SH R. Schroeter R. Schlueter KB KM ML TS MH BV. Performed the experiments: SH R. Schroeter KM R. Schlueter DA. Analyzed the data: SH R. Schroeter HJ SHh KM. Wrote the paper: SH R. Schroeter KM R. Schlueter BV TS MH.

References

1. Schweder T, Hecker M (2004) Monitoring of stress responses. Adv Biochem Eng Biotechnol 89: 47–71.
2. Stadtman ER, Levine RL (2003) Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amin Acids 20(2): 207–218.
3. Farr SB, Kogoma T (1991) Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiol Rev 55: 561–585.
4. Bernardin JN, Sawyer J, Venkateswaran K, Nicholas WL (2003) Spore UV and acceleration resistance of endolic Staphylococcus aureus and Bacillus subtilis isolates obtained from Sororan desert basalt: implications for lithopanspermia. Astrobiology 3: 709–717.
5. Kempf MJ, Chen F, Kern R, Venkateswaran K (2003) Recurrent isolation of hydrogen peroxide-resistant spores of Bacillus pumilus from a spacecraft assembly facility. Astrobiology 3: 391–403.
6. Imlay JA (2008) Cellular defenses against superoxide and hydrogen peroxide. Annu Rev Microbiol 62: 625–657.
7. Bokhina O, Vorilainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot 91 Spec No: 179–194.
8. Imlay JA (2003) Pathways of oxidative damage. Annu Rev Microbiol 57: 395–418.
9. Aronson OF, Hallwell B, Gajewski E, Dizdaroglu M (1991) Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. Biochem J 273 (Pt 3): 601–604.
10. Zuber P (2009) Management of oxidative stress in Bacillus. Annu Rev Microbiol 63: 573–597.
11. Imlay JA (2005) Cellular defenses against superoxide and hydrogen peroxide. Annu Rev Biochem 74: 755–776.
12. Mostert J, Scharf C, Hecker M, Homuth G (2004) Transcriptome and proteome analysis of Bacillus subtilis gene expression in response to superoxide and peroxide stress. Microbiology 150: 497–512.
13. Hoi Le T, Voigt B, Jurgen B, Ehrenreich A, Gotschalk G, et al. (2006) The phosphate-starvation response of Bacillus licheniformis. Proteomics 6: 3582–3601.
14. Buttker K, Bernhardt J, Scharf C, Schimid R, Mader U, et al. (2004) A comprehensive two-dimensional map of cytosolic proteins of Bacillus subtilis. Electrophoresis 25: 2908–2953.
15. Voigt B, Schweder T, Becher D, Ehrenreich A, Gotschalk G, et al. (2004) A proteomic view of cell physiology of Bacillus licheniformis. Proteomics 4: 1465–1480.
16. Wolf C, Hochgräfe F, Kuschi H, Allerbeck D, Hecker M, et al. (2008) Proteomic analysis of antioxidant strategies of Staphylococcus aureus: diverse responses to different oxidants. Proteomics 8: 3139–3153.
17. Volker U, Engelmann S, Maal B, Rieter M, Volker A, et al. (2004) Analysis of the induction of general stress proteins of Bacillus subtilis. Microbiology 150 (Pt 4): 741–752.
18. Homuth G, Masuda S, Mogk A, Kobayashi Y, Schumann W (1997) The dnaK operon of Bacillus subtilis is heat-stable. J Bacteriol 179: 1153–1164.
19. Schworer R, Voigt B, Jurgen B, Mengling K, Pother DG, et al. (2011) The peroxide stress response of Bacillus licheniformis. Proteomics 11: 2581–2596.
20. Delcher AL, Harmon D, Kasif S, White O, Salzberg SL (1999) Improved microbial gene identification with GLIMMER. Nucleic Acids Res 27: 4636–4641.
21. Guo FB, Zhang CT (2006) ZCURVE, V: a new self-training system for recognizing protein-coding genes in viral and plasmid genomes. BMC Bioinformatics 7: 9.
22. Voordovskiy M MR, Besemer J, Lomsadze A (2003) Current Protocols in Bioinformatics.
23. Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, et al. (2010) Prodigal: prokaryote gene recognition and translation initiation site identification. BMC Bioinformatics 11: 119.
24. Wernersson R, Juenger AC, Nissen HB (2007) Probe selection for DNA microarrays using OligoWiz. Nat Protoc 2: 2677–2691.
25. van Hijum SA, de Jong A, Barrecedes RJ, Karsens HA, Kramer NE, et al. (2005) A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA microarray data. BMC Genomics 6: 77.
26. Baldis P, Lang AD (2001) A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes. Bioinformatics 17: 509–519.
27. Pother DG, Lieder B, Hochgräfe A, Antelmann H, Becher D, et al. (2009) Dimane triggers mainly S Thioldem in the cytoprotective protomes of Bacillus subtilis and Staphylococcus aureus. J Bacteriol 191: 7520–7530.
28. Sharma SV, Jothivasan VK, Newton GL, Upton H, Wakabayashi JI, et al. (2011) Chemical and Chemoenzymatic synthesis of bacillithiol: a unique low-molecular-weight thiol among low G+C Gram-positive bacteria. Angew Chem Int Ed Eng 50: 7101–7104.
29. Pother DG, Gieriek P, Harms M, Mosterta J, Hochgräfe F, et al. (2013) Distribution and infection-related functions of bacillithiol in Staphylococcus aurous. Int J Med Microbiol 303: 114–123.
30. Münch R, Hüller K, Bar Y, Hecht D, Lins S, et al. (2003) PRODORIC: prokaryotic database of gene regulation. Nucleic Acids Res 31: 256–260.
31. Fanghong M, Herbig AF, Beir N, Helmann JD (2002) Regulation of the Bacillus subtilis fur and perR genes by PerR: not all members of the PerR regulon and the iron starvation stimulon. Mol Microbiol 45: 1613–1629.
32. Petersohn A, Engelmann S, Setlow P, Hecker M (1999) The katX gene of Bacillus subtilis is essential for hydrogen peroxide resistance of the catalase in spores of Bacillus subtilis. J Bioenerg Biomembr 31: 741–752.
33. Voigt B, Schweder T, Decher D, Ehrenreich A, Gotschalk G, et al. (2004) The peroxide stress response of Bacillus licheniformis. Proteomics 4: 2996–3015.
34. Voigt B, Schweder B, Decher D, Ehrenreich A, Gotschalk G, et al. (2004) A proteomic view of cell physiology of Bacillus licheniformis. Proteomics 4: 1465–1480.
35. Wolf C, Hochgräfe F, Kuschi H, Alberbeck D, Hecker M, et al. (2008) Proteomic analysis of antioxidant strategies of Staphylococcus aurous: diverse responses to different oxidants. Proteomics 8: 3139–3153.
36. Volker U, Engelmann S, Maal B, Rieter M, Volker A, et al. (2004) Analysis of the induction of general stress proteins of Bacillus subtilis. Microbiology 150 (Pt 4): 741–752.
37. Homuth G, Masuda S, Mogk A, Kobayashi Y, Schumann W (1997) The dnaK operon of Bacillus subtilis is heat-stable. J Bacteriol 179: 1153–1164.
38. Schworer R, Voigt B, Jurgen B, Mengling K, Pother DG, et al. (2011) The peroxide stress response of Bacillus licheniformis. Proteomics 11: 2581–2596.
39. Delcher AL, Harmon D, Kasif S, White O, Salzberg SL (1999) Improved microbial gene identification with GLIMMER. Nucleic Acids Res 27: 4636–4641.
40. Guo FB, Zhang CT (2006) ZCURVE, V: a new self-training system for recognizing protein-coding genes in viral and plasmid genomes. BMC Bioinformatics 7: 9.
42. Nakano S, Küster-Schock E, Grossman AD, Zuber P (2003) Spc-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. Proc Natl Acad Sci U S A 100: 13603–13608.

43. Specter A, Yan GZ, Huang RK, McDermott MJ, Gascoyne PR, et al. (1988) The effect of H2O2 upon thioredoxin-enriched lens epithelial cells. J Biol Chem 263: 4984–4990.

44. Chae HZ, Chung SJ, Rhee SG (1994) Thioredoxin-dependent peroxide reductase from yeast. J Biol Chem 269: 27670–27678.

45. Fernandes MR, Nauri H, Yoshizaki S, Nagata-Kano K, Minakami S (1992) Thioredoxin regenerates proteins inactivated by oxidative stress in endothelial cells. Eur J Biochem 209: 917–922.

46. Nakano S, Erwin KN, Ralle M, Zuber P (2003) Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spt. Mol Microbiol 55: 498–510.

47. Veith B, Herzberg C, Steckel S, Fessehe J, Maurer KH, et al. (2004) The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J Mol Microbiol Biotechnol 7: 284–211.

48. Gaballa A, Newton GL, Antelmann H, Parsonage D, Upton H, et al. (2010) Biosynthesis and functions of bacillithiol, a major low-molecular-weight thiol in *Bacillus*. Proc Natl Acad Sci U S A 107: 5617–5622.

49. Lee JW, Sosmanaga S, Helmann JD (2007) A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. Proc Natl Acad Sci U S A 104: 8743–8748.

50. Chi BK, Grolla K, Mader U, Hesling B, Becher D, et al. (2011) S-bacillithiolation protects against hypochlorite stress in *Bacillus subtilis* as revealed by transcriptomics and redox proteomics. Mol Cell Proteomics 10: M111 09506.

51. Chi BK, Roberts AA, Huyten TT, Basel K, Becher D, et al. (2013) S-bacillithiolation protects conserved and essential proteins against hypochlorite stress in *Bacillus subtilis*. Mol Microbiol 86: 1004–1008.

52. Gaballa A, Antelmann H, Hamilton CJ, Helmann JD (2013) Regulation of *Bacillus subtilis* bacillithiol biosynthesis operon by Spc. Microbiology 159: 2025–2035.

53. Miller MC, Rosnick JB, Smith BT, Lovett CM, Jr. (1993) The *Bacillus subtilis* dinR gene codes for the analogue of *Escherichia coli* LexA. Purification and characterization of the DinR protein. J Biol Chem 278: 33502–33508.

54. Love PE, Lyle MJ, Yasbin RE (1983) DNA-damage-inducible (din) loci are transcriptionally activated in competent *Bacillus subtilis*. Proc Natl Acad Sci U S A 82: 6201–6205.

55. Kawai Y, Moriya S, Ogasawara N (2003) Identification of a protein, YneA, responsible for cell division suppression during the SOS response in *Bacillus subtilis*. Mol Microbiol 47: 1113–1122.

56. Krishnamurthy M, Tadesse S, Rothmaier K, Graumann PL (2010) A novel SMC-like protein, SbeE (YhaN), is involved in DNA double-strand break repair and competence in *Bacillus subtilis*. Nucleic Acids Res 38: 453–466.

57. Leichter LI, Scharf C, Hecker M (2003) Global characterization of dmfA mutant of *Bacillus subtilis*. J Bacteriol 185: 1967–1975.

58. Petersohn A, Brigulla M, Haas S, Hoheisel JD, Volker U, et al. (2001) Global analysis of the general stress response of *Bacillus subtilis*. J Bacteriol 183: 5617–5631.

59. Hecker M, Reder A, Fuchu S, Pagen M, Engelmann S (2009) Physiological proteomics and stress/starvation responses in *Bacillus subtilis* and *Staphylococcus aureus*. Mol Microbiol 72: 1119–1132.

60. Tu WY, Pohl S, Gunyski K, Hartwood CR (2012) The iron-binding protein Dps2 confers peroxide stress resistance on *Bacillus anthracis*. J Bacteriol 194: 925–931.

61. Evsen S, Burguier P, August,S, Soultouma O, Danchin A, et al. (2006) Global control of cysteine metabolism by CymR in *Bacillus subtilis*. J Bacteriol 188: 2184–2197.

62. Kajiyama Y, Otagiri M, Sekiguchi J, Kosono S, Kudo T (2007) Complex regulatory network for peroxide stress resistance in *Bacillus subtilis*. Proc Natl Acad Sci U S A 104: 5189–5194.

63. Matuoka H, Hirooka K, Fujita Y (2007) Organization and function of the Ysa regulon of *Bacillus subtilis* involved in fatty acid degradation. J Biol Chem 282: 5100–5114.

64. Perego M, Spiegelman GB, Hoch JA (1988) Structure of the gene for the transition state regulator, ohrA: regulator synthesis is controlled by the ohrA sporulation gene in *Bacillus subtilis*. Mol Microbiol 2: 689–699.

65. Ito M, Guffanti AA, Oudega B, Krulwich TA (1999) *mpA*, a multigene, multifunctional locus in *Bacillus subtilis* with roles in resistance to cationic and in pH homeostasis. J Bacteriol 181: 2394–2402.

66. Kajiyama Y, Onagiri M, Shiguchi J, Kosono S, Kudo T (2007) Complex formation by the *mpAABCDEF* gene products, which constitute a principal Na+/H+ antiporter in *Bacillus subtilis*. J Bacteriol 190: 7511–7514.

67. Kempf B, Bremer E (1993) *OpnA*, an evolutionarily conserved protein, provides a structural scaffold in *Bacillus subtilis*. J Bacteriol 175: 3060–3063.

68. Gaballa A, Antelmann H, Aguilar C, Khakh SK, Song KB, et al. (2008) The *Bacillus subtilis* iron-sparing response is mediated by a Fur-regulated small RNA and three small, basic proteins. Proc Natl Acad Sci U S A 105: 11927–11932.

69. VanBogelen RA, Kelley PM, Neidhardt FC (1987) Differential induction of heat shock, SOS, and osmolarity stress regulons and accumulation of nucleotides in *Escherichia coli*. J Bacteriol 169: 26–32.