Transcriptome analyses of industrially relevant bacteria

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ZUSAMMENFASSUNG

In vielen industriellen Bereichen haben biotechnologische Prozesse rein chemische Verfahren ersetzt bzw. neue umweltschonende Enzym-basierte Verfahren ermöglicht. Besonders bei der industriellen Enzymsynthese kommen Mikroorganismen wie z.B. modifizierte *Bacillus* Stämme zum Einsatz. Die Organismen *Bacillus licheniformis* und *Bacillus pumilus* sind dabei von großer Bedeutung. *B. licheniformis* ist in der Lage Proteine in großen Mengen zu sezernieren, während *B. pumilus* eine hohe Resistenz gegenüber oxidativem Stress aufweist.

Während der Produktionsprozesse im Fermenter können Bedingungen auftreten, die die Physiologie der produzierenden Organismen beeinträchtigen. Dies kann zu einer quantitativen, aber auch qualitativen Minderung der Produkte führen. Dieser Beeinflussung können beispielsweise chemische Prozesse, die Einstellung der Temperatur, des pH-Werts oder der Sauerstoffverfügbarkeit zugrunde liegen. Es kann daher zu verschiedenen Stresssituationen für die produzierenden Bakterien kommen. Zellen reagieren auf veränderte Umweltbedingungen, indem sie die Stressoren wahrnehmen und eine physiologische Schutzreaktion einleiten, was meist durch die Induktion oder Derepression verschiedener Regulons realisiert wird.

Um einen optimalen Produktionsprozess durchführen zu können, sollte man den Stoffwechsel des verwendeten Bakteriums, sowie seine Reaktion auf Stressbedingungen genau kennen. Ziel dieser Arbeit war es, die Stressantwort von *B. licheniformis* auf Hitzestress und Salzstress, sowie von *B. licheniformis* und *B. pumilus* auf oxidativen Stress zu analysieren. Die Untersuchungen erfolgten auf Ebene der Transkription mittels cDNA-Microarrays. Dies ist die unmittelbarste globale Methode, um Änderungen in der Physiologie der Zelle zu analysieren. Die Identifizierung von Stress-spezifischen Markergenen und ihre Unterscheidung von der SigB regulierten generellen Stressantwort war ein weiteres Ziel dieser Arbeit. Die Kenntnis dieser Markergene ermöglicht eine zeitnahe Analyse der Fermentationsbedingungen und damit verbunden eine mögliche Optimierung des Prozesses.

Die Transkriptomanalysen dieser Arbeit zeigten, dass *B. licheniformis* auf Hitzestress mit der Induktion von Hitzeschockgenen reagiert, die verschiedenen Regulons angehören.

Dazu gehören unter anderem das *htpG* Gen, sowie das HrcA Regulon oder das CtsR Regulon, welche vorwiegend Chaperone und Proteasen kodieren, die zur Proteinqualitätskontrolle beitragen. Diese Reaktionen unterscheiden sich nicht signifikant von der des Gram-positiven Modellorganismus *Bacillus subtilis*. Die generelle Stressantwort (SigB Regulon), die durch Hitzestress aktiviert wird, konnte für *B. licheniformis* durch die Untersuchung einer Δ*sigB* Mutante genauer analysiert werden. Auch unter Salzstress konnte eine starke Induktion der generellen Stressantwort bei *B. licheniformis* festgestellt werden. Gene für die Aufnahme und die Synthese kompatibler Solute wurden stark induziert, ebenso wie verschiedene Gene für Transportsysteme mit mehr oder weniger bekannter Funktion. Die Synthese der osmoprotektiven Metabolite Prolin und Glycin-Betain konnte in einer Untersuchung des Metaboloms genauer verifiziert werden. Bei der Reaktion auf oxidativen Stress zeigten sich Unterschiede sowohl zwischen *B. licheniformis* und *B. pumilus*, als auch im Vergleich der oxidativen Stressreaktion beider Organismen zu der Reaktion von *B. subtilis*. In *B. licheniformis* werden bei oxidativen Stress die Gene des Glyoxylatzyklus induziert. Eine Aktivierung des Glyoxylatzyklus unter oxidativen Bedingungen konnte durch eine Analyse des Metaboloms von *B. licheniformis* bekräftigt werden. Zusätzlich ist das PerR Regulon in *B. licheniformis* gegenüber *B. subtilis* um zwei Gene erweitert. Im Gegensatz dazu fehlen mehrere Gene des PerR Regulons im Genom von *B. pumilus*, wie z.B. *katA* (vegetative Katalase) oder *ahpCF* (Alkylhydroperoxidreduktase). Dafür wurden in *B. pumilus* Gene induziert, die unter oxidativen Stressbedingungen weder in *B. subtilis*, noch in *B. licheniformis* hochreguliert wurden. Darüber hinaus wurden bekannte Regulons, reguliert durch z.B. Spx, CtsR oder SOS in beiden Organismen induziert.

Zusammengefasst analysiert diese Dissertation auf Transkriptomebene die Stressantworten von *B. licheniformis* auf Hitze-, Salz- und oxidativen Stress, sowie zusätzlich die oxidative Stressantwort von *B. pumilus*. Es konnten verschiedene Stress-spezifische Regulons sowohl in *B. licheniformis* und auch *B. pumilus* identifiziert werden, die auch der Stressantwort von *B. subtilis* entsprechen. Es war jedoch auch möglich, weitere Gene der Stress-spezifischen Antwort beider Organismen zuzuordnen und Unterschiede zwischen den Stressantworten der Organismen zu finden, wie beispielsweise die Abwesenheit von Teilen des PerR Regulons in *B. pumilus* oder die Aktivierung des Glyoxylat-Wegs in *B. licheniformis* unter oxidativem Stress.

SUMMARY

In many industrial sectors biotechnological production processes have replaced pure chemical methods and allowed new, ecologically friendly and enzyme-based processes. Microorganisms, such as modified *Bacillus* strains are used in particular for the industrial enzyme synthesis. The two organisms *Bacillus licheniformis* and *Bacillus pumilus* are of great industrial importance. *B. licheniformis* is able to secrete proteins in large amounts, while *B. pumilus* shows high resistance to oxidative stress.

During production processes different conditions can occur that affect the physiology of the production hosts and may result in a quantitative, but also a qualitative impairment of the products. This influence is based on e.g. chemical processes, the setting of temperature, pH, or oxygen availability and can lead to various stress situations for the bacteria. Cells respond to changes in their environment by sensing stressors and initiate a response to the stress, which is usually implemented by an induction or derepression of various regulons.

In order to conduct an optimal production process, the metabolism and stress responses of the utilized bacteria should be known exactly. The aim of this study was to analyze of the stress response of *B. licheniformis* to heat and salt stress, and the stress response of *B. licheniformis* and *B. pumilus* to oxidative stress. These analyses were performed at the level of transcriptomics using cDNA microarrays, which is the most direct and global method for the analysis of changes in the physiology of a cell. The identification of stress specific markers genes and their differentiation from the SigB regulated general stress response has been another purpose of this work. Knowledge of these marker genes enables a prompt analysis of the fermentation conditions and thus a possible optimization of the process.

The transcriptome analyses of this work show that *B. licheniformis* responds to heat stress by the induction of heat shock genes belonging to different regulons. These include the *htpG* gene, the HrcA regulon or the CtsR regulon, encoding chaperones and proteases, which mainly contribute to the protein quality control. The heat stress response of *B. licheniformis* revealed no fundamental differences to the heat stress response of the Gram-positive model organism *Bacillus subtilis*. The general stress

response (SigB regulon), which is activated by heat stress, could be analyzed in more detail by the study of a *ΔsigB* mutant of *B. licheniformis*. Salt stress also provokes a strong induction of the general stress response in *B. licheniformis*. Genes for the transport and synthesis of compatible solutes were strongly induced, as well as several genes for transport systems with more or less known functions. The synthesis of the osmoprotective metabolites proline and glycine betaine could be verified in more detail by a metabolomics approach. The response to oxidative stress showed differences between both *B. licheniformis* and *B. pumilus*, and also to the oxidative stress response of *B. subtilis*. In *B. licheniformis*, the genes of the glyoxylate cycle are induced during oxidative stress. An activation of the glyoxylate bypass under oxidative conditions could be confirmed by a metabolome analysis of *B. licheniformis*. In addition, the PerR regulon of *B. licheniformis* is extended to include another two genes compared to *B. subtilis*. In contrast, several genes of the PerR regulon lack in the genome of *B. pumilus*, such as *katA* (vegetative catalase) or *ahpCF* (alkyl hydroperoxide reductase). However, other genes were induced in *B. pumilus* that were upregulated under oxidative stress conditions neither in *B. subtilis* nor in *B. licheniformis*. In addition, known regulons, regulated by e.g. Spx, CtsR or SOS were induced in both organisms.

In summary, this dissertation transcriptionally analyzes the stress responses of *B. licheniformis* to heat, salt and oxidative stress, and in addition the oxidative stress response of *B. pumilus*. Several stress-specific regulons were identified in both, *B. pumilus* and *B. licheniformis*, which also correspond to the stress response of *B. subtilis*. However, it was possible to additionally assign genes to the stress specific responses of both organisms and to find differences, such as the absence of parts of the PerR regulon of *B. pumilus*, or the activation of the glyoxylate pathway in *B. licheniformis* during oxidative stress.

INTRODUCTION

TRANSCRIPTOMICS AND THE METHODOLOGY OF MICROARRAYS

The transcriptome is the entity of all transcripts produced in a cell at a given time. Transcripts are all RNA molecules, i.e. mRNAs, rRNAs, tRNAs and other non-coding RNAs. Unlike the genome, that is more or less static in the entirety of the genetic material of a cell, the transcriptome is dynamic and can vary during different environmental conditions. As the transcriptome comprises all mRNAs, it reflects the genes that are actively expressed at any given time. This is why the term “transcriptomics” is also referred to as gene expression analysis.

In order to analyze the transcriptome, different comprehensive techniques are currently used. In this study the methodology of cDNA microarrays was applied and will be introduced in more detail. Besides microarrays, other approaches like e.g. RNAseq [1] or the “Serial Analysis of Gene Expression” (SAGE) [2] can be utilized to investigate the transcriptome.

Microarrays are the most commonly used technique to measure the expression levels of large numbers of genes, or to genotype multiple regions of a genome. An array consists of a solid support surface, usually a glass or a silicon chip, on which microscopic DNA spots are attached on. Every Spot contains picomoles of a specific DNA sequence, called probes (or reporters/oligos). These probes can be made up of small oligonucleotides, PCR products or other DNA elements. In general, RNA is isolated from two (or more) samples/experimental groups/etc. that should be compared, and converted into either antisense RNA (aRNA) or complementary DNA (cDNA). Through this conversion the target is fluorescently labeled with a fluorochrome. Afterwards, the labeled target is hybridized against the probes. The array is then scanned by a high resolution scanner that is able to quantify the fluorescence signal of the probe-target hybridizations. The data thus obtained is processed by the application of bioinformatics. However, the accomplishment of these steps can vary between different platforms.

A wide range of microarray review articles are available online, see for example [3-7].

The current study uses data obtained from cDNA microarrays (Agilent technologies, Santa Clara, CA, USA) for gene expression analyses of *Bacillus licheniformis* and *Bacillus pumilus*. In cDNA microarrays, both the probes and the targets are cDNAs. RNA from two different samples is reverse-transcribed and simultaneously labeled each with one of two different fluorescent dyes, usually Cyanine-3 (Cy3) and Cyanine-5 (Cy5), and the two samples are co-hybridized to one array. Subsequently, Cy3 and Cy5 fluorescence is measured separately, and captured in two images. These are merged to produce a composite image. The hybridization intensity is represented by the amount of fluorescent emission, which gives an estimate of the relative amounts of the different transcripts that are present. This so called "two-color-hybridization strategy" is often used within cDNA microarrays.

In this study, a common reference design was used for the arrays. This means each experimental sample was hybridized against a common reference sample (e.g. an experimental control sample or a mixture of various samples). This allows derived comparisons between large numbers of samples, without requiring that every pairwise comparison has to be performed or a dye-swap is needed, but maintaining the internal-control aspect of two-color hybridizations [7, 8].

The *B. licheniformis* and *B. pumilus* arrays were designed by Sacha van Hijum (Netherland Bioinformatics Center, Nijmegen, Netherlands) according to the annotation of Veith *et al.* [9] and Handtke *et al.* [10]. The data evaluation was done using the Rosetta Resolver biosoftware (c/o Ceiba Solutions, Boston, MA, USA) or a script obtained from Sacha van Hijum [11, 12].

BACILLUS - A GENUS OF DIVERSITY - UTILIZED BY THE INDUSTRY

Bacillus is a manifold genus of Gram-positive, rod shaped, endospore forming bacteria and part of the family Bacillaceae, order Bacillales, class Bacilli, phylum Firmicutes in the domain Bacteria. It comprises various species with different characteristics, like the pathogens *Bacillus anthracis* or *B. cereus* [13, 14] and also species of industrial relevance, like *B. licheniformis*, *B. pumilus* or *B. subtilis* [15-17].

Nowadays, *B. subtilis* is the best-studied and most widely used model organism for Gram-positive bacteria, as it is *Escherichia coli* for Gram-negatives. *B. subtilis* is categorized as GRAS-organism (Generally Regarded As Safe), easily cultivatable and highly accessible for genetic manipulation. The genome sequence of *B. subtilis* is available since 1997 [18] and was re-sequenced in 2009 [19]. Since years comprehensive studies in terms of molecular biology and cell biology were carried out by diverse study groups. This allowed the global analysis of the adaptation processes of *B. subtilis* to various environmental changes or stresses [20-22]. The two species *B. licheniformis* and *B. pumilus* are closely related to this model organism [23, 24].

The genome of *B. licheniformis* was sequenced and published in 2004 by two different groups [9, 25]. The sequence shows broad similarity with *B. subtilis*, but contains defined differences. Among others, two striking differences are: (i) the presence of the glyoxylate bypass that is lacking in *B. subtilis* and (ii) an anaerobically active ribonucleotide reductase. These findings explain the ability of *B. licheniformis* to grow on acetate or 2,3-butanediol as carbon source and furthermore utilize glucose anaerobically [9]. The other investigated *Bacillus* in this study, *B. pumilus*, showed highly increased resistance against hydrogen peroxide (H_2O_2) and UV radiation compare to other *Bacillus* species [26, 27]. Such strains have been isolated in form of dormant spores in manufacturing systems and surfaces of spacecrafts and at the International Space Station ISS [27-29]. One of these strains, *B. pumilus* SAFR-032, was isolated at the Jet Propulsion Lab in Pasadena, and its genome sequence was published in 2007 [27]. Both species are then as now subject of numerous functional genomics studies [27, 30, 31].

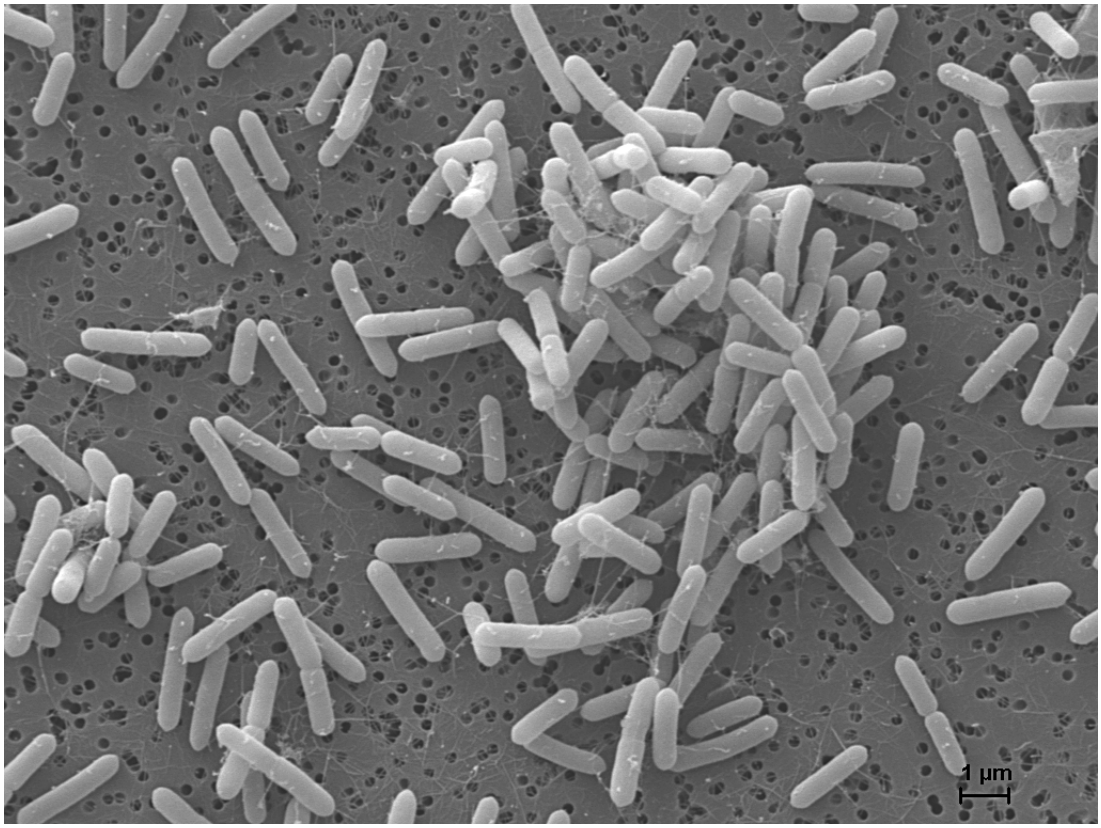

Figure 1. SEM picture of vegetative *Bacillus* cells (*Bacillus pumilus*). Imaging Center Greifswald, Department of Microbiology, University of Greifswald [32]

Besides these specific biochemical properties of both *Bacilli*, their main focus on research is the biotechnological application. Many industrial sectors have established biotechnological production processes in parallel to chemical production. In most cases these products originate from fermentations with bacteria or yeasts. The biggest part is represented by the microbial enzyme production, but also individual amino acids are synthesized by microorganisms. Industrially used microbial synthesis directly competes with the chemical processes in case of cost and yield. The bacterial workhorses are usually optimized in their genetic composition to increase the production and to block undesirable metabolic pathways.

Altogether, modified *Bacillus* strains perform half of the total industrial enzyme production, simply because they show high growth rates and are able to secrete large amounts of extracellular enzymes [17]. Among these are e.g. alkaline proteases, amylases, lipases and many more, which are especially important in the detergents

industry. *Bacillus* species for enzyme production are in use by various companies, e.g. BASF, Novozymes or AB Enzymes. *B. licheniformis* shows the mentioned benefits of *Bacillus* species in enzyme productions, but can reach higher cell densities, and in association yields higher amounts of the product during fermentations, compared to *B. subtilis* [17, 33]. Whereas *B. licheniformis* is already used as production host for years, *B. pumilus* is relatively new in the biotechnological field. Sangeetha *et al*. [34] showed the enormous potential of *B. pumilus* as the strain SG2 produced and secreted both proteases and lipases in large quantities from industrial effluents.

Like most organisms *Bacilli* have to cope with ever changing environmental conditions in their natural habitat. They can be subjected to varying nutrition supplies and starvation or e.g. radiation, shifts in the concentrations of salts or temperature changes. The ability to adapt to unfavorable stress or starvation conditions is essential for the survival of cells and is accomplished by complex regulatory networks. The occurrence of such circumstances during production processes could interfere with the growth of bacteria and also impair product formation or quality. In order to optimize the production process it is necessary to understand the physiology of the production host throughout the fermentation. Specific marker genes, like nutrient-starvation or product-related genes could be identified in different hosts that allow an image of the current physiological and productive state of the cell. However, it is a prerequisite to monitor and analyze these parameters during a fermentation process nearly in real-time, as possible e.g. with electrical biochips [35-37]. Global gene expression analyses in defined cultivations that simulate critical process conditions enable the identification of genes which expression is relevant and specific for selected processes [38]. This study comprehensively analyzes the stress responses of *B. licheniformis* and *B. pumilus* against environmental stresses and identifies stress specific marker genes, e.g. for oxidative-, salt- or heat stress.

HEAT STRESS AND THE ROLE OF THE SIG^B REGULON

In their natural habitat or occasionally during production processes, bacteria can be subjected to temperature shifts. A sudden increase in temperature causes the transient induction of a group of genes (heat shock genes, HSGs), resulting in the synthesis of so-called heat shock proteins (HSPs). These proteins appear to be highly conserved through evolution, indicating similar functions in all organisms [39]. As heat can cause damage to protein structures, leading to misfolding or aggregation of proteins and thus interfere with vital cellular functions, refolding or degradation of involved proteins is a major task of cellular protein quality control systems. Hence, most heat shock proteins belong to either of two classes: molecular chaperones or ATP-dependent proteases [40, 41]. Whereas chaperones ensure that polypeptides fold or assemble properly in the cell, proteases degrade affected proteins, which are unable to refold into their native structure. In many cases of protein quality control, chaperones and proteases collaborate [42, 43]. In the Gram-positive model organism *B. subtilis*, the genes of the heat shock response are organized in several regulons; e.g. σ^B, HrcA, CtsR, CssR and others [40, 44].

One of the strongest reactions of *B. subtilis* towards various stress- or starvation conditions, including heat, is the general stress response controlled by the alternative sigma factor σ^B. The σ^B regulon is one of the most extensive stress- and starvation regulons, as it provides the cell with comprehensive, non-specific, preventive resistances against different stress conditions in response to only one stimulus. Besides heat, e.g. ethanol-, salt- or acid stress or starvation of glucose or phosphate could trigger the general stress response. In *B. subtilis*, more than 150 genes belong to that regulon, and amongst others, genes for unspecific oxidative-, heat- or osmotic stress resistances are regulated by σ^B [45-47]. The *sigB* gene is the penultimate of an eight gene operon encoding seven Rsb-proteins (Regulation of SigmaB). The entire operon is constitutively transcribed from an upstream σ^A promoter and the genes *rsbV-rsbW-sigB-rsbX* can be autoinduced from an internal σ^B promoter [48, 49]. The activity of σ^B is regulated by partner switching mechanisms of alternatively binding proteins, whose interactions are determined by the phosphorylation status of the partners [50, 51]. In brief, σ^B is

sequestered by an anti-sigma factor RsbW, thereby preventing σ^B-interaction with the RNA-polymerase core enzyme. RsbW also possesses a serine kinase activity, which is responsible for the phosphorylation - and thereby reversible inactivation - of RsbV (anti-anti-sigma factor). If *B. subtilis* is exposed to a stimulus of the general stress response, the phosphate is removed from RsbV~P by one of the phosphatases RsbP or RsbU. Dephosphorylated RsbV has the ability to attack the inhibitory complex of RsbW and σ^B and so release the sigma factor. The mentioned phosphatases respond to different situations; RsbP reacts to energy stress situations, RsbU is environmental stress responsive [52-54].

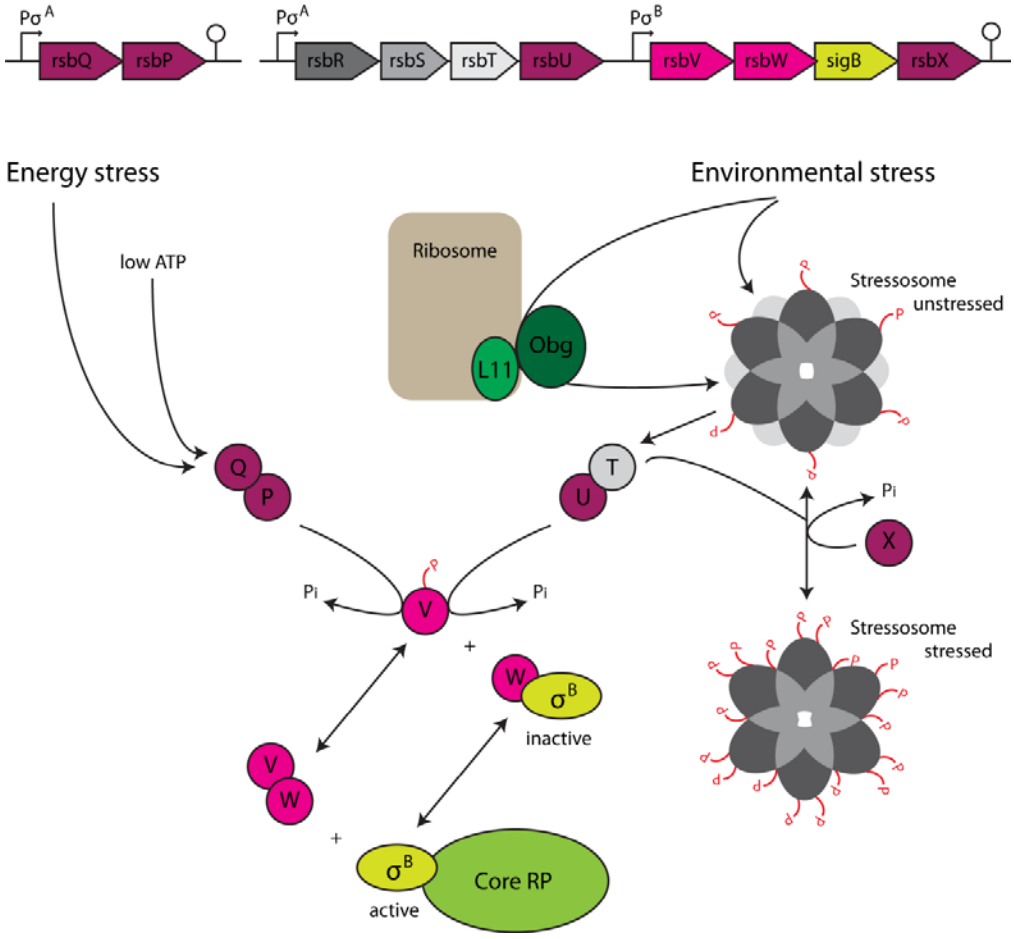

Figure 2. Schematic model of SigB regulation. Chromosomal organization of the *sigB* and *rsbQP* operon and representation of the signal transduction pathways responsible for σ^B activation in *B. subtilis.* The environmental stress pathway is activated by e.g. salt [47, 55], heat [44, 56], or manifold other stimuli [47, 55, 57-60]. The energy stress pathway is activated by different

starvation conditions or limitations [52, 55, 61, 62]. Abbreviations: Core RP (RNA polymerase core enzyme), L11 (ribosomal protein L11), Obg (GTP-binding protein Obg). Modified from [50].

B. licheniformis owns a conserved operon that shows high similarity to the eight-gene-*sigB* operon of *B. subtilis.* Furthermore, a protein that is inducible by different stress conditions and that cross-reacts with a *B. subtilis* anti-SigB antibody is present in *B. licheniformis*, suggesting that the general stress system of *B. licheniformis* is very similar to that of *B. subtilis* [63]. On the other hand, *B. licheniformis* revealed no general stress response after nutrient limitation situations [30, 64], which can be explained by the missing *rsbPQ* operon in the genome of *B. licheniformis* [9, 25]. However, in response to environmental stresses *B. licheniformis* showed the induction of genes belonging to its putative σ^B-dependent general stress response [65-67].

In this study, we were able to further characterize the σ^B regulon of *B. licheniformis* by comparative genomics and the construction and investigation of a *B. licheniformis* Δ*sigB* mutant [66]. Thereby we found several genes regulated by σ^B that are also part of the general stress response in *B. subtilis*, encoding e.g. general stress proteins GspA or YdaG, catalase KatE or σ^B itself. However, among the general stress response in *B. licheniformis* are a number of genes without homologous genes in *B. subtilis* and therefore specific for the general stress response of *B. licheniformis*, e.g. *mcrA* encoding a protein with endonuclease activity, *BLi00576* encoding a fatty acid desaturase or *BLi02212* encoding a Na^+/solute symporter. Many of these genes encode still hypothetical proteins (e.g. *BLi03885* or *BLi01417*). Besides this, there are a lot of genes being σ^B-dependent in *B. subtilis* that are either not σ^B-dependent or under dual control by other regulators in *B. licheniformis*. Due to our study the σ^B regulon of *B. licheniformis* could be described and defined in more detail and enables a better distinction between general and specific stress response, which is important for the definition of stress specific marker genes [66].

Besides the general stress response that we could observe during all our environmental stress experiments there is always a stress specific response towards a stimulus. In

B. subtilis genes of the e.g. HrcA-, CtsR-, CssR regulon are induced specifically in response to heat stress.

The HrcA regulon of *B. subtilis* consists of the heptacistronic *dnaK* operon and the *groEL-groES* operon [40, 53]. These operons encode the major chaperones of the cytosol that prevent protein misfolding and aggregation by stabilization of unfolded or partially folded proteins [68, 69]. Both operons are under negative control of the transcriptional repressor HrcA, encoded by the first gene of the *dnaK* operon. HrcA interacts with a perfect inverted repeat of 9 bp separated by a 9-bp spacer, named CIRCE element (Controlling Inverted Repeat of Chaperone Expression) that precedes both operons [70, 71]. Expression of the HrcA regulon is induced by the presence of denatured proteins in the cytosol and it was postulated that the GroESL system is required to maintain the repressor in its active state [53, 72]. During heat the GroESL system is titrated by non-native proteins, and the repressor is inactivated and dissociates from the promoters of the target genes allowing transient induction of transcription [53]. Nielsen et al. [73] detected CIRCE elements in *B. licheniformis* in front of the *dnaK* and *groEL-groES* operons, observed heat induction of them and revealed an upregulation of the HrcA regulated genes at 37°C in a *ΔhrcA* mutant, thus confirming repression of this operon by HrcA. In the study presented here, heat-dependent induction of the HrcA regulon was observed at the proteome level as well as the transcriptome level for *B. licheniformis* in a *subtilis*-like manner [66].

The CtsR regulon in *B. subtilis* includes the two single genes *clpP* and *clpE* and the tetracistronic *clpC* operon. These genes encode the ATPase subunits ClpC and ClpE, the protease subunit ClpP, the global repressor CtsR with its modulators of activity McsA and McsB. CtsR contains an helix-turn-helix motif and controls the expression of *clpP*, *clpE* and the *clpC* operon by binding specifically to a direct heptanucleotide repeat in their promoter region [74, 75]. Upon heat exposure the regulon is expressed due to a transient, temperature-dependent inactivation of the repressor CtsR, which is an intrinsic thermosensor [76]. Thus, the Clp protease system is upregulated to deal with heat-affected proteins [77-79]. Further regulation at the posttranscriptional and posttranslational level results in an accurate fine-tuning of the amount of the different Clp proteins in the cell depending on growth phase and conditions [80]. For

B. licheniformis, CtsR repression of the regulon could be confirmed based on a *ΔctsR* mutant strain that showed increased expression of the target genes at 37°C in comparison to the wild type strain [73]. Our analysis of *B. licheniformis* corroborates heat induction of the CtsR regulon by strong induction of the CtsR regulated genes and corresponding proteins during heat stress [66].

While the HrcA and CtsR regulons are negatively controlled by two different transcriptional repressors and the σ^B regulon is regulated by this alternative sigma factor, the *htpG* gene represents an own "class" of heat shock genes which is under positive control of an unidentified regulator. The *htpG* gene encodes a protein with chaperone function [81] and was upregulated during heat stress in our study, as it is in *B. subtilis* [40, 66, 81]. Likewise positively regulated by heat stress is the CssRS two-component system with its members *htrA* and *htrB*, encoding putative membrane-anchored proteases and the system itself [82]. This system also responds to secretion stress [82-84]. CssS is a typical sensing kinase and is induced in response to high-level production of homologous or heterologous proteins and by heat stress. Translocation of secreted proteins is required for induction and the response to an activating signal is amplified by positive autoregulation, leading to increased CssRS expression and expression of the chaperones-proteases HtrA and HtrB, that refold or degrade misfolded proteins within the cell envelope [82, 84]. Transcription of the genes *htrA* and *htrB* is upregulated in *B. subtilis* when cells are subjected to a heat shock [40, 53] and could be confirmed for *B. licheniformis* by our study and Nielsen *et al*. [66, 73].

There are a lot of genes induced during heat stress both in *B. subtilis* and *B. licheniformis*, that do not belong to one of the regulons previously discussed, e.g. genes of the σ^I regulon [66, 85]. *B. subtilis* as well as *B. licheniformis* experience a mild oxidative stress response during heat stress, indicated by an induction of genes belonging to the Spx and/or PerR regulon [66, 86]. Furthermore there are genes in *B. licheniformis* that were - or were not - expressed under heat stress, unlike in *B. subtilis*. Various genes belonging to the regulons of ECF type sigma factors were induced in *B. licheniformis* during heat stress, as well as a high affinity phosphate transport system (*pstA*-*S*) or genes encoding tryptophan synthesis enzymes (*trpA*-*F*) [66]. On the other hand, genes belonging to the heat shock stimulon of *B. subtilis* were not

induced in *B. licheniformis*, e.g. the genes *clpX* or *lonA* encoding parts of ATP-dependent proteases [53, 80].

The heat shock stimulon of *B. subtilis* is the largest characterized for this organism with more than 200 heat shock genes described in different studies [40, 53, 87]. In *B. licheniformis* a similar situation became apparent. Some of the regulons involved in the heat shock response also function in other starvation or environmental stress responses and therefore are not solely part of the heat shock stimulon, as described below [66].

OXIDATIVE STRESS AND THE REACTION OF *BACILLI* TO HYDROGEN PEROXIDE

Aerobe living organisms use molecular oxygen (O_2) for respiration or oxidation of nutrients to obtain energy. However, through the use of oxygen reactive by-products can be formed, so-called reactive oxygen species (ROS) [88-90]. ROS comprise superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH·) and in addition ozone (O_3) and singlet oxygen ($^1O_2$) [91, 92]. Superoxide, hydrogen peroxide and the hydroxyl radical are successive one-electron-reduction products of molecular oxygen. Superoxide and hydrogen peroxide are inadvertently produced by enzymes, which usually are capable of univalent electron transfer [93]. Thus, enzymes of electron transport chains are common producers of ROS [90, 94, 95]. Superoxide dismutase, a ubiquitous enzyme among aerobic organisms, is able to convert superoxide into hydrogen peroxide, and this can be detoxified by catalase or peroxiredoxins. The hydroxyl radical is generated from hydrogen peroxide in the Fenton reaction or from hydrogen peroxide and superoxide in the Haber-Weiss reaction. It is highly reactive and cannot be eliminated by an enzymatic reaction (Farr, 1991; Kehrer, 2000}. Singlet oxygen can be formed chemically or in photosynthetic systems [96, 97] and ozone can be generated by UV-light in the atmosphere. If ROS increase intracellularly to a level that exceeds the cells defense capacity, the cellular redox status becomes imbalanced and oxidative stress emerges [91].

All cellular macromolecules can be attacked by ROS such as nucleic acids, proteins or lipids. In nucleic acids, ROS can attack both the bases and the sugar-phosphate backbone of the DNA, breeding strand breaks or the release or oxidation of bases. That in turn can produce 8-hydroxyguanine, urea, hydroxymethyl urea or thymine glycol [98, 99]. Alterations like strand breaks and other lesions that block replication or transcription are likely to contribute more towards cell death than base damage does. Base damages do not hinder the work of polymerases, but they may contribute significantly to mutagenesis [91]. During oxidative stress in lipids primarily poly unsaturated fatty acids are attacked by ROS, resulting in lipid peroxidation and thereby lipid peroxyradicals or hydroperoxydes [100]. The end products of lipid peroxidation include alkanes, ketones, epoxides and aldehydes, what can do further damage to macromolecules e.g. by

interaction with proteins or DNA [91, 101]. In addition, lipid peroxidation can lead to an increased fluidity of the cell membrane and thereby to a loss of structural integrity, which is required e.g. for transport of most nutrients or F_1/F_0 ATPase activity [91]. However, since bacteria mainly assemble mono unsaturated and saturated fatty acids into their membranes, lipid peroxidation seems to play a minor role in bacteria [102]. When proteins are exposed to ROS, the peptide backbone or amino acid side chains of proteins can be differently oxidized. Oxidation of the peptide backbone results in fragmentation of proteins due to back bone cleavage [103, 104]. Furthermore, preferentially sulfur containing and aromatic amino acids are attacked by ROS, e.g. oxidation of methionine and cysteinyl residues form methionine sulfoxide derivatives and disulfide derivatives, respectively [104]. Fe-S clusters can be oxidized by ROS accompanied by the release of free iron [93, 102]. Due to the fact that ROS attack reactive metal centers of redox-active proteins, several enzymes are affected and functionally altered by oxidative stress, as for example dehydratases functioning in aromatic and branched-chain amino acid biosynthesis or the fumarase [105]. Through modifications of amino acids the protein structure is altered, what can lead to targeted proteolytic degradation of the affected proteins [91, 104].

As oxidative stress means a severe impairment of all physiology, cells have developed different strategies to defend themselves against it. One opportunity is (i) the detoxification of the ROS, accomplished by cellular enzymes, such as catalase, superoxide dismutase or alkylhydroperoxide reductase. These enzymes are able to convert ROS into nontoxic metabolites. A second possibility is (ii) the protection of macromolecules, warranted e.g. by DNA-protecting proteins that form biocrystalline complexes with DNA and secure it from ROS [106]. Thiols can be secured from ROS by S-thiolations; where low molecular weight thiols form disulfide bonds to protein thiols, thereby preventing irreversible oxidation of the protein thiols by ROS [107, 108]. In addition, (iii) the repair or removal of damaged molecules is used in defense to ROS [109, 110]. The induction of genes involved in the oxidative stress response is controlled by different regulators which react upon oxidative modification of their amino acid residues.

In low GC, Gram-positive bacteria like *Bacilli* the peroxide stress response is primarily regulated by the repressor PerR. PerR is a dimeric Fur-family repressor that controls the transcription of its regulated genes [111]. Due to peroxide stress a histidine residue in the PerR protein is oxidized to 2-oxohistidine, accompanied by conformational changes and resulting in derepression of the regulon [112]. In *B. subtilis*, the PerR regulon comprises genes encoding catalase (*katA*), alkylhydroperoxide reductase (*ahpCF*), DNA-binding stress protein (*mrgA*), heme biosynthesis (*hemAXCDBL*), regulators (*perR*, *spx* and *fur*) and zinc uptake (*zosA*) [113-115]. In *B. licheniformis* and *B. pumilus*, the PerR proteins of both species show high similarity to the *subtilis*-like PerR (more than 90% identity, respectively). In our analysis of *B. licheniformis* the previously mentioned PerR regulated genes were all induced after peroxide treatment, indicating a similar regulon structure as it is known in *B. subtilis*. In addition, we were able to extend the *B. licheniformis* PerR regulon, as we found the two genes *perR2* (formerly *BLi04114*; transcriptional Fur-family regulator) and *hemH2* (*BLi04115*; Ferrochelatase 2) in an operon with *katA* (catalase), regulated by PerR [65]. On the other hand, the PerR regulon of *B. pumilus* differs from the *B. subtilis* or *B. licheniformis* PerR regulon. Whereas the genes *spx*, *fur*, *zosA* or *hemAXCDBL* showed increased induction rates during peroxide treatment, the genes *katA*, *mrgA* or *ahpCF* are completely missing in the genome of *B. pumilus*. These are genes that exhibit very high induction rates both in *B. subtilis* or *B. licheniformis* cells subjected to hydrogen peroxide. Furthermore, *B. pumilus* has no other gene annotated as alkylhydroperoxide reductase. Instead of catalase KatA, a gene annotated as catalase KatX2 (53% sequence similarity to *B. subtilis* KatX, major spore catalase) was very high induced in peroxide stressed *B. pumilus* cells and is a potential member of the *B. pumilus* PerR regulon [32]. Another repressor involved in the oxidative stress response of *Bacilli* is OhrR. In *B. subtilis* it represses the thiol-dependant peroxidase OhrA [116], but not the ortholog OhrB, which is σ^B regulated [115]. The redox active cysteine of OhrR is initially oxidized to a sulfenic acid and can subsequently react to an S-thiolation, sulfonamide, sulfinic and sulfonic acid in response to organic peroxides [116-119]. Whereas no increased expression of the *ohrA* gene was observed in H_2O_2 stressed *B. subtilis* or *B. licheniformis* cells [65, 120], in our study of *B. pumilus* we noticed a strong induction of this gene in response to H_2O_2, indicating an involvement of this peroxiredoxin in the H_2O_2 resistance in *B. pumilus* [32]. In *B. subtilis*,

the cellular concentration of the regulator Spx is tightly maintained at a low level under reducing conditions by ClpXP-catalyzed proteolysis [121]. The redox state of the cytoplasm is the major effector driving Spx activation. Spx contains a CXXC-motif at its N-terminus, which forms an intramolecular disulfide bond after oxidation [122 2005]. Under oxidative, prevailing disulfide stress, both redox-sensitive PerR and YodB negative regulators of Spx are inactivated [123] and Spx can interact with the C-terminal domain of the α-subunit of the RNA-Polymerase [124]b. The Spx regulon is composed of genes whose product function in thiol homeostasis (thioredoxin TrxA, thioredoxin reductase TrxB), detoxification (thiolperoxidase Tpx, reductase NfrA) or cysteine synthesis (*yrrT* operon, *cysK*), required in the maintenance of the cellular redox balance [125-127]. In *B. licheniformis* and *B. pumilus* genes of the respective Spx regulon were upregulated after H_2O_2 treatment, but with rather moderately increased expression rates [32, 65]. ROS exhibit a high DNA damaging potential. Upon damage of DNA the bacterial SOS response is induced, which allows a cell first to repair the damaged DNA and only after that continue with the cell cycle [128, 129]. The SOS response is regulated by RecA, a protein that recognizes single-strand DNA regions and the repressor LexA. During normal growth, LexA binds to a 20-bp consensus sequence (SOS-box) in the operator region of the SOS specific genes, thereby blocking their transcription. Upon binding of RecA to single-stranded DNA LexA is inactivated leading to the induction of the SOS regulon [130]. The SOS regulon consists of different enzymes function in DNA repair, e.g. the excinuclease UvrABC or the holliday junction helicase RuvAB [131]. Furthermore, the cell division is suppressed in order to permit DNA repair prior to formation of new bacterial generations [132]. In our analyses of hydrogen peroxide stresses *B. licheniformis* and *B. pumilus* cells the SOS response was clearly activated [32, 65], as it was shown for *B. subtilis* elsewhere [120]. ROS also have an impact on the protein quality of the cells. In Gram-positive bacteria protein quality control is exerted amongst others by the global heat shock repressor CtsR, which is activated not only by heat, but also by other environmental stresses. The CtsR regulon comprises chaperones or proteases functioning in the rescue or degradation of misfolded proteins (e.g.ClpC, ClpE, ClpP). During different thiol modulating stresses the CtsR regulon is induced by a different mechanism compared to heat shock in *B. subtilis* [109, 120, 133]. The CtsR regulon of *B. licheniformis* and *B. pumilus* were also induced during peroxide stress [32,

65]. Besides induction of operons more or less directly associated to oxidative stress described above, H_2O_2 treated *Bacilli* exhibited a general stress response [32, 65, 120]. In *B. subtilis*, genes encoding catalases KatX or KatE or the DNA-binding protein Dps are under control of σ^B [45-47], as it is for *B. licheniformis* [66]. As mentioned before, the catalase KatX shows very high expression rates during peroxide treatment, simultanous with the absence of catalase KatA in the genome of *B. pumilus*. Another gene strongly induced in *B. pumilus* under these conditions is *dps*. Dps is a paralog of the DNA-protecting protein MrgA, which is missing in the *B. pumilus* genome as well [32].

In this study we were able to detect other genes upregulated during peroxide stress, either in *B. licheniformis* or *B. pumilus*. In *B. licheniformis* the glyoxylate bypass genes *aceA* and *aceB* (formerly *BLi04207* and *BLi04208*) were heavily induced during peroxide treatment. *B. subtilis* and *B. pumilus* lack the glyoxylate cycle. In contrast *E. coli* induces the glyoxylate shunt during oxidative stress. In parallel, the isocitrate dehydrogenase in *E. coli* can be inactivated by oxidation, accompanied by the fragmentation of the protein and thereby a breakdown of the tricarboxylic acid (TCA) cycle. This redirects the metabolic flux into *E. coli* 's glyoxylate cycle, which supplies malate and NADPH for biosynthetic and regenerating reactions [134, 135]. In our study of *B. licheniformis* we confirmed our transcriptomic results with a metabolomic analysis of the TCA cycle and glyoxylate cycle metabolites. The results supported the idea of an oxidatively damaged isocitrate dehydrogenase and an activated glyoxylate bypass during peroxide stress in *B. licheniformis*.

Bacillithiol (BSH; Cys-GlcN-mal) is a low molecular weight thiol that was identified recently in e.g. in *Bacillus* species, *Staphylococcus aureus* or *Deinococcus radiodurans* [136, 137]. It takes over a similar role as Glutathione (GSH; -L-glutamyl-L-cysteinylglycine), that is the most prominent redox-buffer in many eukaryotes and Gram-negative bacteria. In our study of peroxide stressed *B. licheniformis* cells, we could not detect any upregulation of the genes for Bacillithiol synthesis. However, we were able to observe a distinct upregulation of these genes in *B. pumilus* indicating an involvement of Bacillithiol in the H_2O_2 resistance of that organism. These findings were confirmed by a metabolomics approach concerning the concentration of the cytosolic

thiol compounds. The metabolome analysis showed at least a doubling of the intracellular Bacillithiol concentration after peroxide treatment in *B. pumilus* cells [32].

The peroxide stress response of *B. licheniformis* seems to be quite similar to that of the model organism *B. subtilis*, with the addition of *B. licheniformis* -specific peroxide stress genes and the involvement of the glyoxylate cycle during oxidative stress. The peroxide stress response of *B. pumilus* differs from that, because of its reduced PerR regulon, the lack of alkylhydroperoxide reductases and the strong induction of genes, which were much less induced in *B. subtilis* and *B. licheniformis* [32, 65].

SALT STRESS AND THE OSMOTIC STRESS RESPONSE OF *BACILLI*

The availability of water is indispensable for the survival of cells. An important challenge *Bacilli* have to cope with, both in their natural habitats and in industrial processes, are increases in the external salinity; also referred to as salt stress or osmotic stress. As common soil bacteria, *Bacilli* undergo fluctuations in the water availability of their natural habitat, due to frequent drying or flooding of the soil. This causes sudden changes in the concentrations of salts and osmolarity around the cell. As the bacterial cell wall allows unselective passing of macromolecules, the cytoplasmic membrane represents the most important boundary between cell and environment. This is not permeable for macromolecules or ions, but for water. If the environment outside of the cell evolves a hyper-osmotic character, e. g. by an increased salt concentration, the cell is endangered by the loss of water to the environment, due to diffusion along the osmotic gradient and thereby a loss of the positive cell pressure (turgor) [138]. However, the maintenance of the turgor is essential for survival and growth of cells and therefore effective water management a necessity [139, 140].

Comprehensive studies of *B. subtilis* have shown that salt stress is one of the strongest inducers of the general stress response [141, 142], and members of the σ^B regulon contribute to stress resistance when the cells are exposed to osmotic shocks [143, 144]. But due to the transient nature of the induction of the σ^B regulon in response to acute salt stress, the general stress response system is not crucial for the ability of *B. subtilis* to survive under high salinity growth conditions, but rather a specific stress response that regulates changes in the water economy of the cell [138, 140, 142, 145].

The initial response to a hyper-osmotic surrounding coupled with osmotic stress is the uptake of potassium ions (K^+) to counteract the outflow of water [146]. In *Bacilli*, this uptake is mediated by the potassium transport systems KtrAB and KtrCD [147]. The second step of osmotic adaptation is the accumulation of compatible solutes thereby permitting a reduction in the cellular potassium level [140, 146]. Compatible solutes are osmoprotective substances that comprehensively occur in all three domains of life (bacteria, archea and eukaryotes) [148-150]. Compatible solutes comprise classes of sugars and polyols, amino acids and their derivates, trimethyl ammonium compounds,

methyl sulfonium compounds and sulphate esters. Some examples of them are shown exemplarily in Figure 3. Usually, compatible solutes are water-soluble, polar, unable to cross cell membranes rapidly without transport systems and most of them are not charged at a pH around 7. Besides their ability to hydrate the cell, compatible solutes can act as chemical chaperones, stabilizing proteins or other cell components against denaturing effects of high ionic strength [151-153]. In a hyper osmotic environment, the cell is able to synthesize large amounts of compatible solutes, but with external availability the uptake of osmoprotectants is preferred, due to rapidness and energy efficiency [154]. The uptake from the environment is carried out by osmotically regulated transport systems, in *Bacilli* named Opu-transporter ("osmoprotectant uptake").

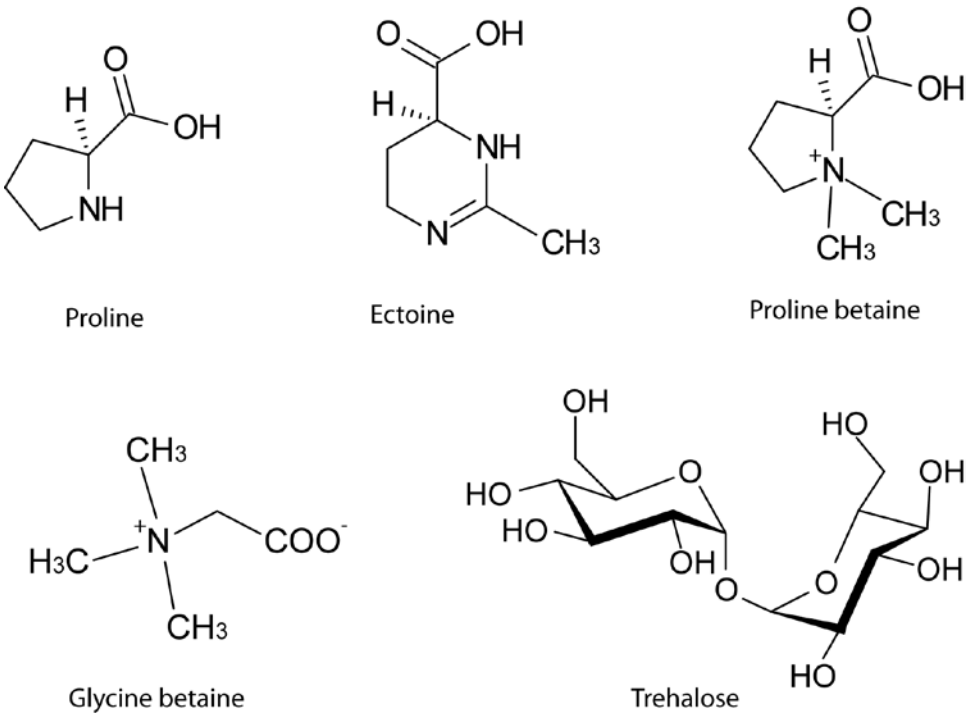

Figure 3: Structures of selected osmoprotectants.

In our analysis of salt-stressed *B. licheniformis* cells, we found no significant changes in the transcriptional expression of potassium transporter genes (e.g. *ktrA*, *ktrB*, *ktrC*, *ktrD*, *yubG*) [67]. This is consistent with investigations of *B. subtilis*, where neither the

potassium concentration of the environment, nor the salt concentration of the medium influences expression of the *ktr*-transcripts [147].

B. subtilis possesses five Opu-transport systems [155-158]. OpuA, OpuB and OpuC are multi-component ABC-type transporter, whereas the substrate specificity is comparatively low for OpuA and especially for OpuC. A broad spectrum of osmoprotectants is transported by this system (see Figure 4). On the other hand, OpuB is a highly specific transporter solely for the osmoprotectant choline. OpuD and OpuE are single component transporters with high substrate specificity, for glycine betaine or proline, respectively. *B. licheniformis* completely lacks the OpuB transporter for the transport of choline, but the expression rates of the other Opu-transporter genes were highly induced during salt stress [67]. In addition, other genes encoding transport systems of more or less known functions were induced in salt-stressed *B. licheniformis* cells. Among them, the two genes *BLi03671* (putative ABC transporter) and *BLi03672* (transmembrane protein) showed heavy induction rates. A protein sequence comparison (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed a high similarity with a not further characterized transport system of *B. pumilus*, not existing in *B. subtilis*. Besides induction through salt stress, ECF sigma factor independent induction of these two genes was shown in *B. licheniformis* by treatment with vancomycin [159].

In addition to the uptake of compatible solutes, cells enable the possibility to synthesize them *de novo* or by transformation of precursors; primarily proline and glycine betaine [146, 148]. During physiological growth conditions, synthesis of the amino acid proline for anabolic purposes is carried out by the enzymes ProBAI in *B. subtilis*. The corresponding genes are induced in response to proline limitation via a T-box regulatory mechanism [160]. On the other hand, the synthesis of proline as an osmoprotectant is mediated by the enzymes ProJAH. In *B. subtilis* expression of the *proHJ* genes is induced during increased external salinity but *proA* is not [161]. The γ-glutamyl phosphate reductase ProA represents an interface between both proline synthesis pathways in *B. subtilis*, and disruption of the gene leads to abolishment of the osmoadaptive proline synthesis and also to proline auxotrophy [161]. The genetic composition, together with our analysis of *B. licheniformis*, displayed a different situation for this organism compared to *B. subtilis*. In *B. licheniformis*, there are two homologous genes named

proA1 and *proA2*, encoding each a γ-glutamyl phosphate reductase. ProA1 takes part in the anabolic pathway of proline synthesis. The gene *proA2* is part of the *proHB2A2* operon, which is the "genetic block" for the osmoprotective synthesis of proline in *B. licheniformis* [67]. Moreover, in the context of synthetic microbiology this operon provides the opportunity to engineer salt stress resistance in salt-susceptible microorganisms.

Besides Proline, glycine betaine, a trimethylated derivative of the amino acid glycine, is widely used as compatible solute in nature and used by *B. subtilis* under challenging high-osmolarity conditions. Microorganisms can synthesize glycine betaine by one of two different routes: (i) through a stepwise methylation of the amino acid glycine [162, 163] or (ii) through the import and subsequent oxidation of choline [164-166]. In *B. subtilis*, choline itself has no osmoprotective function *per se* [164, 167], but glycine betaine is produced through the uptake of the precursor via the OpuB and OpuC ABC transporters and a subsequent two-step oxidation process by the type III alcohol dehydrogenase GbsB and the glycine betaine aldehyde dehydrogenase GbsA, with glycine betaine aldehyde as intermediate product [164, 168]. An induction of the *gbsAB* operon in response to a salt shock was shown previously [169]. Recent studies revealed that glycine betaine production is regulated by the choline - and not osmotic - sensitive GbsR repressor in *B. subtilis*, controlling expression of the *opuB* and *gbsAB* operons [170]. Apart from the OpuB transporter, *B. licheniformis* possesses homologs of the glycin betaine synthetic genes, hypothesizing a similar regulation. However, our transcriptomic analysis of salt stressed *B. licheniformis* cells showed increased expression of the genes *gbsAB*, in spite of a lack of choline in our growth medium.

Thereupon we performed a metabolomic analysis to check a possible different regulation. Therefore, *B. licheniformis* cells were cultivated in (i) synthetic medium, containing added (ii) choline, (iii) NaCl or (iv) both. The metabolomic analysis revealed that external provided choline plays no significant role under normal growth conditions, but with its external availableness under salt stress conditions choline is taken up into the cell and subsequently used for betaine synthesis. Salt stressed cells grown in media without choline showed no changes in the betaine levels compared to unstressed cells, but increased amounts of proline and glutamate. On the other hand, salt stressed cells

grown in choline-containing medium showed control level proline concentrations. This argues for a *B. subtilis* -like GbsR regulation in *B. licheniformis* with choline dependency. These findings also confirm the preference of uptake compared to synthesis of compatible solutes [67, 154, 170].

As expected from investigations of *B. subtilis* [141, 142], *B. licheniformis* also showed a strong induction of the general stress response after salt stress [67]. In addition to the σ^B regulon, *Bacilli* possess sigma factors with extracytoplasmic function (ECF sigma factors), which specifically govern the physiological response to cell envelope stress [171]. Among *B. subtilis'* seven ECF sigma factor regulons [171], three are affected by a sudden increase in the external osmolarity in *B. subtilis*; σ^M, σ^W and σ^X [141, 169, 172]. Due to the high similarity of the consensus promoter sequences and diverse overlaps between the members of the four ECF sigma factor regulons σ^M, σ^V, σ^W and σ^X it is hard to distinguish between them [173-175]. In salt stressed *B. licheniformis* cells, only genes of the two ECF regulons σ^W and σ^X showed increased induction rates [67]. We did not find any σ^M-dependent gene induced in *B. licheniformis* after salt stress, whereas the σ^M regulon in *B. subtilis* is known to be essential for prolonged growth and survival in a high salt containing environment [172].

The other way round, during low osmotic conditions these osmoprotectants are a threat to the integrity of cell. Despite the existence of aquaporins in many microorganisms, no bacterial cell can actively pump water across the cytoplasmic membrane to compensate for water influx or efflux [140, 176, 177]. During an osmotic down-shock, water-attracting ions and compatible solutes are therefore rapidly released through mechanosensitive channels (e.g. MscL and MscS). This is important to curb the inflow of water and to prevent an undue increase in turgor [178-181].

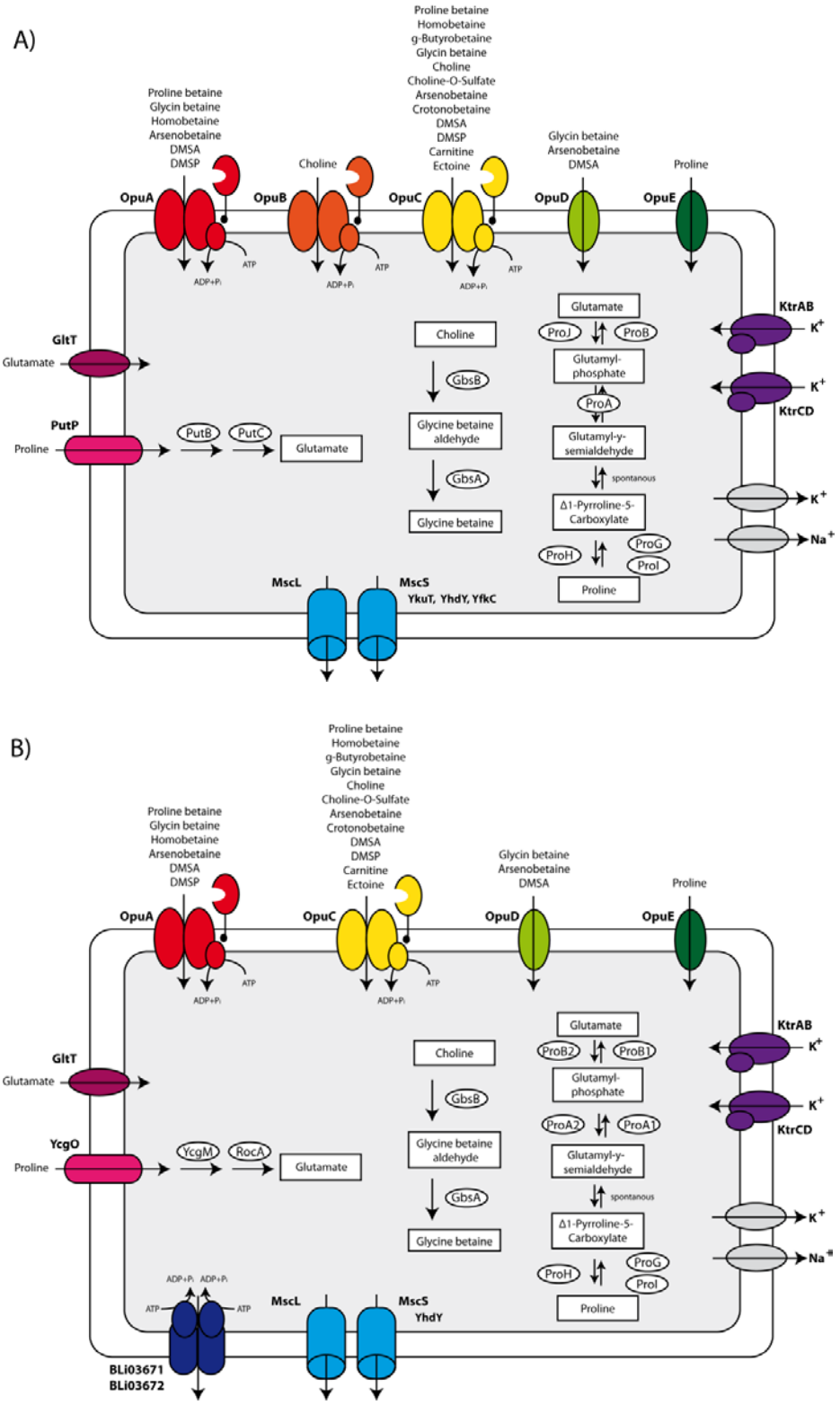

Figure 4. Schematic overview of the osmotic stress response of A) *B. subtilis* and B) *B. licheniformis*. Uptake and/or synthesis of potassium and compatible solutes like proline or glycine betaine is carried out via the Opu-transporters or, in case of glycine betaine and proline, synthesis within the cell. Modified from [67, 138].

CONCLUDING REMARKS

In this dissertation a comprehensive dataset on global gene expression patterns during selected physical stress situations of the industrially relevant bacterium *B. licheniformis* could be established. Furthermore, the stress response against hydrogen peroxide was analyzed in both, *B. licheniformis* and *B. pumilus*. By this approach specific marker genes for the investigated stresses could be identified. In addition, individual differences of the stress adaptation of *B. licheniformis* and *B. pumilus* in comparison to the Gram-positive model organism *B. subtilis* could be revealed. This supports the necessity of analyzing the stress response of each organism of interest rather than relying on the information derived from model organisms in the specific family.

In response to high osmotic conditions specific Opu-transporters were induced in *B. licheniformis*, as well as other transport systems of so far unknown functions, like e.g. the system BLi03671/03672. Also the proline synthesis specific for salt stress was induced. Other genes could be detected, which were not specific for salt stress but rather general stress genes, like the SigB regulon or genes controlled by ECF sigma factors.

For peroxide stress in *B. licheniformis* and *B. pumilus* significant differences were determined also in respect to *B. subtilis*. In *B. licheniformis* the induction of the glyoxylate shunt was detected during oxidative stress and also the PerR-response in this organism seemed to be extended compared to *B. subtilis*. On the other hand, parts of the PerR regulon are missing in *B. pumilus*, but other specific genes like *katX* or *dps* are strongly upregulated. Furthermore, known regulons like the Spx, CtsR or SOS regulon were induced in both organisms.

B. licheniformis responds to heat by the induction of many different regulons. Some of them are not specific to this stress, like the general stress response or the CtsR regulon. In general these regulons do not differ significantly from the response of *B. subtilis*. However, the heat stress response is mainly conducted by chaperones and proteases that also do not differ in many eukaryotic organisms.

The ascertained stress specific marker genes can further be utilized to develop assays that allow an improved monitoring of critical stress situations during fermentation processes in order to be able immediately adjust fermentations to ensure optimal

production conditions. Thus, this study provides fundamental information for the improvement of the fermentation processes for both organisms analyzed.

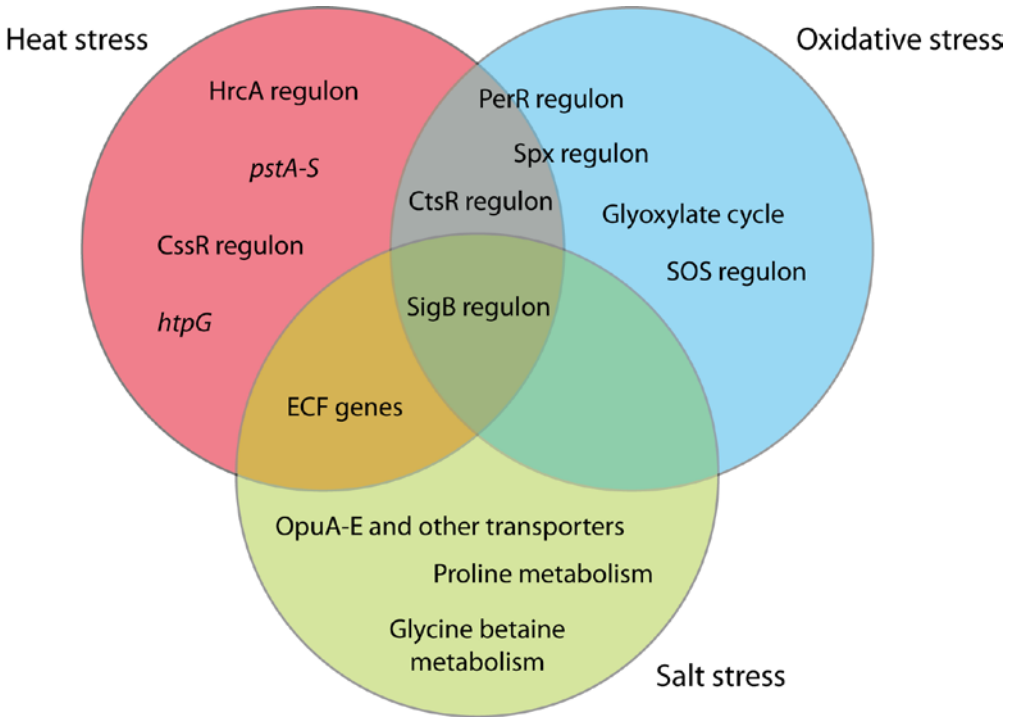

Figure 5. VENN-Diagram of genes and regulons belonging to either the heat stress (red), the oxidative stress (blue) or the salt stress (green) response of *B. licheniformis*. Inside the intersection of the circles (overlapping areas) regulons / genes are listed that belong to at least two - or all - stress responses investigated in this study (purple, turquois, orange, brown areas) [65-67].

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THE PEROXIDE STRESS RESPONSE OF *BACILLUS LICHENIFORMIS*

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Author's contribution

The experimental design was developed by RS, BV, BJ, KM, DCP, ML, MH and TS. Bacterial cultivations were performed by RS and BV. RNA isolations, microarray experiments, Northern Blots and transcriptome analysis were carried out by RS. 2D-PAGE and protein analysis was done by BV. Thiol modification assays were carried out by HS in assistance with DCP. Mass spectrometry analysis was done by DA. Metabolite extractions were performed by RS and KM. GC-MS and NMR analysis of metabolites were done by KM. Comparative genomics was performed by TM. The manuscript was written by all authors.

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RESEARCH ARTICLE

The peroxide stress response of *Bacillus licheniformis*

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The oxidative stress response of *Bacillus licheniformis* after treatment with hydrogen peroxide was investigated at the transcriptome, proteome and metabolome levels. In this comprehensive study, 84 proteins and 467 transcripts were found to be up or downregulated in response to the stressor. Among the upregulated genes were many that are known to have important functions in the oxidative stress response of other organisms, such as catalase, alkylhydroperoxide reductase or the thioredoxin system. Many of these genes could be grouped into putative regulons by genomic mining. The occurrence of oxidative damage to proteins was analyzed by a 2-DE-based approach. In addition, we report the induction of genes with hitherto unknown functions, which may be important for the specific oxidative stress response of *B. licheniformis*. The genes *BLi04114* and *BLi04115*, that are located adjacent to the catalase gene, were massively induced during peroxide stress. Furthermore, the genes *BLi04207* and *BLi04208*, which encode proteins homologous to glyoxylate cycle enzymes, were also induced by peroxide. Metabolomic analyses support the induction of the glyoxylate cycle during oxidative stress in *B. licheniformis*.

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1 Introduction

Bacillus licheniformis, a widespread soil bacterium, is capable of producing and secreting a high yield of numerous hydrolytic enzymes, which renders this organism an interesting host for industrial purposes [1–3]. However, little is known about the physiology and stress responses of *B. licheniformis* compared with its close relative *Bacillus subtilis*. During the fermentation process a variety of stresses could impair the fitness of the host organism and the quality of the fermentation product, as was previously described [4–7].

ROS, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$) or the hydroxyl radical ($^{\bullet}OH$) occur during incomplete electron transfer in all aerobic living organisms [7–9]. Increased ROS production that exceeds the capacities of cellular defense systems leads to oxidative stress in the cell and to the oxidation of proteins, lipids and nucleic acids [6, 7, 10–12].

Cellular defense systems can be divided into three groups: (i) detoxification of harmful agents (e.g. KatA or SodA), (ii) protection of macromolecules (Dps or MrgA) and (iii) repair or removal of damaged molecules (AhpCF, TrxAB). These systems are regulated by specific transcriptional regulators, such as SigB, LexA/RecA, Spx, OhrR and PerR in *B. subtilis*, which have been described in detail [7, 13–20].

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Abbreviation: TCA, tricarboxylic acid

Colour Online: See the article online to view Fig. 4 in colour.

To understand the oxidative stress response of *B. licheniformis* we performed a comprehensive analysis of the transcriptome and of the proteome during peroxide stress. Our results indicate that the oxidative stress response of *B. licheniformis* is quite similar to that of *B. subtilis*. However, there are distinct differences between the two organisms. Several proteins/genes involved in the oxidative stress response are present in one but not in the other organism and the transcription pattern of genes differs between both organisms. The results we present here will allow deeper insights into the stress physiology of *B. licheniformis* and will therefore provide regulation patterns and biomarkers that can be used for efficient monitoring of industrial *B. licheniformis* fermentation processes.

2 Materials and methods

2.1 Strain, media and growth conditions

All experiments were performed using the strain *B. licheniformis* DSM13 (equivalent to ATCC 14580, type strain from the German Collection of Microorganisms and Cell Cultures, DSMZ). Cells were cultivated in synthetic Belitzky minimal medium [21] with 0.2% w/v glucose. Growth was monitored by measuring the optical density at 500 nm ($OD_{500\,nm}$). *B. licheniformis* from overnight cultures was used to inoculate pre-warmed growth medium to obtain a starting $OD_{500\,nm}$ of 0.04. Cultures were routinely grown in 500 mL Erlenmeyer flasks in a shaking water bath at 180 rpm and 37°C.

2.2 Exposure to stress and cell sampling

Oxidative stress was provoked by the addition of H_2O_2 to a final concentration of 50 μM to exponentially growing cells at an $OD_{500\,nm}$ of 0.4. For survival tests, samples from bacterial cultivations were diluted and 100 μL were plated onto LB agar plates. After overnight incubation at 37°C colony-forming units were counted and subsequently the weighted arithmetic mean was determined. Samples for RNA extraction were taken from unstressed cultures before (control) and 5, 10 and 20 min after addition of H_2O_2. Cell samples for RNA extraction were mixed with 0.5 volumes of ice-cold killing buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM NaN_3), and immediately harvested at 10 000 × *g* for 5 min at 4°C. For the preparation of cytoplasmic proteins, bacteria were pulse-labeled with 556 Bq/mL L-[^{35}S]-methionine for 5 min, as described by Hoi et al. [22]. Samples were labeled during exponential growth ($OD_{500\,nm}$ 0.4, control), and 10 and 30 min after addition of H_2O_2. For preparative gels, unlabeled protein samples were prepared using cells treated with H_2O_2 for 30 and 60 min as described by Hoi et al. [22]. For the analysis of oxidative damage to extracellular proteins, cells were grown 1 h into stationary growth phase and then stressed with 300 μM hydrogen peroxide for 30 min. Cells were removed by centrifugation and extracellular proteins were precipitated using trichloroacetic acid as described before [1]. A fluorescence thiol modification assay with extracellular proteins was performed according to Hochgräfe et al. [23]. Samples for the metabolome analysis were harvested from unstressed cultures before (control) and 10, 30, 60 and 180 min after addition of peroxide by filtration, as described by Meyer et al. [24]. Samples for the different analyses were taken at different time points to reflect the successive synthesis and half-life of these molecules.

2.3 2-DE, protein identification and imaging

Protein extracts (80 μg protein for radiolabeled samples, 500 μg for preparative gels, 100 μg for fluorescence thiol modification assay gels) were loaded onto commercially available IPG-strips (pH 4–7 for cytosolic proteins, pH 3–10 for extracellular proteins, GE Healthcare) and gel electrophoresis was performed as described previously [25]. Autoradiography of labeled gels and staining of preparative gels was carried out according to Hoi et al. [22]. Following fluorescence detection of the BODIPY FL C_1-IA (Invitrogen) label, thiol modification assay gels were stained with Flamingo fluorescent gel stain (Bio-Rad) according to the instructions of the manufacturer. Induced proteins, cut from the preparative Coomassie-stained gels, and most extracellular proteins were identified by mass spectrometry (MS) as described by Liedert et al. [26]. Repressed proteins and prominent extracellular spots were not identified by MS, but the labels were conveyed from the corresponding *B. licheniformis* master gels. Quantification of protein spots and calculation of synthesis rates were done using the Delta 2D software (Decodon). The labeling experiments were repeated three times and the synthesis rates given in Supporting Information Table S1 were derived from these independent experiments. The thiol modification assay was repeated twice and technical replicates were included in the quantification.

2.4 RNA isolation

Cells were mechanically disrupted by a single run with the RiboLyser (Thermohybaid, Thermo Scientific) and RNA was extracted with the KingFisher mL (Thermo Labsystems), as described previously in detail [27]. The isolated RNA was treated with DNase (RNase-free DNase Set, Qiagen) and subsequently concentrated and cleaned (RNA cleanup and concentration Kit, Norgen Biotek). Quantity of RNA was determined on a microscale spectrophotometer (Nanodrop ND-1000, Peqlab Biotechnologie) and RNA integrity was

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analyzed using a capillary electrophoresis system (Bioanalyser 2100, Agilent Technologies).

2.5 Microarray experiments

Synthesis and purification of fluorescent-labeled cDNA was carried out according to Charbonnier et al. [28] with minor modifications. Aliquots of 10 μg total RNA were mixed with random primers (Promega). For hybridization the control transcript A (for Cy3 hybridization) or alternatively B (for Cy5 hybridization) of the Two-Color RNA Spike in Kit (Agilent Technologies) was added and incubated at 70°C for 10 min. For reverse transcription 10 μL 5 × 1st strand buffer (Invitrogen), 5 μL 0.1 M DTT, 0.5 μL dNTP Mix (10 mM dATP, dGTP and dTTP); 2.5 mM dCTP (Roche Diagnostics), 7 μL nuclease-free water (Ambion), 1.25 μL Cy3-dCTP or Cy5-dCTP (GE Healthcare) and 2 μL Superscript II reverse transcriptase (Invitrogen) were added, incubated for 1 h at 42°C and afterwards for 10 min at 70°C. An aliquot of 1 μL of RNase H (Invitrogen) was added and the mixture was incubated in dark at room temperature for 30 min. The labeled cDNA was purified using the CyScribe GFX Purification Kit (GE Healthcare) as described by the manufacturer. Incorporation efficiency and yield were determined using the Nanodrop ND-1000 (Peqlab Biotechnologie). An aliquot of 0.4 μg of Cy3- and Cy5-labeled cDNA (ad. 19.2 μL), respectively, was denatured for 2 min at 95°C. After that, 4.8 μL pre-warmed blocking agent and 24 μL hybridization buffer (Gene expression hybridization kit, Agilent Technologies) were agitated with the denatured cDNA-mix and 40 μL of the emerging mixture were used for hybridization.

Custom-made *B. licheniformis* DSM13 8x15K gene expression arrays were obtained from Agilent Technologies (https://earray.chem.agilent.com/earray/). Probe design was performed on the annotated open reading frames of *B. licheniformis* DSM13 strain according to Veith et al. [29].

The arrays were hybridized and washed according to the manufacturer's instructions (Two-Color Microarray-based Gene Expression Analysis Protocol, Agilent Technologies), followed by a last washing step with ACN (Carl Roth) for 30 s. Microarrays were scanned using the Agilent scanner Type G2565CA with high-resolution upgrade G2539A and the software Scan Control 8.4.1 (Agilent Technologies).

2.6 Microarray data extraction, processing and analysis

Data were extracted from scanned images using Agilent's Feature Extraction Software (version 10.5.1.1) (Agilent Technologies) using default settings. Gene expression data were loaded into the Rosetta Resolver® Gene Expression Analysis System 7.2. (Rosetta Inpharmatics c/o Ceiba Solutions). A common reference type of design was employed, and data from three biological replicate hybridizations were combined using an error-weighted average. Genes showing significant differences in expression were identified by error-weighted ANOVA analysis, with a Benjamini–Hochberg false discovery rate multiple test correction. Only genes, which were at least threefold induced (fold change above 3) or threefold repressed (fold change below −3) and for which an ANOVA $p<0.01$ was obtained by statistical testing for at least one time point throughout the experiment were considered as differentially expressed and were used for further evaluation.

Cluster analysis was generated with the Rosetta Resolver® Gene Expression Analysis System 7.2, and was performed using an agglomerative hierarchical clustering algorithm and a cosine correlation metric type.

2.7 Northern blot experiments

Northern blot analyses were performed according to the method of Homuth et al. [30], using 1.5% agarose–2.1 M formaldehyde–MOPS gels. Each RNA blot was stained with methylene blue prior to hybridization in order to check the RNA quality and to ensure that equal amounts of RNA were loaded and blotted for each lane. Digoxigenin-labeled RNA probes for *katA*, *BLi04114* and *BLi04115* were obtained by in vitro transcription with T7 RNA polymerase (Roche Diagnostics) from PCR products of the respective genes that were fused to a T7 promoter. The following primers were used for generation of RNA probes, respectively:

katA–for (5′GACAACCAAAACTCAATGAC3′), *katA–revT7* (5′CTAATACGACTCACTATAGGGAGATCATAGTTCCCTTCCTCTG3′), *BLi04114–for* (5′TACGAATTACAGCTCAGCG3′), *BLi04114–revT7* (5′CTAATACGACTCACTATAGGGAGACTTTGAGGTGAGGATAGTC3′), *BLi04115–for* (5′ATATCCGTCATGGAAAACG′3), *BLi04115–revT7* (5′CTAATACGACTCACTATAGGGAGAATTGTATACTTCGGTGCTG3′). The filters were prehybridized, hybridized and washed following Homuth et al. [30]. Northern blots were developed using a digoxigenin-specific antibody conjugated with alkaline phosphatase (Roche Diagnostics) and CDP-Star (Applied Biosystems) as a chemiluminescence substrate. Signal detection and quantitation were done with the ChemoCam HR16 (INTAS). Transcript sizes were determined by comparison with the RNA molecular weight marker I (Roche Diagnostics).

2.8 Analysis of intracellular and extracellular metabolites

Samples for intracellular metabolite analysis were harvested according to the fast filtration approach described by Meyer et al. [24], but without washing due to a decelerated filtration rate. Metabolite extraction was carried out in two steps. Ethanol extraction was done by alternate shaking and vortexing of the filters ten times in the extraction solution (5 mL of 60% (w/v) ethanol, ≤4°C) followed by a centrifu-

gation step (5 min, 8500 rpm, 4°C). The supernatant, containing intracellular metabolites, was transferred into a new Falcon tube. For water extraction, 5 mL of ice-cold water were added to the disrupted filters and the cell pellet, and shaken, vortexed and centrifuged as described above. The aqueous and the ethanolic supernatants from the same sample were combined and used for lyophilization till complete dryness. Intracellular metabolites were analyzed by a modified GC-MS method described earlier [31]. Quantitative analyses were performed using the software package ChemStation (Agilent). Ribitol and D-norvaline were used as internal standard compounds.

For the analysis of extracellular metabolites 2 mL of cell suspension were sterile-filtered and samples were analyzed by NMR, with a method described by Liebeke et al. [31].

2.9 Comparative genomics and regulon mining

Putative regulons involved in the oxidative stress response of *B. licheniformis* were predicted based on the information on target genes and regulator binding sites available for *B. subtilis*. For *in silico* regulon mining, FASTA-formatted lists of known regulator binding sites were generated for LexA, Fur, PerR and CtsR, based on experimentally verified operator sequences extracted from the available literature (see Supporting Information data for details). These lists were used as custom input files to generate Position Weight Matrices to subsequently screen the genome of *B. licheniformis* for putative target genes with the help of the virtual footprint algorithm [32], implemented into the Prodoric database [33] at http://www.prodoric.de/vfp/. In case of SigB, the collection of pre-existing position weight matrices was directly used for screening.

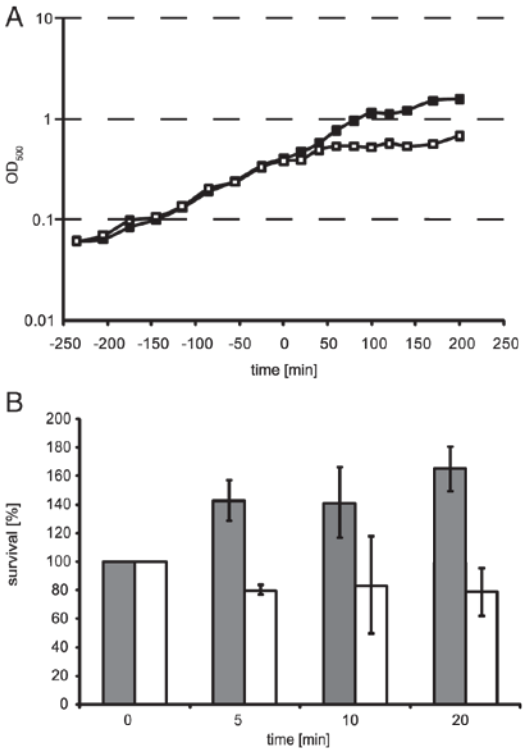

Figure 1. Growth and survival of *B. licheniformis* stressed with H_2O_2. *B. licheniformis* cells were cultivated in a chemically defined medium under control conditions (filled squares) and stressed with 50 µM H_2O_2 at $OD_{500\,nm}$ 0.4 (empty squares) (A). Directly before the stress and at given time points samples were taken to determine colony-forming units of stressed (white bars) and unstressed (grey bars) cultures, from which the survival rate was calculated (B).

3 Results and discussion

3.1 Adaption of *B. licheniformis* to H_2O_2

B. licheniformis was treated with 50 µM H_2O_2, which diminished the growth rate from 0.82 h^{-1} in the exponential phase to 0.32 h^{-1} (Fig. 1A). Colony-forming units showed a survival rate of *B. licheniformis* of about 80% (Fig. 1B).

We analyzed the oxidative stress response of *B. licheniformis* at the transcription level by DNA microarrays and on the translation level by 2-D PAGE. We found 18 proteins to be upregulated and 66 proteins to be downregulated by H_2O_2. The analysis of DNA microarrays showed the induction of 254 genes and repression of 213 genes after exposure to H_2O_2. The expression data for up and downregulated proteins and mRNAs are summarized in Table 1, and Supporting Information Tables S1 and S2, respectively. We classified up- and downregulated proteins and genes of *B. licheniformis* in putative regulons according to the known regulons of *B. subtilis* and/or the presence of relevant regulator-binding sites in the genome of *B. licheniformis*, as described in Section 2.9. The latter allowed us to assign additional genes to the putative *B. licheniformis* regulons. Moreover, proteins and genes that were assumed to belong to certain regulons in *B. subtilis* could be confirmed based on their detected regulator-binding site (Supporting Information Table S3, Fig. S1).

3.2 Induction of the PerR-regulon

In *B. subtilis*, the repressor of the peroxide regulon PerR is known to control the expression of at least eight genes important for detoxification, repair and protection of molecules during oxidative stress [16, 17, 34–37]. The PerR-

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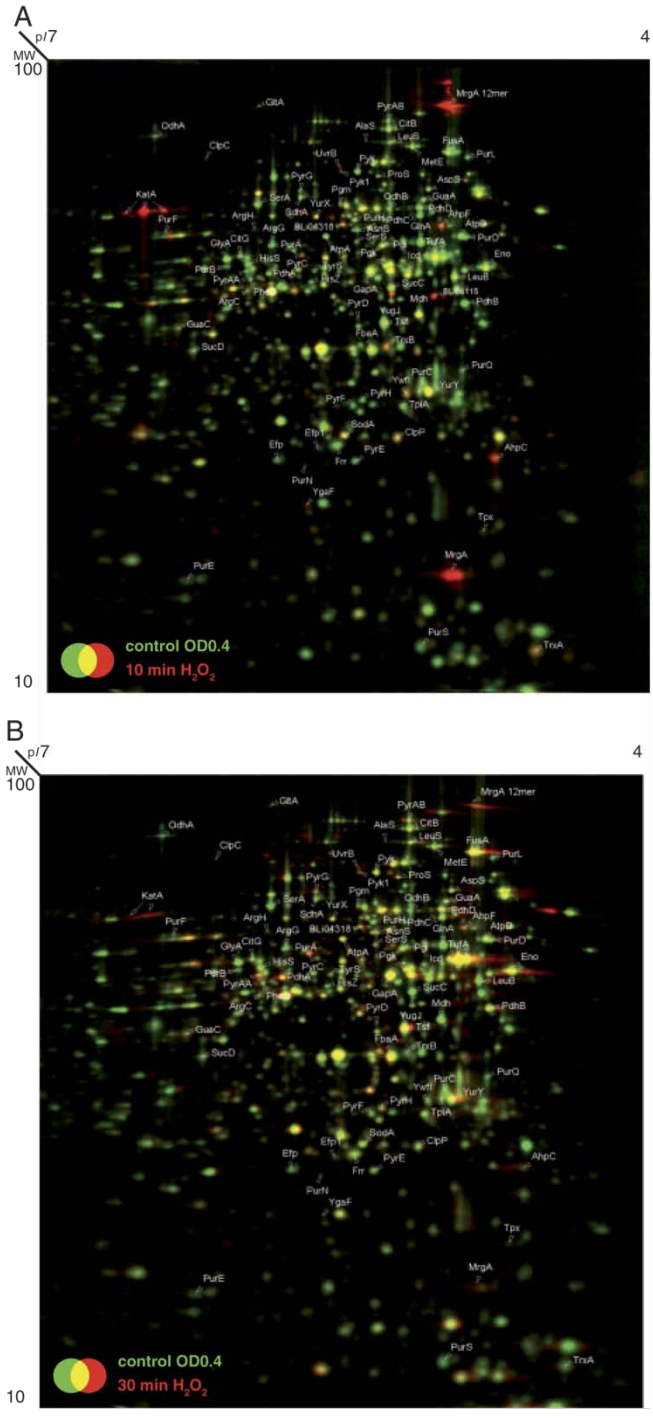

Figure 2. The cytosolic proteome of H_2O_2-stressed *B. licheniformis* cells 10 min after H_2O_2 addition (A), 30 min after H_2O_2 addition (B). The dual channel images were created by the Delta 2D software (Decodon). Cell samples were labeled with L-[^{35}S]-methionine during the exponential growth phase ($OD_{500\,nm}$ 0.4), and 10 and 30 min after H_2O_2 addition. Proteins were separated in a pH gradient 4–7.

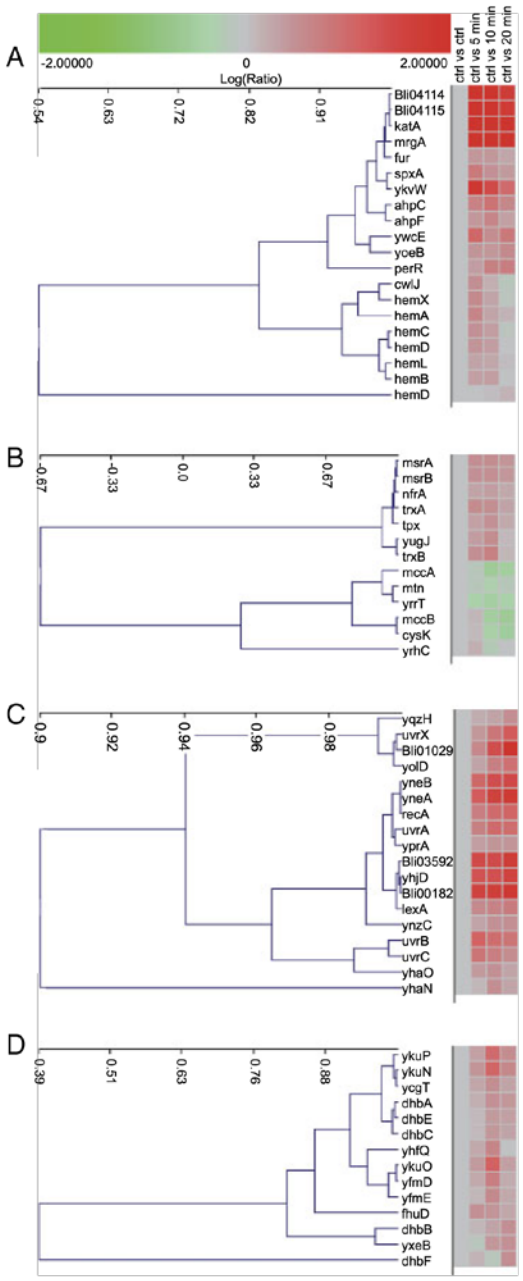

Figure 3. Cluster analysis of different putative regulons of *B. licheniformis* in response to H_2O_2 stress. Different genes were clustered according to known oxidative stress response regulons from *B. subtilis*: PerR (A), Spx (B), SOS (C) and Fur (D). Correlation of the transcription patterns of the different clustered genes is represented on the *x*-axis (cosine correlation).

protein of *B. licheniformis* has high identity to the *B. subtilis* PerR-protein (91%), supporting the idea of the same mode of de-repression as reported by Lee and Helmann [38]. In our analysis of *B. licheniformis*, MrgA (DNA-protection protein) and KatA (vegetative catalase A) were among the most highly induced proteins after peroxide treatment (Fig. 2, Table 1 and Supporting Information Table S1). MrgA was present in a monomeric and in a multimeric form. The higher induction rate of the multimeric form (45-fold) compared with the monomeric form (10-fold) suggests a fast assembly of MrgA-monomers to the multimer. Induction of MrgA was transient, after 30-min exposure of the cells to H_2O_2 synthesis of this protein was almost back to control level. Induction of the alkylhydroperoxide reductase protein AhpCF could be identified as well during oxidative stress in *B. licheniformis*. The induction of *katA*, *mrgA* and *ahpCF* was also observed on the mRNA level. The genes *katA* (100–200-fold) and *mrgA* (200–800-fold) were the most highly induced genes during peroxide treatment. Additionally, other members of the *B. subtilis*-like PerR-regulon could be identified as induced genes: *zosA* (zinc-uptake protein ZosA), *perR* and *spxA* (regulator proteins PerR and SpxA). Parts of the heme biosynthesis operon *hemAXCDBL* were also significantly upregulated after H_2O_2 treatment. The *fur*-gene (iron-uptake regulator Fur) was induced as well, but did not reach the threshold of significance, which resembles the situation in *B. subtilis* [16]. Upregulation of the genes *yoeB*, *cwlJ* and *ywcE* in a PerR-mutant of *B. subtilis* was reported by Helmann et al. [17]. In *B. licheniformis*, these genes were found to be induced after treatment with H_2O_2, indicating direct or indirect regulation by PerR. In summary, our results strongly suggest an induction of a putative PerR-regulon in *B. licheniformis* similar to that of *B. subtilis*. However, direct control by PerR in *B. licheniformis* has to be verified.

Interestingly, two genes of unknown function, *BLi04114* and *BLi04115*, showed a massive induction after H_2O_2 treatment in this study. Induction of BLi04115 was also observed at the protein level. Hierarchical clustering of the transcriptional data indicated co-regulation of these genes together with a *katA*-specific mRNA (Figs. 3A and 4A). Strikingly, *BLi04114* and *BLi04115* are located directly downstream of the *katA* gene (*BLi04113*) in the genome of *B. licheniformis*, whereas in *B. subtilis katA* is monocistronically transcribed. Northern blots indicated a co-transcription of *Bli04114* and *Bli04115* with the *katA* gene after peroxide treatment (Fig. 4B, C and D). For the *katA* gene itself, both a single *katA* transcript and a *katA-BLi04114-BLi04115* co-transcript were detected in peroxide-stressed cells, but no transcript at all was detectable in exponentially growing cells. *BLi04114* exhibits high similarity to a transcriptional regulator of the Fur-family, whereas *BLi04115* shares identity with the ferrochelatase HemH. *BLi04115* might therefore aid the heme biosynthesis.

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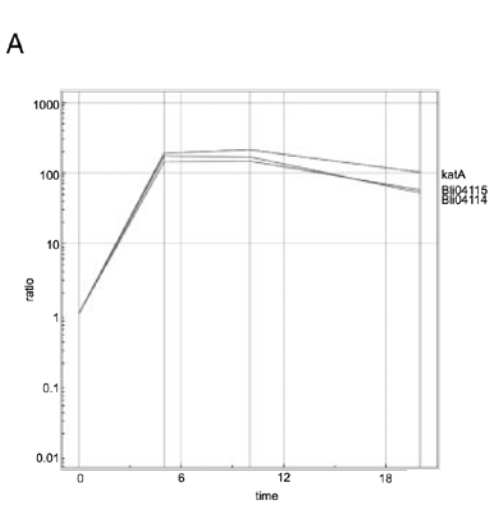

Figure 4. Analysis of *katA*, *BLi04114* and *BLi04115* expression. Transcription pattern of the three genes in response to H_2O_2 (A). The graphs show the similar transcription pattern of the genes *katA* (*BLi04113*), *BLi04114* and *BLi04115* after treatment with H_2O_2 in *B. licheniformis*. Northern blot analysis from *katA* (B), *BLi04114* (C) and *BLi04115* (D) of unstressed (ctrl) and H_2O_2-stressed (5, 10 and 20 min) cells were performed to identify a possible co-transcription of the three genes (5 µg per lane). During stress all three genes are co-transcribed (3 kb band), whereas *katA* also shows a single transcript band (1.5 kb band).

3.3 Induced genes and proteins regulated by Spx

In *B. subtilis*, Spx regulates genes, which cover different functions, such as thiol homeostasis (*trxA*, *trxB*), detoxification (*nfrA*, *tpx*), cysteine biosynthesis (*cysK*, *trxA* and the *yrrT*-operon: *yrhC*, *mccB* (*yrhB*), *mccA* (*yrhA*), *mtn* and *yrrT*) and NADPH production (*yugJ*) [39, 40]. All these genes are also present in *B. licheniformis*. Recent results revealed that Spx also mediates the regulation of the methionine sulfoxide reductase operon *msrAB* in *B. subtilis* and the repression of genes responsible for competence and metabolism, as for example the *srf*-operon [14, 40, 41], which is not present in the *B. licheniformis* genome [29].

In *B. licheniformis*, four proteins were induced following peroxide treatment, which belong to the putative Spx-regulon: Tpx (thiol peroxidase), TrxA (thioredoxin), TrxB (thioredoxin reductase) and YugJ (similar to NADH-dependent butanol dehydrogenase). These upregulated proteins could also be found as induced genes in the transcriptomic approach (Fig. 3B, Table 1). Additionally, induction of the genes encoding the methionine sulfoxide reductases *msrAB* could be observed. The induction of these genes suggests a severe impact on sulfur-containing amino acids due to H_2O_2. Interestingly, as also observed in *B. subtilis*, cysteine biosynthesis genes were not induced by peroxide stress in *B. licheniformis* [9, 16, 42, 43].

3.4 The SOS-regulon is induced during peroxide stress

The SOS-regulon in *B. subtilis* is regulated by RecA/LexA [44] and controls a cellular response to DNA damage [45]. A total of 33 transcriptional units belong to this regulon in *B. subtilis* [46] and 25 of them can be found as homologs in *B. licheniformis*. By proteomics we only found one protein, which was upregulated after peroxide treatment: the excinuclease subunit UvrB. The gene *uvrB* was heavily transcribed at the early time point and transcription diminished over time. In addition, the genes encoding the corresponding excinuclease subunits (*uvrA* and *uvrC*) were also induced (Fig. 3C). The genes for the SOS-response regulators (*recA* and *lexA*) were induced as well as a gene encoding a putative regulator similar to LexA: *BLi03592*. The gene cluster *yhaON*, probably involved in repair of DNA double-strand breaks in *B. subtilis* [47], showed a significantly increased expression rate after peroxide treatment in *B. licheniformis*. The operon *yneAB–ynzC* was induced during peroxide stress in *B. licheniformis*; it participates in the suppression of cell division during the SOS-response in *B. subtilis* [48]. Other members of the putative *B. licheniformis* SOS-regulon, like *dinB*, were slightly induced by H_2O_2, but did not reach the threshold of significance. Similar results were shown for the peroxide stress response of *B. subtilis* [16].

With the LexA-consensus sequence from Au et al. [46] we were able to identify six additional genes with a LexA-binding site that were significantly induced in *B. licheniformis* after peroxide treatment. The genes *Bli00182*, *Bli01029*, *yolD* and *yqzH* encode hypothetical proteins with unknown function, whereas *uvrX* encodes a UV-damage repair protein. The gene *ybbJ* encodes a protein that shows high similarity to an acyltransferase of *B. subtilis*.

3.5 Induction of iron uptake by derepression of Fur

In *B. subtilis* the Fur regulator mediates the iron-dependent repression of at least 20 transcriptional units encoding more than 40 genes [49, 50], of which eight are not present in the *B. licheniformis* genome. Of these putative Fur-regulated transcriptional units six showed a significantly increased

expression rate after addition of H_2O_2 (*dhbACEBF*, *fhuD*, *ycgT*, *yhfQ-yfmDE*, *ykuNOP*, *yxeB*) (Fig. 3D), however, none of these inductions could be observed at the protein level. Compared with *B. licheniformis*, for *B. subtilis* twice the number of induced Fur-regulated transcriptional units have been reported after peroxide treatment [16].

In addition, we found that the PerR-regulated *fur* gene for the iron-uptake regulator was induced by H_2O_2 in *B. licheniformis*, but it did not reach the threshold of significance (see Supporting Information data). Induction of Fur and thereby repression of iron uptake genes could prevent $^{\bullet}$OH-production catalyzed by free iron in the Fenton reaction. Surprisingly, three Fur-repressed genes appeared to be induced in H_2O_2-stressed *B. licheniformis* cells, possibly as a response to iron limitation caused by H_2O_2. A screening of the *B. licheniformis* genome identified the three genes *ykuN*, *yhfQ* and *BLi02844* (putative iron-binding protein) with a Fur- and additionally a potential PerR-binding site. This observation could be viewed as an indication for a regulatory overlap between the PerR- and the Fur-regulon in *B. licheniformis* (Table 1; Supporting Information Fig. S1). The induction of Fur-repressed genes could also argue for an oxidatively damaged Fur-protein, which cannot fulfill its function, leading to the derepression of Fur-regulated genes and iron uptake. D'Autreaux et al. found an inhibition of Fur activity by Fe-nitrosylation in *Escherichia coli* [51]. This supports the idea that H_2O_2 could also oxidize the Fur protein leading to a non-functional protein.

3.6 Control of protein quality by the regulator CtsR and others

In *B. subtilis* the CtsR regulon is induced by various oxidative stressors [16, 52]. In our analysis of *B. licheniformis*, we found an induction of the proteolytic complex ClpCP by the proteomic approach. However, the transcriptome analysis showed only the proteolytic subunit *clpP* as significantly induced. Other members of the CtsR-regulon were only marginally upregulated by H_2O_2. Four other genes encoding for proteases were found to be induced by peroxide stress: *ispA*, *ykvY*, *yugP* and *BLi03556*. Probably these proteases participate in the degradation of oxidatively damaged proteins.

The analysis of the intracellular metabolome revealed an increase in free amino acids at the early time points after the stress (see Supporting Information Table S4). This strongly indicates proteolytic degradation of proteins after exposure to H_2O_2.

3.7 The general stress response regulated by SigB

The general stress response has been extensively studied in *B. subtilis* [19, 53, 54], but so far little is known about the SigB-dependent general stress response of *B. licheniformis*. In our analysis, the predicted SigB-regulated superoxide dismutase SodA was upregulated during peroxide stress at the protein level. However, no induction was identified at the mRNA level. The screening of the *B. licheniformis* genome revealed 19 genes harboring SigB promoters with an at least threefold increased mRNA amount after peroxide treatment (Supporting Information Table S3, Fig. S1). Among them were *mgsR*, *ydaG* and *yvyD*. Many other SigB-dependent genes were only slightly induced and did not match the criterion of significance. The gene for the organic hydroperoxide resistance reductase OhrB was induced, too. However, it is not clear whether this gene is SigB-dependently expressed, as it contains a mismatch in an otherwise invariant residue of the −10 promoter region. This observation is consistent with the results of Helmann et al. [17] and Mostertz et al. [16], who reported a weak induction of the SigB-regulon after peroxide treatment in *B. subtilis*.

3.8 Other proteins and genes upregulated by oxidative stress

Few other proteins, belonging to different regulation units, were synthesized at an elevated level following the exposure to H_2O_2. Among them were YgaF, a putative peroxiredoxin, and YwfI, a putative oxidoreductase (Table 1; Supporting Information Table S1).

After peroxide treatment, six genes with a putative gene-regulatory function were significantly upregulated: *Bli01319*, *Bli04167*, *yobU*, *yuxN*, *Bli02274* and *Bli03704*.

For some upregulated genes a detoxifying or protective role in the oxidative stress management was predicted. The expression of the putative oxidoreductase genes *BLi01190*, *BLi02273*, *ydeQ* and *ywrO* was found to be significantly increased after peroxide treatment, as well as the expression of the following genes: *yazB*, *ykuU* and the Gat1-superfamily genes *yoaZ1* and *yoaZ2*.

SufB and SufC, both proteins belonging to the recently described iron/sulfur cluster biogenesis system [55], were induced by H_2O_2 stress at the protein level. At the mRNA level the *sufCDSUB* operon was significantly induced except for *sufB*. The SUF (sulfur mobilization) system has been described as induced by oxidative stress in *E. coli* replacing the components of the housekeeping iron/sulfur cluster biogenesis system (ISC system) under such conditions [55–57].

Interestingly, we observed a strong induction of *BLi04207* and *BLi04208* encoding for the isocitrate lyase and the malate synthase of the glyoxylate cycle in *B. licheniformis*. An investigation of *E. coli* showed a repression of the tricarboxylic acid (TCA) cycle under oxidative stress conditions, most likely to avoid an accompanying reduction of reducing equivalents and the possible generation of ROS in the oxidative phosphorylation [58]. Murakami [59] revealed that *E. coli*'s isocitrate dehydrogenase requires divalent metals such as Fe^{2+}, which may generate ROS by the

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Table 1. Selected induced genes in response to H_2O_2 as revealed by transcriptome and proteome analyses

ORF ID	Gene[a]	Transcriptome[b]			Proteome[c]		Gene product function	Regulon in *Bacillus subtilis*	Regulon prediction in *B. licheniformis*[d]
		5 min	10 min	20 min	10 min	30 min			
BLi00014	***guaB***	−1.75	1.67	3.81			InosineMonoPhosphate dehydrogenase, synthesis of GMP		
BLi00101	*ctsR*	2.40	2.21	1.14			Transcriptional regulator, negative regulation of class III stress genes (clpC, clpP, clpE)	CtsR, SigB	CtsR
BLi00102	*mcsA*	2.31	2.11	−1.08			Modulator of CtsR repression	CtsR, SigB	CtsR
BLi00103	*mcsB*	2.00	2.16	1.04			Modulator of CtsR repression; ATP:guanido phosphotransferase	CtsR, SigB	CtsR
BLi00104	***clpC***	1.67	2.09	1.41	2.25	0.67	Class III stress response-related ATPase	CtsR, SigB	CtsR
BLi00182	***none***	33.9	42.0	67.0			Hypothetical protein		SOS
BLi00345	***cwlJ***	3.31	1.47	−1.18			Cell wall hydrolase (sporulation); sigE-reg.	PerR	
BLi00512	***ydaG***	3.59	1.96	1.33			BLAST similar to general stress protein of *B. subtilis*	SigB	SigB
Bli00637	***dltE***	2.18	3.36	1.38			Involved in lipoteichoic acid biosynthesis		SigB
BLi00867	***ydeQ***	1.53	3.36	1.05			Similar to NAD(P)H oxidoreductase		
BLi00899	***ygaF***	1.42	1.82	2.23	3.77	0.95	Putative peroxiredoxin		
BLi00900	***perR***	2.3	5.0	5.5			Transcriptional repressor of the peroxide regulon	PerR	PerR
BLi00946	***yazB***	4.28	1.09	−1.30			Putative DNA binding protein		
BLi01029	***none***	4.3	23.7	124.8			Hypothetical protein		SOS
BLi01068	***yhaO***	2.6	3.1	2.0			Similar to DNA repair exonuclease of *B. cereus*	SOS	SOS
BLi01069	***yhaN***	1.3	3.3	2.0			Unknown	SOS	SOS
BLi01070	*yhaM*	1.4	2.3	1.7			3′-5′ Exoribonuclease	SOS	SOS
BLi01111	***yhfQ***	1.75	4.38	1.00			Similar to iron(III) dicitrate-binding protein	Fur	Fur, PerR
BLi01112	***yfmD***	1.68	5.59	1.59			Similar to ferrichrome ABC transporter (permease)	Fur	Fur, PerR
BLi01113	***yfmE***	1.26	3.95	1.68			Similar to ferrichrome ABC transporter (permease)		Fur, PerR
BLi01120	***yhjD***	21.3	22.3	33.8			Unknown	SOS	SOS
BLi01189	***yitI***	4.29	5.62	11.93			BLAST similar to acetyltransferase		
BLi01190	***none***	5.79	10.01	18.14			Putative oxidoreductase		
BLi01238	***spxA***	6.6	3.2	2.5			Transcriptional regulator	PerR	
Bli01240	***mecA***	3.41	2.54	3.26			Negative regulator of competence		SigB
BLi01249	***yjbH***	4.42	2.43	1.38			BLAST similar to dithiol-disulfide isomerase of *B. clausii*/similar to Thioredoxin of *B. cereus*		SigB
BLi01250	***yjbI***	4.98	2.30	1.65			BLAST similar to thiol management oxidoreductase component of *B. subtilis*		SigB
BLi01308	***yoeB***	3.0	2.6	3.9			BLAST similar to inhibitor of autolysins of *B. subtilis*, protection against cell envelope stress	PerR	
BLi01319	***none***	1.73	1.76	27.55			Putative repressor protein		
BLi01401	***ykgA***	1.27	2.02	3.69			BLAST putative transferase, sigmaB dependent	SigB	SigB
BLi01416	***ykzA***	3.18	1.81	1.73			Similar to organic hydroperoxide resistance protein OhrB, sigmaB regulon	SigB	SigB
BLi01423	***ispA***	5.26	2.47	3.68			Major intracellular serine protease		
BLi01491	***ykzB***	1.99	2.24	3.17			Unknown		SigB
BLi01593	***zosA (ykvW)***	73.60	28.00	9.67			Similar to heavy metal-transporting ATPase	PerR	PerR, SOS
BLi01594	***ykvY***	5.01	3.34	1.27			Similar to Xaa-Pro dipeptidase		SigB
BLi01629	***ykuN***	2.87	11.54	4.23			Similar to flavodoxin	Fur	Fur, PerR
BLi01630	***ykuO***	2.42	13.80	2.15			BLAST similar to glycoside hydrolase	Fur	
BLi01631	***ykuP***	2.12	9.67	3.48			Similar to flavodoxin	Fur	

Table 1. Continued.

ORF ID	Gene[a]	Transcriptome[b]			Proteome[c]		Gene product function	Regulon in *Bacillus subtilis*	Regulon prediction in *B. licheniformis*[d]
		5 min	10 min	20 min	10 min	30 min			
BLi01635	***ykuU***	3.06	1.88	−2.03			Similar to 2-cys peroxiredoxin; BLAST Thioredoxin_like superfamily		
BLi01645	***none***	3.44	2.50	2.11			Hypothetical protein		SigB
BLi01918	***recA***	5.6	10.1	12.7			Multifunctional protein involved in homologous recombination and DNA repair	SOS	SOS
BLi02032	***lexA***	3.9	4.7	5.9			Transcriptional repressor of the SOS regulon	SOS	SOS
BLi02033	***yneA***	15.4	36.4	59.7			Similar to peptidoglycan-binding protein	SOS	SOS
BLi02034	***yneB***	11.1	23.7	30.0			Similar to resolvase	SOS	
BLi02035	***ynzC***	2.0	3.1	3.4			Unknown	SOS	
BLi02068	***none***	2.22	2.01	4.07			Hypothetical protein		
BLi02196	***yobU***	7.15	3.40	2.25			BLAST putative regulatory protein		
BLi02273	***none***	3.50	1.80	1.07			Short-chain dehydrogenase/reductase protein/putative oxidoreductase		
BLi02274	***none***	3.37	1.67	1.12			BLAST similar to DNA-binding protein, WHTH_GntR domain		
BLi02302	***msrB***	3.21	3.33	2.74			Methionine sulfoxide reductase B	Spx	
BLi02303	***msrA***	3.42	3.51	2.87			Peptidyl methionine sulfoxide reductase	Spx	
BLi02535	***yolD***	2.0	5.1	8.5			Hypothetical protein, similar to proteins from *B. subtilis*		SOS
BLi02536	***uvrX***	2.8	7.1	16.7			UV-damage repair protein		SOS
BLi02537	***yqzH***	1.6	2.0	3.3			Hypothetical protein		SOS
BLi02620	*yqhQ*	2.74	1.83	2.22			BLAST similar to membrane protein YqhQ	SigB	
BLi02621	***yqhP***	3.37	1.73	2.29			BLAST similar to membrane protein YqhP		
BLi02651	***mgsR***	3.69	2.26	2.89			BLAST similar to transcriptional regulator, controls a subset of stress genes	SigB	SigB
BLi02658	***fhuD***	3.21	2.82	1.87			Ferrichrome ABC transporter (ferrichrome-binding protein)	Fur	
BLi02679	***sodA***	1.88	1.86	−1.13	2.24	0.87	Superoxide dismutase	SigB	
BLi02680	***yqgC***	1.75	3.16	3.19			BLAST similar to membrane protein YqgC		SigB
BLi02810	***ycgT***	1.85	3.35	1.99			Similar to thioredoxin reductase	Fur	Fur
BLi02844	***none***	1.41	4.14	2.42			BLAST similar to peripasmic iron-binding protein		Fur, PerR
BLi02942	*hemL*	2.0	1.9	1.1			Glutamate-1-semialdehyde 2,1-aminotransferase	PerR	PerR, SOS
BLi02943	*hemB*	2.2	2.3	1.0			δ-Aminolevulinic acid dehydratase	PerR	PerR, SOS
BLi02944	*hemD*	3.0	2.3	1.0			Uroporphyrinogen III cosynthase	PerR	PerR, SOS
BLi02945	***hemC***	3.2	2.3	−1.0			Porphobilinogen deaminase	PerR	PerR, SOS
BLi02946	***hemX***	4.0	2.0	−1.1			Negative effector of the concentration of HemA	PerR	PerR, SOS
BLi02947	***hemA***	4.5	2.1	1.4			Glutamyl-tRNA reductase	PerR	PerR, SOS
BLi02997	***uvrC***	7.3	5.4	3.5			Excinuclease ABC subunit C	SOS	
BLi02998	***trxA***	3.50	3.39	1.94	5.50	1.19	Thioredoxin	Spx	
BLi03075	***none***	1.36	2.26	3.38			Hypothetical protein		SigB
BLi03088	***tpx***	2.09	3.16	1.69	3.07	0.73	Putative peroxiredoxin	Spx	
BLi03138	***ytzB***	1.95	2.53	3.89			Hypothetical protein		
BLi03306	***yugP***	6.78	5.47	2.07			BLAST similar to metal-dependent protease/peptidase of *B. subtilis*		
BLi03317	***yugJ***	2.39	3.68	1.18	3.15	0.42	Putative NADH-dependent butanol dehydrogenase	Spx	
BLi03447	*sufB (yurU)*	2.56	2.29	2.14			BLAST similar to FeS assembly protein SufB; forms a cytosolic complex SufBCD		

Table 1. Continued.

ORF ID	Gene[a]	Transcriptome[b]			Proteome[c]		Gene product function	Regulon in *Bacillus subtilis*	Regulon prediction in *B. licheniformis*[d]
		5 min	10 min	20 min	10 min	30 min			
BLi03448	***sufU (yurV)***	2.69	3.02	2.17			BLAST similar to SUF system FeS assembly protein of *B. pumilus*		
BLi03449	***sufS (csd)***	2.69	3.27	2.23			BLAST similar to cysteine desulfurase		
BLi03450	***sufD (yurX)***	3.23	4.12	2.32	2.98	2.09	BLAST similar to FeS assembly protein SufD of *B. subtilis*		
BLi03451	***sufC (yurY)***	2.82	4.06	2.33	3.11	3.11	BLAST similar to FeS assembly ATPase SufC of *B. cereus*		
BLi03480	***mrgA***	812.5	212.8	126.2	37.64	1.68	Metalloregulation DNA binding stress protein	PerR	PerR
BLi03485	***yuxN***	8.36	3.57	2.03			BLAST putative transcriptional regulator		
BLi03556	***none***	4.40	3.58	1.04			Putative intracellular proteinase I		
BLi03592	***none***	27.8	26.1	49.3			Putative transcriptional repressor protein, similar to LexA		SOS, SigB
BLi03659	***yoaZ1***	52.97	17.16	30.40			Similar to putative factor of the oxidative stress response of *B. subtilis*		
BLi03660	***yoaZ2***	57.79	26.52	25.78			Similar to putative factor of the oxidative stress response of *B. subtilis*		
BLi03696	***ywrO***	3.17	5.50	2.89			Similar to NAD(P)H oxidoreductase		
BLi03704	***none***	3.21	4.47	2.05			Putative transcriptional regulator; BLAST HTH_ARSR superfamily		
BLi03710	***clpP***	2.19	3.13	2.05	2.43	0.42	ATP-dependent Clp protease proteolytic subunit (class III heat-shock protein)	CtsR, SigB	CtsR, SigB
BLi03727	***none***	2.82	3.14	1.07			Hypothetical protein		SigB
BLi03728	***trxB***	3.24	5.36	1.27	2.70	0.87	Thioredoxin reductase	Spx	
BLi03730	***ybbJ***	4.62	5.59	11.68			BLAST similar to putative acyltransferase [Bacillus subtilis]		SOS
BLi03758	***uvrA***	5.0	9.9	9.0			Excinuclease ABC subunit A	SOS	SOS
BLi03759	***uvrB***	13.6	9.5	7.6	5.23	6.35	Excinuclease ABC subunit B	SOS	SOS
BLi03774	***yvyD***	16.35	7.21	7.08			Similar to sigma-54 modulating factor of Gram-negative bacteria	SigB	SigB
BLi03892	***spoIIQ***	3.28	1.91	1.02			Required for completion of engulfment		
BLi03894	***ywnF***	7.10	5.00	1.77			Hypothetical protein		SigB
BLi03898	***dhbF***	1.39	−1.22	3.28			Involved in 2,3-dihydroxybenzoate biosynthesis	Fur	Fur
BLi03899	***dhbB***	1.25	1.97	3.18			Isochorismatase	Fur	Fur
BLi03900	*dhbE*	1.24	2.49	2.22			2,3-Dihydroxybenzoate-AMP ligase	Fur	Fur
BLi03901	*dhbC*	1.21	2.68	1.94			Isochorismate synthase	Fur	Fur
BLi03902	***dhbA***	1.48	3.18	2.66			2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase	Fur	Fur
BLi03998	***ywfI***	2.32	2.31	1.14	2.87	0.90	Putative oxidoreductase/oxygenase/dismutase		
BLi04028	***ywcE***	10.96	3.72	6.36			Unknown	PerR	
BLi04042	***none***	−1.09	1.60	3.29			Hypothetical protein		SigB
BLi04091	***yxzF***	2.02	2.71	3.11			Unknown	SigB	SigB
BLi04113	***katA***	184.5	208.5	100.7	148.40	12.95	Vegetative catalase A	PerR	PerR
BLi04114	***none***	168.3	166.6	51.4			Putative transcriptional repressor of the peroxide regulon		PerR
BLi04115	***none***	141.6	145.5	56.2	24.36	3.75	Close homolog to HemH ferrochelatase		PerR
BLi04150	***none***	2.63	2.12	4.16			Hypothetical protein		SigB
BLi04167	***none***	6.42	2.61	1.46			Putative transcriptional regulator of anerobic genes		
BLi04206	***none***	14.20	5.55	6.72			Hypothetical protein		
BLi04207	***none***	2.33	3.16	19.62			Isocitrate lyase		
BLi04208	***none***	7.63	20.07	142.41			Malate synthase		
BLi04262	***yxeB***	−1.08	2.63	3.20			Similar to ABC transporter (binding protein)	Fur	
BLi04291	***ahpC***	4.9	8.2	4.2	12.63	3.59	Alkylhydroperoxidreductase, small subunit	PerR	PerR

Table 1. Continued.

ORF ID	Gene[a]	Transcriptome[b]			Proteome[c]		Gene product function	Regulon in *Bacillus subtilis*	Regulon prediction in *B. licheniformis*[d]
		5 min	10 min	20 min	10 min	30 min			
BLi04292	***ahpF***	2.6	4.5	2.2	5.28	2.91	Alkylhydroperoxidreductase, large subunit	PerR	PerR
BLi04318	***none***	1.50	1.03	−1.19	2.55	1.94	Putative type I site-specific deoxyribonuclease		

Genes are listed, which could be either assigned to a regulon or are known to be involved in the oxidative stress response of other organisms. For complete lists of induced proteins and genes see Supporting Information Tables S1 and S2. For transcriptome analyses, values were calculated by the Rosetta Resolver software from three independent array hybridizations. For proteome analyses, values were calculated by the Delta 2D software (Decodon) from three biological replicates. Quantitation of induced proteins represents mean values. Detailed protein quantification is presented in Supporting Information Table S1. Boldface type indicates significant upregulation with a cutoff of 3 at the mRNA level or a cutoff of 2 at the protein level under stress conditions for at least one time point. The information given in the column "gene product function" is according to the NCBI GenBank AE017333.1 (http://www.ncbi.nlm.nih.gov/nuccore/AE017333) and partly also to BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi), then indicated by "BLAST". Regulon prediction for *B. licheniformis* was done with the help of the virtual footprint algorithm [32] of the Prodoric database (http://www.prodoric.de/vfp/) [33].

a) The indentions indicate downstream genes in an operon, as deduced from the genomic organization. Note that the first (not indented) gene could be located either in the line(s) above or below, depending on the orientation of the operon on the chromosome.

b) Transcriptome fold change according to Rosetta Resolver® Gene Expression Analysis System 7.2. (Rosetta Inpharmatics c/o Ceiba Solutions).

c) Proteome ratio according to the Delta 2D software (Decodon).

d) Prediction is based on the identification of a putative regulator binding site by regulon mining and the operon structure deduced from the genomic organization (as indicated by the indention in the gene column).

Fenton reaction. This results in an enzyme inactivation accompanied by the fragmentation of this protein, which leads to a redirection of the metabolic flux into the glyoxylate cycle, which supplies malate and NADPH for biosynthetic and regenerating reactions [59, 60].

To further investigate these transcriptomic results, we performed a metabolomic study with special attention to the TCA cycle and the glyoxylate cycle metabolites. Unfortunately, it was not possible to detect glyoxylate in our biological samples. However, we were able to identify the majority of TCA cycle metabolites (Fig. 5A). At later time points unstressed cells are in the stationary phase in which the TCA cycle is induced and less amino acids originating from α-ketoglutarate are needed. Thus, TCA cycle metabolites increase over time. During stress the concentration of *cis*-aconitate and isocitrate increased intracellularly, whereas the subsequent TCA metabolites did not. A massive increase of the *cis*-aconitate concentration during stress can be found in the extracellular medium as well, whereas the extracellular α-ketoglutarate concentration decreases compared with the control conditions (Fig. 5B). This strongly supports the idea of an oxidatively damaged isocitrate dehydrogenase, because precursors of this enzyme increase and products decrease during stress. On the other hand, the malate concentration under stress reached the control level. One molecule isocitrate used in the TCA cycle results in one molecule malate, whereas it yields two molecules of malate in the glyoxylate cycle. Hence, the high concentration of malate during stress supports the idea of an activated glyoxylate cycle.

3.9 Proteins and genes downregulated by H_2O_2

The addition of 50 μM H_2O_2 to exponentially growing cells not only induced gene expression and subsequent protein synthesis, but also caused repression of several *B. licheniformis* proteins and genes (see also Supporting Information Tables S1 and S2). Most prominent was the repression of genes belonging to the synthesis of purines and pyrimidines, which was found at the protein as well as at the mRNA level. Repression of the pyridine synthesis was only transient and expression increased again after 20 min (mRNA) and 30 min (protein), respectively. A repression of genes responsible for synthesis of arginine and histidine was also detectable at the transcriptomic level. Genes of the *cysH* operon were repressed after peroxide treatment in *B. licheniformis* as well as in *B. subtilis* [16]. In *B. subtilis* this operon encodes several proteins involved in the conversion of sulfate into sulfide and in the incorporation of sulfide into cysteine [61]. Probably, the cells concomitantly reduce the sulfur assimilation via the *cysH*-operon in order to reduce free cysteine in the cell.

Investigations of *B. subtilis* and *E. coli* indicate that oxidative stress induces ppGpp accumulation and results in a positive and negative stringent response, e.g. repression of

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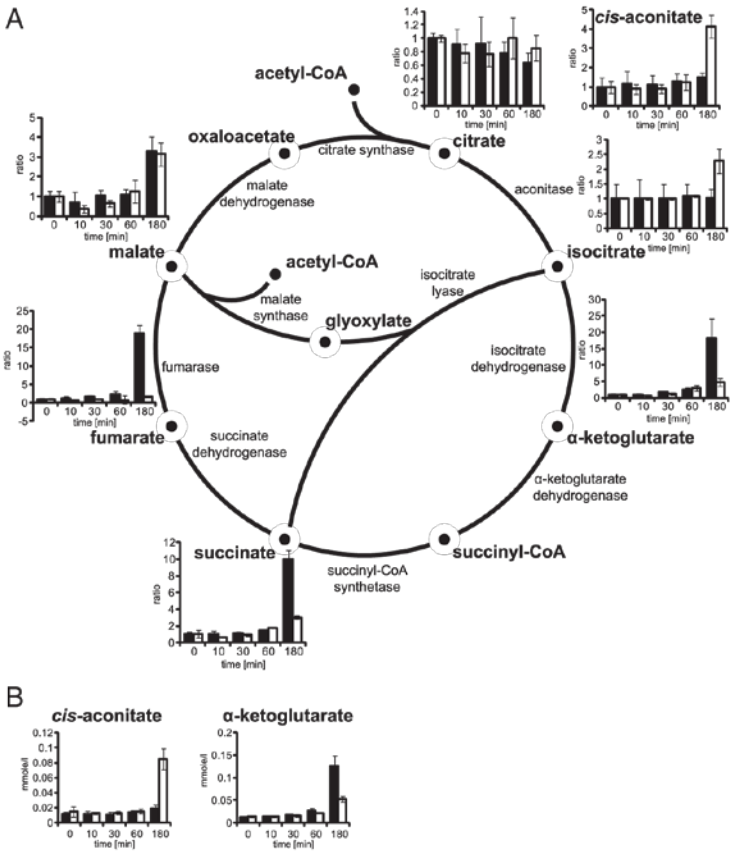

Figure 5. Changes of intra- and extracellular TCA-intermediates during oxidative stress. Intracellular metabolites from unstressed (black bars) and hydrogen peroxide-treated cells (white bars) were analyzed by GC-MS (A), whereas the medium of these cells was analyzed by NMR for extracellular metabolites (B). Samples from t_0 (stressed and unstressed) were set to 1 and ratios of the following samples are shown at their sample points. The data are shown in the context of the TCA-cycle with metabolites as dots and enzyme reactions as lines.

ribosomal proteins and translation factors [16, 52, 62]. We found various downregulated proteins involved in translation, e.g. most aminoacyl-tRNA synthetases and some translation elongation factors, which may be regulated by the stringent response in *B. licheniformis*, as for example FusA, Tsf or TufA. In contrast to that, only a slight, but not significant up or downregulation of genes belonging to the putative stringent response of *B. licheniformis* could be detected after peroxide treatment by the transcriptome analysis.

3.10 Oxidation of extracellular proteins

To study oxidation of extracellular proteins, a thiol modification assay indicating oxidation of the thiol groups of the cysteines and subsequent disulfide bond formation was performed. Although it is known that *Bacilli* favor exclusion of cysteine from their secreted proteins [63], there is still a considerable number of extracellular proteins in *B. licheniformis* that contain cysteines allowing this approach. Some of the proteins containing two or more cysteines are already oxidized under control conditions (Supporting Information Table S5, Fig. S2). For some proteins, higher oxidation levels could be shown during the stress. However, not all proteins containing cysteine in their sequence show oxidation upon application of hydrogen peroxide. This could be due to differences in protein structures. In some proteins the cysteines might be concealed deeply within the structure and thereby at least partially protected from oxidation. The data indicate that oxidative stress also influences the quality of secreted proteins.

4 Concluding remarks

The present study revealed a quite similar response to peroxide stress as known for *B. subtilis*. However, *B. licheniformis* reveals also a specific oxidative stress response, which involves the induction of several new genes (e.g. *BLi04114* and *BLi04115*) with as yet unknown functions. Future detailed analyses of these genes are required to gain

better insights into the specific oxidative stress physiology of *B. licheniformis*. Additionally, the induction of the glyoxylate cycle, supported by transcriptomic and metabolomic analyses, is an interesting response of *B. licheniformis* to oxidative stress. The data of this study indicate that oxidative stress could have a negative effect on the fitness and productivity of *B. licheniformis* cells in industrial fermentation processes and could compromise the quality of overproduced proteins. Understanding the oxidative stress response and defining marker genes and proteins for this stress can help to optimize the industrial use of *B. licheniformis*.

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The authors have declared no conflict of interest.

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THE RESPONSE OF *BACILLUS LICHENIFORMIS* TO HEAT AND ETHANOL STRESS AND THE ROLE OF THE SIGB REGULON

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Author's contribution

The experimental design was developed by BV, RS, BJ, TS and MH. Bacterial cultivations were performed by BV and RS. 2D-PAGE and protein analysis was done by BV. RNA isolations, microarray experiments and transcriptome analysis were carried out by RS. Mutant construction was performed by RS. Mass spectrometry analysis was done by DA. The manuscript was written by all authors.

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RESEARCH ARTICLE

The response of *Bacillus licheniformis* to heat and ethanol stress and the role of the SigB regulon

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The heat and ethanol stress response of *Bacillus licheniformis* DSM13 was analyzed at the transcriptional and/or translational level. During heat shock, regulons known to be heat-induced in *Bacillus subtilis* 168 are upregulated in *B. licheniformis*, such as the HrcA, SigB, CtsR, and CssRS regulon. Upregulation of the SigY regulon and of genes controlled by other extracytoplasmic function (ECF) sigma factors indicates a cell-wall stress triggered by the heat shock. Furthermore, tryptophan synthesis enzymes were upregulated in heat stressed cells as well as regulons involved in usage of alternative carbon and nitrogen sources. Ethanol stress led to an induction of the SigB, HrcA, and CtsR regulons. As indicated by the upregulation of a SigM-dependent protein, ethanol also triggered a cell wall stress. To characterize the SigB regulon of *B. licheniformis*, we analyzed the heat stress response of a *sigB* mutant. It is shown that the *B. licheniformis* SigB regulon comprises additional genes, some of which do not exist in *B. subtilis*, such as *BLi03885*, encoding a hypothetical protein, the Na/solute symporter gene *BLi02212*, the arginase homolog-encoding gene *BLi00198* and *mcrA*, encoding a protein with endonuclease activity.

Keywords:
Bacillus licheniformis / Ethanol stress / Heat stress / Microbiology / *sigB* mutant / Transcriptome

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

The preferred natural habitats of *Bacillus licheniformis*, a bacterium closely related to *Bacillus subtilis*, are bird feathers [1] and soil. Especially soil habitats are prone to rapidly changing conditions, such as shifts in nutrient availability, osmolarity, pH, or temperature. Bacteria living in such environments had to develop strategies to cope with such unfavorable conditions. A considerable proportion of the genomic information of these organisms is therefore dedicated to adaptation networks evolved to survive physical stress or limitation of nutrients.

Response to heat stress of mesophilic bacteria is well characterized [2–5]. In *B. subtilis*, the HrcA regulon, the SigB regulon, the CtsR regulon, the HtpG operon, and the CssSR regulon are all upregulated in response to a heat shock [4]. The heat shock stimulon of *B. subtilis* contains a number of additional heat-inducible genes. Response to ethanol exposure is less well studied. It is known that ethanol targets the cell envelope and induces the SigM regulon [6, 7]. Furthermore, it has been described that ethanol stress is a strong inducer of the SigB regulon in *B. subtilis* [8].

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Abbreviation: WT, wild type

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Accumulation and aggregation of denatured, misfolded proteins is an important aspect of heat and ethanol stress [9]. Denatured proteins can, on one hand, be recovered by chaperones, such as GroEL or DnaK, which assist in refolding [10]. On the other hand, such damaged proteins can be refolded or targeted by the Clp ATPase subunits (e.g., ClpC, ClpE) and degraded in cooperation with the proteolytic ClpP subunit [11, 12]. The ClpCP and ClpEP complexes are the major ATPase/protease complexes for degradation of heat aggregated cellular protein in *B. subtilis* [11, 12].

In this study, we comprehensively analyzed the response of *B. licheniformis* to heat and ethanol stress by a combination of transcriptome and proteome analyses. Furthermore, genes/proteins belonging to the general stress SigB regulon were identified by means of construction and analysis of a *sigB* mutant. With this approach, we were able to confirm SigB-dependent regulation of many genes and detect putative new members of the *B. licheniformis* SigB regulon. The analysis of specific stress related genes allows for the determination of critical process-relevant genes of this important industrial host. A detailed understanding of the stress responses of *B. licheniformis* might enable the development of improved production strains.

2 Material and methods

2.1 Strains, media, and growth conditions

The bacterial strains used were the *B. licheniformis* wild-type strain DSM13 (equivalent to ATCC 14580, type strain from the German Collection of Microorganisms and Cell Cultures, DSMZ, Braunschweig, Germany), MW3 (Δ*hsdR1*, Δ*hsdR2*) [13], carrying the competence plasmid pMMcomK with a tetracycline resistance cassette [14], and the mutant strain Δ*sigB* (Δ*hsdR1*, Δ*hsdR2*, Δ*rsbWsigBrsbX*, with a spectinomycin resistance cassette) [this work]. Cells were cultivated in defined medium [15] containing 0.2% w/v glucose unless otherwise stated. Growth was monitored by measuring the OD at 500 nm (OD_{500nm}). Antibiotics were used at the following concentrations: 12.5 μg/mL tetracycline (Tet) and 200 μg/mL spectinomycin (Sp). *B. licheniformis* from overnight cultures was used to inoculate prewarmed growth medium to obtain a starting OD_{500nm} of 0.04. Cultures were routinely grown in 500 mL Erlenmeyer flasks in a shaking water bath at 180 rpm and 37°C.

2.2 Exposure to stress, survival test, and cell sampling

At an OD_{500nm} of 0.4 exponentially growing cells of *B. licheniformis* were exposed to heat stress by transferring the culture flasks from 37°C to 54°C. This temperature was chosen since it still allows growth of the bacteria—a temperature of 55°C results in an almost complete inhibition of growth (data not shown). Temperature was controlled by measuring the temperature in the shaking water bath. Ethanol stress was provoked by adding 6% ethanol (resulting in a half-maximal growth rate) to the medium at an OD_{500nm} of 0.4. For analyzing the survival of cells after stress, cell samples (control/0 min; before stress; and 30, 60, and 120 min after stress, three biological replicates) were diluted and appropriate concentrations were plated on LB agar plates. Colonies were counted after about 16 h incubation at 37°C. Samples for RNA extraction (only for heat stressed cells) were taken from unstressed cultures before (control/0 min) and 5, 10, and 20 min after exposure to heat stress. Since changes in gene expression occur very fast but often transiently, two time points relatively short and one time point later after the stress were chosen. Cell samples for RNA extraction were mixed with 0.5 volumes of ice-cold killing buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl, 20 mM NaN_3), and immediately harvested at 10 000 × *g* for 5 min at 4°C. Labeled cytoplasmic proteins were prepared by incubating the bacteria with 556 Bq/mL L–[^{35}S]–methionine for 5 min in 5 mL culture, as described before [16]. Samples were labeled during exponential growth (OD_{500nm} 0.4, control), and 10 and 30 min after heat or ethanol stress to allow the newly synthesized proteins to accumulate to detectable levels. For identification of proteins, preparative gels with unlabeled proteins were prepared using cells heat or ethanol stressed for 30 and 60 min as described by Hoi et al. [16].

2.3 Construction of a Δ*sigB* mutant

To construct a deletion of *sigB* in *B. licheniformis*, the *sigB* gene was substituted for a spectinomycin resistance cassette (Sp^R) amplified with primers 1 and 2 (Supporting Information Table 1) from pUS19 [17]. For the crossover event, flanking regions of *sigB* from *B. licheniformis* MW3 were fused to Sp^R by a modified two-step fusion PCR protocol [18]. For amplification of the upstream region primers 3 and 4 and for the downstream region primers 5 and 6 were used (Supporting Information Table 1). Due to the gene arrangement in the *sigB* operon of *B. licheniformis*, the deletion of *sigB* caused a truncation of the upstream gene *rsbW* and the downstream gene *rsbX*. To circumvent a read-through of *rsbW*, a stopcodon was introduced before Sp^R. All PCR products were separated from template and primer DNA by agarose gel electrophoresis, cut and purified with the Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany). The linear PCR fusion product was methylated (DNA-methylase, Invitrogen, Carlsbad, NM) and afterwards used for transformation of *B. licheniformis* MW3 cells carrying the pMMcomK plasmid, as described earlier [14]. Mutants were selected on LB agar plates containing spectinomycin. To verify deletion of the *sigB* gene, the area of the SigB operon was sequenced (Agowa, Berlin, Germany).

2.4 Comparative genomics of the SigB regulon

The putative SigB regulon of *B. licheniformis* was predicted based on the information on target genes and regulator binding sites available for *B. subtilis*, with the help of the Virtual Footprint algorithm [19], implemented into the Prodoric database [20] at http://www.prodoric.de/vfp/. For in silico regulon mining of SigB, the collection of preexisting position weight matrices was directly used to subsequently screen the genome of *B. licheniformis* for putative target genes.

2.5 Preparation of protein extracts, 2DE, and protein identification

Protein extracts were prepared as described in Hoi et al. [16]. Gel electrophoresis with 80 μg protein for radiolabeled samples and 300 μg for preparative gels in a pH gradient of 4–7 was done according to Büttner et al. [21]. Autoradiography of labeled gels and Coomassie staining of preparative gels was performed as described earlier [16]. Labeling experiments were done in triplicates. Upregulated proteins were cut from the preparative Coomassie-stained gels and identified by MS according to Liedert et al. [22] (Supporting Information Table 2). Downregulated proteins were labeled according to the *B. licheniformis* master gel [23]. Delta 2D software (Decodon, Greifswald, Germany) was used for quantification of protein spots and calculation of ratios. First, fusion gels were created from all images included in the quantification analysis. These fusion gels contained all spots present on the single gels. The fusion gels were used for automatic spot detection. When necessary, spots were edited manually. The detected and edited spots were transferred to the single gels and used for quantification. Normalization to the total spot volume including all spots present in a gel was done by the software. Ratios were calculated on basis of the relative spot volume (representing the relative portion of an individual spot of the total protein present on the gel). Statistical analysis was done using the *t*-test in the Delta2D software (Supporting Information Table 3A–C).

2.6 RNA isolation and microarray experiment

RNA isolation, RNA integrity and quantity check, and Microarray experiments were carried out according to Schroeter et al. [24]. Custom-made *B. licheniformis* DSM13 8×15K gene expression arrays were obtained from Agilent Technologies (Santa Clara, CA) (https://earray.chem.agilent.com/earray/). Probe design was performed on the annotated ORFs of *B. licheniformis* DSM13 strain according to Veith et al. [25]. Microarrays were scanned using the Agilent scanner Type G2565CA with high resolution upgrade G2539A and the software Scan Control 8.4.1 (Agilent Technologies). Data were extracted from scanned images using Agilent's Feature Extraction Software (version 10.5.1.1) (Agilent Technologies) using default settings including a Linear/Lowess data normalization (for detailed Information see Agilent's Feature extraction manual). Gene expression data were loaded into the Rosetta Resolver® Gene Expression Analysis System 7.2 (Rosetta Inpharmatics c/o Ceiba Solutions, Boston, MA). A common reference type of design was employed, and data from three biological replicates were combined using an error-weighted average (Rosetta Resolver error model [26]). Genes showing significant differences in expression were identified by error-weighted ANOVA analysis included in the Rosetta Resolver software, with a Benjamini–Hochberg false discovery rate multiple test correction. Only genes for which an ANOVA $p < 0.01$ was obtained by the statistical testing, and which were at least fourfold induced (fold change above 4) or fourfold repressed (fold change below −4) for at least one time point throughout the experiment were considered as differentially expressed and were used for further evaluation.

3 Results and discussion

3.1 Growth and survival of *B. licheniformis* wild-type DSM 13 and *sigB* mutant subjected to heat and ethanol stress

Growth of the *B. licheniformis* wild type was only slightly impaired when the cells were transferred to 54°C (Fig. 1A). Addition of ethanol had a more profound impact on growth of the wild type. The growth behavior of the *sigB* mutant was very similar to that of the wild type (Fig. 1B). Survival of the cells was analyzed directly before the stress (0 min) and 30, 60, and 120 min after stress (Supporting Information Fig. 1). Survival before the stress was set to 100 and survival at the later time points was calculated accordingly. In both strains, the survival after ethanol addition was better than the survival after heat stress. Only minor differences in the survival behavior between the wild-type and the *sigB* mutant could be detected in the analyzed time window. These results are in contrast to a comparative analysis of the stress resistance of the wild-type *B. subtilis* strain 168 and its isogenic *sigB* mutant [27], where pronounced differences in the survival rates of the wild type and the *sigB* mutant after exposure to ethanol (10%) or heat stress (54°C) could be detected. A comparison of the survival experiments of both studies indicates that *B. licheniformis* DSM13 is more stress resistant than *B. subtilis* 168. However, the only slightly impaired survival rate of the *sigB*-deficient *B. licheniformis* strain is surprising. It could be speculated that some crucial genes are either not SigB dependent in *B. licheniformis* or are under dual control by other regulators (see also Section 3.8), which could compensate the loss of the SigB regulator.

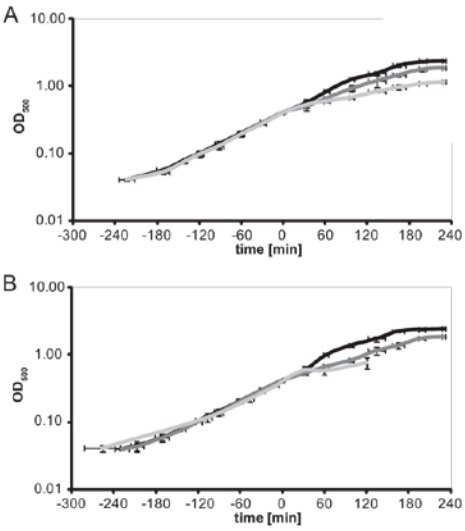


Figure 1. Growth of *Bacillus licheniformis* DSM13 (A) and the *sigB* mutant (B) under control conditions (black lines) and during heat (dark gray lines) and ethanol stress (light gray lines). Cells were cultivated in a chemically defined medium under control conditions and either transferred to 54°C or stressed with 6% ethanol at OD_{500nm} 0.4. Means of three biological replicates are shown with SD.

3.2 Response of *B. licheniformis* cells to heat stress

The response of *B. licheniformis* to a temperature up shift from 37°C to 54°C during exponential growth was studied at the level of the transcriptome (5, 10, and 20 min after stress) and proteome (10 and 30 min after stress). Although the growth of the bacterium was only slightly impaired (Fig. 1), the stress had a very profound effect on the cellular physiology. About 60 upregulated proteins could be identified (Fig. 2, Table 1). It should be noted that this upregulation could be the result of an elevated protein synthesis. However, different turn-over rates of proteins could also mimic this effect. More than 1000 genes were up- or downregulated (level of significance four-fold, Table 2, Supporting Information Table 4). When possible, differentially expressed genes/proteins were classified into putative regulons based on knowledge and gene ontology of *B. subtilis*.

3.3 Genes/proteins specifically upregulated during heat shock in *B. licheniformis*

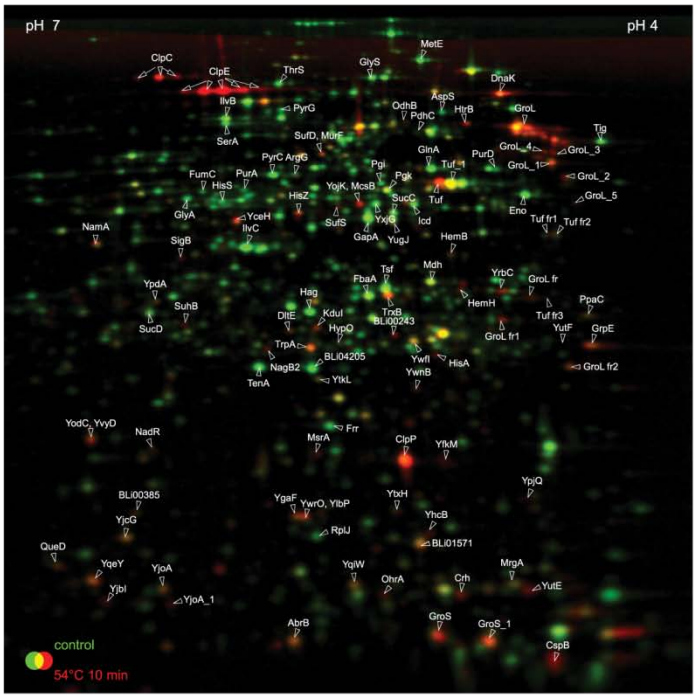
3.3.1 HrcA, CtsR, and HtpG operon

The HrcA regulon in *B. subtilis* consists of nine genes organized in two operons—the *dnaK* operon with seven genes and the *groEL-groES* operon [2, 4]. HrcA is a transcriptional repressor binding to sequences denominated as CIRCE elements [28]. After a heat shock, the repressor dissociates from the promoters of the target genes allowing transient induction of transcription. Strong heat induction of transcription was shown only for the first three genes of the *dnaK* operon, *hrcA*, *grpE*, and *dnaK* [2]. Nielsen et al. [5] observed heat induction of the putative HrcA regulon in *B. licheniformis*. They revealed an upregulation of these genes at 37°C in a *hrcA* mutant and could thus confirm repression of this operon by HrcA. In the study presented here, heat-dependent induction of the HrcA regulon was observed at the proteome level with DnaK, GrpE, GroL, and GroS being synthesized at up to 14-fold higher levels (Fig. 2, Table 1). Induction of transcription was observed for *groEL*, *groES*, *hrcA*, *grpE*, and *dnaK* (Table 2).

The CtsR regulon in *B. subtilis* includes the genes encoding the subunits of the ATP-dependent proteases ClpC and ClpE (ATPase subunits) and ClpP (protease) [2, 4]. In *B. subtilis*, *clpC* is part of an operon together with *ctsR* and two genes, *mcsA* and *mcsB*, encoding proteins, which modulate CtsR activity. ClpE and ClpP are encoded in monocistronic operons. Upon exposure to increased temperatures, the repressor CtsR is transiently inactivated in a temperature-dependent manner leading to the expression of the regulon [29]. Thus, the Clp protease system is upregulated after heat shock to deal with heat-denatured proteins [11, 12]. Further regulation at the posttranscriptional and posttranslational level results in a very accurate fine-tuning of the amount of the different Clp proteins in the cell depending on growth phase and conditions [30]. Synthesis of some of the CtsR regulon proteins was upregulated in our study for *B. licheniformis* (ClpC, ClpE—the protein with the highest induction, ClpP and McsB, Fig. 2, Table 1). Although the induction rate is considerably higher for ClpE than for ClpP (80–240-fold for ClpE and 13–16-fold for ClpP after 10 min heat shock), the spot volume for both proteins in the gels differs not as much (5–13% spot volume for ClpE and 4–5% for ClpP). This is due to the fact that the ClpP protein is present at a higher basal level in control cells. Heat induction of the CtsR regulon at the transcriptomic level was also observed with *clpE* being the gene with the highest induction after heat shock (Table 2). Nielsen et al. [5] confirmed CtsR repression of the target genes in *B. licheniformis*, since deletion of *ctsR* leads to an enhanced expression of the genes at 37°C compared to the wild type.

The *htpG* gene is the only member of the class IV heat shock genes described so far. The HtpG protein functions as a molecular chaperone. The *htpG* gene is induced in *B. subtilis* when cells are subjected to a heat shock [2, 4]. In our study, transcription of *htpG* is induced about tenfold at all time points (Table 2). Heat inducible induction of *htpG* transcription in *B. licheniformis* was also described by Nielsen et al. [5].

A



B

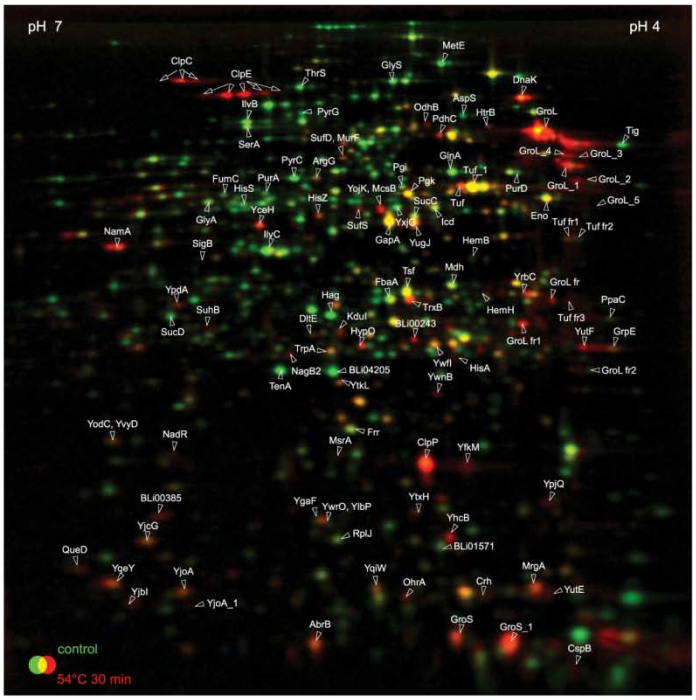


Figure 2. Proteome of *Bacillus licheniformis* DSM13 cells under heat stress conditions. (A) 10 min heat stress, (B) 30 min heat stress. Cell samples were labeled with L–[^{35}S]–methionine during the exponential growth phase (OD_{500nm} 0.4), and after 10 and 30 min of heat stress. Proteins were separated in a pH gradient 4–7. The dual channel images were created with the Delta 2D software (Decodon).

Table 1. Proteins upregulated during heat stress

Name	Function	Accession number	Regulon	10 min heat shock WT		30 min heat shock WT		Array WT	10 min heat shock Δ*sigB*		30 min heat shock Δ*sigB*	
				Mean ratio	SD	Mean ratio	SD		Mean ratio	SD	Mean ratio	SD
HrcA regulon												
DnaK	Chaperone protein	BLi02739		13.01	1.88	8.20	1.31	x	4.62	2.56	9.79	3.66
GroL	60 kDa chaperonin	BLi00624		11.48	1.67	12.60	1.79	x	7.99	4.36	24.19	4.76
GroL_1	60 kDa chaperonin	BLi00624		8.57	1.98	7.14	2.25	x				
GroL_2	60 kDa chaperonin	BLi00624		5.00	2.21	3.47	0.51	x				
GroL_3	60 kDa chaperonin	BLi00624		16.49	2.98	12.42	1.78	x				
GroL_4	60 kDa chaperonin	BLi00624		10.33	3.75	11.62	2.29	x			10.75	1.43
GroL_5	60 kDa chaperonin	BLi00624		7.01	2.39	7.27	3.87	x				
GroL fr	60 kDa chaperonin	BLi00624		5.33	0.65	6.13	1.21	x	6.67	3.07	7.50	0.83
GroL fr1	60 kDa chaperonin	BLi00624		7.11	1.96	6.73	2.39	x	6.78	1.41	14.86	2.02
GroL fr2	60 kDa chaperonin	BLi00624		6.12	0.53	2.77	0.73	x				
GroS_1	10 kDa chaperonin	BLi00623		3.48	0.69	5.58	0.94	x				
GroS	10 kDa chaperonin	BLi00623		5.44	0.64	4.63	0.99	x				
GrpE	Nucleotide exchange factor for DnaK activity	BLi02740		6.73	1.90	5.47	2.04	x	3.28	1.86	5.45	1.05
CtsR regulon												
ClpC	Class III stress response-related ATPase	BLi00104		30.68	7.20	16.43	2.58	x	6.46	5.70	1.39	0.75
ClpE	ATP-dependent Clp protease-like (class III stress gene)	BLi01525		162.11	64.12	30.99	7.51	x	37.35	23.33	1.14	0.34
ClpP	ATP-dependent Clp protease proteolytic subunit 1 (EC 3.4.21.92)	BLi03710		14.78	1.24	11.79	0.67	x	9.57	0.68	4.54	1.38
McsB (YjoK)	Putative ATP:guanido phosphotransferase	BLi00103		3.22	0.82	5.41	0.65	x				
SigB regulon												
SigB	RNA polymerase sigma factor	BLi00560		7.58	4.69	4.24	0.44	x				
SodA	Superoxide dismutase (EC 1.15.1.1)	BLi02679							1.40	0.09	1.03	0.32
SodA_1	Superoxide dismutase (EC 1.15.1.1)	BLi02679							4.95	0.50	1.83	0.20
YceH	Putative signal peptide binding protein	BLi00361		5.48	1.07	5.22	0.17	x				
YfkM	General stress protein	BLi00815		11.08	1.58	18.06	8.83	x				
YflT (Crh)	Unknown	BLi00779		4.82	0.54	2.42	0.52	x				
YtkL	Metal-dependent hydrolase	BLi03080		1.43	0.10	2.21	0.13	2–3 times				
YtxH	Unknown	BLi03129		3.55	0.21	3.04	0.61	-				
CssRS regulon												
HtrB	Putative serine protease	BLi03481		11.74	0.75	3.86	0.41	x				
Other												
AbrB	Transcriptional pleiotropic regulator of transition state genes	BLi00050	AbrB	3.08	0.52	4.38	1.23	x				
AlsS	Alpha-acetolactate synthase	BLi03848	AlsR, Rex								11.02	3.87
AroE	Shikimate 5-dehydrogenase (EC 1.1.1.25)	BLi02759	TRAP						5.50	2.45	3.33	1.40
BLi00243	Unknown	BLi00243		8.57	1.16	12.47	2.58	x				
BLi00385	NAD(P)H dehydrogenase	BLi00385		2.67	0.75	5.63	1.61	x				
BLi01571	Unknown	BLi01571		2.78	0.24	0.54	0.08	2–3 times				
CodY	GTP-sensing transcriptional pleiotropic repressor	BLi01837	CodY						3.59	0.42	2.95	0.87
Crh (YflT)	Catabolite repression HPr-like protein	BLi03722		4.82	0.54	2.42	0.52	x				
CspB	Cold-shock protein	BLi02332		12.56	3.42	2.40	0.36	-				
Ddl	D-Alanyl-D-alanine ligase A (EC 6.3.2.4)	BLi00543	SigM						1.53	0.55	2.79	0.35
DltE	Involved in lipoteichoic acid biosynthesis	BLi00637	SigD, M,X	3.23	0.16	2.05	0.32	x				
GuaB	Inosine-monophosphate dehydrogenase (EC 1.1.1.205)	BLi00014	CodY						13.23	5.47	2.32	0.80

Table 1. Continued

Name	Function	Accession number	Regulon	10 min heat shock WT		30 min heat shock WT		Array WT	10 min heat shock Δ*sigB*		30 min heat shock Δ*sigB*	
				Mean ratio	SD	Mean ratio	SD		Mean ratio	SD	Mean ratio	SD
HemH	Ferrochelatase (EC 4.99.1.1)	BLi01093		2.56	0.22	1.49	0.28	2–3 times				
HemQ	Putative oxidoreductase/oxygenase/dismutase	BLi03998		8.57	1.16	12.47	2.58	2–3 times	2.04	0.11	2.15	0.52
HisA	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase (EC 5.3.1.16)	BLi03733		5.36	0.07	2.26	0.86	-				
HisZ	Histidyl-tRNA synthetase (EC 6.1.1.21)	BLi03738		4.48	0.38	2.09	0.59	-				
HypO	Similar to NAD(P)H-flavin oxidoreductase	BLi00813	HypR	0.99	0.22	4.81	0.49	x	1.79	0.22	5.56	1.09
KduI	4-Deoxy-L-threo-5-hexosulose-uronate ketol-isomerase (EC 5.3.1.17)	BLi03829	CcpA, KdgR	3.21	0.16	2.77	0.24	x	2.94	0.16	4.30	0.72
MurAA	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	BLi03922							4.64	1.74	4.98	1.09
MurF (SufD)	UDP-*N*-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanine ligase (EC 6.3.2.15)	BLi00544	SigM	3.87	0.27	2.40	0.24	x	3.03	0.77	4.30	0.29
NadA	Quinolinate synthetase (EC 4.1.99.-)	BLi02912	NadR						2.73	0.65	2.22	0.58
NadR	Transcriptional repressor of de novo NAD biosynthesis	BLi02916	NadR	4.41	1.72	4.53	1.39	2–3 times				
NagB2	Glucosamine-6-phosphate deaminase	BLi04349	NagR	3.12	0.30	2.27	1.13	x				
NamA	NADH dehydrogenase (EC 1.6.99.1)	BLi02551		2.30	0.45	9.72	2.20	x	10.72	2.68	8.00	0.29
OdhB	2-Oxoglutarate dehydrogenase complex (dihydrolipoamide transsuccinylase, E2 subunit) (EC 2.3.1.61)	BLi02259		3.31	0.77	3.53	0.39	Twice				
PdhC	Pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit) (EC 2.3.1.12)	BLi01676		0.97	0.04	5.73	0.33	-				
PdxT	Glutamine amidotransferase (EC 2.6.-.-)	BLi00017	Spo0A						4.92	1.18	2.68	0.54
PpaC	Manganese-dependent inorganic pyrophosphatase (EC 3.6.1.1)	BLi03882		2.26	0.26	2.24	0.99	-				
Sat	Sulfate adenylyltransferase (EC 2.7.7.4)	BLi01780	CymR, S-box						5.03	1.92	6.15	2.24
SdhA	Succinate dehydrogenase (flavoprotein subunit) (EC 1.3.99.1)	BLi02993							4.81	1.60	2.86	0.65
SecA	Translocase binding subunit (ATPase)	BLi03773							2.11	0.77	2.90	0.70
SuhB	Myo-inositol-1(or 4)-monophosphatase (EC 3.1.3.25)	BLi01685		4.71	1.46	3.39	0.35	2–3 times				
TrpA	Tryptophan synthase (alpha subunit) (EC 4.2.1.10)	BLi02398	TRAP	2.97	0.02	1.40	0.18	x	4.09	0.72	3.64	0.74
Tuf	Elongation factor Tu	BLi00131		9.94	1.89	4.19	1.15	-				
Tuf_1	Elongation factor Tu	BLi00131		0.55	0.03	0.80	0.04	-	1.06	0.23	2.10	0.26
Tuf fr1	Elongation factor Tu	BLi00131		2.80	0.55	3.06	1.10	-				
Tuf fr2	Elongation factor Tu	BLi00131		2.23	0.17	2.06	0.20	-				
Tuf fr3	Elongation factor Tu	BLi00131		3.06	0.88	5.74	1.67	-				
YgaF	Peroxiredoxin Q/BCP (EC 1.11.1.15)	BLi00899		4.86	0.39	2.78	0.76	x				

Table 1. Continued

Name	Function	Accession number	Regulon	10 min heat shock WT		30 min heat shock WT		Array WT	10 min heat shock Δ*sigB*		30 min heat shock Δ*sigB*	
				Mean ratio	SD	Mean ratio	SD		Mean ratio	SD	Mean ratio	SD
YhcB	Putative Flavodoxin/nitric oxide synthase	BLi00966		8.54	0.69	16.30	3.89	x				
YjbG	Oligoendopeptidase F (EC 3.4.24.-)	BLi01247							2.50	0.46	0.55	0.15
YjbI	Truncated form of bacterial hemoglobin	BLi01250		10.45	3.76	16.20	4.84	x				
YjcG	Putative RNA ligase or phosphoesterase	BLi01284		2.85	0.36	2.89	0.90	x				
YjoA_1	Unknown	BLi02905		17.95	2.19	10.54	2.98	2–3 times				
YjoA	Unknown	BLi02905		2.53	0.39	3.34	0.38	2–3 times				
YlbP (YwrO)	Putative acetyltransferase	BLi01727	CcpA	4.73	1.10	2.55	0.29	x				
YojK (McsB)	Unknown	BLi00529		3.22	0.82	5.41	0.65	x				
YpdA	Thioredoxin reductase (NADPH) (EC 1.8.1.9)	BLi02434		1.74	0.39	3.04	0.20	x				
YpjQ	Putative phosphatidylglycerophosphatase	BLi02320		4.25	0.83	3.37	0.77	2–3 times				
YqeY	Unknown	BLi02731		3.66	0.76	4.29	1.12	-				
YqiW	Unknown	BLi02578		2.31	0.24	2.09	0.29	-				
YrbC	Unknown	BLi02909		4.55	0.98	2.71	0.86	x				
YrvO	Cysteine desulfurase (EC 2.8.1.7)	BLi02876							2.57	0.56	3.63	0.48
YutE	Unknown	BLi03419		12.89	0.72	7.36	1.30	-				
YutF	4-Nitrophenyl phosphatase (EC 3.1.3.41)	BLi03418		3.51	0.69	24.64	25.26	-				
YwnB	Putative oxidoreductase	BLi03911		6.53	0.49	7.15	0.52	x				
YwrO (YlbP)	NAD(P)H dehydrogenase	BLi03696		4.73	1.10	2.55	0.29	x				
Oxidative stress												
HemB	Delta-aminolevulinic acid dehydratase (porphobilinogen synthase) (EC 4.2.1.24)	BLi02943	PerR	2.53	0.49	1.36	0.45	-				
MrgA	Metalloregulation DNA-binding stress protein	BLi03480	PerR	1.83	0.06	2.97	0.97	x				
MsrA	Peptide methionine sulfoxide reductase (EC 1.8.4.11)	BLi02303	Spx	5.95	0.55	3.79	0.64	x				
OhrA	Organic hydroperoxide resistance protein	BLi01414	OhrR	6.03	1.18	7.67	1.27	x				
SufD (MurF)	FeS assembly protein	BLi03450		3.87	0.27	2.40	0.24	2–3 times	8.98	1.91	11.61	0.44
SufD_1	FeS assembly protein	BLi03450							2.95	1.21	2.05	0.17
SufS	Cysteine desulfurase (EC 2.8.1.7)	BLi03449		2.81	0.42	3.57	0.53	2–3 times				
TrxB	Thioredoxin reductase (EC 1.6.4.5)	BLi03728	Spx	0.84	0.38	2.37	0.53	x	2.02	0.40	0.76	0.04
YugJ	Iron-containing alcohol dehydrogenase (EC 1.1.1.-)	BLi03317	Spx	0.71	0.05	1.89	0.13	x	2.28	0.11	1.50	0.10

Array WT: gene regulation determined with the microarray for the wild type; x: upregulation more than fourfold; -: no upregulation at the transcriptional level; regulon classification mainly according to *Bacillus subtilis*; protein names in brackets: two proteins identified in one spot.

3.3.2 Further genes/proteins upregulated in heat-shocked *B. licheniformis* cells

The CssRS two component system of *B. subtilis* is known to be induced by secretion stress [31] and has also been described as being heat inducible [32]. Transcription of the CssRS-regulated genes *htrA* and *htrB* is upregulated in *B. subtilis* and *B. licheniformis* when cells are subjected to a heat shock [2, 4, 5, 32]. In the study presented here, we could only observe a strong upregulation of *htrA* (up to 50-fold, Table 2). The *htrB* (*yvtA*) gene is also transcribed at an elevated level, but the transcription declines at later time points (10 and 20 min). The same induction pattern was found for the respective protein HtrB (Fig. 2, Table 1).

Table 2. Transcriptome data of genes belonging to known heat shock induced regulons.

Name	Function	Accession number	5 min			10 min			20 min			Regulon	SigB membership determined by:
			Ratio	log (ratio)[a]	log (error)	Ratio	log (ratio)[a]	log (error)	Ratio	log (ratio)[a]	log (error)		
HrcA regulon													
groES	10 kDa chaperonin	BLi00623	**16.55**	**1.22**	**0.11**	**13.81**	**1.14**	**0.17**	2.52	0.40	0.20	HrcA	
groEL	60 kDa chaperonin	BLi00624	**11.07**	**1.04**	**0.02**	**11.66**	**1.07**	**0.04**	**5.91**	**0.77**	**0.06**	HrcA	
yqeV	YqeV	BLi02735	2.18	0.34	0.03	2.76	0.44	0.04	2.58	0.41	0.04	HrcA	
yqeU	YqeU	BLi02736	1.58	0.20	0.03	1.67	0.22	0.03	1.53	0.18	0.03	HrcA	
prmA	Ribosomal protein L11 methyltransferase	BLi02737	2.57	0.41	0.02	2.91	0.46	0.02	2.96	0.47	0.02	HrcA	
dnaJ	Chaperone protein DnaJ	BLi02738	3.15	0.50	0.04	3.62	0.56	0.05	3.41	0.53	0.03	HrcA	
dnaK	Chaperone protein DnaK	BLi02739	**10.71**	**1.03**	**0.02**	**10.07**	**1.00**	**0.04**	**8.79**	**0.94**	**0.05**	HrcA	
grpE	Protein grpE	BLi02740	**7.49**	**0.87**	**0.02**	**6.59**	**0.82**	**0.03**	**4.94**	**0.69**	**0.06**	HrcA	
hrcA	Heat-inducible transcription repressor HrcA	BLi02741	**14.33**	**1.16**	**0.02**	**15.82**	**1.20**	**0.02**	**14.84**	**1.17**	**0.04**	HrcA	
SigB regulon													
yacK	DNA integrity scanning protein DisA	BLi00106	**17.86**	**1.25**	**0.02**	**28.84**	**1.46**	**0.02**	**22.44**	**1.35**	**0.03**	SigB	BS
yacL	Putative uncharacterized protein yacL	BLi00107	**13.26**	**1.12**	**0.07**	**19.48**	**1.29**	**0.07**	**13.02**	**1.11**	**0.08**	SigB	BS
ispD	2-C-Methyl-D-erythritol 4-phosphate cytidylyltransferase	BLi00108	**5.64**	**0.75**	**0.04**	**6.31**	**0.80**	**0.03**	**5.83**	**0.77**	**0.03**	SigB, SigM	BS
ispF	2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase	BLi00109	3.53	0.55	0.03	**4.02**	**0.60**	**0.02**	**4.05**	**0.61**	**0.02**	SigB	BS
ybyB	Putative uncharacterized protein ybyB	BLi00231	**4.86**	**0.69**	**0.04**	**4.74**	**0.68**	**0.06**	**7.23**	**0.86**	**0.10**	SigB	Mutant, BS
none	Putative uncharacterized protein	BLi00243	**4.69**	**0.67**	**0.03**	**46.13**	**1.66**	**0.06**	**111.77**	**2.05**	**0.07**	SigB	Mutant
ycbP	Putative uncharacterized protein ycbP	BLi00264	3.74	0.57	0.04	**4.36**	**0.64**	**0.02**	3.09	0.49	0.05	SigB	BS
yjgA	Putative uncharacterized protein yjgA	BLi00273	**4.18**	**0.62**	**0.08**	2.60	0.41	0.05	2.87	0.46	0.08	SigB	BS
ycdF	Glucose 1-dehydrogenase II	BLi00341	**4.51**	**0.65**	**0.12**	**9.90**	**1.00**	**0.11**	**13.24**	**1.12**	**0.16**	SigB	Mutant, VF, BS
none	ABC transporter, ATP-binding protein	BLi00349	**7.10**	**0.85**	**0.05**	**37.83**	**1.58**	**0.10**	**81.93**	**1.91**	**0.08**	SigB	Mutant
yceC	Putative stress response protein YceC	BLi00354	**8.55**	**0.93**	**0.03**	**8.82**	**0.95**	**0.04**	**8.06**	**0.91**	**0.04**	SigB	BS
yceD	Putative stress response protein YceD	BLi00355	**6.85**	**0.84**	**0.08**	**6.83**	**0.83**	**0.04**	**6.64**	**0.82**	**0.06**	SigB	BS
yceE	Putative stress response protein YceE	BLi00356	**16.47**	**1.22**	**0.06**	**12.82**	**1.11**	**0.05**	**13.12**	**1.12**	**0.04**	SigB	BS
yceF	Integral membrane protein TerC family, YceF	BLi00357	**20.11**	**1.30**	**0.06**	**12.11**	**1.08**	**0.04**	**13.07**	**1.12**	**0.04**	SigB	BS
yceG	Putative uncharacterized protein yceG	BLi00360	**5.40**	**0.73**	**0.07**	**4.64**	**0.67**	**0.06**	**5.28**	**0.72**	**0.07**	SigB	BS
yceH	Putative signal peptide binding protein YceH	BLi00361	**9.61**	**0.98**	**0.03**	**7.56**	**0.88**	**0.03**	**9.06**	**0.96**	**0.02**	SigB	BS
yclO	Putative transport system permease protein	BLi00464	**0.03**	**−1.55**	**0.05**	**0.03**	**−1.50**	**0.07**	**0.10**	**−0.98**	**0.04**	SigB	Mutant
ycsD	YcsD	BLi00490	**6.25**	**0.80**	**0.05**	**8.47**	**0.93**	**0.09**	**8.28**	**0.92**	**0.10**	SigB	BS
ydaD	Putative short-chain dehydrogenase/reductase YdaD	BLi00509	**15.91**	**1.20**	**0.11**	**59.15**	**1.77**	**0.12**	**75.67**	**1.88**	**0.13**	SigB	Mutant, BS
ydaE	YdaE	BLi00510	1.41	0.15	0.04	**26.18**	**1.42**	**0.27**	**5.20**	**0.72**	**0.38**	SigB	Mutant, BS
ydaG	FMN-binding split barrel domain protein YdaG	BLi00512	**57.25**	**1.76**	**0.07**	**136.49**	**2.14**	**0.04**	**234.73**	**2.37**	**0.15**	SigB	Mutant, VF, BS
ydaP	Pyruvate decarboxylase	BLi00520	2.00	0.30	0.07	3.21	0.51	0.07	**4.90**	**0.69**	**0.06**	SigB	Mutant, VF, BS
ydaS	Transglycosylase-associated protein	BLi00527	2.15	0.33	0.09	**5.96**	**0.78**	**0.05**	**8.76**	**0.94**	**0.14**	SigB	Mutant, BS

Table 2. Continued

Name	Function	Accession number	5 min			10 min			20 min			Regulon	SigB membership determined by:
			Ratio	log (ratio)[a)]	log (error)	Ratio	log (ratio)[a)]	log (error)	Ratio	log (ratio)[a)]	log (error)		
ansB	Aspartate ammonia-lyase	BLi00528	1.68	0.22	0.06	**4.14**	**0.62**	**0.07**	**4.78**	**0.68**	**0.08**	SigB	Mutant
ydaT	Conserved protein YdaT	BLi00530	**5.47**	**0.74**	**0.15**	**13.82**	**1.14**	**0.12**	**18.96**	**1.28**	**0.19**	SigB	Mutant, BS
gsiB	General stress protein	BLi00534	**10.58**	**1.02**	**0.03**	**23.83**	**1.38**	**0.05**	**56.18**	**1.75**	**0.08**	SigB, SigI	Mutant, VF, BS
rsbW	Serine-protein kinase RsbW	BLi00559	2.77	0.44	0.01	3.97	0.60	0.03	**4.51**	**0.65**	**0.04**	SigB	BS
sigB	RNA polymerase sigma factor SigB	BLi00560	2.84	0.45	0.04	**4.09**	**0.61**	**0.04**	**5.04**	**0.70**	**0.03**	SigB	Mutant, BS
rsbX	Phosphoserine phosphatase rsbX	BLi00561	3.37	0.53	0.02	**5.38**	**0.73**	**0.04**	**5.47**	**0.74**	**0.03**	SigB	BS
none	Fatty acid desaturase	BLi00576	**12.39**	**1.09**	**0.09**	**13.59**	**1.13**	**0.07**	**16.66**	**1.22**	**0.05**	SigB	Mutant, VF
none	Putative uncharacterized protein	BLi00709	**4.13**	**0.62**	**0.16**	**16.16**	**1.21**	**0.09**	**7.49**	**0.87**	**0.14**	SigB	Mutant
none	Putative uncharacterized protein	BLi00719	**8.80**	**0.94**	**0.07**	**10.26**	**1.01**	**0.07**	**13.42**	**1.13**	**0.08**	SigB	Mutant
none	Putative uncharacterized protein	BLi00720	**8.48**	**0.93**	**0.04**	**9.84**	**0.99**	**0.08**	**13.22**	**1.12**	**0.10**	SigB	Mutant
yflT	Putative uncharacterized protein yflT	BLi00779	**33.58**	**1.53**	**0.20**	**194.67**	**2.29**	**0.10**	**448.39**	**2.65**	**0.12**	SigB	Mutant, VF, BS
yvaZ	Conserved membrane protein SdpI	BLi00786	**24.13**	**1.38**	**0.02**	**35.84**	**1.55**	**0.03**	**27.60**	**1.44**	**0.05**	SigB, AbrB, SdpR	Mutant
yfkM	General stress protein YfkM	BLi00815	**22.07**	**1.34**	**0.20**	**27.41**	**1.44**	**0.27**	**59.36**	**1.77**	**0.18**	SigB, Fur	Mutant, VF, BS
yfkE	H+/Ca2+ exchanger YfkE	BLi00823	3.29	0.52	0.06	3.81	0.58	0.09	**4.13**	**0.62**	**0.06**	SigB	BS
yfkD	Conserved protein YfkD	BLi00824	**4.01**	**0.60**	**0.02**	**5.42**	**0.73**	**0.02**	**5.86**	**0.77**	**0.02**	SigB	Mutant, BS
yhcU	Putative uncharacterized protein yhcU	BLi00985	0.39	−0.41	0.02	**0.23**	**−0.63**	**0.05**	**0.30**	**−0.53**	**0.09**	SigB	Mutant
yhdF	Short-chain dehydrogenase/reductase SDR YhdF	BLi01012	3.34	0.52	0.05	**6.92**	**0.84**	**0.05**	**10.52**	**1.02**	**0.05**	SigB	Mutant, BS
nhaX	NhaX	BLi01046	**17.05**	**1.23**	**0.05**	**51.02**	**1.71**	**0.03**	**74.19**	**1.87**	**0.08**	SigB	Mutant, BS
cotJA	CotJA	BLi01145	2.04	0.31	0.04	**6.91**	**0.84**	**0.08**	**5.50**	**0.74**	**0.04**	SigB	Mutant
cotJB	CotJB	BLi01146	2.53	0.40	0.04	**5.77**	**0.76**	**0.07**	**5.90**	**0.77**	**0.09**	SigB	Mutant
yjzB	Putative uncharacterized protein yjzB	BLi01220	1.61	0.21	0.04	**4.17**	**0.62**	**0.05**	3.73	0.57	0.05	SigB	Mutant
cotY	CotY	BLi01269	1.29	0.11	0.05	3.06	0.49	0.05	**4.79**	**0.68**	**0.11**	SigB, SigK	Mutant
none	Hypothetical DNA-binding protein, putative transcriptional regulator	BLi01292	**0.25**	**−0.61**	**0.03**	**0.13**	**−0.90**	**0.04**	**0.09**	**−1.03**	**0.09**	SigB	Mutant
ykgA	Putative uncharacterized protein ykgA	BLi01401	**5.40**	**0.73**	**0.04**	**9.05**	**0.96**	**0.04**	**12.28**	**1.09**	**0.03**	SigB	Mutant, VF, BS
ykzA	Organic hydroperoxide resistance protein, sigmaB regulon	BLi01416	**79.97**	**1.90**	**0.09**	**118.63**	**2.07**	**0.10**	**257.67**	**2.41**	**0.14**	SigB	Mutant, BS
none	Putative uncharacterized protein	BLi01417	**4.82**	**0.68**	**0.05**	**9.21**	**0.96**	**0.05**	**12.83**	**1.11**	**0.07**	SigB	Mutant
none	Putative uncharacterized protein	BLi01488	2.34	0.37	0.04	3.02	0.48	0.05	1.90	0.28	0.04	SigB	VF
ykzB	Putative uncharacterized protein ykzB	BLi01491	**13.75**	**1.14**	**0.04**	**19.91**	**1.30**	**0.09**	**24.02**	**1.38**	**0.14**	SigB	Mutant, VF
none	Putative uncharacterized protein	BLi01622	**4.89**	**0.69**	**0.04**	**20.53**	**1.31**	**0.08**	**36.47**	**1.56**	**0.09**	SigB	Mutant, VF
ykuP	Similar to flavodoxin	BLi01631	**0.09**	**−1.06**	**0.05**	**0.05**	**−1.27**	**0.08**	**0.12**	**−0.93**	**0.05**	SigB	Mutant
ykpC	Putative uncharacterized protein ykpC	BLi01661	**4.72**	**0.67**	**0.03**	**33.61**	**1.53**	**0.05**	**19.58**	**1.29**	**0.13**	SigB	Mutant
ykzI	Putative uncharacterized protein ykzI	BLi01684	**5.71**	**0.76**	**0.13**	**8.10**	**0.91**	**0.06**	**14.07**	**1.15**	**0.11**	SigB	Mutant, VF, BS

Table 2. Continued

Name	Function	Accession number	5 min			10 min			20 min			Regulon	SigB membership determined by:
			Ratio	log (ratio)[a]	log (error)	Ratio	log (ratio)[a]	log (error)	Ratio	log (ratio)[a]	log (error)		
murG	UDP-*N*-acetylglucosamine–*N*-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase	BLi01739	0.59	−0.23	0.22	**0.24**	**−0.61**	**0.23**	**0.28**	**−0.55**	**0.19**	SigB	Mutant
pyrD	Dihydroorotate dehydrogenase 1	BLi01774	2.03	0.31	0.03	0.38	−0.42	0.07	**0.24**	**−0.63**	**0.09**	SigB, PyrR	Mutant
ylqH	Putative uncharacterized protein ylqH	BLi01828	**0.08**	**−1.11**	**0.06**	**0.06**	**−1.22**	**0.06**	**0.12**	**−0.91**	**0.06**	SigB	Mutant
cwlC	*N*-Acetylmuramoyl-L-alanine amidase CwlC	BLi01974	**4.80**	**0.68**	**0.03**	**6.61**	**0.82**	**0.03**	**4.22**	**0.63**	**0.08**	SigB, SigK	Mutant
none	Trp repressor binding protein, putative	BLi02026	1.07	0.03	0.11	2.90	0.46	0.03	**5.12**	**0.71**	**0.04**	SigB	Mutant, VF
dctB	C4-dicarboxylate binding protein	BLi02120	**5.14**	**0.71**	**0.14**	**25.76**	**1.41**	0.16	**69.37**	**1.84**	**0.19**	SigB	Mutant
ydeT	Hypothetical DNA-binding protein	BLi02124	2.27	0.36	0.04	**4.38**	**0.64**	0.05	**4.04**	**0.61**	**0.05**	SigB, AseR	Mutant
yvmB	YvmB	BLi02188	**7.37**	**0.87**	**0.05**	**11.18**	**1.05**	**0.04**	**10.84**	**1.04**	**0.07**	SigB	Mutant
none	Na+/solute symporter	BLi02212	**9.89**	**1.00**	**0.08**	**9.28**	**0.97**	**0.08**	**14.76**	**1.17**	**0.09**	SigB	Mutant, VF
yjgD	Putative uncharacterized protein yjgD	BLi02221	**11.79**	**1.07**	**0.09**	**22.14**	**1.35**	**0.04**	**20.77**	**1.32**	**0.03**	SigB	Mutant, BS
yjgC	Formate dehydrogenase	BLi02222	**7.52**	**0.88**	**0.07**	**11.39**	**1.06**	**0.05**	**13.14**	**1.12**	**0.05**	SigB	Mutant, BS
yocK	General stress protein	BLi02246	**12.96**	**1.11**	**0.04**	**24.27**	**1.39**	**0.03**	**32.65**	**1.51**	**0.05**	SigB	Mutant, VF, BS
none	Putative uncharacterized protein	BLi02272	**5.60**	**0.75**	**0.03**	**8.65**	**0.94**	**0.06**	**6.92**	**0.84**	**0.11**	SigB	Mutant
none	Putative uncharacterized protein	BLi02422	**0.29**	**−0.54**	**0.12**	**0.15**	**−0.82**	**0.09**	**0.17**	**−0.76**	**0.07**	SigB	Mutant
seaA	Putative uncharacterized protein seaA	BLi02423	**0.24**	**−0.61**	**0.05**	**0.13**	**−0.89**	**0.04**	**0.11**	**−0.96**	**0.05**	SigB	Mutant
ypuD	YpuD	BLi02476	3.06	0.49	0.03	**5.26**	**0.72**	**0.07**	**6.15**	**0.79**	**0.13**	SigB	BS
yqjL	Putative hydrolase	BLi02552	**23.11**	**1.36**	**0.04**	**16.63**	**1.22**	**0.03**	**22.78**	**1.36**	**0.03**	SigB	BS
bmrU	Multidrug resistance protein	BLi02579	**7.14**	**0.85**	**0.11**	**22.11**	**1.34**	**0.09**	**15.27**	**1.18**	**0.19**	SigB	Mutant, VF, BS
comGB	Late competence protein ComGB/DNA transport protein ComGB	BLi02647	2.79	0.45	0.09	**4.77**	**0.68**	**0.13**	**4.30**	**0.63**	**0.13**	SigB	Mutant
comGA	Late competence protein ComGA	BLi02648	**4.95**	**0.69**	**0.13**	**10.82**	**1.03**	**0.08**	**6.36**	**0.80**	**0.09**	SigB	Mutant
yqhA	Putative sulfate transporter YqhA	BLi02649	**11.89**	**1.08**	**0.10**	**45.71**	**1.66**	**0.05**	**34.78**	**1.54**	**0.06**	SigB	Mutant, BS
yqgZ	(mgsR) transcriptional regulator	BLi02651	**78.84**	**1.90**	**0.10**	**161.43**	**2.21**	**0.06**	**269.75**	**2.43**	**0.04**	SigB	Mutant, VF, BS
bmrR	Transcriptional activator	BLi02784	**0.20**	**−0.71**	**0.06**	**0.19**	**−0.73**	**0.06**	**0.23**	**−0.64**	**0.05**	SigB, Mta	BS
yrkE	YrkE	BLi02794	0.73	−0.13	0.03	2.33	0.37	0.05	**7.00**	**0.84**	**0.03**	SigB	BS
yrhK	YrhK	BLi02834	**6.39**	**0.81**	**0.12**	**18.03**	**1.26**	0.10	**13.36**	**1.13**	**0.05**	SigB, SigV	Mutant
none	Putative uncharacterized protein	BLi02908	3.26	0.51	0.02	**8.06**	**0.91**	0.03	**7.63**	**0.88**	**0.02**	SigB	Mutant
yshC	Putative DNA polymerase YshC	BLi03006	3.17	0.50	0.02	**4.59**	**0.66**	0.04	3.60	0.56	0.03	SigB	BS
ysdB	Conserved protein YsdB	BLi03031	2.46	0.39	0.01	**5.54**	**0.74**	0.07	**10.12**	**1.01**	**0.07**	SigB	BS
none	Small acid-soluble spore protein (Beta-type SASP)	BLi03099	1.65	0.22	0.03	**3.97**	**0.60**	**0.03**	**5.24**	**0.72**	**0.09**	SigB	Mutant
ytzE	Probable transcriptional regulator YtzE	BLi03153	**3.44**	**0.54**	**0.05**	**6.15**	**0.79**	**0.05**	**5.50**	**0.74**	**0.10**	SigB	BS

Table 2. Continued

Name	Function	Accession number	5 min			10 min			20 min			Regulon	SigB membership determined by:
			Ratio	log (ratio)[a)]	log (error)	Ratio	log (ratio)[a)]	log (error)	Ratio	log (ratio)[a)]	log (error)		
glgC	Glucose-1-phosphate adenylyltransferase	BLi03229	0.46	−0.34	0.06	**0.24**	**−0.63**	**0.12**	**0.24**	**−0.63**	**0.09**	SigB	Mutant
yfiZ	ABC transport system permease protein	BLi03474	**0.10**	**−0.99**	**0.02**	**0.14**	**−0.85**	**0.04**	**0.26**	**−0.58**	**0.03**	SigB	Mutant
uxaC	Uronate isomerase	BLi03516	**4.28**	**0.63**	**0.06**	**19.61**	**1.29**	**0.07**	**72.69**	**1.86**	**0.07**	SigB, ExuR	Mutant
hisB	Imidazoleglycerol-phosphate dehydratase	BLi03735	**0.13**	**−0.89**	**0.05**	0.36	−0.44	0.11	0.43	−0.37	0.09	SigB	Mutant
hisG	ATP phosphoribosyl-transferase	BLi03737	**0.19**	**−0.72**	**0.09**	0.40	−0.40	0.07	0.54	−0.26	0.07	SigB	Mutant
csbA	CsbA	BLi03760	3.98	0.60	0.03	**4.28**	**0.63**	**0.04**	**4.57**	**0.66**	**0.05**	SigB	BS
yvjB	Peptidase S41A, C-terminal protease	BLi03765	0.91	−0.04	0.03	1.55	0.19	0.07	**4.72**	**0.67**	**0.19**	SigB, SigE, SigG	Mutant
ywtD	Gamma-DL-glutamyl hydrolase	BLi03835	**0.20**	**−0.71**	**0.05**	**0.24**	**−0.63**	0.03	0.38	−0.41	0.02	SigB, SigD	Mutant
ydbD	General stress protein, putative manganese-containing catalase	BLi03883	**4.57**	**0.66**	**0.15**	**28.74**	**1.46**	**0.15**	**41.41**	**1.62**	**0.13**	SigB	Mutant, BS
yqjF	Conserved protein YqjF	BLi03884	**7.12**	**0.85**	**0.13**	**13.77**	**1.14**	**0.12**	**24.72**	**1.39**	**0.10**	SigB	Mutant, VF
none	CBS domain protein	BLi03885	**14.52**	**1.16**	**0.09**	**95.94**	**1.98**	**0.06**	**155.71**	**2.19**	**0.08**	SigB	Mutant
yqxL	Mg^{2+} transporter protein, CorA-like	BLi03886	**17.04**	**1.23**	**0.10**	**47.20**	**1.67**	**0.06**	**63.10**	**1.80**	**0.04**	SigB	Mutant, VF, BS
yqhB	YqhB	BLi03887	**36.42**	**1.56**	**0.10**	**64.21**	**1.81**	**0.05**	**79.40**	**1.90**	**0.06**	SigB	Mutant, BS
ywnB	YwnB	BLi03911	1.25	0.10	0.02	**10.35**	**1.02**	**0.03**	**33.00**	**1.52**	**0.06**	SigB	Mutant
ywcE	Spore morphogenesis and germination protein ywcE	BLi04028	3.58	0.55	0.05	**5.45**	**0.74**	**0.05**	**4.85**	**0.69**	**0.09**	SigB, AbrB	Mutant
none	Putative uncharacterized protein	BLi04033	**0.19**	**−0.72**	**0.07**	**0.14**	**−0.84**	**0.05**	**0.17**	**−0.77**	**0.06**	SigB	Mutant
none	Putative uncharacterized protein	BLi04034	**0.13**	**−0.89**	**0.05**	**0.10**	**−1.00**	**0.02**	**0.13**	**−0.90**	**0.08**	SigB	Mutant
gspA	General stress protein A	BLi04064	**51.61**	**1.71**	**0.04**	**138.23**	**2.14**	**0.02**	**187.81**	**2.27**	**0.06**	SigB	Mutant, VF, BS
yxzF	YxzF	BLi04091	**6.48**	**0.81**	**0.04**	**10.86**	**1.04**	**0.04**	**10.27**	**1.01**	**0.08**	SigB	BS
yxlJ	Putative 3-methyladenine DNA glycosylase	BLi04092	**5.87**	**0.77**	**0.02**	**9.77**	**0.99**	**0.04**	**9.32**	**0.97**	**0.03**	SigB	BS
none	Putative transcriptional regulator	BLi04093	**4.22**	**0.63**	**0.03**	**9.25**	**0.97**	**0.07**	**8.60**	**0.93**	**0.08**	SigB	Mutant
mmgD	Citrate synthase III	BLi04094	**5.73**	**0.76**	**0.05**	**12.76**	**1.11**	**0.06**	**16.43**	**1.22**	**0.05**	SigB, SigE	Mutant
none	Transcriptional regulator Fnr family protein	BLi04167	**8.59**	**0.93**	**0.03**	**18.03**	**1.26**	**0.04**	**18.35**	**1.26**	**0.03**	SigB	Mutant
katE1	sigB dep. Catalase	BLi04196	**17.26**	**1.24**	**0.04**	**26.11**	**1.42**	**0.02**	**32.29**	**1.51**	**0.03**	SigB	Mutant, BS
katE2	sigB dep. Catalase	BLi04197	**18.65**	**1.27**	**0.05**	**21.97**	**1.34**	**0.06**	**31.66**	**1.50**	**0.04**	SigB	Mutant, VF, BS
iolF	Inositol transport protein	BLi04246	**6.11**	**0.79**	**0.10**	**14.93**	**1.17**	**0.15**	**39.77**	**1.60**	**0.12**	SigB, IolR	Mutant
hsdS	HsdS	BLi04316	3.51	0.55	0.07	3.21	0.51	0.09	**6.66**	**0.82**	**0.05**	SigB	Mutant
none	Putative uncharacterized protein	BLi04317	**5.27**	**0.72**	**0.03**	**5.40**	**0.73**	**0.02**	**7.52**	**0.88**	**0.02**	SigB	Mutant
none	Putative type I restriction-modification system M subunit	BLi04318	3.76	0.58	0.06	**4.45**	**0.65**	**0.05**	**5.46**	**0.74**	**0.05**	SigB	Mutant
yycD	YycD	BLi04344	1.61	0.21	0.03	3.15	0.50	0.11	**4.25**	**0.63**	**0.18**	SigB	BS
cotF	Spore coat protein F	BLi04356	**7.42**	**0.87**	**0.10**	**10.64**	**1.03**	**0.08**	**13.50**	**1.13**	**0.09**	SigB	Mutant, VF
yybO	Major facilitator superfamily	BLi04360	**4.46**	**0.65**	**0.12**	**11.82**	**1.07**	**0.12**	**14.97**	**1.18**	**0.14**	SigB	Mutant, VF, BS

Table 2. Continued

Name	Function	Accession number	5 min			10 min			20 min			Regulon	SigB membership determined by:
			Ratio	log (ratio)[a]	log (error)	Ratio	log (ratio)[a]	log (error)	Ratio	log (ratio)[a]	log (error)		
CtsR regulon													
ctsR	Transcriptional regulator, negative regulation of class III stress genes (clpC, clpP, clpE)	BLi00101	**25.92**	**1.41**	**0.03**	**28.38**	**1.45**	**0.04**	**30.31**	**1.48**	**0.06**	CtsR	
mcsA	Modulator of CtsR repression	BLi00102	**35.69**	**1.55**	**0.04**	**39.42**	**1.60**	**0.05**	**48.02**	**1.68**	**0.06**	CtsR	
mcsB	Modulator of CtsR repression; ATP:guanido phosphotransferase	BLi00103	**47.74**	**1.68**	**0.05**	**63.95**	**1.81**	**0.06**	**53.19**	**1.73**	**0.07**	CtsR	
clpC	Class III stress response-related ATPase	BLi00104	**45.17**	**1.65**	**0.04**	**67.18**	**1.83**	**0.03**	**71.98**	**1.86**	**0.03**	CtsR	
radA	DNA repair protein RadA	BLi00105	**22.47**	**1.35**	**0.08**	**34.86**	**1.54**	**0.11**	**34.39**	**1.54**	**0.10**	CtsR, SigB	
clpE	ATP-dependent Clp protease-like (class III stress gene)	BLi01525	**1068.27**	**3.03**	**0.05**	**2593.88**	**3.41**	**0.08**	**4044.45**	**3.61**	**0.05**	CtsR	
clpP	ATP-dependent Clp protease proteolytic subunit (class III heat-shock protein)	BLi03710	**26.62**	**1.43**	**0.07**	**51.47**	**1.71**	**0.08**	**92.34**	**1.97**	**0.08**	CtsR	
HtpG operon													
htpG	HSP 90	BLi04256	**11.85**	**1.07**	**0.07**	**10.75**	**1.03**	**0.10**	**8.39**	**0.92**	**0.08**	HtpG	
CssR regulon													
htrA	Serine protease Do (heat-shock protein)	BLi01390	**53.57**	**1.73**	**0.04**	**21.42**	**1.33**	**0.03**	**8.41**	**0.92**	**0.04**	CssR	
htrB (yvtA)	Similar to HtrA-like serine protease (htrB)	BLi03481	**10.21**	**1.01**	**0.07**	**6.94**	**0.84**	**0.05**	3.07	0.49	0.06	CssR	

Selected genes are shown for 5, 10, and 20 min after stress compared to the control conditions (0 min). For a complete list of induced and repressed genes see Supporting Information Table 2. Genes were considered as significantly upregulated by heat stress when an ANOVA $p < 0.01$ was obtained by statistical testing (included in the Rosetta resolver software package), and if for at least one time point their fold change reached the significance cutoff of 4 at the mRNA level. All values were calculated by the Rosetta Resolver software from three independent array hybridizations. The information given in the column "gene product function" is according to the UniProt Database (http://www.uniprot.org/). In case of heat shock class II (SigB), members were predicted by (A) the *Bacillus licheniformis* MW3.1 Δ*sigB* heat stress experiment (this work, indicated by "mutant"), (B) the Virtual Footprint algorithm of the Prodoric database (http://www.prodoric.de/vfp/) [19, 20], indicated by "VF," and (C) members of the known SigB regulon of *Bacillus subtilis*, indicated by "BS".
a) The log(ratio) displays the $\log_{10}$ of the expression ratio for each gene, where the ratio is equal to the normalized signal intensity in channel 2 divided by the normalized signal intensity in channel 1: log(ratio) = $\log_{10}$ (r/g); note that this calculation is made using program-specific error weighting [26, 45]. The log error then displays the error of the log ratio: log(error) = |log(ratio)/xdev|.
Resolver defines a measure of differential expression "xdev" as follows: $xdev = (r - g)/(\sigma_r^2 + \sigma_g^2)^{1/2}$, where r = red intensity and g = green intensity after normalization and σ_r and σ_g = the estimated errors in the red and green intensity. This method of determining if a gene is differentially expressed is insensitive to low intensity in any one channel and hence is preferable to methods that are ratio based. Bold indicates all values reaching the significance cutoff of 4.

For *B. subtilis*, it has been shown that the alternative sigma factor SigI belongs to the heat induced proteins [33]. It could be shown that a *sigI* mutant does not grow at higher temperatures [33]. The *sigI* gene of *B. licheniformis* was transcribed at a higher level during heat shock (Supporting Information Table 4). Some genes of the putative SigI regulon were also upregulated (*mreBH*, *ykrI*, *rsgI*, *gsiB*—also SigB-dependent); whereas two others were downregulated (*lytE*, *ywoA*). In *B. subtilis lytE* expression is controlled by SigI and is heat inducible. A *lytE* mutant is not able to grow at higher temperatures, as is the *sigI* mutant [34]. However, in our heat shock experiment with *B. licheniformis*, *lytE* expression was reduced up to threefold during heat shock, although a SigI promoter sequence is present in front of the *lytE* gene of *B. licheniformis* [34]. Other heat shock genes described for *B. subtilis* like *clpX* (inducible only at the transcriptional level [30]) or *lonA* are not heat inducible in *B. licheniformis* [4] (Supporting Information Table 4).

B. licheniformis cells subjected to heat shock experience a mild oxidative stress indicated by upregulation of genes and proteins belonging to the PerR and the Spx regulon (Table 1, Supporting Information Table 4). Induction of the Spx regulon by heat stress has been demonstrated for *B. subtilis* as well [35]. Furthermore, the heat stress seems to cause a cell wall stress. Synthesis of two proteins and transcription of about 20 genes of different ECF type sigma factor regulons

(SigM, SigV, SigW, SigY) was elevated (Table 1, Supporting Information Table 4). The sigma factor genes themselves were not upregulated except for *sigM* and *sigY*. Surprisingly, we observed an upregulation of enzymes involved in tryptophan synthesis (*trpA-F*, pathway from chorismate to tryptophan, elevated synthesis of the TrpA protein after 10 min) during heat shock (Table 1, Supporting Information Table 4). Induction was strongest (sixfold to 14-fold) after 5 and 10 min and decreased after 20 min heat shock. However, adding tryptophan to cultures at the time of the heat stress did not improve growth or survival of the cells (data not shown). It is interesting to note that a number of regulons involved in usage of alternative carbon and nitrogen sources were also induced (AnsR, IolR, FruR, GabR, GntR, KdgR, LacR, MalR, NagR, PutR, RocR; Supporting Information Table 4). This is surprising since the heat shock experiment was conducted with exponentially growing cells that should not experience any nutrient starvation. Elevated transcription of these regulons might result from heat provoked conformational changes of the corresponding transcriptional regulators, in most cases repressors. In addition, genes encoding components of the high affinity phosphate transporter (*pstA-S*) also displayed elevated transcription especially 5 and 10 min after heat shock (Supporting Information Table 4). This induction is PhoPR-independent, since the genes *phoP* and *phoR* are not upregulated. Two genes with elevated transcription, *yvaZ* and *yvbA* (*sdpI* and *sdpR* in *B. subtilis*), are involved in a cannibalism mechanism in *B. subtilis* in which cells secrete killing factors to lyse sibling cells [36] (Supporting Information Table 4). However, in *B. subtilis* this system comprises additional genes for which no homologs have been found in the *B. licheniformis* genome. The relevance of induction of these two genes is therefore unknown.

3.4 The SigB dependent general stress response during heat stress

The SigB regulon of *B. subtilis* comprises more than 150 genes [37–39] all of which are under the positive control of the alternative sigma factor SigB. Since the genes of this regulon are induced under a variety of conditions, such as heat, ethanol, salt, or acid stress and starvation for glucose or phosphate, they are called general stress genes [40]. Contribution of the SigB regulon to resistance to different stresses including heat stress has been demonstrated [27, 41]. Induction of more than 80 SigB regulated genes in *B. subtilis* during heat shock was shown to be transient [2]. The SigB regulon of *B. licheniformis* has not yet been elucidated in detail. However, Brody and Price [42] analyzed the *B. licheniformis sigB* operon and found a conserved operon that equals the eight-gene-operon of *B. subtilis*. Brody and Price [42] also found a protein that cross-reacts with a *B. subtilis* anti-SigB antibody and that is inducible by different stress conditions like salt, heat, and ethanol stress. They concluded that the general stress system of *B. licheniformis* is very likely similar to that of *B. subtilis*.

In our study more than 50 putative members of the *B. licheniformis* SigB regulon showed elevated transcription during all time points and several others were induced but did not reach the threshold level of fourfold induction (Table 2, Supporting Information Table 4). Among the induced genes were the sigma factor *sigB* itself and the three *rsb* genes (*rsbV*, *rsbW*, *rsbX*), which are in *B. subtilis* and likely also in *B. licheniformis* transcribed from the internal SigB-dependent promoter [42]. The four *rsb* genes (*rsbR*, *rsbS*, *rsbT*, *rsbU*) encoded in the upstream part of the operon and probably transcribed from a SigA-dependent promoter are induced only slightly (1.5–2.5-fold, Supporting Information Table 4) as described also by Helmann et al. [2] for *B. subtilis*. The gene encoding the RsbR paralog YqhA (RsbRD [43]) is strongly induced, too (Supporting Information Tab 2). On the 2D gels elevated synthesis of SigB itself and of some putative SigB-dependent proteins (e.g., YceH, YfkM, YflT) could be shown (Fig. 2, Table 1).

3.5 Genes/proteins downregulated in heat-shocked *B. licheniformis* cells

A multitude of vegetative genes and proteins were downregulated following the heat shock, for example, genes and proteins involved in synthesis of different amino acids, such as arginine, histidine (Supporting Information Tables 4 and 5). Furthermore, mRNA amounts of most genes encoding enzymes of the nucleotide metabolism were clearly lowered particularly late during the heat shock (10 and 20 min). Synthesis of the corresponding proteins was also downregulated (Supporting Information Table 5). Regulation of glycolysis and TCA cycle enzymes was antithetic at the transcriptional and translational level. Transcription of the genes was mostly not regulated or only slightly downregulated. Synthesis of the proteins, however, was diminished especially after 10 min heat shock. Genes with functions in translation were mostly slightly downregulated. For example, the elongation factor Tuf was found to be downregulated at the transcriptional and also at the protein level (Supporting Information Table 2). An additional Tuf protein spot with a more alkaline p*I* appeared on the 2D gels after heat shock—this could be due to PTM of a portion of the newly synthesized protein (Table 1). Most aminoacyl-tRNA synthetases were downregulated at the transcriptional as well as at the translational level. However, six aminoacyl-tRNA synthetase genes were upregulated (*cysS*, *gltX*, *lysS*, *metS*, *thrZ*, *tyrZ*). Transcription of transporters for compatible solutes (*opuAA-E*) was up to 50 times downregulated. Furthermore, there is a strong reduction in transcription of genes involved in motility and chemotaxis (SigD regulon). The Hag protein was one of the proteins which were synthesized at a much lower level. Nielsen et al. [5] observed a downregulation of the Ytr operon and found that a *ytrEF* deletion mutant is impaired in growth at higher temperatures. In our heat shock experiment, only the genes *ytrA* and *ytrB* showed strong downregulation 20 min after heat shock (Supporting Information Table 4).

The other three genes of the operon were only slightly downregulated. Furthermore, Nielsen et al. [5] found an upregulation of many genes involved in iron metabolism. Most of these genes showed strongly decreased transcription in our experiment (up to 30-fold). This could be due to the different media used—a complex medium by Nielsen et al. [5] and a minimal medium in our experiment.

3.6 Response of *B. licheniformis* cells to ethanol stress

Ethanol stress was studied only at the proteome level. The stress was provoked by adding 6% ethanol to the cultures during exponential growth. Addition of ethanol diminished growth of *B. licheniformis* slightly (Fig. 1). About 40 upregulated proteins could be identified (Table 3).

3.7 Proteins regulated during ethanol stress

Cells subjected to ethanol stress show a reaction similar to that to heat stress (Fig. 3, Table 3). For example, there is an upregulation of synthesis of proteins of the HrcA and CtsR regulon, although induction is weaker than by heat shock. Seydlova et al. [6] found recruitment of some chaperones (DnaK and GroEL) to cell membranes in ethanol stressed *B. subtilis* cells. If a similar mechanism exists in *B. licheniformis*, the level of synthesis of these proteins might be underestimated since only soluble proteins were studied. On the other hand, many more SigB-dependent proteins are synthesized at a higher level compared to control than during heat shock. In protein samples from heat-shocked cells, we identified only six SigB-regulated proteins with higher synthesis, whereas in samples from ethanol stressed cells we found 15 such proteins. Upregulation of the SigB protein itself was similar during both stresses (mean ratios between 7.58 and 4.24). That the SigB regulon contributes considerably to protection against ethanol stress in *B. subtilis* was shown by Höper et al. [27]. They analyzed the survival and growth of individual mutants in SigB regulon genes when subjected to different stresses. Many mutants displayed higher sensitivity to ethanol stress than the wild type. Among them were mutants in genes conferring resistance to oxidative stress, such as *dps*, *ohrB* (*ykzA*) *sodA*, and *ycdf*. The corresponding proteins Dps, OhrB, SodA, and YcdF were found to be induced in ethanol stressed *B. licheniformis* cells (Fig. 3, Table 3). However, Höper et al. [27] observed elevated transcription of oxidative stress-related genes known to be regulated SigB independently, such as *katA* and *ahpC*. There was no elevated synthesis of such proteins in *B. licheniformis* following ethanol stress. Further, SigB-dependent genes important for resistance to ethanol stress [27], which we found induced at the protein synthesis level in *B. licheniformis*, were *yfkM* (general stress protein) and *yflT* (hypothetical protein).

It is well established that the main target of ethanol is the cell envelope [44]. In *B. subtilis* ethanol stress induced the expression of the extracytoplasmic sigma factor SigM and some genes of the SigM regulon [7]. In our stress experiment, we found only two SigM-dependent proteins synthesized at an elevated level (YwaC, DltE—also SigD- and SigX-dependent, Fig. 3, Table 3).

As during heat shock, many vegetative proteins are downregulated, such as proteins involved in translation, amino acid synthesis, and nucleotide synthesis (Supporting Information Table 5). However, contrary to heat shock, there is no downregulation of glycolysis and TCA cycle enzymes but the ATPase subunits AtpA and AtpD are strongly downregulated.

3.8 Analysis of a *B. licheniformis sigB* mutant

To further characterize the SigB regulon in *B. licheniformis*, we constructed a *sigB* mutant and compared the response of wild type and mutant to heat shock at the transcriptome and proteome level and to ethanol stress at the proteome level.

The transcription of 38 genes was more than ten times lower in heat-shocked *sigB* mutant cells when compared to the wild type (Supporting Information Table 6). The gene with the highest reduction in transcription was the *sigB* gene itself (more than 500-fold). The genes, which are transcribed at a lower level in the mutant, are putatively SigB-dependent and for most of them this dependency has been described in *B. subtilis*. Analysis of these *B. licheniformis* genes with Virtual Footprint (http://prodoric.tu-bs.de/vfp/vfp_promoter.php) resulted in detection of typical SigB promoter sequences in front of 17 of these genes (using *B. subtilis* consensus sequences given on the Virtual Footprint website). Among the genes not upregulated in the mutant are several genes for which no homologs exist in *B. subtilis* (Supporting Information Table 5). These might be SigB-dependent genes specific for *B. licheniformis*. Many of these genes encode as yet uncharacterized hypothetical proteins (e.g., *BLi03885*, *BLi01417*, Supporting Information Table 5). Yet, the SigB regulon of *B. licheniformis* includes genes with additional (putative) functions like *mcrA*, encoding a protein with endonuclease activity, *BLi02212*, encoding a Na/solute symporter, *BLi00198*, encoding an arginase homolog or *BLi00576*, encoding a fatty acid desaturase. None of the SigB-dependent proteins synthesized at an elevated level in the wild type could be detected in the mutant. However, the superoxide dismutase (SodA) was identified in two spots in the proteome of the mutant and one of these spots was induced in heat-stressed cells (Fig. 4,Table 1). About 25 putative SigB-dependent genes induced during heat shock in the wild type are not downregulated in the *sigB* mutant. These genes are either not SigB dependent in *B. licheniformis* or are under dual control by other regulators (Supporting Information Table 5).

Heat stress-related induction of the genes of the HrcA, CtsR, CssR regulon and the HtpG operon was similar in the

Table 3. Proteins upregulated during ethanol stress

Name	Function	Accession number	Regulon	10 min ethanol WT		30 min ethanol WT		10 min ethanol Δ*sigB*		30 min ethanol Δ*sigB*	
				Mean ratio	SD	Mean ratio	SD	Mean ratio	SD	Mean ratio	SD
HcrA regulon											
DnaK	Chaperone protein	BLi02739		2.55	0.25	1.31	0.53	0.85	0.07	1.49	0.22
GroL	60 kDa chaperonin	BLi00624		6.39	0.19	11.59	3.78	2.70	0.11	12.92	2.45
GroL_1	60 kDa chaperonin	BLi00624		6.66	0.85	7.92	2.06				
GroL_2	60 kDa chaperonin	BLi00624		6.95	0.26	7.85	2.19				
GroL_4	60 kDa chaperonin	BLi00624		8.72	0.62	14.18	2.43	2.29	0.16	9.66	2.83
GroL fr	60 kDa chaperonin	BLi00624		6.32	0.66	11.88	2.11	2.83	0.79	6.95	3.16
GroL fr1	60 kDa chaperonin	BLi00624		2.09	0.39	2.22	0.49	2.41	0.90	7.96	3.26
GroL fr2	60 kDa chaperonin	BLi00624		2.81	0.29	3.91	0.60				
GroS	10 kDa chaperonin	BLi00623		0.09	0.01	0.12	0.03				
GroS_1	10 kDa chaperonin	BLi00623		2.09	0.13	6.42	1.36				
CtsR regulon											
ClpC	Class III stress response-related ATPase	BLi00104		4.74	1.41	2.45	0.08	1.93	1.53	2.42	1.46
ClpE	ATP-dependent Clp protease-like (class III stress gene)	BLi01525		5.19	2.21	1.27	0.10	1.56	0.66	2.06	0.39
ClpP	ATP-dependent Clp protease proteolytic subunit 1 (EC 3.4.21.92)	BLi03710		5.05	0.53	6.66	1.89	4.60	0.92	8.16	2.10
SigB regulon											
Dps	DNA-protecting protein	BLi03207		7.39	0.72	20.44	2.83				
GsiB	General stress protein	BLi00534		2.68	0.12	75.98	7.32				
GspA (DegU, YqfL)	Glycosyl transferase family 8	BLi04064		9.27	1.46	15.25	9.75				
OhrB	Organic hydroperoxide resistance protein	BLi01416		4.17	0.42	8.88	1.64				
RsbV	Antisigma factor antagonist	BLi00558		2.07	1.18	6.22	1.46				
RsbW	Serine-protein kinase (EC 2.7.11.1)	BLi00559		4.72	0.26	2.04	1.68				
SigB	RNA polymerase sigma factor	BLi00560		4.87	1.00	4.96	1.35				
SodA	Superoxide dismutase (EC 1.15.1.1)	BLi02679		1.44	0.16	6.53	1.23	1.63	0.26	3.75	0.17
SodA_1	Superoxide dismutase (EC 1.15.1.1)	BLi02679						3.47	1.11	6.24	2.69
SodA_2	Superoxide dismutase (EC 1.15.1.1)	BLi02679						3.36	0.49	4.09	0.75
SodA_3	Superoxide dismutase (EC 1.15.1.1)	BLi02679						3.17	0.08	9.07	0.70
YcdF	Glucose 1-dehydrogenase II	BLi00341		2.67	0.37	6.73	1.34				
YdbD	General stress protein, putative manganese-containing catalase	BLi03883		2.27	0.34	18.73	3.06				
Ycec (Upp)	Putative stress response protein	BLi00354	SigM,W,X					4.00	0.75	4.04	0.37
YfkM	General stress protein	BLi00815		3.86	0.42	11.13	1.50				
YflT (Crh)	Unknown	BLi00779		0.77	0.08	9.77	1.34				
YtkL	Metal-dependent hydrolase	BLi03080		1.01	0.10	3.25	0.51				
YtxH	Unknown	BLi03129		5.08	0.54	10.17	1.15				
YvyD	Ribosome-associated sigma 54 modulation protein	BLi03774	SigH	2.30	0.42	5.22	0.27				
CssRS regulon											
HtrB	Putative serine protease	BLi03481		3.29	0.31	2.47	1.15				
Other											
BLi00328	Penicillin-binding protein	BLi00328		6.36	1.66	7.87	0.83	1.94	1.09	10.64	11.50
BLi00385	NAD(P)H dehydrogenase	BLi00385		0.80	0.16	2.75	0.73				
Crh (YflT)	Catabolite repression HPr-like protein	BLi03722		0.77	0.08	9.77	1.34				
CysC	Adenylylsulfate kinase (EC 2.7.1.25)	BLi01781	CymR, S-box	10.54	0.82	12.74	1.31				
Ddl	D-Alanyl-D-alanine ligase A (EC 6.3.2.4)	BLi00543	SigM					1.35	0.29	2.44	0.52

Table 3. Continued

Name	Function	Accession number	Regulon	10 min ethanol WT		30 min ethanol WT		10 min ethanol Δ*sigB*		30 min ethanol Δ*sigB*	
				Mean ratio	SD	Mean ratio	SD	Mean ratio	SD	Mean ratio	SD
DltE	Involved in lipoteichoic acid biosynthesis	BLi00637	SigD, M,X	2.12	0.93	3.28	0.51				
Hag	Flagellin protein	BLi03780		3.57	0.60	0.51	0.11	3.19	0.47	0.47	0.09
HemQ	Putative oxidoreductase/oxygenase/dismutase	BLi03998						3.61	1.02	7.32	1.09
HisC	Histidinol-phosphate aminotransferase and tyrosine/phenylalanine aminotransferase (EC 2.6.1.9 and EC 2.6.1.5)	BLi02397	TRAP					4.00	0.90	16.25	3.25
HsdM	Type I restriction-modification system M subunit (EC 2.1.1.72)	BLi04318		3.43	0.46	1.60	0.04				
MtnD (YpjQ)	1,2-Dihydroxy-3-keto-5-methylthiopentene dioxygenase	BLi01517	S-box	3.05	0.25	7.40	0.81				
NamA	NADH dehydrogenase (EC 1.6.99.1)	BLi02551		1.53	0.56	4.44	1.10	2.54	1.07	13.42	5.91
NusA	Transcription termination	BLi01885		5.96	0.33	2.55	0.26				
OdhB	2-Oxoglutarate dehydrogenase complex (dihydrolipoamide transsuccinylase, E2 subunit) (EC 2.3.1.61)	BLi02259		3.99	0.01	2.24	0.79				
OdhB_1	2-Oxoglutarate dehydrogenase complex (dihydrolipoamide transsuccinylase, E2 subunit) (EC 2.3.1.61)	BLi02259	CcpA	4.97	0.09	1.82	0.67				
PdhC	Pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit) (EC 2.3.1.12)	BLi01676		3.65	0.31	1.40	0.34				
PdxT	Glutamine amidotransferase (EC 2.6.-.-)	BLi00017	Spo0A					1.97	0.10	2.82	0.47
RapA	Response regulator aspartate phosphatase	BLi02480	CodY, ComA, Spo0A	3.93	0.44	3.28	1.34				
SalA	Mrp family regulator	BLi00172	SalA	6.60	0.71	4.01	0.40				
SdhA	Succinate dehydrogenase (flavoprotein subunit) (EC 1.3.99.1)	BLi02993						1.25	0.60	4.17	0.49
SecA	Translocase binding subunit (ATPase)	BLi03773						0.71	0.30	2.01	0.86
TrpE	Anthranilate synthase (EC 4.1.3.27)	BLi02403	TRAP	2.40	0.41	1.24	0.30				
Upp (YceC)	Undecaprenyl pyrophosphate synthetase	BLi01874						4.00	0.75	4.04	0.37
YcdC	Unknown	BLi00343		5.87	0.37	1.60	0.56				
YdfG	Unknown	BLi04370		0.95	0.06	2.56	0.38				
YgaF	Peroxiredoxin Q/BCP (EC 1.11.1.15)	BLi00899		1.11	0.23	2.73	0.55				
YisK (BLi04205)	Fumarylacetoacetate (FAA) hydrolase	BLi01170		5.16	0.90	3.52	0.29				
Yjbl	Truncated form of bacterial hemoglobin	BLi01250		1.92	0.44	3.44	0.54				
YjcH	Putative carbohydrate esterase family 1	BLi01285		2.45	1.23	2.59	0.08				
YjoA	Unknown	BLi02905		1.06	0.06	2.10	0.50				
YjoA_1	Unknown	BLi02905		4.87	0.78	1.94	0.44				
YkwC	3-Hydroxyisobutyrate dehydrogenase (EC 1.1.1.31)	BLi01606		2.51	0.13	3.10	0.10	2.66	0.25	4.50	0.09
YlbP (YwrO)	Putative acetyltransferase	BLi01727		1.61	0.17	2.57	0.44				
YmfG	Putative peptidase	BLi01909		2.11	0.12	2.05	0.76				

Table 3. Continued

Name	Function	Accession number	Regulon	10 min ethanol WT		30 min ethanol WT		10 min ethanol Δ*sigB*		30 min ethanol Δ*sigB*	
				Mean ratio	SD	Mean ratio	SD	Mean ratio	SD	Mean ratio	SD
YpfD	RNA degradation presenting factor (ribosomal protein S1 homolog)	BLi02427						1.95	0.15	6.80	0.85
YpjQ (MtnD)	Unknown	BLi02320	S-box	3.05	0.25	7.40	0.81				
YpqE	Similar to phosphotransferase system enzyme II	BLi02359		1.01	0.05	3.13	0.18				
YukE	Unknown	BLi03379		1.04	0.18	3.68	0.39				
YwaC	Similar to GTP-pyrophosphokinase	BLi04078	SigM, W	6.23	2.26	13.78	4.44				
YwlF	Ribose 5-phosphate isomerase B (EC:5.3.1.6) (pentose phosphate)	BLi03937	TnrA	1.07	0.13	3.54	0.84				
YwrO (YlbP)	NAD(P)H dehydrogenase	BLi03696		1.61	0.17	2.57	0.44				
YxeH	Putative hydrolase	BLi04258						2.52	0.62	4.09	1.10
Oxidative stress											
SufD	FeS assembly protein	BLi03450						2.67	0.14	5.03	0.57
TrxB	Thioredoxin reductase (EC 1.6.4.5)	BLi03728	Spx	2.60	0.49	3.60	0.63	1.41	0.52	1.89	0.18
YugJ	Iron-containing alcohol dehydrogenase (EC 1.1.1.-)	BLi03317						2.26	0.33	8.78	1.82

Regulon classification mainly according to *Bacillus subtilis*, protein names in brackets: two proteins identified in one spot.

mutant and in the wild type. The *clpE* gene was the gene with the highest induction, as it was in the wild type (Supporting Information Table 7). However, analysis of the proteome revealed differences in the synthesis of the ClpC and ClpE proteins between wild type and mutant (Fig. 4, Table 1). Whereas in the wild type these proteins were still synthesized at an elevated level after 30 min of stress, synthesis was reduced to control level in the *sigB* mutant at this time. Synthesis of the HcrA-dependent proteins DnaK and GroL was similar in wild type and mutant. Furthermore, the mutant exhibited upregulation of some proteins involved in oxidative stress response, as does the wild type. Some proteins were synthesized at a higher level in the heat stressed *sigB* mutant but not in the wild type, including the repressor protein CodY, the D-alanyl-D-alanine ligase A Ddl, and the cysteine desulfurase YvrO. On the other hand, not all proteins upregulated during heat stress in the wild type were also upregulated in heat stressed cells of the *sigB* mutant.

As the wild type, the *B. licheniformis sigB* mutant reacted to ethanol stress with an induction of proteins from the HcrA and CtsR regulon (Fig. 4, Table 3). However, the induction of GroL was delayed in the mutant, after 5 min stress the protein synthesis was only slightly increased. Synthesis of the proteins ClpC and ClpE was only slightly induced, the strongest induction for proteins from the CtsR regulon was found for ClpP (Table 3). As expected, synthesis of SigB-dependent proteins was not detected in the ethanol stressed mutant. The exception was the superoxide dismutase (SodA) which was also found to be upregulated in heat-stressed mutant cells (Fig. 4, Table 3). Under ethanol stress conditions, this protein occurred in four different spots, all of which were newly synthesized; obviously this protein was extensively modified after translation resulting in multiple spots. In the proteome of the *sigB* mutant, two additional SigM-dependent spots (YceC—which is also SigB-dependent and Ddl, Table 3) were found to be upregulated (Table 3). Not all proteins synthesized at a higher level in the wild type could be found in the mutant. However, some additional proteins were upregulated in the ethanol stressed *sigB* mutant (e.g., the putative hydrolase YxeH, the ribosomal protein S1 homolog YpfD, and the glutamine amidotransferase PdxT, Table 3). Among the additionally upregulated proteins were two proteins (YugJ and SufD) indicating oxidative stress. Downregulation of protein synthesis was similar in wild type and mutant cells subjected to heat or ethanol stress (Supporting Information Table 5).

4 Concluding remarks

In this study, it is shown that *B. licheniformis* cells react to a heat shock with induction of many regulons well known from *B. subtilis*, such as the HrcA, CtsR, and SigB regulon. However, the heat shock stimulon of *B. licheniformis* includes also regulons and genes, which seem to be specific for this bacterium. This includes the SigY regulon and genes from other ECF type sigma factor regulons indicating a cell wall stress triggered by elevated temperatures similar to ethanol stress. Surprisingly, genes encoding tryptophan synthesis enzymes were upregulated in heat stressed *B. licheniformis*

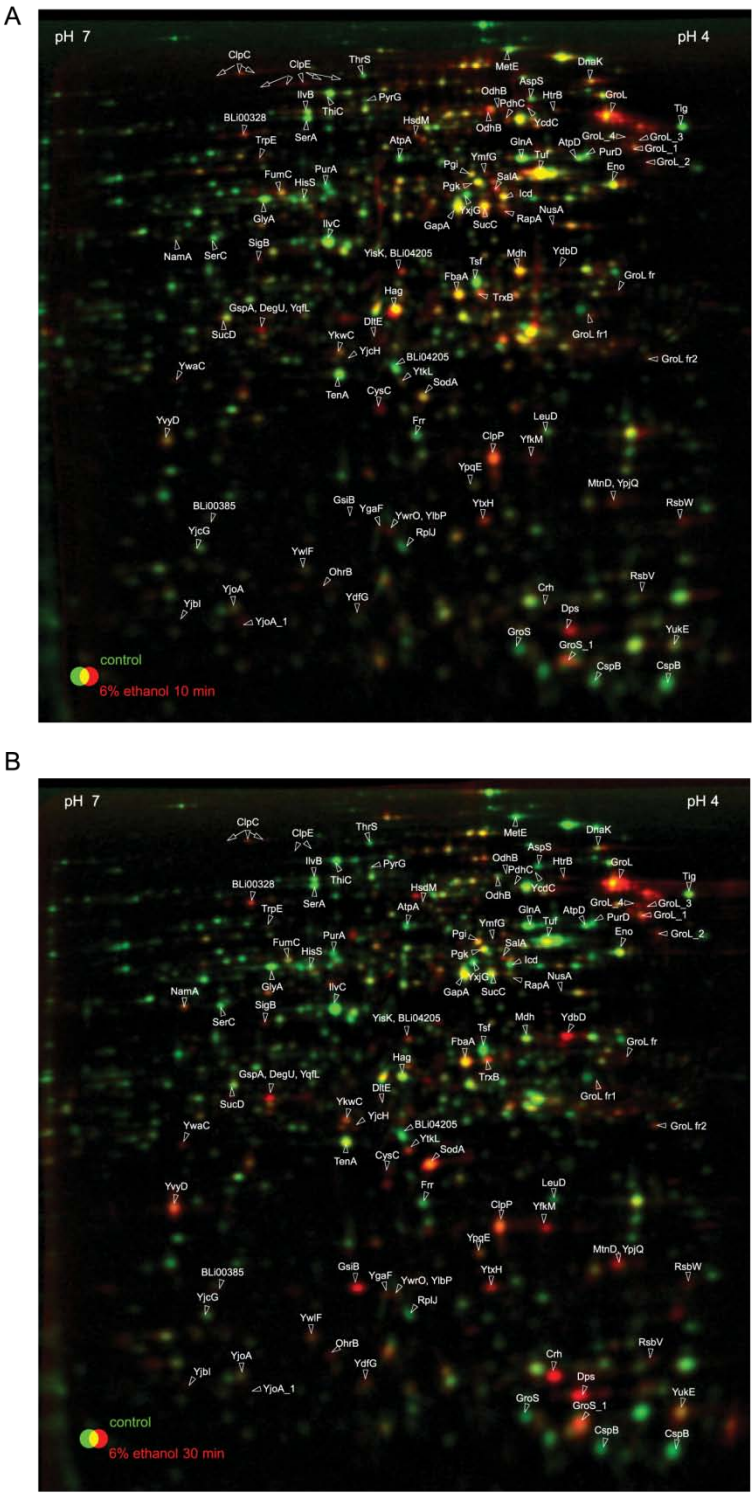


Figure 3. Proteome of *Bacillus licheniformis* DSM13 cells under ethanol stress conditions. (A) 10 min after addition of 6% ethanol, (B) 30 min after addition of 6% ethanol. Cell samples were labeled with L–[^{35}S]–methionine during the exponential growth phase (OD_{500nm} 0.4), and 10 and 30 min after ethanol addition. Proteins were separated in a pH gradient 4–7. The dual channel images were created with the Delta 2D software (Decodon).

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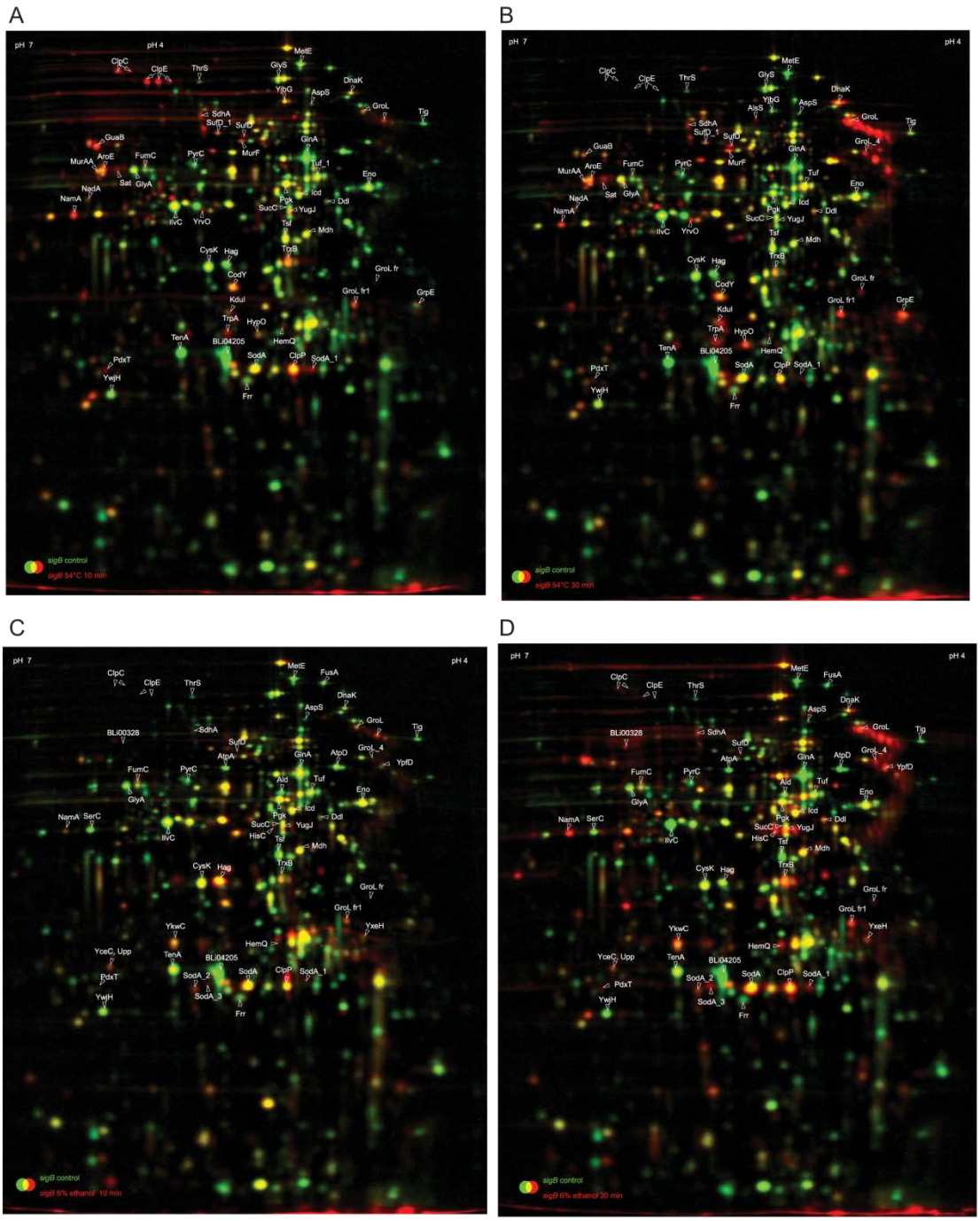

Figure 4. Proteome of *Bacillus licheniformis sigB* mutant cells under heat and ethanol stress conditions. (A) 10 min heat stress, (B) 30 min heat stress. (C) 10 min after addition of 6% ethanol, (D) 30 min after addition of 6% ethanol. Cell samples were labeled with L–[^{35}S]–methionine during the exponential growth phase (OD_{500nm} 0.4), and 10 and 30 min after stress. Proteins were separated in a pH gradient 4–7. The dual channel images were created with the Delta 2D software (Decodon).

cells as well as a number of regulons involved in usage of alternative carbon, phosphate, and nitrogen sources. The analysis of the SigB regulon of *B. licheniformis* using a *sigB* mutant showed that many genes known to be SigB-dependent in *B. subtilis* are also members of the *B. licheniformis* SigB regulon. However, the *B. licheniformis* SigB regulon comprises some genes for which no homologs exist in *B. subtilis*.

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The authors have declared no conflict of interest.

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BACILLUS PUMILUS REVEALS A REMARKABLY HIGH RESISTANCE TO HYDROGEN PEROXIDE PROVOKED OXIDATIVE STRESS

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Author's contribution

The experimental design was developed by SH, ReS, BJ, KM, RaS, ML, TS, MH and BV. Bacterial cultivations were performed by SH and ReS. 2D-PAGE and protein analysis was done by SH. RNA isolations and microarray experiments were done by ReS. Transcriptome analyses were carried out by ReS and SvH. Mass spectrometry analysis was done by DA. HPLC analysis of metabolites was performed by KM and ML. Electron microscopy was done by RaS. The manuscript was written by all authors.

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Bacillus pumilus reveals a remarkably high resistance to hydrogen peroxide provoked oxidative stress

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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*.

1. 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 resistances of *B. pumilus* could be a major benefit for the improvement of industrial production strains, since oxidative stress can occur in all phases of fermentation processes.

Reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH·) 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. subtilis* are missing in the genome of *B. pumilus*, such as the catalase KatA or alkyl hydroperoxide reductase AhpCF. For some of these genes no homologs could be found in the *B. pumilus* genome. This leads to the questions, which genes compensate the missing genes and are thus responsible for the oxidative stress resistance of *B. pumilus*. In this study we used a combination of proteomics, transcriptomics and metabolomics to investigate the individual peroxide stress response of *B. pumilus*.

2. Material and methods

2.1 Strain, media, growth and cell sampling

Bacillus pumilus Jo2 (DSM 14395) was used for all experiments described in this study. Cells were grown aerobically at 37°C and 180 rpm in minimal medium containing 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$ x $7H_2O$, 27 mM KCl, 7 mM Na-citrate x $2H_2O$, 50 mM Tris-HCl (pH 7.5) supplemented with 1.8 mM KH_2PO_4, 2 mM $CaCl_2$, 1 µM $FeSO_4$ x $7H_2O$, 10 µM $MnSO_4$ x $4H_2O$, 4.5 mM glutamate, 0.2 % w/v glucose and 0.04 µM biotin. Exponentially growing cells at an OD_{500nm} of 0.6 were exposed to a final concentration of 2 mM hydrogen

peroxide. Proteome samples were taken from unstressed cultures before and 10 as well as 30 minutes after exposure to hydrogen peroxide. Samples were pulse-labeled with L-[^{35}S]-methionine for 5 min, as described by Hoi *et al.* [14]. Samples for preparative gels were prepared from unlabeled cells 30 and 60 min after exposure to H_2O_2 [14].
Samples for RNA extraction were taken before (control) and 3 and 8 min after addition of H_2O_2. Cell samples for RNA extraction were mixed with 0.5 volumes of ice-cold killing buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl, 20 mM NaN_3), and immediately harvested at 10000 x g for 5 min at 4°C.

2.2 Electron Microscopy

2.2.1 Scanning electron microscopy

For the scanning electron microscopy, the cells were separated from the culture medium by filtration through a 0.2 µm pore size polycarbonate filter. The filter were placed in fixation solution (1 % glutaraldehyde, 4 % paraformaldehyde, 50 mM NaN_3 in 5 mM HEPES [pH 7.4]) for 1 h at room temperature and 4°C over night. After fixation, the samples were treated with 2 % tannic acid for 1 h, 1 % osmium tetroxide for 2 h, 1 % thiocarbohydrazide for 30 min, 1 % osmium tetroxide over night, and 2 % uranyl acetate for 30 min with washing steps in between. The samples were dehydrated in a graded series of aqueous ethanol solutions (10 – 100 %) and then critical point-dried. Finally, filter were mounted on aluminum stubs, sputtered with gold/palladium and examined in a scanning electron microscope EVO LS10 (Carl Zeiss microscopy GmbH, Oberkochen, Germany).

2.2.2 Transmission electron microscopy

Cells were fixed in 1 % glutaraldehyde, 4 % paraformaldehyde, 50 mM NaN3 in 5 mM HEPES for 1 h at room temperature and then at 4°C over night. Subsequent to embedding the cells in low gelling agarose, cells were postfixed in 2 % osmium tetroxide for 2 h at 4°C. After dehydration in graded series of ethanol (20 – 100 %) for 10 min each step with 0.5 % uranyl acetate in 70 % ethanol for 30 min (at 4°C) in between, the material was embedded in Epon. Sections were cut on an ultramicrotome (Reichert Ultracut, Leica UK Ltd, Milton Keynes, UK), stained with uranyl acetate and lead citrate and analyzed with a transmission electron microscope LEO 906 (Carl Zeiss microscopy GmbH, Oberkochen, Germany).

2.3 2D-Gel electrophoresis

Cytosolic protein extracts were loaded on 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 Büttner *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 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 *B.*

pumilus Jo2 (DSM 14395) database as described by Wolf *et al.* [17]. Protein quantification was done with the help of Delta2D proteome software (Decodon, Germany).

2.4 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 subsequently. 600 ng of Cy3- and Cy5 -labeled cDNA (ad. 44 µl), respectively, was denaturated and mixed with 11 µl pre-warmed blocking agent and 60 µl hybridization buffer (both Gene expression hybridization kit, Agilent Technologies, USA). 100 µl of the emerging cDNA mixture were used for hybridization. Custom-made *B. pumilus* Jo2 4x44K gene expression microarrays were obtained from Agilent Technologies (https://earray.chem.agilent.com/earray/). Probe design was performed on the annotated open reading frames of *B. pumilus* Jo2 strain according to [21]. 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. Spot signals were normalized using Lowess as described earlier [22]. 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 [23] by means of multiple testing correction [22]. Differential regulation was defined as a two-fold or higher differential expression with a FDR cut-off value of 0.05 or lower.

2.5 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 [24]. 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, 25]. For detection and quantification of LMW-thiols, ion pairing HPLC was performed as described before [26]. 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.

2.6 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 [27] using the consensus sequence as described by Fuangthong *et al.* [28].

3. Results and Discussion

3.1 Effects of H_2O_2 on growth and cell morphology

Exponentially growing *B. pumilus* cells were treated with 2 mM hydrogen peroxide. Thus, the concentration of H_2O_2 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]. This indicates a striking resistance of *B. pumilus* to peroxide stress. Compared to unstressed cells, growth was significantly impaired for a short time (approximately 15 min) after the H_2O_2 treatment (Figure 1). However, after that time, cells continued to grow with a reduced growth rate. An electron microscopy analysis indicated that after exposure to H_2O_2 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 (Figure 2B) indicating an impact of hydrogen peroxide stress on processes involved in cell division.

3.2 Global expression profile

The analysis of the soluble intracellular proteome of *B. pumilus* revealed 54 significantly upregulated and 111 downregulated proteins 10 min after H_2O_2 treatment (with a threshold of two-fold, Table 1, Table S1, Figure 3). For the visualization of the fast and early response on proteome level, a labeling with ^{35}S-methionine was necessary. 30 minutes after initiating the stress, 73 proteins were up- and 59 proteins downregulated (Table 1, Table S1, Figure 4). Transcriptome analysis revealed an at least two-fold increased transcription of 181 genes three minutes after treatment with H_2O_2; 76 of them were more than three-fold upregulated. Eight minutes after treatment, the transcription of 558 genes appeared at least two-fold increased (307 genes with an at least three-fold increased transcription). Three minutes after the stress, 266 genes were transcribed with an at least three-fold lower rate than under control conditions, for 296 genes this decreased transcription rate has been shown eight minutes after treatment.

To compare the physiological changes in H_2O_2 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].

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 paraquat [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 H_2O_2 treated *B. pumilus* cells that is similar to the de-repression model reported for *B. subtilis* [29].
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 HemABCD2LX and the general stress protein YjbC 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, were absent from the genome of the *B. pumilus* strain used in our study, as well as from a previously published *B. pumilus* genome [30]. 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 *ahpC* and *ahpF*, 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, KatX was one of the proteins with the highest induction rates detected. However, compared to the KatA induction rates shown for *B. subtilis* and *B. licheniformis* subjected to hydrogen peroxide of more than 100-fold, induction of KatX2 was rather moderate [13, 20]. In *B. subtilis*, KatX is the major spore catalase and under control of SigB and SigF [31, 32]. We detected a *B. subtilis* PerR consensus sequence [28] containing two mismatches about 90 bases in front of the start codon indicating a possible involvement of PerR in its regulation. Vegetative *B. pumilus* cells expressed this gene weakly already under exponential growth conditions. The abundance of KatX2 at control conditions is comparable to the abundance of KatA in *B. subtilis* and *B. licheniformis* control cells [16, 33].

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 [28]. The regulator protein Fur of *B. subtilis* controls the expression of genes responsible for iron uptake [34]. Immediately after exposure to H_2O_2, cytosolic iron concentration is considerably reduced to prevent the formation of $OH^{\bullet}$ by the Fenton reaction [13]. Upregulation of the Fur-controlled genes may be a reaction of the cells to face the resulting iron limitation.
Nine genes of a putative Fur regulon showed a significantly increased expression in *B. pumilus* cells after H_2O_2 treatment, including the ABC transporter system *fhuB1C1G1*

(Table 1). The *fhuC* gene was induced by H_2O_2 in *B. subtilis* and *B. licheniformis*, too [13, 20]. Further Fur regulon member genes known to be induced by H_2O_2 in *B. subtilis* showing an induction in our study were *ykuN*, *ykuP* (flavodoxins) and the hypothetical protein *ykuO*. With an about 30-fold higher mRNA level eight minutes after treatment, these were among the highest upregulated genes in this putative regulon. The putative nitroreductase YfhC, also induced in H_2O_2 stressed *B. subtilis* cells, was the only member of the putative Fur regulon we observed to be upregulated at translational level.

The gene *ywjA*, encoding another ABC transporter of yet unknown function, the peptidase encoding gene *yfkM* 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_2O_2 in this organism [13, 35]. In *B. subtilis* and *B. licheniformis*, the siderophore biosynthesis complex encoded by *dhbACEBF* was strongly upregulated by H_2O_2. In our study, these genes showed no significant changes in their expression level.

Other genes that exhibited higher transcription rates after H_2O_2 treatment were the iron ABC transporter protein encoding gene *feuA* and its upstream-located regulator *ybbB* [36]. 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 *fhuG2* and *fhuD* immediately after subjecting the cells to the stress. Two further putative iron transporter systems, *bpj35830-bpj35840-bpj35850* and*bpj08420-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 RhbA, RhbE and RhbF, encoded by the *rhbABCDEF*-operon (Table 1). A rather slight induction at the translational level was shown for the iron/sulfur cluster biogenesis proteins SufB, SufS, SufD and SufC 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

Another regulator protein assigned to the putative PerR regulon is SpxA, controlling the expression of the Spx regulon in *B. subtilis* [37, 38]. This gene exhibited an about 4-fold increased transcription rate in H_2O_2 stressed *B. pumilus* cells. Some of the genes and proteins attributed to a putative Spx regulon in *B. pumilus* appeared to have rather moderately increased expression rates or were not induced after H_2O_2 treatment.

In our study we detected six genes of a putative Spx regulon to be induced following H_2O_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_2O_2 treated cells, too. Upregulation of *msrAB* (methionine sulfoxide reductase 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 [39-41]. Cystathionine gamma-lyase MccB and DinB-like domain-containing protein YuaE showed an induction only at proteome level.

The Spx-regulated *srf* operon, mediating competence and metabolic functions in *B. subtilis*, is absent in the *B. pumilus* genome as shown before for *B. licheniformis* [38, 42, 43].

3.6 SOS regulon

H_2O_2 treatment leads to the formation of $OH^{\bullet}$ 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 H_2O_2, induced the SOS regulon, regulated by the proteins RecA and LexA, responsible for repair of DNA [13, 20, 44, 45].
The proteomic analysis displayed the induction of two proteins, excinuclease subunit UvrB and the recombinase RecA, assigned to a putative SOS regulon in *B. pumilus* following H_2O_2 treatment (Table 1). The transcriptomic approach added further 13 upregulated genes belonging to this putative regulon; among them the excinuclease subunits encoding genes *uvrA* and *uvrC*. The operon *yneABynzC*, induced by H_2O_2 and involved in suppression of cell division in *B. subtilis*, was also strongly induced in our study [13, 46].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 *yhaONM* exhibited a significantly higher transcription rate following H_2O_2 addition [47].

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, 48]. In our study, we detected an upregulation of nine genes assigned to a putative CtsR regulon in *B. pumilus* indicating a significant impact of H_2O_2 on protein quality (Table 1). The operon *ctsR-mcsAB-clpC* was transcribed with significantly higher intensity after the addition of H_2O_2 as well as the genes *clpE*, *clpX* and *clpP*, 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, H_2O_2 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) [49, 50]. 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* [51].
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 *mgsR* and *ydaG* (general stress protein), both also detected to be induced in H_2O_2 stressed *B. licheniformis* cells [20]. The upregulated genes *mgsR* 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 YtxH 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 H_2O_2 treated *B. pumilus* cells, too [52].

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 [53]. 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 H_2O_2 [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 adenylyltransferase (Sat) catalyzing sulfate assimilation to 3'-phospho-adenylylsulfate was also detected (Table 1). Further proteins involved in cysteine biosynthesis were not significantly upregulated. The third upregulated protein is the uroporphyrin-3 C-methyltransferase (CysG). This enzyme catalyzes a reaction in a branch in the heme pathway producing precorrin2. An induction of the enzymes that continue the pathway from precorrin2 to siroheme could not be detected.

3.10 Other *B. pumilus* upregulated genes/proteins

The OhrR-regulated peroxiredoxin-encoding gene *ohrA* is reported to be involved in organic peroxide resistance in *B. subtilis* [54]. Following H_2O_2 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 H_2O_2 resistance of *B. pumilus* (Table 1). Transcription of the other organic peroxide resistance peroxiredoxin (*ohrB*) as well as their regulator gene *ohrR* was also slightly induced in hydrogen peroxide treated *B. pumilus* cells.
H_2O_2 treatment induced some additional regulator genes. One of them is *fadR*, encoding a regulator protein mediating fatty acid degradation in *B. subtilis* [55]. Two genes putatively controlled by FadR, *etfAB* - encoding the electron transfer flavoprotein alpha and beta subunit, were also induced (Table S1). Another regulator, AbrB1, controlling the expression of genes induced by transition from exponential to stationary growth in *B. subtilis* [56], 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 thiol-disulfide oxidoreductase YkuV. Furthermore, several putative regulator genes with still unknown targets were observed to be upregulated. *Bpj13620*, *bpj17020* and *ydcI* 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 H_2O_2 treatment. Its function is also unknown.
Several genes and proteins involved in transport processes were detected to be upregulated following H_2O_2 stress (Table 1, S1). H_2O_2 treatment caused an upregulation of the sodium uptake system *natAB* and the *mrpABCDEFG* 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* [57, 58].
Strikingly, transcription of the glycine betaine uptake system consisting of *opuAA-AB-AC* and *opuCA-CB-CC-CD* was observed to be significantly induced after treatment, indicating that H_2O_2 impacts osmotic homeostasis in *B. pumilus* cells [59]. Furthermore, it is worth to mention that H_2O_2 induced expression of a putative TRAP regulon in *B. pumilus* cells. An upregulation of the tryptophan-synthesis operon *trpABFCDE* 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.

3.11 Bacillithiol

We noticed an increased transcription of *ypdA* and *yqiW* as well as an induction of the *yphP* gene product (Table 1). The proteins encoded by these genes are involved in the synthesis of bacillithiol (Cys-GlcN-malate, BSH) synthesis [60]. 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, 61, 62]. The induction of these genes suggests an involvement of bacillithiol in the H_2O_2 resistance of *B. pumilus*. For further investigation, we analyzed the cytosolic metabolome of H_2O_2 treated *B. pumilus* cells concerning the concentration of thiol compounds. Our analysis revealed a surprisingly high bacillithiol level of 2.6 nmol already under control conditions. Ten minutes after H_2O_2 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. These results support the thesis of an involvement of bacillithiol in the H_2O_2 resistance of *B. pumilus*.

3.12 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 metabolic 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 S2) [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 H_2O_2 treatment. Furthermore, we observed a reduced expression of most of the aminoacyl-tRNA-synthetases, with the exception of tryptophanyl-tRNA-synthetase *trpS*, which matched the upregulation of the tryptophan operon.
Strikingly, a stringent response, i.e. a downregulation of ribosomal proteins or elongation factors like *fusA*, *tsf* or*tufA*, as described for other organisms (*B. subtilis*, *B. licheniformis*, *E. coli*) could not be detected in *B. pumilus* [13, 20, 63].

4. Conclusion

The combination of proteomics and transcriptomics revealed a specific adaptation of *B. pumilus* cells caused by the oxidative stress trigger H_2O_2. 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 seems to be involved in the remarkable oxidative stress resistance of *B. pumilus*. The concentration of H_2O_2 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 to 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 bacillithiol 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 cytosolic bacillithiol concentration during peroxide stress. Furthermore, a considerable set of H_2O_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 role in the oxidative stress adaptation of this organism. Finally, since *B. pumilus* is an organism of industrial interest, understanding its oxidative stress response and defining marker genes for the analysis of fermentation processes is important to prevent possible negative influences on the process and the product quality.

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Figure legends

Figure 1. Growth of *B. pumilus*.
Growth of *B. pumilus* under control conditions (filled squares) and stressed with 2mM H_2O_2 at OD_{500nm} 0.6 (empty squares).

Figure 2. Electron microscopy micrographs.
Scanning (A,B) and transmission (C,D) electron microscopy micrographs of *B. pumilu*s cells under control conditions (A,C) and 30min after treatment with 2 mM H_2O_2 (B, D

Figure 3. Cytosolic proteome 10 min after H_2O_2 treatment.
The cytosolic proteome of *B. pumilus* cells 10 min after H_2O_2 treatment. Cell samples were labeled with L–[^{35}S]-methionine during the exponential growth phase (OD_{500nm} 0.6), and 10 min after H_2O_2 addition. Proteins were separated in a pH gradient 4 (right) – 7 (left).

Figure 4. Cytosolic proteome 30 min after H_2O_2 treatment.
The cytosolic proteome of *B. pumilus* cells 30 min after H_2O_2 treatment. Cell samples were labeled with L–[^{35}S]-methionine during the exponential growth phase (OD_{500nm} 0.6), and 30 min after H_2O_2 addition. Proteins were separated in a pH gradient 4 (right) – 7 (left).

Figure 5: Concentration of thiol compounds in *B. pumilus* cells.
Cytosolic concentration of Bacillithiol (BSH), CoA and cysteine (Cys) per mg cell dry weight (CDW) during the exponential growth phase (OD_{500nm} 0.6 at 0 min) and 10, 30 and 60 min after H_2O_2 treatment.

Table 1. Selected induced genes and proteins.

Selected genes and proteins that are induced in H_2O_2 treated *B. pumilus* cells.

ORF ID	gene		transcriptome		proteome		Regulon in other *Bacilli*
			3 min	8 min	10 min	30 min	
BPJ13600	zinc-transporting ATPase ZosA	*zosA*	12.74	28.72			perR
BPJ25410	glutamyl-tRNA reductase HemA	*hemA*	3.44	3.99			perR
BPJ25390	porphobilinogen 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 ferric uptake regulation protein Fur	*fur*	1.92	3.62			perR
BPJ11620	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
BPJ34450	putative ABC transporter permease YwjA	*ywjA*	1.57	4.47			fur
BPJ30810	hydroxamate siderophore ABC transporter ATP-binding protein FhuC	*fhuC1*	1.51	2.46			fur
BPJ30830	hydroxamate siderophore ABC transporter permease FhuB	*fhuB1*	1.52	4.01			fur
BPJ30820	hydroxamate siderophore ABC transporter permease FhuG	*fhuG1*	1.53	3.20			fur
BPJ08440	ABC transport system permease	*bpj08440*	4.11	7.49			fur
BPJ08430	putative iron complex transport system substrate binding protein	*bpj08430*	4.54	7.43			fur
BPJ08420	putative HTH-type transcriptional regulator	*bpj08420*	3.58	5.59			fur
BPJ08580	putative nitroreductase YfhC	*yfhC*		2.67	5.00	1.10	fur
BPJ08410	ferredoxin--NADP reductase 2	*bpj08410*	3.90	3.83			fur

BPJ37570	AraC family transcriptional regulator/putative FeuA-like substrate-binding domain ybbB	*ybbB*	4.93	12.84			fur
BPJ37580	iron complex ABC transporter substrate-binding protein FeuA	*feuA*	3.21	10.04			fur, btr, citB
BPJ37590	putative bacillibactin esterase YbbA	*ybbA*	5.24	18.42			fur/btr/citB
BPJ07970	C56 family peptidase YfkM	*yfkM*	2.94	7.61	7.39	3.09	fur/sigB
RBPU30260	FeS cluster assembly protein SufB	*sufB*	1.87	2.10	1.73		Fe/S cluster biogenesis
RBPU30280	cysteine desulfurase SufS	*sufS*			-1.77	2.69	Fe/S cluster biogenesis
RBPU30290	FeS cluster assembly permease SufD	*sufD*			1.73		Fe/S cluster biogenesis
RBPU30300	FeS cluster assembly ATPase SufC	*sufC*			2.52	2.13	Fe/S 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
BPJ11090	rhizobactin siderophore biosynthesis protein RhbF	*rhbF*			-1.72	3.02	siderophore synthesis
BPJ35800	iron complex ABC transporter ATP-binding protein FhuC	*fhuC2*	3.88	7.85			iron uptake
BPJ35810	iron complex ABC transporter permease FhuB	*fhuB2*	3.32	7.15			iron uptake
BPJ35770	putative iron complex ABC transporter permease FhuG	*fhuG2*	2.31	4.39			iron uptake
BPJ35780	putative iron complex ABC transporter substrate-binding protein FhuD	*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	*bpj35840*	4.91	7.62			iron uptake
BPJ35850	putative iron transport-associated protein	*bpj35850*	3.89	6.47			iron uptake
BPJ28430	DinB-like domain-containing protein YuaE	*yuaE*			2.25	2.87	spx
BPJ31980	thioredoxin-disulfide reductase TrxB	*trxB*		3.97	3.93	3.59	spx
BPJ29110	putative NADH-dependent butanol dehydrogenase YugJ	*yugJ*		2.32	1.08	4.60	spx
BPJ19830	methionine sulfoxide reductase MsrA	*msrA*	1.46	2.24			spx
BPJ19820	peptide-methionine sulfoxide reductase MsrB	*msrB*	1.48	2.27			spx
BPJ25870	thioredoxin TrxA	*trxA*	1.40	2.58			spx/ctsR/sigB
BPJ35200	NADPH-dependent nitro/flavin reductase NfrA	*nfrA*		2.50	2.47	5.21	spx/sigD/spo0A

BPJ24450	cystathionine gamma-lyase MccB	*mccB*			-1.58	7.58	spx/cymR
BPJ17710	putative cell division suppressor protein YneA	*yneA*	2.24	44.25			lexA/SOS
BPJ10180	3'-5' exoribonuclease YhaM	*yhaM*	0.71	2.81			lexA/SOS
BPJ21860	DNA polymerase 4	*polY1*		10.68			lexA/SOS
BPJ32300	excinuclease ABC subunit B	*uvrB*		7.22	2.52	4.29	lexA/SOS
BPJ32290	excinuclease ABC subunit A	*uvrA*	1.49	6.75			lexA/SOS
BPJ25860	excinuclease ABC subunit UvrC	*uvrC*		3.65			lexA/SOS
BPJ17700	repressor LexA	*lexA*	1.55	5.66			lexA/SOS
BPJ17730	DUF896 family protein YnzC	*ynzC*	0.65	8.85			lexA/SOS
BPJ12460	phage-like PBSX protein XkdA	*xkdA*	3.10	17.84			lexA/SOS
BPJ17720	resolvase-like protein YneB	*yneB*	1.38	17.03			lexA/SOS
BPJ10160	putative exonuclease YhaO	*yhaO*		8.76			lexA/SOS
BPJ16880	recombinase RecA	*recA*	1.63	7.22	4.94	9.58	lexA/SOS/comK
BPJ35170	minor extracellular serine protease Vpr	*vpr*	1.58	2.23			lexA/SOS/phoP
BPJ21470	hypothetical protein YpuD	*ypuD*	1.93	7.12			lexA/SOS/sigB/sigM
BPJ10170	putative ATPase YhaN	*yhaN*		8.73			lexA/SOS
BPJ13450	ATP-dependent Clp protease ATP-binding subunit ClpE	*clpE*	2.78	45.41			ctsR
BPJ25460	ATP-dependent protease ATP-binding subunit ClpX	*clpX*		2.67			ctsR
BPJ00800	DNA repair protein RadA	*radA*		10.02			ctsR/sigB
BPJ00760	transcriptional regulator CtsR	*ctsR*		9.40			ctsR/sigB
BPJ00770	transcriptional regulator McsA	*mcsA*		10.26			ctsR/sigB
BPJ31850	ATP-dependent Clp protease proteolytic subunit ClpP	*clpP*	1.79	4.26	8.74	1.73	ctsR/sigB
BPJ00780	putative ATP:guanido 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	adenylyl-sulfate kinase CysC	*cysC*			23.93	1.60	cymR
BPJ15480	uroporphyrin-3 C-methyltransferase CysG	*cysG*			1.49	10.53	cymR
BPJ15460	sulfate adenylyltransferase 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-(5'-phosphoribosyl)anthranilate 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
BPJ12970	peroxiredoxin OhrA	*ohrA*	10.66	9.88	6.29	1.30	ohrR
BPJ12990	peroxiredoxin OhrB	*ohrB*		2.10			sigB/ohrR
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
BPJ02950	glycine betaine ABC transporter ATP-binding protein OpuAA	*opuAA*	2.76	10.75			glycine betaine transport
BPJ02960	glycine betaine ABC transporter membrane protein	*opuAB*	2.41	10.19			glycine betaine transport
BPJ02970	glycine betaine ABC transporter substrate-binding protein	*opuAC*	2.37	7.48			glycine betaine transport
BPJ29360	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
BPJ29400	Na+/H+ antiporter subunit MrpE	*mrpE*	1.51	3.20	sodium transport
BPJ29410	Na+/H+ antiporter subunit MrpF	*mrpF*	1.72	3.38	sodium transport
BPJ29420	Na+/H+ antiporter subunit MrpG	*mrpG*	1.96	2.60	sodium transport

Genes and proteins are listed, which could be assigned to putative regulons known from other *Bacilli*. Complete lists of upregulated as well as downregulated genes/proteins is given in supporting information Tables S1 and S2. Protein quantification was performed by the Delta 2D software (Decodon) from three biological replicates.

Figure 1

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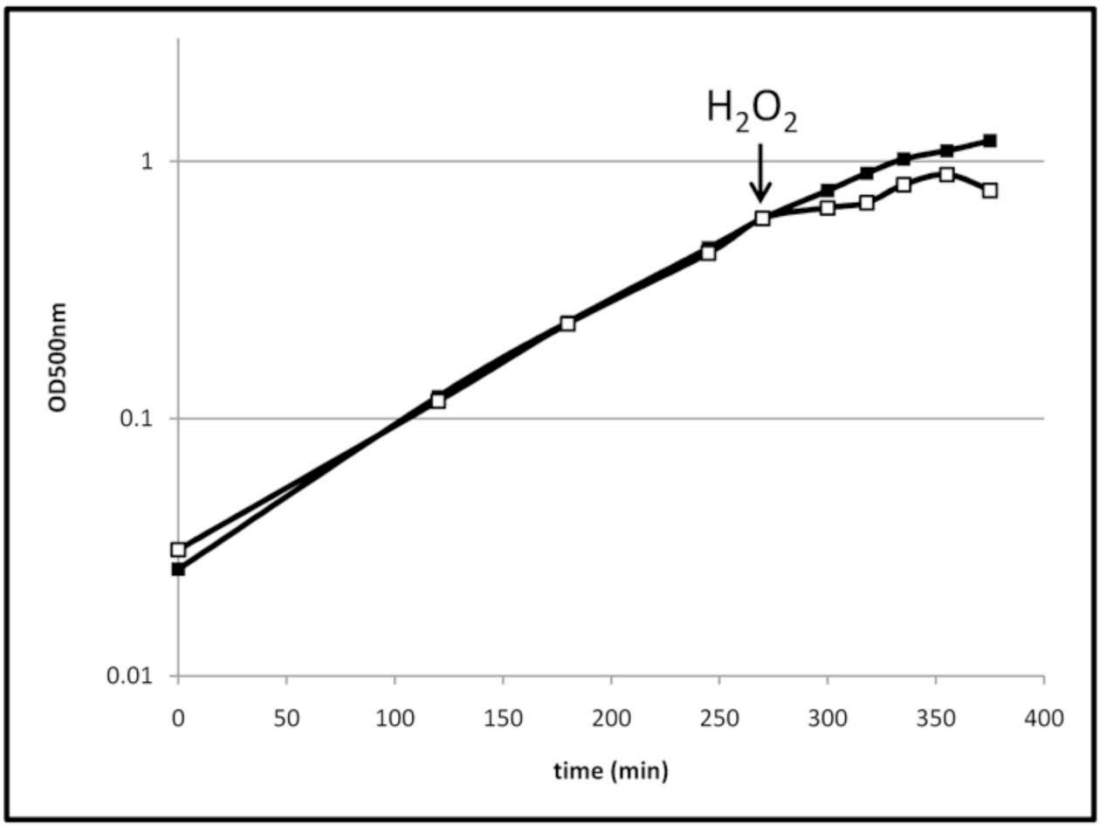

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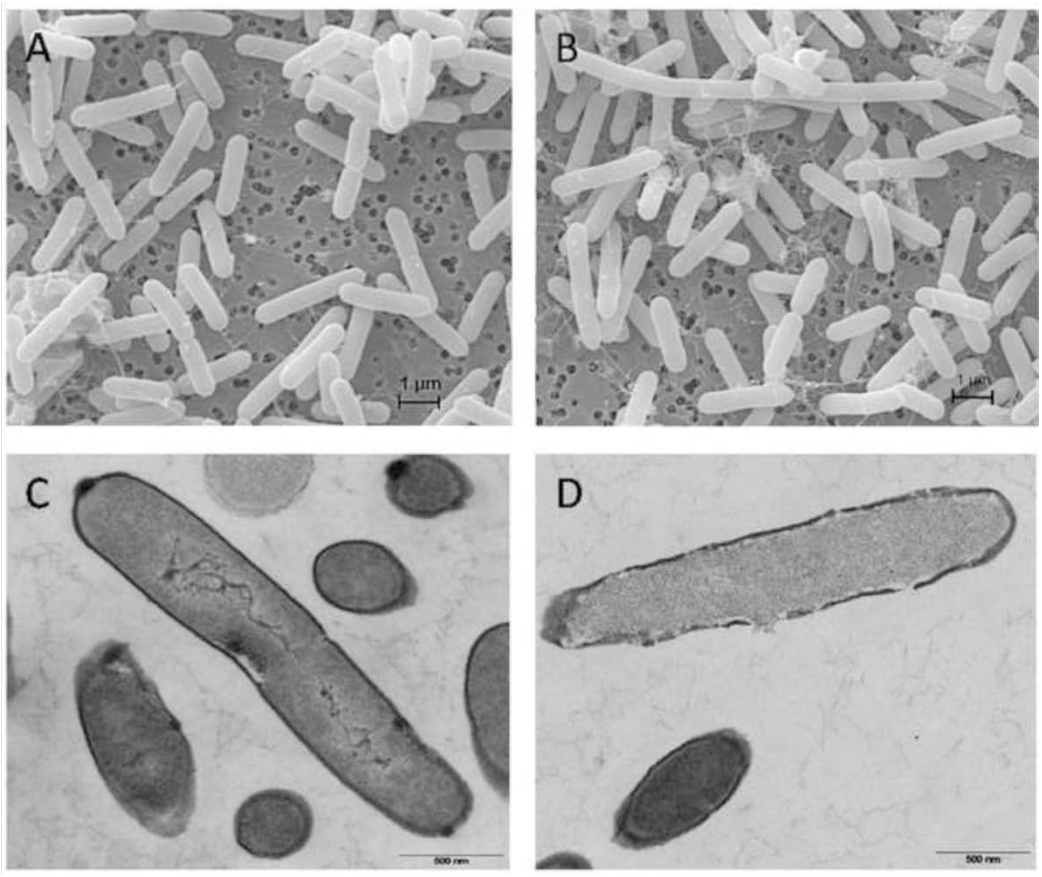

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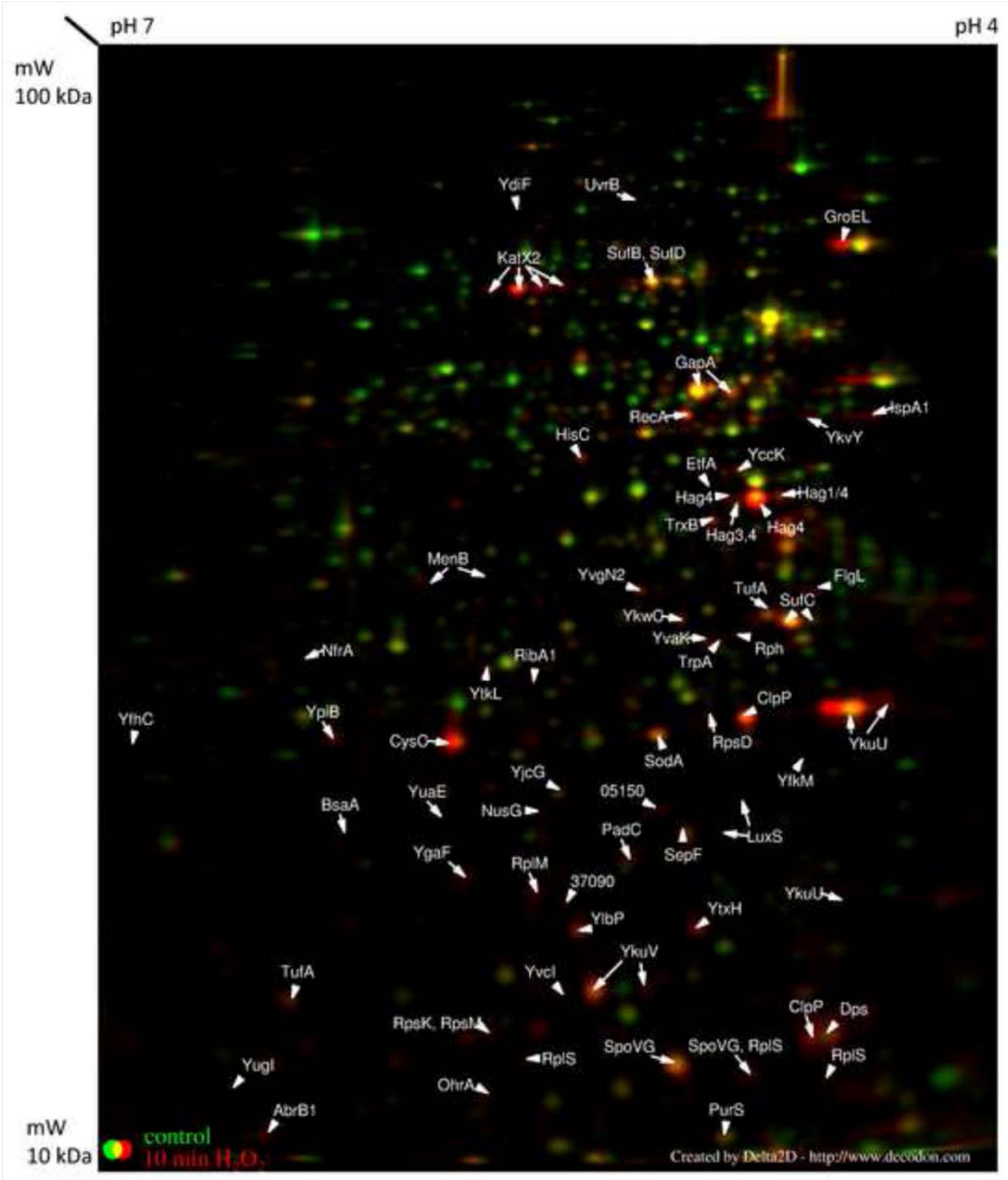

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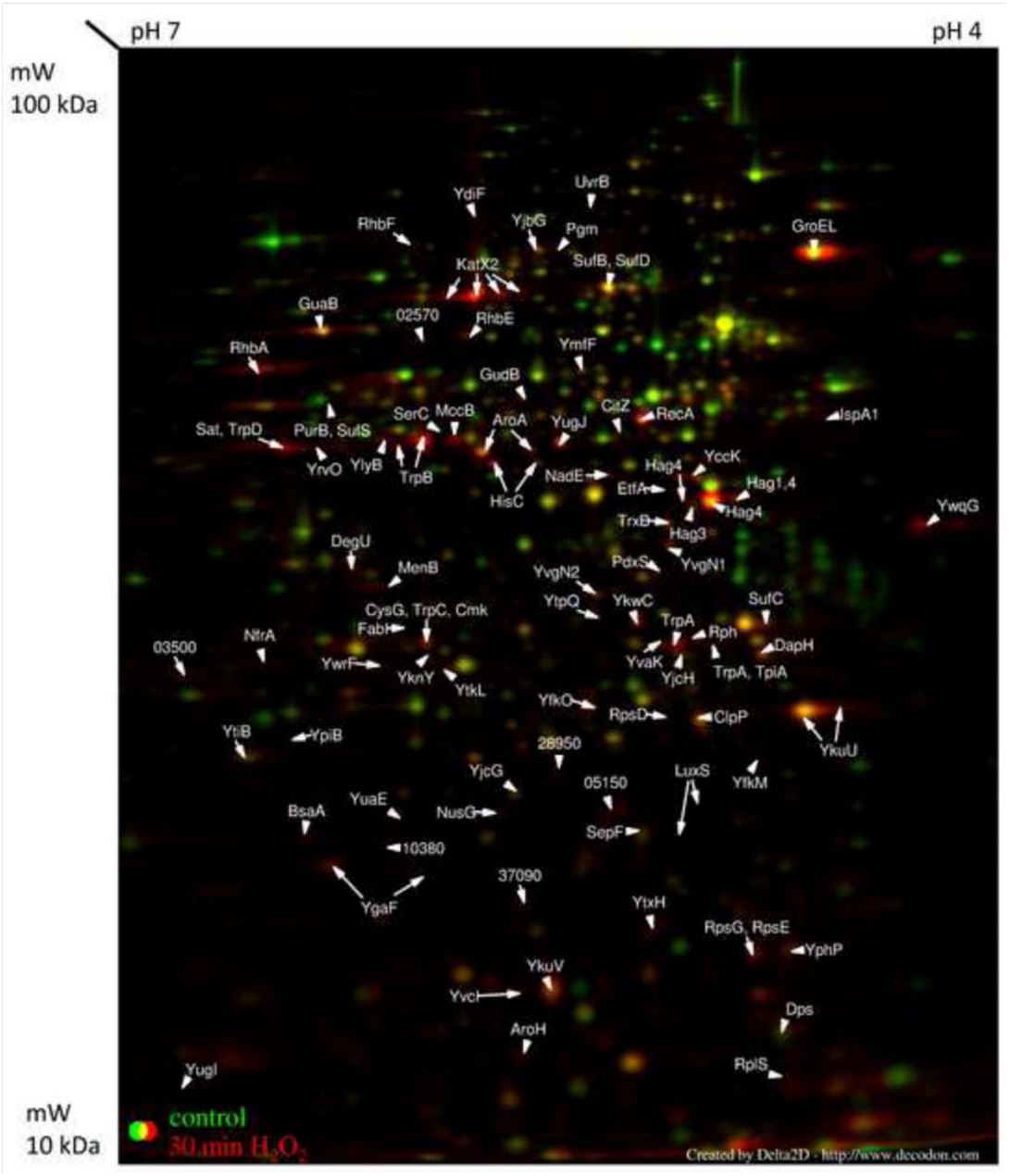

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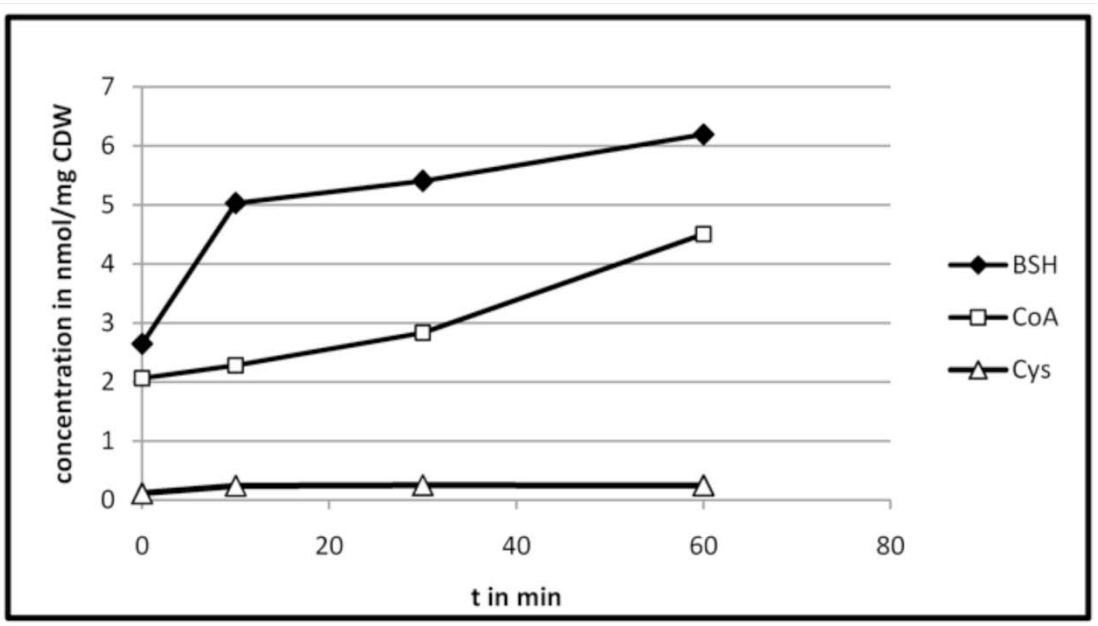

STRESS RESPONSES OF THE INDUSTRIAL WORKHORSE *BACILLUS LICHENIFORMIS* TO OSMOTIC CHALLENGES

Rebecca Schroeter, Tamara Hoffmann, Birgit Voigt, Hanna Meyer, Monika Bleisteiner, Jan Muntel, Britta Jürgen, Dirk Albrecht, Dörte Becher, Michael Lalk, Stefan Evers, Johannes Bongaerts, Karl-Heinz Maurer, Harald Putzer, Michael Hecker, Thomas Schweder and Erhard Bremer

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Author's contribution

The experimental design was developed by RS, TH, BV, TS, MH and EB. Bacterial cultivations were performed by RS, TH and BV. RNA isolations, microarray experiments and transcriptome analysis were carried out by RS. 2D-PAGE and protein analysis was done by BV. Mass spectrometry analysis was done by JM, DB and DA. Extraction and analysis of metabolites was performed by RS, HM, ML, TH, MB and EB. Secondary structure prediction was done by TH, EB and HP. Analysis of *proHJAA* and proline as nutrient was done by MB, TH and EB. The manuscript was written by all authors.

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Stress responses of the industrial workhorse Bacillus licheniformis to osmotic challenges

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Abstract:	The Gram-positive endospore-forming bacterium Bacillus licheniformis can be found widely in nature and it is exploited in industrial processes for the manufacturing of antibiotics, specialty chemicals, and enzymes. Both in its varied natural habitats and in industrial settings, B. licheniformis cells will be exposed to increases in the external osmolarity, conditions that trigger water efflux, impair turgor, cause the cessation of growth, and negatively affect the productivity of cell factories in biotechnological processes. Despite the obvious importance of this parameter for the cell's well-being and its performance as a microbial production factory, little is known about the cellular adjustment processes and stress responses of B. licheniformis to high-osmolarity surroundings. We have taken here both systems-wide and targeted physiological approaches to unravel the core of the osmostress responses of B. licheniformis. Cells were suddenly subjected to an osmotic upshift of considerable magnitude (with 1 M NaCl), and their transcriptional profile was then recorded in a time-resolved fashion on a genome-wide scale. A bioinformatics cluster analysis was used to group the osmotically up-regulated genes into categories that are functionally associated with the synthesis and import of osmostress-relieving compounds (compatible solutes), the SigB-controlled general stress response, and genes whose functional annotation suggests that salt stress triggers secondary oxidative stress responses in B. licheniformis. The data set focusing on the transcriptional profile of B. licheniformis was enriched by proteomics aimed at identifying those proteins that were accumulated by the cells through increased biosynthesis in response to osmotic stress. Furthermore, these global approaches were augmented by a set of experiments that addressed the synthesis of the compatible solutes proline and glycine betaine and assessed the growth-enhancing effects of various osmoprotectants. Combined, our data provide a blueprint of the cellular adjustment processes of B. licheniformis to both sudden and sustained osmotic stress.
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Financial Disclosure Describe the sources of funding that have supported the work. Please include relevant grant numbers and the URL of any funder's website. Please also include this sentence: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript." If this statement is not correct, you must describe the role of any sponsors or funders and amend the aforementioned sentence as needed. * typeset	This study was financially supported by grants from the German Ministry of Education and Research (BMBF) via the Bacell-SysMo2 consortium (to M.L., M.H. and E.B) and the competence network "Genome Research in Bacteria" (to M.L., M.H., K.H.M., and T.S.). Additional Funds were provided through the LOEWE program of the State of Hessen (via the Centre for Synthetic Microbiology; SynMicro, Marburg) (to E.B.), the Fonds der Chemischen Industrie (to E.B) and the CNRS (UPR 9073), Université Paris VII-Denis Diderot (to H.P).
Ethics Statement All research involving human participants must have been approved by the authors' institutional review board or equivalent committee(s) and that board must be named by the authors in the manuscript. For research involving human participants, informed consent must have been obtained (or the reason for lack of consent explained, e.g. the data were analyzed anonymously) and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should submit a statement from their ethics committee or institutional review board indicating the approval of the research. We also encourage authors to submit a sample of a patient consent form and may require submission of completed forms on particular occasions. All animal work must have been conducted according to relevant national and international guidelines. In accordance with the recommendations of the Weatherall report, "The use of non-human primates in research" we specifically require authors to include details of animal welfare and steps taken to ameliorate suffering in all work involving non-human primates. The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement. Please enter your ethics statement below and place the same text at the beginning of the Methods section of your manuscript (with the subheading Ethics Statement). Enter "N/A" if you do not require an ethics statement.	N/A

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Stress responses of the industrial workhorse *Bacillus licheniformis* to osmotic challenges

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Abstract

The Gram-positive endospore-forming bacterium *Bacillus licheniformis* can be found widely in nature and it is exploited in industrial processes for the manufacturing of antibiotics, specialty chemicals, and enzymes. Both in its varied natural habitats and in industrial settings, *B. licheniformis* cells will be exposed to increases in the external osmolarity, conditions that trigger water efflux, impair turgor, cause the cessation of growth, and negatively affect the productivity of cell factories in biotechnological processes. Despite the obvious importance of this parameter for the cell's well-being and its performance as a microbial production factory, little is known about the cellular adjustment processes and stress responses of *B. licheniformis* to high-osmolarity surroundings. We have taken here both systems-wide and targeted physiological approaches to unravel the core of the osmostress responses of *B. licheniformis*. Cells were suddenly subjected to an osmotic upshift of considerable magnitude (with 1 M NaCl), and their transcriptional profile was then recorded in a time-resolved fashion on a genome-wide scale. A bioinformatics cluster analysis was used to group the osmotically up-regulated genes into categories that are functionally associated with the synthesis and import of osmostress-relieving compounds (compatible solutes), the SigB-controlled general stress response, and genes whose functional annotation suggests that salt stress triggers secondary oxidative stress responses in *B. licheniformis*. The data set focusing on the transcriptional profile of *B. licheniformis* was enriched by proteomics aimed at identifying those proteins that were accumulated by the cells through increased biosynthesis in response to osmotic stress. Furthermore, these global approaches were augmented by a set of experiments that addressed the synthesis of the compatible solutes proline and glycine betaine and assessed the growth-enhancing effects of various osmoprotectants. Combined, our data provide a blueprint of the cellular adjustment processes of *B. licheniformis* to both sudden and sustained osmotic stress.

Introduction

Bacillus licheniformis is a Gram-positive endospore-forming microorganism that is widely distributed in nature and can readily be isolated from soils and animal and plant material [1-5]. It is extensively exploited in industrial processes [6-8]. In particular, the excellent protein secretion capacities of *B. licheniformis* [9,10] have made it an attractive host for the large-scale production of commercially employed enzymes (e.g., amylases, phytases, proteases). It is generally regarded as safe, and since some strains of this species are considered as probiotic, *B. licheniformis* is also used in the food and feed industry [11,12], but it can also be considered as a food spoilage bacterium [5].

The sequencing of the genomes of two closely related *B. licheniformis* strains, *B. licheniformis* DSM 13^T [13] and *B. licheniformis* ATTC 14580 [14], has provided a blueprint for further in-depth studies of the physiology of this industrial workhorse and incentives for the rational design of industrial relevant strains with improved production capabilities [15-18] and enhanced biosafety [19]. Genome-wide transcriptomic and proteomic investigations of stressed *B. licheniformis* cells have allowed detailed insights into its genetic regulatory circuits, metabolic networks, biosynthetic capabilities, and cellular stress adaptation responses [10,20-26]. These studies have provided valuable knowledge when one considers that large-scale and high-density fermentation processes impose considerable constraints on microbial cells and can impair their fitness and capacity to produce biotechnologically valuable products efficiently [27-29].

An important challenge with which *B. licheniformis* has to cope, both in its varied natural habitats [1-5] and in industrial settings [30], are fluctuations in the external osmolarity. In its soil ecosystem, *B. licheniformis* will be frequently exposed on one hand to high osmolarity micro-niches that are caused by desiccation processes and increases in salinity; on the other hand, rainfall and flooding of the soil habitat will confront the cell with rapid osmotic down-shifts. During biotechnological applications, high-level excretion of metabolites and substrate feed will increase the osmolarity of the growth medium [27,30,31], a process leading to water efflux from microbial cells that causes a reduction in turgor and eventually leads to cessation of growth [32]. Hence, the overall productivity of *B. licheniformis* cell factories will be negatively affected by high-osmolarity growth

89 conditions. However, the cellular adjustment process to either suddenly imposed or sustained osmotic
90 stress is not well studied in this Bacillus species [33,34].

91 As its cousins *Bacillus subtilis* [35,36] and *Bacillus cereus* [37], *B. licheniformis* possesses a
92 general stress response system that is under the control of the alternative transcription factor SigB
93 [37,38]. Members of the SigB-controlled regulon provide pre-emptive stress resistance to a multitude
94 of environmental insults, various cellular constraints, and nutritional limitations [35-37]. Detailed
95 studies in *B. subtilis* have shown that high salinity is one of the most-strongest inducers of the general
96 stress response [39,40], and many members of its SigB regulon contribute to survival when the cells
97 are exposed to severe and growth limiting osmotic up-shocks [41,42]. However, due to the transient
98 nature of the induction of the SigB-regulon in response to acute salt stress [40,43,44], this general
99 stress response system is not crucial for the ability of *B. subtilis* to strive under sustained high-salinity
100 growth conditions [43]. Under these circumstances, an effective cellular water management is key to
101 ascertaining a physiologically adequate level of hydration of the cytoplasm and magnitude of turgor in
102 order to sustain growth [32,45].

103 Despite the existence of water-conduction channels, the aquaporins, in a considerable number
104 of microorganisms [46], it is worth recalling that no bacterial cell can actively pump water across the
105 semi-permeable cytoplasmic membrane to compensate for the water influxes or effluxes instigated by
106 changes in the external osmolarity [32,47]. As a consequence, microorganisms have to balance the
107 vital osmotic gradient across their cytoplasmic membrane indirectly by influencing the osmotic
108 potential of the cytoplasm to direct the flux of water in or out of the cell [48,49]. They accumulate
109 water-attracting ions and organic osmolytes (compatible solutes) when they face hyperosmotic
110 conditions to prevent cellular dehydration [32,48], and they rapidly expel these compounds through
111 the transient opening of mechanosensitive channels to avert cell rupture when the osmolarity suddenly
112 drops [50-52].

113 The cellular stress response to high osmolarity is typically a multi-phasic process [32]. It
114 initially entails in many microorganisms the rapid uptake of K^+ ions as an emergency reaction [53] and
115 the subsequent replacement of this ion with a class of organic osmolytes that are highly compliant
116 with cellular physiology, the compatible solutes [48]. Synthesis and uptake of the compatible solutes

glycine betaine and proline play a key role in the defense of *B. subtilis* against the insults of high salinity [53-64]. A qualitative assessment by natural abundance ^{13}C-NMR spectroscopy has previously revealed that *B. licheniformis* belongs to the group of Bacilli that synthesize large amounts of proline when they are continuously exposed to high salinity surroundings [33,34].

While an effective water management is certainly the cornerstone for the cellular response to high salinity by most microorganisms [32,47], the overall adjustment process to this environmental challenge is a rather complex process. This is evident from genome-wide transcriptomic and proteomic assessments of the responses of *B. subtilis* and of the food pathogen *B. cereus* to acute and sustained high salinity environments [39,41,65,66]. Here we have combined physiological approaches, multi-omics techniques and genome mining to derive a comprehensive picture of the molecular and cellular events that allow the adaptation of *B. licheniformis* to high osmolarity surroundings.

Results and Discussion

Assessment of the resistance of B. licheniformis against salt stress

To assess the resistance of *B. licheniformis* DSM 13^T against the growth-inhibiting effects of high salinity, we grew cells in a chemically defined medium (SMM) with glucose as the carbon source and different salinities in shake-flask experiments at 37° C for 14 h and then determined the growth yield of the cultures by measuring their optical densities (OD_{578}). As shown in Fig. 1A, *B. licheniformis* DSM 13^T can readily withstand salt concentrations up to 1 M NaCl but a further increase in the external salinity rapidly leads to a strong decline in growth yield; the presence of 1.3 M NaCl in the minimal medium resulted to a complete inhibition of growth. This osmotic stress resistance profile of *B. licheniformis* DSM 13^T is similar, but not identical, to that of *B. subtilis* [60]. *B. licheniformis* DSM 13^T is thus a representative of the group of Bacilli exhibiting an intermediate degree of osmotic stress resistance, and most of these species synthesize proline as their dominant osmoprotectant [33,34]. Bacilli that exhibit a considerably higher degree of salt tolerance (e.g., *Virgibacillus salexigens*) than *B. licheniformis* DSM 13^T or *B. subtilis* typically synthesize the compatible solute ectoine, whereas those Bacilli that synthesize only glutamate as their osmoprotectant (e.g., *B. cereus*) are rather salt-sensitive species [33,34,66].

High salinity triggers a finely tuned adjustment in the cellular proline pool

Using natural abundance ^{13}C-NMR spectroscopy, it has previously been found that *B. licheniformis* DSM 13^T produces increased amounts of proline when cells are grown in SMM containing 1 M NaCl [34]. To investigate this salt-stress responsive *de novo* proline biosynthesis in greater detail, we grew cultures of *B. licheniformis* DSM 13^T to the same optical density (OD_{578} = 2) in SMM with different salinities and then measured the proline content of the cells by HPLC analysis (Fig. 1B). An increase of the external salinity up to 0.2 M NaCl had little effect on the proline content of the cells, but further increases led to a graded rise in the cellular proline pool in a fashion that was directly proportional to the degree of the imposed osmotic stress (Fig. 1B). Hence, it can be inferred from this experiment that *B. licheniformis* DSM 13^T must have the ability to detect incremental increases in the external salinity and that the cell is then able to genetically convert this information into the build-up of a situation-conform pool of an osmostress-relieving compound. The size of the proline pool at a given external salinity was strongly influenced by the presence of 1 mM glycine betaine in the growth medium. Externally provided glycine betaine is an excellent osmoprotectant for *B. licheniformis* DSM 13^T (Fig. 1C) and its presence in the growth medium repressed the build-up of the osmostress responsive proline pool entirely (Fig. 1B). Hence, osmotically challenged *B. licheniformis* DSM 13^T cells preferred the import of a preformed osmoprotectant such as glycine betaine over the synthesis of proline, the only compatible solute that they can produce *de novo* [33,34]. The same phenomenon has also been observed in *B. subtilis* [54,57] and it might be connected with the cell's attempt to cope with bioenergetic constraints under high-salinity growth conditions [67] or to optimize the solvent properties of the cytoplasm [47].

Osmotic stress induced changes in the transcriptome and proteome of B. licheniformis

To assess the cellular response of *B. licheniformis* DSM 13^T cells to high salinity on a systems-wide level, we performed both a transcriptome (Fig. 2) and a proteome (Fig. 3) analysis. For these experiments, we grew the cells in minimal medium and then subjected them to a strong osmotic up-shift by suddenly exposing them to 1 M NaCl. Total RNA was extracted from cells cultivated under control and salt-stress conditions, and changes in the transcriptional profile were analyzed in a

time-resolved fashion (0, 5, 10, 20 min) by custom-designed DNA microarrays. From the 4178 *B. licheniformis* DSM 13^T genes covered by the DNA-array slides, we found 246 genes to be significantly up-regulated (at least 3-fold) (Suppl. Table S1) and 166 genes to be significantly down-regulated (at least 3-fold) (Suppl. Table S1) in response to the suddenly imposed salt stress. A summary of all genome-wide expression data collected in this study is given in supplemental Table S2. For the analysis of those genes whose transcription was affected by the increase in the external salinity, putative regulons were predicted from the annotation of the *B. licheniformis* DSM 13^T genome sequence [13] as detailed by Schroeter *et al.* [68]. The transcriptome data were then subjected to a cluster analysis and were binned according to known salt-stress response gene groups of *B. subtilis* [39,65]. These gene clusters comprised those that are functionally connected with: (i) the synthesis and uptake of compatible solutes, (ii) SigB-controlled general stress responses, (iii) ECF sigma factor dependent genes, and (iv) salt-stress-elicited secondary oxidative stress responses. Heat-maps representing the transcriptional profile of these four groups in *B. licheniformis* DSM 13^T are shown in Fig. 2.

In parallel to the analysis of the transcriptional response of *B. licheniformis* DSM 13^T to salt stress, we also analyzed its cellular response to this environmental cue at the level of the proteome by 2D-gel analysis (Fig. 3). The synthesis of proteins subsequent to the imposition of salt stress was monitored by labeling the cells with [^{35}S]-methionine and assessing the incorporation of radiolabeled methionine at time points 10 min and 30 min after an osmotic up-shock with 1M NaCl. This analysis revealed 59 proteins whose synthesis was up-regulated at least at one time point during the imposed osmotic stress (Fig. 3) (Suppl. Table S3). In addition, the accumulation of specific proteins at a time of 2 hours subsequent to the inflicted salt stress was determined. A higher accumulation in stressed cells compared to control cells was found for about 300 proteins (Suppl. Table S4), indicating that the adjustment of *B. licheniformis* DSM 13^T cells to a suddenly inflicted severe salt stress is a dynamic process that extends over a considerable time period.

The initial salt stress response: uptake of potassium

The initial physiological adjustment reaction of *B. subtilis* to a sudden up-shift in the external osmolarity consists of a rapid import of potassium ions to curb the outflow of water [53,69]. Mining of the *B. licheniformis* DSM 13^T genome sequence [13] showed that the two potassium importers (KtrAB and KtrCD), critical for the initial stress salt stress response of *B. subtilis* [69], are also present in *B. licheniformis* DSM 13^T (Fig. 4). However, the expression of their structural genes, as reported for *B. subtilis* [69], is not up-regulated in response to osmotic stress (Fig. 2A). A third, low level, potassium import activity can be detected in *B. subtilis* strains defective in the KtrAB and KtrCD systems [69]. It might be mediated by YugO (Fig. 4), a functionally poorly characterized system that is related to potassium channels and that has recently been shown to be involved in biofilm formation in *B. subtilis* [70]. There was no up-regulation in the expression of the *yugO* gene in *B. licheniformis* DSM 13^T (Suppl. Table S2).

Sodium ions are highly cytotoxic and *B. subtilis* up-regulates genes encoding several exporters for this ion, including Mrp, NhaK and NhaC, when it is suddenly exposed to high NaCl concentrations [39]. *B. licheniformis* DSM 13^T possesses homologs to some of these sodium exporters (e.g., Mrp and NhaC) (Fig. 4), but the transcription of the corresponding structural genes were not up-regulated in response to salt stress (Suppl. Table S2).

The sustained salt stress response: uptake of osmoprotectants and synthesis of glycine betaine

We observed a strong up-regulation in the transcription of the genes encoding transport systems (Fig. 4) for compatible solutes: the expression levels of the genes for the OpuA (*opuA-opuAB-opuAC*), OpuC (*opuCA-opuCB-opuCC-opuCD*) and OpuE (*opuE*) transporters [32,45,55,62-64,71] were 3- to 90-times higher than found under control conditions (Fig. 2) (Suppl. Table S1 and S2). Transcription of the *opuD* gene [63] was also induced but its induction ratio did not match the criterion chosen in this study to represent a significant fold of induction (3-fold) in response to salt stress (Suppl. Table S2). We tested the functionality of compatible solute import systems through osmostress protection assays [60] where we strongly impaired the growth of *B. licheniformis* DSM 13^T by the addition of 1.3 M NaCl to SMM and then monitored the stress-relieving effects of various types

of compatible solutes on cell growth. As shown in Fig. 1C, *B. licheniformis* DSM 13^T can derive osmoprotection from a spectrum of externally provided compatible solutes (e.g., glycine betaine, proline, carnitine), osmoprotectants that it will likely encounter in its varied soil habitats and through its association with plant and animal tissues [1-5]. The spectrum of the osmoprotectants that can be exploited by *B. licheniformis* DSM 13^T (Fig. 1C) is similar, but not identical, to those usable by *B. subtilis* [45,71].

In *B. subtilis*, two very closely related osmoprotectant uptake systems, OpuB and OpuC, exist that are members of the ABC superfamily of transporters. They probably evolved through a gene duplication event [64] with a subsequent evolution of their strikingly different substrate specificities [71]. The OpuB and OpuC ABC transport systems can be distinguished, however, based on the amino acid sequence of their extracellular ligand-binding proteins (OpuBC and OpuCC) that are tethered to the cytoplasmic membrane via an N-terminally attached lipid moiety [64]. OpuBC and OpuCC are the least conserved component (69% amino acid sequence identity) of the OpuB and OpuC transporters [64]. Based upon this criterion, *B. licheniformis* DSM 13^T does not possess an OpuB-type transporter (Fig. 4), a system that is highly specific in *B. subtilis* for the import of choline, the precursor for glycine betaine synthesis [59-61,64], whereas OpuC is an uptake system with a highly promiscuous substrate profile that also includes choline [45,71].

Glycine betaine accumulation via synthesis from the precursor choline is an effective osmoprotection mechanism in *B. subtilis*. Choline is imported via OpuB and OpuC and subsequently oxidized by the alcohol dehydrogenase GbsB and the glycine betaine aldehyde dehydrogenase GbsA (Fig. 5A). [60,61,64]. Expression of the genes encoding the choline-specific importer OpuB and the GbsAB glycine betaine biosynthetic enzymes are controlled by the choline-responsive repressor protein GbsR [59], whose structural gene is found in a divergent orientation from the *gbsAB* operon, both in *B. subtilis* [59] and in *B. licheniformis* (Fig. 5B). We found a very strong increase in the expression level of the *gbsAB* genes in *B. licheniformis* DSM 13^T (up to 90-fold) (Fig. 2) (Suppl. Table S1 and S2) in response to acute salt stress and also observed that the transcription of the *gbsR* regulatory gene was significantly induced (five-fold) (Fig. 2) (Suppl. Table S1 and S2) under these conditions as well.

The strong transcriptional induction of the OpuC transport system that is expected to mediate choline import in *B. licheniformis* DSM 13^T and that of the GbsAB glycine betaine biosynthetic enzymes in response to high osmolarity strongly suggested that this Bacillus species should be able to synthesize glycine betaine though the oxidation of choline (Fig. 5A). To test this directly, we performed a metabolome analysis and monitored the cellular pools of choline and glycine betaine in cells that were grown in the presence or absence of choline (1 mM) and either at low or high (1 M NaCl) osmolarity. Cells grown at low osmolarity did not accumulate choline nor contained glycine betaine. In contrast, those cells that were cultured at high salinity had high glycine betaine pools and possessed moderate intracellular levels of choline, provided that choline had been present in the growth medium. Glycine betaine production by *B. licheniformis* was thus dependent on both the presence of the precursor molecule choline and high-salinity growth conditions (Fig. 5C and D), cues that also determine glycine betaine synthesis in *B. subtilis* [59] .

Synthesis of proline by B. licheniformis for anabolic purposes

Both *B. subtilis* and *B. licheniformis* DSM 13^T produce strongly elevated levels of proline when they are osmotically stressed (Fig. 1B) [33,34]; the genetics and the enzymes involved in this process have been intensively studied in *B. subtilis* [54,72-74]. As in many other bacteria [75], proline biosynthesis in *B. subtilis* [54,72,73] proceeds from the precursor glutamate [73]. It was therefore somewhat surprising that the synthesis of the GltA protein, a subunit of the glutamate synthase converting 2-oxoglutarate to glutamate, was down-regulated in salt-stressed *B. licheniformis* DSM 13^T cells (Fig. 3) (Suppl. Table S2). The same phenomenon has also been observed in *B. subtilis* [41].

The two proline biosynthetic routes operating in *B. subtilis* are the ProB-ProA-ProI [72] and the ProJ-ProA-ProH [54] pathways. These pathways serve two different physiological functions, anabolism and osmostress adjustment. The transcriptional control of the involved genes reflects the task to provide the cell with different pools sizes of proline under different growth conditions [53,54,72]. The expression of the genes (*proBA*; *proI*) for the anabolic proline biosynthetic route (ProB-ProA-ProI) are regulated by a *cis*-acting mRNA device, a T-box system [76], that allows enhanced gene expression only when the cells are actually starving for proline [72]. On the other hand,

the genes (*proHJ*) for two of the enzymes mediating the osmostress-adaptive proline biosynthetic route ((ProJ-ProA-ProH) are osmotically inducible. These two routes are interlinked in B. subtilis pry the *proA*-encoded γ-glutamyl-phosphate reductase (ProA). The *proA* gene is part of the T-box regulated proBA operon and is clearly not under osmotic control [72].

Mining of the *B. licheniformis* DSM 13^T genome [13] revealed the presence of the full set of genes involved in anabolic proline production by *B. subtilis* [72,73] (Fig. 4). As outlined above, the transcription of the anabolic *proBA* and *proI* proline biosynthetic genes are controlled via a T-Box system [72], a regulatory device that relies on different conformations of the untranslated 5'-region (UTR) of the mRNA [76,77]. Folding of the 5'-UTR into mutually exclusive terminator and anti-terminator structures is governed by the relative amounts of a given set of charged and non-charged tRNAs. Specificity to a given T-Box system is conferred by a codon, the so-called specifier, present in a bulged region of the folded 5'-UTR of the mRNA with which a cognate tRNA can interact [76-78]. We inspected the UTR regions in front of the *proBA* and *proI* genes for DNA signature sequences that are characteristic for T-box controlled genes [76] and readily detected them (Fig. 6A). In Fig. 6B and C we present the predicted terminator and anti-terminator configurations of the 5'-UTR for the *proBA* and *proI* mRNA species, respectively. Both the *proBA* and the *proI* sequences contain a proline-specific codon (CCU) in a region of the folded mRNA where the specifier codon is typically located (Fig. 6B and C) [76]. We thus surmise that the anabolic proline biosynthetic route (ProB-ProA-ProI) of *B. licheniformis* DSM 13^T is genetically controlled by a T-Box system. Like *B. subtilis* [73], *B. licheniformis* DSM 13^T possesses genes for multiple Δ^1-pyrroline-5-carboxylate reductases; one of them is ProG (Fig. 4). However, in contrast to the *proBA* and *proI* genes, *proG* does not possess the signature sequence of a T-Box system. The precise physiological function of ProG in both *B. subtilis* and in *B. licheniformis* DSM 13^T is unresolved; expression of *proG* was not osmotically inducible under the conditions tested (Fig. 2A).

Osmostress-adaptive proline biosynthetic route

The genetics of the regulatory switch of the T-Box system controlling the expression of the anabolic *proHJ* and *proI* genes is set such that a wasteful overproduction of proline is prevented

through the coupling of the size of the cellular proline pool to the ongoing protein biosynthetic activities of the cell [72]. It is obvious from this type of genetic control that the ProB-ProA-ProI proline biosynthetic route (Fig. 4) is unsuited for the biosynthesis of the very large amounts of proline required to achieve osmostress protection (Fig. 2A) [53,54,57,58]. Mining of the *B. licheniformis* DSM 13^T genome [13] revealed an important difference between *B. subtilis* and *B. licheniformis* concerning the genes for the osmostress-adaptive proline biosynthetic route, since a gene cluster (*proH-proJ-proAA*) was detected that encodes the full complement of enzymes required for proline biosynthesis (Fig. 4). This latter group of genes, in contrast to those involved in the anabolic proline production (ProB-ProA-ProI), was found to be osmotically inducible in our DNA transcriptional profiling study (Fig. 2A).

A Northern blot analysis revealed that the *proH*, *proJ,* and *proAA* genes are co-transcribed as an osmotically inducible operon (Fig. 7A and B). Inspection of the DNA sequence in the presumed regulatory region of the *B. licheniformis* DSM 13^T *proH-proJ-proAA* gene cluster revealed a putative SigA-dependent promoter (Fig. 7C), whose -10, -16 and -35 regions [79] closely resemble those identified for the osmotically inducible *B. subtilis proHJ* operon through primer-extension analysis [54]. To study the osmotic control of the *proH-proJ-proAA* operon in greater detail, we fused a 130-bp DNA segment encompassing the promoter region to a promoterless *treA* reporter gene and then inserted the resulting *proH-treA* transcriptional fusion in a single copy at the non-essential *amyE* site of the *B. subtilis* genome. A sudden exposure of the *proH-treA* reporter strain to increased salinity (0.4 M NaCl) led to a strong induction of gene expression (Fig. 8A). Under sustained high-salinity growth conditions, the level of transcription of the *proH-treA* reporter fusions was sensitively linked to the severity of the imposed osmotic stress (Fig. 8B). Hence, osmotic control of *proH-proJ-proAA* operon transcription was maintained across the species boundaries of *B. licheniformis* DSM 13^T and *B. subtilis*.

To further characterize the functionality of the *proH-proJ-proAA*-encoded enzymes from *B. licheniformis* DSM 13^T within the physiological framework of osmostress adaptation, we cloned this gene cluster into a plasmid vector (pX) [80] that allows the integration of cloned genes as single copies into the *amyE* site of the *B. subtilis* genome. In this way we inserted the *B. licheniformis* DSM

341 13^T *proH-proJ-proAA* operon into a *B. subtilis* strain that was proficient in osmostress adaptive proline
342 biosynthesis and one that was defective in this stress response. We then tested both the growth of the
343 resulting recombinant strains under high-salinity conditions and measured their proline pools. As
344 summarized in Fig. 9, the *B. licheniformis* DSM 13^T *proH-proJ-proAA* gene cluster rescued the salt-
345 sensitive growth phenotype [54] of a *B. subtilis proHJ* mutant strain (Fig. 9A) and restored osmostress
346 responsive proline production (Fig. 9B). Hence, there can be no doubt the *proH-proJ-proAA*-encoded
347 enzymes (Fig. 4) are responsible for the osmostress adaptive proline biosynthesis (Fig. 1B) of *B.*
348 *licheniformis* DSM 13^T.

349 An *in silico* analysis of the genome sequence of the industrially relevant species *Bacillus*
350 *megaterium* has previously suggested the existence of two complete proline biosynthetic production
351 routes (Fig. 4) with distinctly different physiological functions [81]. However, the transcriptomic (Fig.
352 2A) and physiological data (Fig. 9) reported here for *B. licheniformis* DSM 13^T provide now for the
353 first time direct experimental evidence for this notion. Hence, in both *B. licheniformis* DSM 13^T and in
354 *B. megaterium*, the curious interconnection observed in *B. subtilis* between the anabolic and
355 osmostress-relieving proline biosynthetic routes via a single ProA enzyme [54,72] is avoided.

356

357 *Use of proline as a nutrient by B. licheniformis DSM 13^T*

358 In *B. subtilis*, proline not only serves as an osmostress protectant [53,54,58] but can also be
359 exploited by the cell as sole source of energy, carbon, or nitrogen [82]. This is accomplished through
360 proline import via the OpuE-related proline transporter PutP and its subsequent catabolism to
361 glutamate through the sequential actions of the PutB and PutC enzymes [82] (Fig. 10A). The
362 corresponding gene cluster (*putBCP*) (*ycgMNO*) is also present in the genome sequence of *B.*
363 *licheniformis* DSM 13^T [13] and is followed by the *putR* (*ycgP*) gene (Fig. 10A) that encodes the
364 proline-responsive transcriptional activator protein of the *putBCP* operon of *B. subtilis* [83,84]. As
365 found in *B. subtilis* [82], none of these genes is osmotically inducible in *B. licheniformis* (Suppl. Table
366 S2). We tested the use of proline as nutrient by *B. licheniformis* DSM 13^T and found that it can be
367 used both as sole carbon and nitrogen source (Fig. 10C and D). This contrasts with our findings
368 concerning the use of the osmoprotectant glycine betaine as a nutrient, a compound that is

metabolically inert not only in *B. licheniformis* DSM 13^T (Fig. 10C and D) but in *B. subtilis* as well [60].

General stress responses and ECF controlled systems

Our transcriptome and proteome analysis showed that salt stress caused a strong induction of the σ^B-controlled general stress regulon of *B. licheniformis* DSM 13^T [37,38] (Fig. 2B and Fig. 3) (Suppl. Table S1 and S2). At least 23 putative σ^B-dependent genes revealed a more than ten-fold higher mRNA level after the osmotic up-shock; hence, σ^B-responsive genes are among the most highly salt-stress inducible genes of *B. licheniformis* DSM 13^T. This is fully consistent with studies of *B. subtilis*, where salt stress is also one of the most potent inducers of the SigB-dependent general stress response system [39,40]. Among the σ^B-controlled genes identified in this study (Fig. 2B), one can correlate the predicted function(s) of the encoded proteins with particular types of stress-relieving properties for some of them. These are: *bmrU,* a gene that encodes a multidrug resistance protein; *gsiB,* a gene encoding a putative desiccation resistance protein; and *dps*, a gene encoding a DNA-protecting protein. The strong up-regulation of these and other SigB-regulated genes (Fig. 2B) clearly indicates that the induction of the general stress response system is an important aspect of the cells attempt to cope with a suddenly imposed increase in the external salinity.

The most strongly induced genes 20 min after the salt shock encode a FMN-binding split barrel domain protein (YdaG), a so-called general stress protein (GspA, a putative glycosyl transferase), and a putative organic hydroperoxide resistance protein (OhrB or YkzA) (Fig. 2B). However, the contribution of a particular σ^B-controlled gene in coping with high salinity can be difficult to understand in physiological terms. The same experience was true for *B. subtilis*, where a comprehensive deletion and functional analysis study has been conducted to elucidate the contribution of individual genes of the σ^B-regulon to the cell's ability to resist the detrimental effects of strong up-shifts in the external salinity [41,42].

The high-level induction of the SigB-controlled hydroperoxide resistance gene *ohrB* and the catalase genes *katE1* and *katE2* indicate that a secondary oxidative stress is triggered by the sudden increase in the external salinity (Fig. 2B) (Suppl. Table S1 and S2). This assumption is supported by

397 the increased synthesis of further distinct oxidative stress marker proteins, such as KatA (catalase),
398 SodA (superoxide dismutase), and AhpCF (alkyl hydroperoxide reductase) (Fig. 3) (Suppl. Table S3).
399 The concentration of the oxidative stress-specific PerR-regulated DNA-binding protein MrgA was
400 increased more than 20-fold at the protein level after the imposition of the salt stress. It is interesting
401 to note that this protein was detected on the 2D-gels only in the monomeric form in salt-stressed cells,
402 although it occurs in both a monomeric and in multimeric forms in *B. licheniformis* DSM 13^T cells
403 subjected to a H_2O_2-triggered oxidative stress [22].

404 The *sufCDSUB* operon, which encodes a biogenesis system for an iron-sulfur cluster that is
405 essential for the assembly or repair of Fe/S proteins [85], was significantly induced at the
406 transcriptional and translational level in response to high salinity (Fig. 2D and Fig. 3). This increased
407 demand for iron-sulfur cluster biogenesis might in part be due to oxidative stress provoked by salt
408 challenges and the damage to Fe/S cluster proteins by reactive oxygen species (ROS). Furthermore, in
409 the proteome of salt-stressed cells a higher concentration of several enzymes of the pentose phosphate
410 pathway could be detected 2 h after the beginning of the salt stress. This could also be related to the
411 onset of a secondary oxidative stress, as many ROS-detoxifying enzymes rely on NADPH [86], which
412 is produced in the pentose phosphate pathway.

413 In *B. subtilis* a secondary oxidative stress response is linked to the MgsR transcriptional
414 regulator [87,88]. This σ^B dependent regulator seems to sense and integrate the secondary oxidative
415 stress signal and controls a specific subregulon within the σ^B dependent general stress regulon. It is
416 interesting to note that the *mgsR* homologue gene *yqgZ* in *B. licheniformis* belongs to the strongest
417 induced genes triggered by salt stress. It has been suggested that members of this MgsR subregulon
418 are linked to oxidative stress management [87,88]. In our study we found an induction of several
419 putative MgsR controlled genes with in most cases unknown functions (e.g. *gsiB*, *ydaD*, *ydaE*, *yhdF*,
420 or *ydbD*).

421 The elevated transcription and synthesis of the general stress proteins ClpC, ClpE and ClpP or
422 DnaK, GroEL and GrpE could also indicate damage or misfolding of proteins that might be caused
423 either directly by the osmotic stress or indirectly by the elicited secondary oxidative stress responses
424 (Fig. 3) (Suppl. Tables S1, S2 and S3). Consistent with this idea are reports that show an induction of

425 members of the Clp system in *B. licheniformis* DSM 13^T cells directly subjected to oxidative stress
426 [89]. Furthermore, it has already been shown that ClpC is required in *B. subtilis* for resistance to salt
427 stress, as the growth of a *clpC* mutant was significantly impaired under high saline conditions [90].
428 The two sigma factors σ^W and σ^X with extracytoplasmic function (ECF sigma factors), which
429 govern physiological response to cell envelope stress in *B. subtilis* [91], were affected by salt stress in
430 *B. licheniformis* DSM 13^T Among the seven ECF sigma factor regulons present in *B. subtilis* [91],
431 three are affected by a sudden increase in the external osmolarity: σ^M, σ^W, and σ^X [39,92,93]. While
432 the σ^M regulon in *B. subtilis* is known to be crucial for prolonged survival in a high-salt-containing
433 environment due to its central role in cell-wall maintenance systems [93], we did not find any σ^M-
434 dependent gene induced in *B. licheniformis* after salt stress. In *B. licheniformis* DSM 13^T cells, only
435 genes of the two ECF regulons σ^W and σ^X showed increased induction rates (Fig. 2C) (Suppl. Table S1
436 and S2). At least 26 ECF-regulated genes of *B. licheniformis* DSM 13^T whose transcription was
437 responsive to salt stress could be identified in this study. In addition to the up-regulation of the ECF
438 sigma-factor-encoding genes *sigW* and *sigX*, enzymes involved in cell wall metabolism had higher
439 expression levels at the transcriptional level. This includes, for example, an increased transcription of
440 the intra-membrane protease *yqeZ* and the membrane protein *yvlD*. None of these cell-wall-associated
441 proteins could be identified in the 2D-gel-based proteomic approach employed in this study. The
442 membrane-bound nature of these proteins could readily explain their absence from the soluble
443 cytosolic proteome. However, a higher rate of synthesis and protein level could be detected for the
444 cell-wall-related protein UTP-glucose-1-phosphate uridylyltransferase (GtaB) and the penicillin-
445 binding proteins PbpC and PbpX. Up-regulation of penicillin-binding proteins at the transcriptional
446 level in salt-shocked cells was also observed for *B. subtilis* [39,94].
447 One of the strongest gene clusters of *B. licheniformis* DSM 13^T inducible by high salinity (Fig.
448 2A) encode for the components (BLi03671 and BLi03672) of a transport system that belongs to the
449 exporter sub-family of the ABC super-family (Fig. 4). Expression of the genes for the membrane
450 component (BLi03672) and ATPase subunit (BLi03671) was about 250- and 136-fold up-regulated in
451 response to high salinity (Suppl. Table S1 and S2). Homologs of this presumed multi-drug export
452 system do not exist in *B. subtilis* but can be found in the genome sequences of *B. megaterium* [81] and

453 *Bacillus pumilus* [95]. Wecke *et al*. [20] have shown that the expression of the structural genes for the
454 BLi03671/BLi03672 ABC-type export system from *B. licheniformis* is up-regulated in response to the
455 cell-wall damaging antibiotic vancomycin in a fashion that is independent of ECF-type sigma factors,
456 indicating that its strong salt-stress-mediate induction might reflect cell-wall-damaging effects exerted
457 by severe salt shocks.

458 We observed that proteins involved in chemotaxis (CheV, CheW, CheY, Yfms) and motility
459 (MotB, SwrC) were accumulated 120 min subsequent to the imposed salt stress and that the flagellin
460 protein Hag was also present at higher concentrations in salt stressed cells (Suppl. Table S3). This
461 latter observation conflicts with data from the transcriptome analysis where the *hag* mRNA levels
462 were found to be strongly down-regulated in response to salt stress. However, it needs to be kept in
463 mind that the latest time point taken for the transcriptome analysis was 20 min after the salt stress was
464 imposed, whereas the accumulation of proteins in salt-stressed cells was analyzed 2 h subsequent to
465 the imposition of the salt stress. These findings indicate perhaps a progressive response to osmotic
466 stress as far as the motility and chemotactic systems of *B. licheniformis* are concerned. It should be
467 noted in this context that a sustained rise in the external salinity strongly negatively affects the
468 chemotaxis and motility systems of *B. subtilis* and strongly down-regulate the production of the Hag
469 protein [39,65].

470

471 *Concluding remarks*

472 Our study offers an in-depth analysis of the salt-stress response of *B. licheniformis* at the
473 transcriptome and proteome level, and we have combined this system-wide assessment with
474 physiological approaches that address the synthesis and uptake of osmoprotectants. Combined with
475 genome mining of systems known to be important for the management of osmotic stress in *B. subtilis*
476 [32,41,45,52,54,69,71], the data presented here provide a detailed view of the *B. licheniformis* cell's
477 attempt to cope with and ameliorate the negative effects of high salinity on its physiology. A
478 considerable overlap with the salt stress response of *B. subtilis* was evident but also stress reactions
479 that are specific to *B. licheniformis* were found.

480 It well established that large-scale growth conditions of microbial cells influence the outcome
481 of industrial size bioprocesses [96,97]. High-level excretion of the desired product from the microbial
482 producer cell into the culture broth will successively increase the osmolarity of the medium [30,31]
483 and such an increase can limit cell density and volumetric productivity. The feeding of osmoprotective
484 compounds such as glycine betaine, choline, carnitine, proline or proline-containing peptides to
485 osmotically stressed cells [58,60,71] will likely ameliorate such negative effects (Fig. 1C) [30].
486 Microorganisms used in large-scale reactor environments are continuously exposed to various
487 types of gradients [96,98]. This comprises also the development of osmotic gradients as a result of the
488 feeding of high concentrated nutrient solutions and insufficient mixing of the culture broth. Since such
489 osmotic gradients will certainly induce stress response in the microbial cell factory [28,31], the data
490 presented here will help to understand such processes in *B. licheniformis* on a much more solid
491 footing. Furthermore, the ability to distinguish between essential osmotic-stress-relieving pathways
492 and dispensable regulons, which are not required or are even perturbing under industrial scale process
493 conditions, could help to design more robust and efficient production strains of *B. licheniformis*.
494 We surmise that the *B. licheniformis* DSM 13^{T}-derived *proH-proI-proAA* operon for the
495 osmostress-adaptive proline biosynthesis (Fig. 4) might be exploited in the context of synthetic
496 microbiology as a *bio-brick* to engineer salt stress resistance in salt-susceptible microorganisms.
497 Furthermore, the salient features of the osmotically inducible promoter driving the expression of this
498 gene cluster (Fig. 7 and 8), and the regulatory elements of other osmotically controlled *B.*
499 *licheniformis* genes identified in our study (Fig. 2) (Suppl. Table S1), might turn out to be useful tools
500 in developing novel types of environmentally responsive expression systems for Bacilli.
501
502 **Materials and Methods**
503 *Bacterial strains, media and growth conditions*
504 The *Escherichia coli* K-12 strain DH5α (Invitrogen, Carlsbad, CA, USA) was used for routine
505 cloning purposes, maintenance of cloning vectors, and recombinant plasmids. These strains were
506 maintained on Luria-Bertani agar plates [99]. For physiological studies that involved either *B. subtilis*
507 or *B. licheniformis*, the cells were cultured in Spizizen´s minimal medium (SMM) [100], with 0.5%

(w/v) glucose as the carbon source. In experiments that employed the *B. subtilis* strain JH642 (*trpC2 pheA1*) [101] or any of its derivatives, the minimal medium was supplemented with L-tryptophan (20 mg l^{-1}) and L-phenylalanine (18 mg l^{-1}) to satisfy the auxotrophic growth requirements of JH642. A solution of trace elements [100] was added to SMM to improve the growth of *B. subtilis* and *B. licheniformis* strains. The growth of bacterial cultures was monitored by measuring their optical densities at a wavelength of 578 nm (OD_{578}). In physiological experiments, cultures were inoculated from exponentially growing pre-cultures into pre-warmed minimal media (20-ml culture volumes in 100- Erlenmeyer flasks) to optical densities OD_{578nm} of 0.1, and the cultures were then further propagated at 37°C in a shaking water bath set to 220 rpm. The osmolarity of SMM was increased by adding NaCl to it from a 5 M stock solution to the final concentration indicated in the individual experiments. Solutions of the compatible solutes glycine betaine, choline, proline, proline betaine, carnitine, choline-*O*-sulfate, dimethylsulfoniopropionate (DMSP), ectoine, and hydroxyectoine were sterilized by filtration and added to the growth media from 100 mM stock solutions (the final concentration in the medium was 1 mM). The antibiotics ampicillin, chloramphenicol, and tetracycline were used in the final concentrations of 100 µg ml^{-1}, 5 µg ml^{-1}, and 10 µg ml^{-1}, respectively.

For all experiments that involved *B. licheniformis*, we used strain DSM 13^T (equivalent to strain ATCC 14580), the *B. licheniformis* type strain of the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). Its genome sequence has been established [13]. To perform proteomic and transcriptomic analyses of *B. licheniformis* DSM 13^T, the cells were cultivated in synthetic Belitzky minimal medium (BMM) [102] with 0.2% (w/v) glucose as the carbon source. Growth of these cultures was monitored by measuring their optical density at 500 nm (OD_{500nm}). Cultures of *B. licheniformis* were grown in 500-ml Erlenmeyer flasks in a shaking water bath at 180 rpm and 37°C and were inoculated to a starting OD_{500nm} of 0.04 from overnight cultures.

Heterologous expression studies involving the *B. licheniformis proHJAA* operon were performed in the *B. subtilis* strain JH642 [101] as the host, or its corresponding *proHJ* mutant derivative, strain JSB8 [Δ(*proHJ*::*tet*)1] [54,72]. Strain FSB1 [*(treA::neo)*1] [43] is a derivative of JH642 and was used as a host for the chromosomal integration of the [Φ*(proHB.li 130′-treA)*16 *cat*] reporter construct at the *amyE* region.

536 The antibiotics ampicillin, chloramphenicol, and tetracycline and the artificial substrate for the
537 TreA enzyme, *para*-nitrophenyl-α-D-glucopyranoside (α-PNPG), were all purchased from Sigma-
538 Aldrich (Steinheim, Germany). 9-fluorenyl-methoxycarbonyl chloride (FMOC) and 1-
539 adamanatanamine (ADAM) for the derivatization of amino acids quantitated by HPLC analysis were
540 obtained from Grom Analytic (Rottenburg-Hailfingen; Germany); sodium 3-trimethylsilyl-[2,2,3,3-
541 D4]-1-propionic acid (TSP) as a standard for NMR analysis were obtained from Aldrich
542 (Deisenhofen, Germany). All compatible solutes used in this study were from laboratory stocks.
543
544 *Construction of bacterial strains and plasmids*
545 A 3 616-bp DNA fragment containing the *B. licheniformis* DSM 13^{T} *proHJAA* operon region
546 was amplified by PCR from chromosomal DNA using DNA primers [forward primer proH-for: 5'-
547 AAAACTAGTCCAAAGGCTGTTGATCTCC-3'; reverse primer proAA-rev: 5'-
548 AAAACTAGTGGTGTCTGACAAACCAGGTG-3'] that contained artificially added *Spe*I restriction
549 sites at their ends. The amplified genomic region contained the *proHJAA* coding region and a DNA
550 segment (314 bp upstream of the *proH* start codon) that is predicted to contain the *proHJAA* promoter
551 region; it was cloned into the *B. subtilis* vector pX (*cat*) [80], and this yielded plasmid pTMB20.
552 pTMB20 carries the *B. licheniformis proHJAA* genes in an anti-linear orientation to the P_{XylA} promoter
553 that is present in plasmid pX [80]. DNA of plasmid pTMB20 and the empty pX cloning vector were
554 linearized by restriction digestion and used to transform competent cells of the *B. subtilis* strain JH642
555 and its [Δ(*proHJ*::*tet*)1] mutant derivative strain JSB8 [72] to stably integrate the cloned *B.*
556 *licheniformis proHJAA* genes into the *B. subtilis* chromosome at the *amyE* gene via a double
557 recombination event. The resulting strains were TMB134 [*proHJ*$^{+}$ *amyE*::pX-*cat*], TMB135 [*proHJ*$^{+}$
558 *amyE*::*proHJAA-cat*], TMB136 [Δ(*proHJ*::*tet*)1 *amyE*::pX-*cat*], and TMB137 [Δ(*proHJ*::*tet*)1
559 *amyE*::*proHJAA-cat*]. For the construction of a transcriptional *proH-treA* reporter gene fusion, we
560 amplified a chromosomal 130-bp fragment carrying the predicted *B. licheniformis proH* promoter
561 region [forward primer: B.li 130 SmaproHJAAtreA 5´- cgccccgggcaaacttgacataatgatctaaagtg-3';
562 reverse primer: B.li BamproHJAAtreA 5´- cgcggatcctgatcccgcccctataaaagctacg-3']; the used DNA
563 primers carried artificially introduced *Sma*I and *Bam*HI restriction sites at their ends. The resulting

PCR fragment was cut with the restriction enzymes *Sma*I and *Bam*HI and ligated into the low-copy-number *treA* operon fusion vector pJMB1 (M. Jebbar and E. Bremer, unpublished results), thereby yielding plasmid pMD30. The *treA* gene encodes a highly salt-tolerant phospho-α-(1,1)-glucosidase [103] whose enzyme activity can be quantitated using the chromogenic substrate PNPG [104]. DNA of plasmid pMD30 [Φ*(proHB.li 130′-treA)16 cat*] was linearized by digestion with a restriction enzyme and used in a transformation experiment to insert the reporter gene fusion construct as a single copy into the chromosomal *amyE* locus of the *B. subtilis* strain FSB1 [*(treA::neo)*1] [43] via a double recombination event by selecting for chloramphenicol-resistant colonies; this yielded strain MDB60. Loss of AmyE function caused by the integration of the [Φ*(proHB.li 130′-treA)16 cat*] construct into *amyE* was assessed by flooding *B. subtilis* colonies that were grown on agar plates containing 1 % starch with Gram´s iodine stain and scoring the size of the zone around individual colonies where starch hydrolysis had occurred [105].

TreA enzyme assay

To determine the transcriptional activity of the *B. licheniformis proHJAA* promoter in a heterologous *B. subtilis* host, we grew the [Φ*(proHB.li 130′-treA)16 cat*] reporter strain MDB60 in SMM with various NaCl concentrations and in the absence or presence of 1 mM glycine betaine. When the cells reached mid-exponential growth phase (OD_{578} of about 1.5), 1.5 ml of each culture was harvested by centrifugation and assayed for TreA reporter enzyme activity using the chromogenic substrate PNPG as described [99,104]. TreA enzyme activity is represented in units per mg of protein, and the protein concentrations of the samples were estimated from the optical density of the cell [99].

HPLC analysis of intracellular proline pools in cells adapted to hyperosmotic stress

For the quantification of the newly synthesized compatible solute proline, *B. subtilis* cultures were grown in SMM in the presence of various NaCl concentrations. After they reached mid-exponential growth phase (OD_{578} of about 2.0), the cells were harvested by centrifugation and lyophilized; the cell dry weight (cdw) was then determined. Soluble compounds were extracted from the dried cells by the Blight and Dyer technique using a methanol-chloroform-water mixture (10:5:4) (vol/vol/vol) [34]. The amino acids present in the extracts were separated as FMOC (9-fluorenyl-methoxycarbonyl chloride)-derivatives in an HPLC analysis and using a GROM-SIL 11 Amino-1PR

column (GROM, Herrenberg, Germany) coupled to a fluorescence detector. Quantification of the L-proline concentration of the samples was carried out using a standard curve derived from the parallel measurement of various defined standard solutions of FMOC-derivatized L-proline.

Northern blot analysis

Total RNAs were isolated from *B. licheniformis* DSM 13^T cultures that were grown in SMM either in the absence or in the presence of 0.8 M NaCl to mid-exponential growth phase (OD_{578} of approximately 1.5) by the acidic-phenol method [106]. 15 µg of total RNAs were denatured by heating (70°C, 5 minutes) in the presence of 50 % formamide and separated according to size on a 1.4 % agarose gel. Digoxygenin-labeled single-stranded antisense RNA probes covering internal 500-bp regions of the *proH*, *proJ*, and *proAA* genes were generated using the *in vitro* transcription system StripEZ-kit (Ambion, Austin, Texas, USA). PCR fragments of the above-indicated gene regions carrying an artificial T7 promoter sequence at one of their ends were used as DNA templates for the *in vitro* transcription reaction to generate the single-stranded anti-sense RNA probes. Northern blot analysis of the denatured RNAs from *B. licheniformis* DSM 13^T was performed as described [107].

Exposure of B. licheniformis to salt shock conditions and cell sampling for proteome and transcriptome analysis

Exponentially growing *B. licheniformis* cells (OD_{500nm} of about 0.4) were subjected to a strong salt shock by the addition of NaCl to a final concentration of 1.0 M (6 g of solid NaCl was added per 100 ml of cell culture). Cell samples for RNA extraction were taken from unstressed cultures before (control) and 5, 10, and 20 min after NaCl was added to the cultures. The cells withdrawn from the cultures were mixed with 0.5 volumes of ice-cold killing buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl, 20 mM NaN_3), and the cells were then immediately harvested at 10 000 x g for 5 min at 4°C. Labeled cytoplasmic proteins were prepared by incubating the bacteria with 556 Bq ml^{-1} L-[^{35}S]-methionine (specific activity 37 TBq/ mmol; Hartmann Analytic GmbH, Braunschweig, Germany) for 5 min, as described [21]. Cells were labeled during exponential growth phase (OD_{500nm} 0.4, control) of the *B. licheniformis* culture, and 10 and 30 min after the addition of NaCl

(final concentration 1.0 M). For the identification of proteins, preparative SDS gels with unlabeled proteins were prepared using cells treated with NaCl for 30 and 60 min as described by Hoi *et al*. [21]. In addition, cell samples were harvested 2 h after the osmotic up-shock (the control was harvested at an OD_{500nm} of 0.4 before the salt stress) for a label-free quantification method by using the Synapt G2 mass spectrometer technique (Waters; Milford, MA; USA).

RNA isolation and DNA microarray experiments

To monitor gene expression before and subsequent to a suddenly imposed salt stress, total RNA was isolated from *B. licheniformis* DSM 13^T cultures and used for genome-wide transcriptional profiling experiments. The isolation of RNA, the determination of RNA quality and quantity and the microarray experiments were all carried out according to procedures detailed by Schroeter *et al.* [22]. Custom-made *B. licheniformis* DSM 13^T gene expression arrays were prepared by and purchased from Agilent Technologies (Santa Clara, CA; USA) (https://earray.chem.agilent.com/earray/). The design of the hybridization probes was conducted on the basis of the annotated open reading frames of the genome sequence of the *B. licheniformis* DSM 13^T strain [13]. Hybridization conditions for the DNA array experiments were as previously described [22]. Microarrays were scanned using the Agilent scanner Type G2565CA combined with the high-resolution upgrade G2539A and the software Scan Control 8.4.1 (Agilent Technologies; Santa Clara, CA; USA). Data were extracted from scanned images using Agilent's Feature Extraction Software (version 10.5.1.1) (Agilent Technologies; Santa Clara, CA; USA) using default settings. Gene expression data were loaded into the Rosetta Resolver® Gene Expression Analysis System 7.2. (Rosetta Inpharmatics c/o Ceiba Solutions; Boston, MA; USA). A common reference type of design was employed, and data from three biological replicate hybridizations were combined using an error-weighted average (Rosetta Resolver error model [108]). Genes showing significant differences in expression were identified by error-weighted ANOVA analysis, with a Benjamini–Hochberg FDR (false discovery rate) multiple test correction. Only genes for which an ANOVA $p<0.01$ was obtained by statistical testing and whose transcription was at least three-fold induced (fold change above + 3) or three-fold repressed (fold change below -3) for at least

one time point throughout the conducted transcriptional profiling experiment were considered as differentially expressed and were used for further evaluation.

2D-PAGE and protein identification

Gel electrophoresis was performed as described previously [109] using 80 µg of protein extract L-[^{35}S]-methionine-labeled protein extract and 500 µg of unlabeled protein extract for preparative gels. In the first dimension IPG-strips (GE Healthcare; Freiburg, Germany) in the pH range 4-7 were applied. Autoradiography of labeled gels and staining of preparative gels was carried out as described by Hoi *et al.* [21]. Proteins, which were found to be up-regulated in response to salt-stress were cut out from preparative Coomassie-blue-stained gels and identified by mass spectrometry according to Liedert *et al.* [110]. The identification of proteins down-regulated in response to salt stress conducted by consulting a *B. licheniformis* proteome reference map established by Voigt *et al.* [23]. Spot quantification and calculation of synthesis rates of proteins were performed with the Delta 2D software (Decodon GmbH, Greifswald, Germany). The protein-labeling experiments were repeated twice and the synthesis rates (Suppl. Table S1) were calculated from these two independent experiments. Furthermore, technical replicates were included for the quantification of proteins and their synthesis rates.

Sample preparation for label-free protein quantification

Prior to the digestion of proteins with Trypsin, 500 µg of protein was diluted to a final concentration of 5 µg µl^{-1} in 50 mM TEAB/0.1 % RapiGest™ SF buffer (Waters; Milford, MA,; USA). The samples were reduced with 500 µM TCEP (Tris-(2-carboxyethyl)phosphine hydrochloride, Invitrogen) for 45 min at 60°C. The cysteine residues were blocked by alkylation with iodacetamide (Sigma-Aldrich; Steinheim, Germany) for 15 min at room temperature in the dark. Trypsin (Promega; Madison, WI; USA) was added to a final enzyme/protein ratio of 1:20. Subsequently, RapiGest™ was removed from the samples according to manufacturer's instructions (Waters; Milford, MA; USA). Finally, the protein samples were further cleaned with C18 – StageTips (Proxeon; Odense, Denmark).

676 *Multiplexed LC/MS analysis (LC/MS^E)*

677 The digested protein mixture (5 µg) was separated using the nanoACQUITY™ UPLC™
678 system (Waters; Milford, MA; USA) without a trapping column by direct injection. The protein
679 sample was loaded within a timeframe of 35 min onto the column (nanoACQUITY™ UPLC™
680 column, BEH130 C18, 1.7 µm, 75 µm x 200 mm, Waters; MA; USA) with 99 % buffer A (0,1%
681 acetic acid in H_2O_2), 1 % buffer B (0,1% acetic acid in acetonitrile) at a flow rate of 300 nL/min. The
682 peptides were separated within a 265 min time-frame by applying the following solvent gradient: in
683 165 min to 18 % buffer B, in 60 min to 26 % buffer B, in 40 min to 60 % buffer B, in 1 min on 99 %
684 buffer B for 10 min, and equilibration for 15 min with 99 % buffer A. The nanoHPLC was coupled
685 online to a Synapt G2 mass spectrometer equipped with a NanoLockSpray source and operated with
686 the MassLynx V4.1 software (Waters; Milford, MA; USA). For all measurements, a mass range of 50-
687 2000 was used and the analyzer was set to resolution mode. For lock mass correction, [Glu1]-
688 fibrinopeptide B solution (GluFib, m/z: 785.8426 Da, ERA, 500 fmol/µL in 50 % (v/v) acetonitrile,
689 0.1 % (v/v) formic acid) was infused through the reference fluidics system of the Synapt G2 at a
690 constant flow rate of 500 nl/min and sampled every 30 s. The mass spectrometer was run in the
691 LC/MS^E mode in which collision energy was alternated between 5eV in the precursor ion trace and a
692 ramp from 10-40 eV for fragment ion trace. All scan times were set to 2 s. All samples were analyzed
693 in triplicate, leading to nine replicate runs per growth state of the *B. licheniformis* culture under study.

694 *Data evaluation and label-free quantification*

695 For data processing, protein identification and label free quantification, raw data were
696 imported into ProteinLynx Global Server 2.4 (PLGS) (Waters, Milford, MA; USA) and processed via
697 the Apex3D algorithm with the following parameters. Chromatographic peak width: automatic; MS
698 ToF resolution: automatic; lock mass for charge 2: 785.8426 Da/e; lock mass window: 0.25 Da; low
699 energy threshold: 250 cts; elevated energy threshold: 30 cts; retention time window: automatic;
700 intensity threshold of precursor/fragment ion cluster: 1000 cts. Searches for peptides was carried out
701 by the ion accounting algorithm [111] against a randomized protein database predicted from the *B.*
702 *licheniformis* DSM 13^T genome sequence [13] with added common laboratory contaminants (8 478
703 entries) using the following parameters. Peptide tolerance: automatic; protein tolerance: automatic;

minimal fragment ions matches per peptide: 3; minimal fragment ions matches per protein: 7; minimal peptide matches per protein: 2; primary digest reagent: trypsin; missed cleavages: 2; variable modifications: carbamidomethylation C (+57.0215), deamidation N, Q (+0.9840), oxidation M (+15.9949), pyrrolidone carboxylacid N-TERM (-27.9949); false discovery rate (FDR): 5 %; calibration protein: yeast ADH1.

Label-free relative quantification was carried out by Expression^{E} software included in PLGS 2.4 (Waters, Manchester, UK). The quantification was achieved on basis of the observed peptide ion peaks intensities. Normalization was performed on all identified peptide signals using the autonormalization function. Proteins were taken as significantly changed in their amount if regulation probability was below 0.05 or higher than 0.95. To increase the confidence of protein identification and quantification, proteins were filtered out which were identified only once in the nine replicates per growth state of the *B. licheniformis* DSM 13^{T} culture studied. This resulted in a FDR of 3.5 % for the whole dataset used for the analysis.

Analysis of intracellular metabolites of salt-shocked B. licheniformis cells

For the metabolome analysis [112] of salt-stressed *B. licheniformis* DSM 13^{T} cells, cultures growing in BMM without or with 1 mM glycine betaine received a strong salt shock by the addition of NaCl to a final concentration of 1.0 M (6 g of solid NaCl was added per 100 ml of cell culture). Control cultures remained unshocked. In some experiments, the cells were also grown in the presence of 1 mM choline, the precursor for glycine betaine biosynthesis in *B. subtilis* [60,61]. Samples (100 ml at an OD_{500} of about 0.4) of the cell culture were harvested by filtration from unstressed and osmotically stressed cultures as described by Meyer *et al.* [113]. Cells used for the assessment of intracellular metabolite analysis were washed twice with 2.5 ml isoosmotic saline solution, based on the osmolarity of the unstressed or salt-stressed growth medium. Metabolite extraction was carried out in two steps. Extraction by ethanol was done by alternating shaking and vortexing the filters ten times in the extraction solution (5 ml of 60% (w/v) ethanol, ≤ 4°C) followed by a centrifugation step (5 min, 8500 rpm, 4°C). The supernatant, containing the intracellular metabolites, was transferred into a new Falcon tube. Extraction by water was done by adding 5 ml of ice-cold water to the disrupted filters and

732 the cell pellet, which were then shaken, vortexed, and centrifuged as described above. The aqueous
733 and the ethanolic supernatant from the same sample were combined and were then lyophilized to
734 complete dryness. The identity of intracellular metabolites was analyzed by a modified ^{1}H-NMR
735 method as described by Liebeke *et al*. [114]. Dried samples were resuspended in 500 µl double-
736 distilled water, and 400 µl of each sample were used for subsequent analysis. A "noesypresat" pulse
737 sequence with water presaturation and a total number of 1024 free induction decays (FID scans) were
738 used. Spectral referencing and quantification were done relative to 1 mM sodium 3-trimethylsilyl-
739 [2,2,3,3-D4]-1-propionic acid (TSP) in 0.2 M phosphate buffer.
740
741 *In silico analysis of the B. licheniformis genome sequence and of the folding of the proBA and proI 5'-*
742 *UTR mRNA sequences*
743 The mining of the *B. licheniformis* DSM 13^{T} genome [13] for genes involved in osmostress
744 responses were done using the web-server available from the Joint Genomic Institute homepage
745 (http://img.jgi.doe.gov/cgi-bin/w/main.cgi) using the "Find genes-by BLAST" and the "gene
746 neighborhood analysis" tools. The structures of the 5'-untranslated regions (UTR) of the *B.*
747 *licheniformis proI* and *proBA* genes were predicted using the Mfold algorithm [115]. The mRNA
748 secondary structures suggested by the program (http://mfold.rna.albany.edu/) corresponding to the T-
749 box control element [76] were then further manually adjusted based on phylogenetic considerations of
750 other T-box regulated genes [72,116].
751
752 **Supporting Information**
753 **Table S1 Overview of transcriptionally induced and repressed genes in response to salt stress.**
754 Genes are shown that were significantly induced or repressed at the mRNA level (with a cutoff of +3
755 or -3, respectively) and for which an ANOVA of $p<0.01$ was obtained by statistical testing. The
756 information listed in the column ''gene product function'' was compiled according to functional
757 annotations given by NCBI GenBank AE017333.1 (http://www.ncbi.nlm.nih.gov/nuccore/AE017333)
758 and/or SwissProt (http://www.uniprot.org/uniprot/?query=bacillus+licheniformis&sort=score).
759

Table S2 Summary of all transcriptome data. For a genome-wide transcriptome analyses of *B. licheniformis* DSM 10^T, values for each gene and analyzed time point subsequent to the imposition of the salt stress were calculated by the Rosetta Resolver software from three independent array hybridizations against a reference sample taken just before the salt shock (0 min). All values were calculated by the Rosetta Resolvers Gene Expression Analysis System 7.2. (Rosetta Inpharmatics c/o Ceiba Solutions).

Table S3 Proteome data for 2D gel analysis (10 and 30 min after addition of NaCl).

Table S4 Proteome data for label free quantification (2 h after addition of NaCl).

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Autors Contributions

Conceived and designed the experiments: ML K-HM MH TS EB. Performed the experiments and analyzed data: RS TH BV HM MB JM BJ DA DB SE JB HP. Wrote the paper: TS EB.

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Legends to figures

Figure 1. Growth yields, proline production and osmoprotection of *B. licheniformis* DSM 13^T by compatible solutes. (A) Cultures of *B. licheniformis* DSM13^T were grown at 37° C in SMM with glucose as the carbon source in the presence of the indicated NaCl concentrations. Growth yields of the cultures (as assessed by measuring the OD_{578}) were determined after 14 h of incubation. (B) Proline content of osmotically stressed *B. licheniformis* DSM 13^T cells. Cultures were grown in SMM with the indicated salinities either in the absence (●) or in the presence (●) of 1 mM of the osmoprotectant glycine betaine to an optical density (OD_{578}) of approximately 2. The proline content of the cells was determined by HPLC analysis. The data shown represent one typical experiment. (C) Salt-stress protection of *B. licheniformis* DSM 13^T by exogenously provided compatible solutes. Cultures of *B. licheniformis* DSM 13^T were grown in SMM either in the absence (hatched bar) or in the presence of 1.3 M NaCl (black bars) in the absence (-) or in the presence of various compatible solutes. GB: glycine betaine; Cho: choline; Pro: proline; PB: proline betaine; Car: carnitine; COS: choline-*O*-sulfate; DMSP: dimethylsulfoniopropionate; Ect: ectoine; OHEct: hydroxyectoine. The compatible solutes were added to the growth medium at a final concentration of 1 mM. Growth yields of the cultures were measured after 14 h of incubation at 37 °C in a shaking water bath.

Figure 2. Cluster analysis of transcriptional changes in response to a sudden salt challenge. Cells of *B. licheniformis* DSM 13^T were cultivated in BMM with glucose as the carbon source until they reached early exponential growth phase (OD_{500nm} of about 0.4) when they were exposed to a sudden salt shock (the final NaCl concentration in the growth medium was 1.0 M). Immediately before and at the indicated time intervals subsequent to the imposed increase in the external salinity, cells were withdrawn and used for the isolation of total RNA for a genome-wide transcriptional analysis. The derived data were then subjected to a cluster analysis and grouped according to known salt stress response clusters from *B. subtilis* [39,65]: (A) Synthesis and transport of compatible solutes, (B) general stress responses, (C) ECF-sigma factor genes, and (D) secondary oxidative stress response. The correlation of the transcription patterns of the different clustered genes is represented on the X-axis (cosine correlation). Detailed values for the transcriptional profile of individual genes are given in Suppl. Table S1. Genes marked in red represents those whose transcription is up-regulated in response to osmotic stress.

Figure 3. The cytosolic proteome of salt-stressed *B. licheniformis* DSM 13^T cells 30 min after the imposition of a NaCl shock. Cells of *B. licheniformis* DSM 13^T were cultivated in BMM with glucose as the carbon source until they reached early exponential growth phase (OD_{500nm} of about 0.4) when they were exposed to a sudden salt shock (the final NaCl concentration in the growth medium was 1.0 M). 30 min after the exposure to the salt stress, samples of cells were harvested and processed for 2D-PAGE analysis; proteins were separated in a pH gradient 4-7. The control sample of the cells

was harvested just prior to the imposed salt shock. Cell samples were labeled with L-[^{35}S]-methionine during the exponential growth phase (control, OD_{500nm} 0.4) and 30 min after the addition of NaCl. Dual channel images were created from the 2D-gels with the Delta 2D software (Decodon GmbH, Greifswald, Germany).

Figure 4. Cellular components involved in the osmostress response of *B. licheniformis* DSM 13^T. The genome of the *B. licheniformis* DSM 13^T strain [13] was mined for transporters and channels that could potentially contribute to osmostress resistance. Membrane-localized proteins involved in uptake or release of osmotically relevant compounds, as well as cytoplasmic enzymes catalyzing the synthesis of the compatible solute glycine betaine and the catalytic steps for the anabolic (green enzyme symbols) and osmo-stress-adaptive (red enzyme symbols) proline biosynthetic routes are presented. These systems were identified based on their homology to their functionally characterized counterparts in *B. subtilis* [32,45,52,54,61,69,71,72]. In addition, the system responsible for the uptake (PutP) and catabolism (PutB-PutC) of externally provided L-proline [82] is shown. The physiological function of the potential exporter system Bli03671/Bli03672 [13], a member of the ABC-superfamily, whose transcription was drastically induced in response to salt stress (Suppl. Table S1 and S2), is as yet unknown. MscS and MscL are mechanosensitive channels whose transient gating subsequent to an osmotic down-shock prevents cell lysis [52] since they release non-specifically water-attracting ions and organic compounds to reduce the osmotic potential of the cytoplasm and thereby curb water influx [50,51].

Figure 5. Synthesis of glycine betaine from the precursor choline by *B. licheniformis* DSM 13^T in response to high salinity. (A) Externally provided choline is predicted to be taken up via the ABC-transporter OpuC [64] and then converted into the compatible solute glycine betaine in a two-step oxidation reaction that involves the GbsB (choline dehydogenase) and GbsA (glycine betaine aldehyde dehydrogenase) enzymes in *B. subtilis* [61] and their counterparts in *B. licheniformis* DSM 13^T. (B) Genetic organization of the osmotically inducible *opuC* [*opuCA-opuCB-opuCC-opuCD*] cluster encoding the OpuC transporter, the *gbsAB* biosynthetic operon, and the *gbsR* regulatory gene [59] in the genome of *B. licheniformis* DSM 13^T [13]. (C) The intracellular choline and (D) glycine betaine content of the cells were analyzed by ^{1}H-NMR spectroscopy from unstressed and NaCl-stressed cells (the final NaCl concentration in the growth medium was 1 M). For this experiment, the cells were grown to mid-exponential growth phase in BMM or BMM with 1 M NaCl either in the absence (-) or in the presence of 1 mM choline (+ Cho). Intracellular choline and glycine betaine concentrations were absolutely quantified and normalized to cell dry weight (CDW) [nmol/mg CDW]. The error bars give the standard deviation of three independently grown cultures.

Figure 6. Predicted secondary structures of the *B. licheniformis* DSM 13^T *proI* and *proBA* mRNA leader transcripts. (A) Overview of the genetic organization of the structural genes in the genome of *B. licheniformis* DSM 13^T [13] for the ProB-ProA-ProI anabolic proline biosynthetic route. The predicted secondary structures of the non-coding 5'-regions of the *proI* (B) and *proBA* (C) mRNA leader sequences were generated with the Mfold algorithm [115] and edited manually for their termination and anti-termination configurations. The suggested proline-specific specifier codons (CCU) [72] in the T-box element for the *proI* and *proBA* mRNA leader sequences are shown in green, and the T-box signature sequences are marked in red. Asterisks indicate other short sequences conserved in the T-box gene family [76,116].

Figure 7. Induction of *proHJAA* transcription in *B. licheniformis* DSM 13^T in response to salt stress. (A) Genetic organization of the *B. licheniformis* DSM 13^T *proHJAA* locus with its indicated promoter and transcriptional terminator regions. The localization of the single-stranded anti-sense RNA´s used as probes in the Northern blot analysis of the *proHJAA* gene cluster are indicated as black bars below the individual gene symbols. (B) Northern blot analysis of the *proHJAA* transcript. Total RNA was isolated from cultures of *B. licheniformis* DSM 13^T that were grown in SMM either in the absence (-) or the presence (+) of 0.8 M NaCl. Gene-specific RNA transcripts were identified by hybridization of total RNA to DIG-labeled single-stranded anti-sense RNA probes. The arrow indicates the position of an approximately 3 400 nucleotide mRNA species that corresponds to the full-length mRNA of the *proHJAA* operon. (C) DNA sequence of the *proH* promoter regions of the *B. subtilis* and *B. licheniformis* DSM 13^T chromosomes. The start site (indicated by an arrow) mapped for the *B. subtilis proHJ* mRNA transcript via primer extension analysis [54] revealed a SigA–type promoter (shown in red, with boxed -10, -16 and -35 sequences [79]) and a putative ribosome-binding site (RBS) located upstream of the predicted ATG start codon of the *proH* coding region. DNA sequences resembling those of the *B. subtilis proHJ* promoter [54] can be found in the *B. licheniformis* DSM 13^T *proHJAA* promoter region.

Figure 8. Osmotic induction of *proHJAA* promoter activity in response to an osmotic up-shock and under continuous stress conditions. A 130-base pair DNA fragment covering the predicted promoter of the *proHJAA* operon from *B. licheniformis* DSM 13^T was fused to a promoterless *treA* reporter gene [Φ*(proHB.li 130´-treA) cat*] and inserted into the chromosome of the heterologous *B. subtilis* host strain FSB1 [*(treA::neo)*1] [43], thereby yielding the reporter strain MDB60. (A) The expression of the [Φ($proH_{130}$-*treA*) *cat*] reporter fusion was monitored by measuring the TreA activity of strain MDB60 cells cultivated in SMM that were subjected either to a sudden osmotic up-shock (indicated by an arrow) with 0.4 M NaCl (●) or that were not subjected to an osmotic up-shift (●). (B) Cultures of the Φ($proH_{130}$-*treA*) reporter fusion strain MDB60 were grown in SMM with different salinities either in the absence (●) or in the presence of 1 mM of the osmoprotectant glycine betaine

(●). They were assayed for TreA reporter enzyme activity when the *B. subtilis* cells reached mid-exponential growth phase (OD_{578} of about 1.5). The values given for the TreA enzyme activity represent the averages of two independently grown cultures. For each sample analyzed, the TreA activity was determined twice.

Figure 9. Physiological complementation of a *B. subtilis proHJ* mutant strain by the heterologous *proHJAA* operon of *B. licheniformis* DSM 13^T. The *proHJAA* operon of *B. licheniformis* DSM 13^T was cloned into plasmid pX, yielding plasmid pTMB20. pTMB20 and the empty cloning vector pX (used as a control) were recombined in a single copy into the chromosomal *amyE* sites of the *B. subtilis* wild-type strain JH642 and its [Δ(*proHJ*::*tet*)1] mutant derivative JSB8 [72]. This resulted in the construction of the following *B. subtilis* strains: TMB134 [*proHJ* wild type and *amyE*::pX], TMB135 [*proHJ* wild type and *amyE::proHJAA*], TMB136 [Δ(*proHJ*::*tet*)1 and *amyE*::pX], and TMB137 [Δ(*proHJ*::*tet*)1 and *amyE::proHJAA*]. (A) Cultures of these strains were grown in SMM without (black bars) or with 0.8 M NaCl (hatched bars). Their growth yields (OD_{578}) were measured after 16 h of incubation at 37 °C. (B) Proline content of recombinant *B. subtilis* strains grown in SMM without (black bars) or with 0.8 M NaCl (hatched bars). When the cultures had reached mid-exponential growth phase (OD_{578} of about 2), the cells were harvested, their total solute pool was extracted and the intracellular proline concentrations were determined by HPLC analysis. The error bars represent the standard deviations of the proline pools found in three independently grown cultures. The same set of strains as that shown in panel (A) was used for this experiment.

Figure 10. Use of L-proline as a nutrient by *B. licheniformis* DSM 13^T. The proline catabolic system has been studied in *B. subtilis* where a high-affinity proline transporter (PutP), two proline catabolic enzymes (PutB-PutC) [82], and a proline-responsive activator protein (PutR) [83,84] have been functionally studied. (A) This proline catabolic pathway is predicted from the genome sequence to be present in *B. licheniformis* DSM 13^T as well [13]. (B) Genetic organization of the *put* locus of *B. licheniformis* DSM 13^T. (C) *B. licheniformis* DSM 13^T cells were grown in SMM with glucose as the carbon source without the addition of a nitrogen (N) source (-) or in the presence of 15 mM $(NH_4)_2SO_4$ [NH_4^+, 30 mM L-proline [Pro], or 30 mM glycine betaine [GB], respectively. Growth yields of the cultures were measured after 16 h of incubation at 37 °C in a shaking water bath. (D) *B. licheniformis* DSM 13^T cells were grown in SMM with $(NH_4)_2SO_4$ [15 mM] in the absence (-) of a carbon source or in the presence of 27 mM glucose [Gluc], 32.4 mM L-proline [Pro] or 32.4 mM glycine betaine [GB]. Growth yields of the cultures were measured after 16 h of incubation at 37 °C in a shaking water bath. The error bars give the standard deviation of three independently grown cultures.

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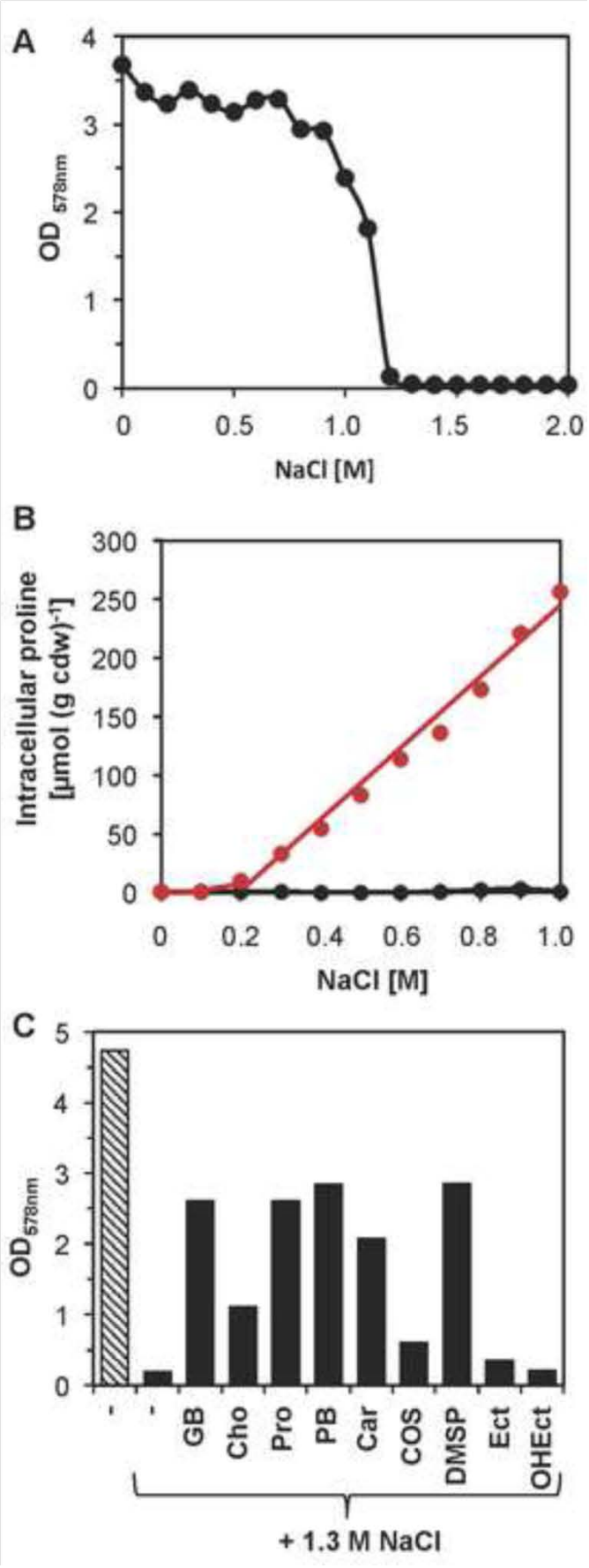

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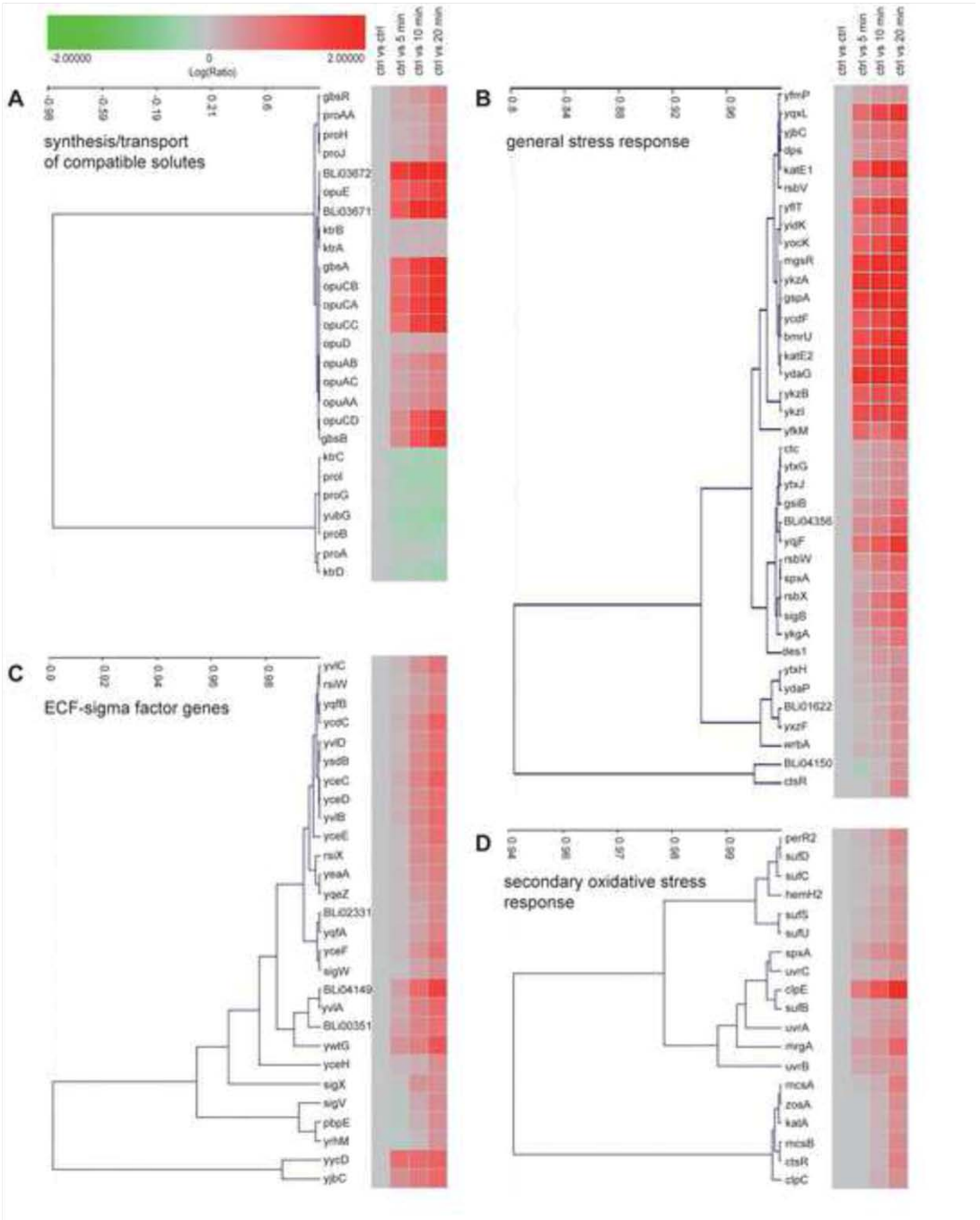

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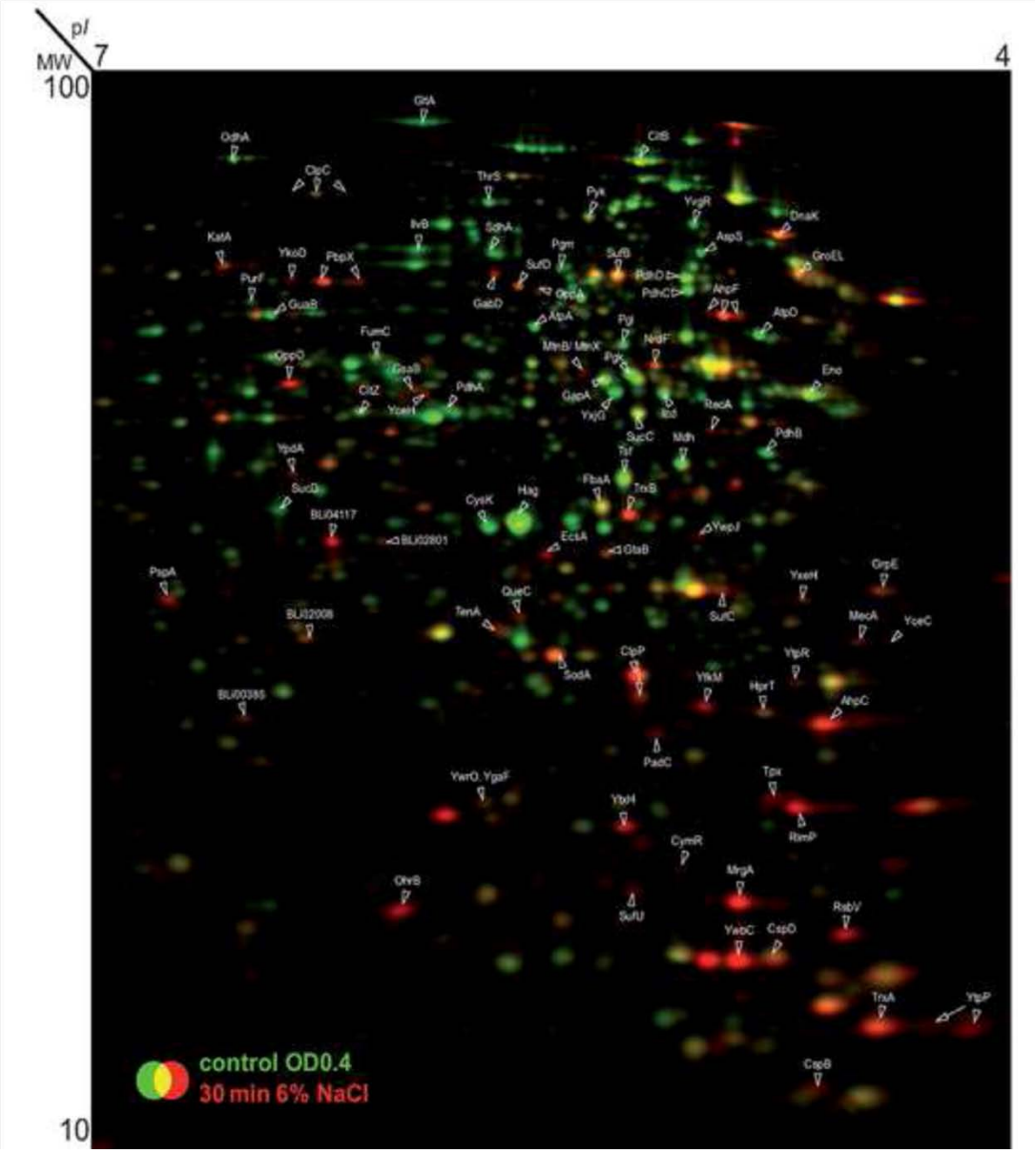

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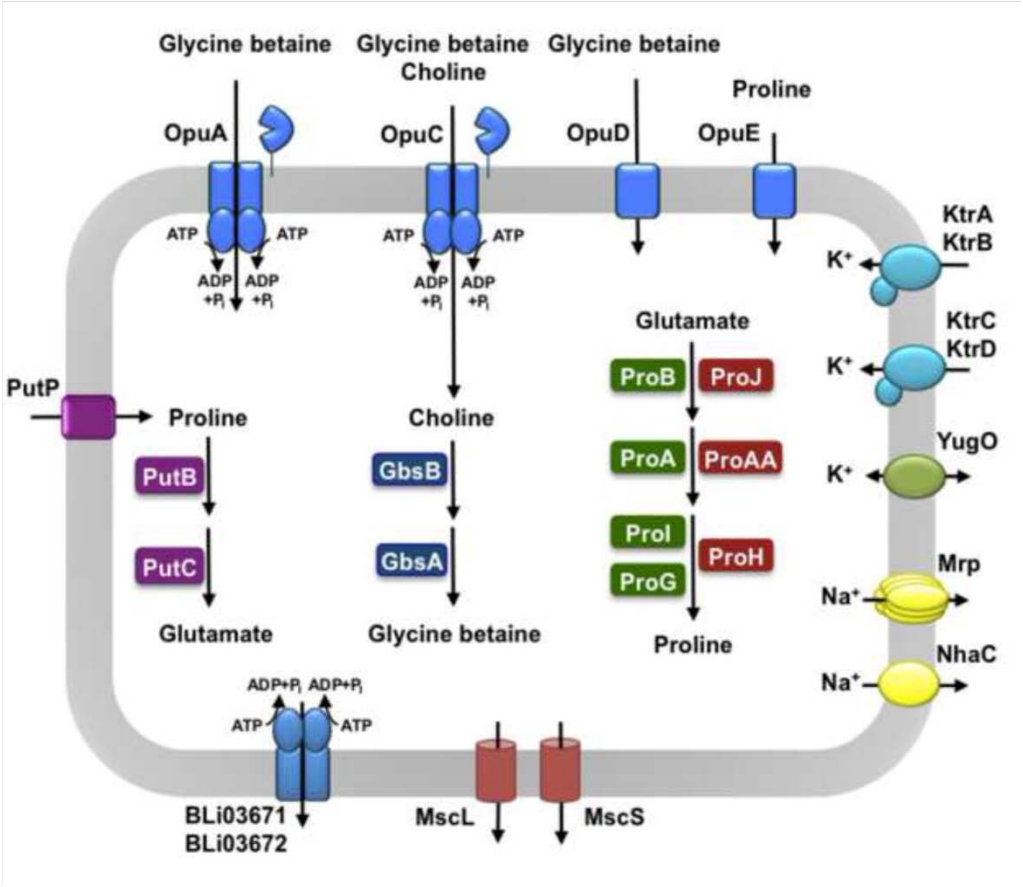

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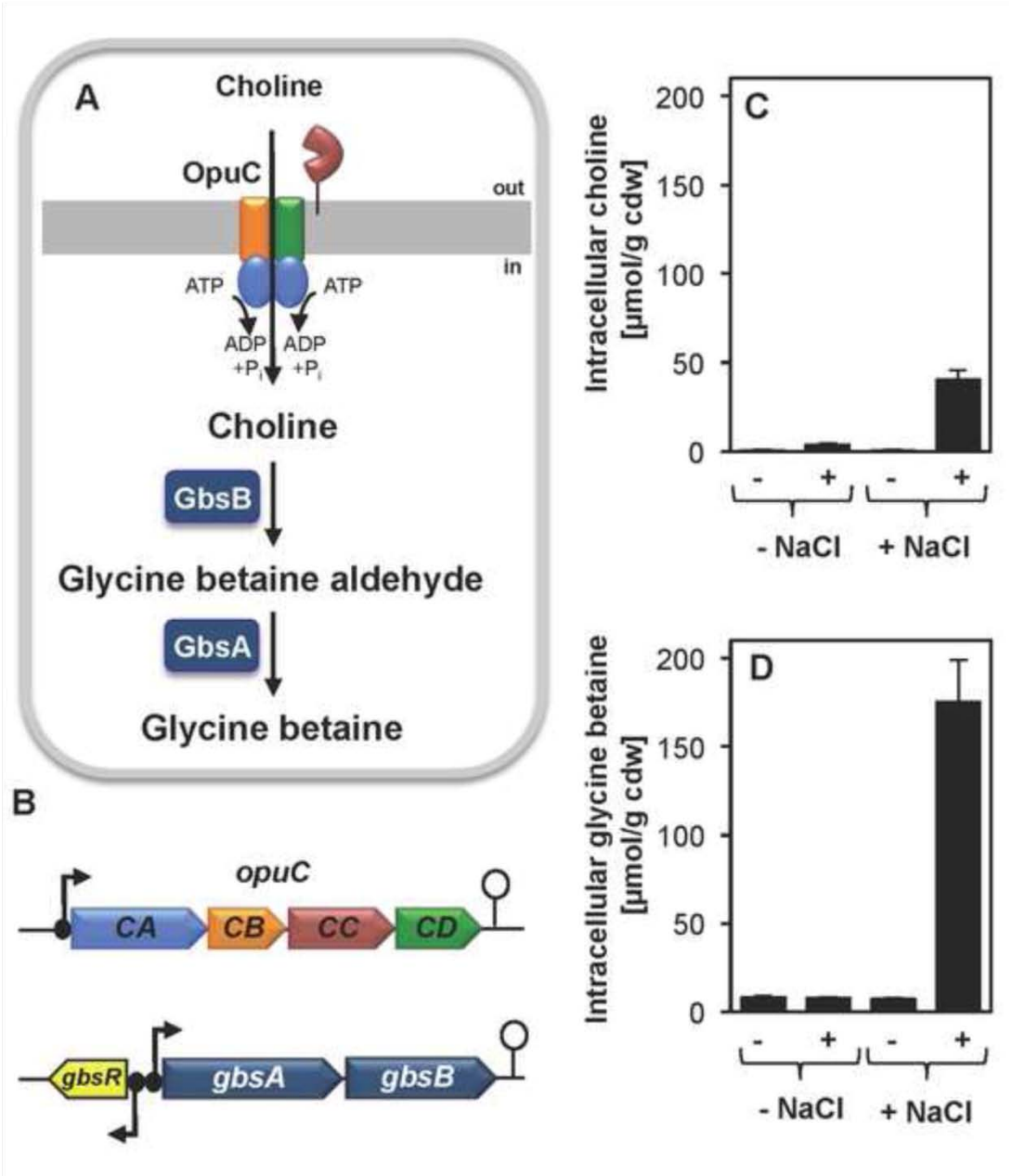

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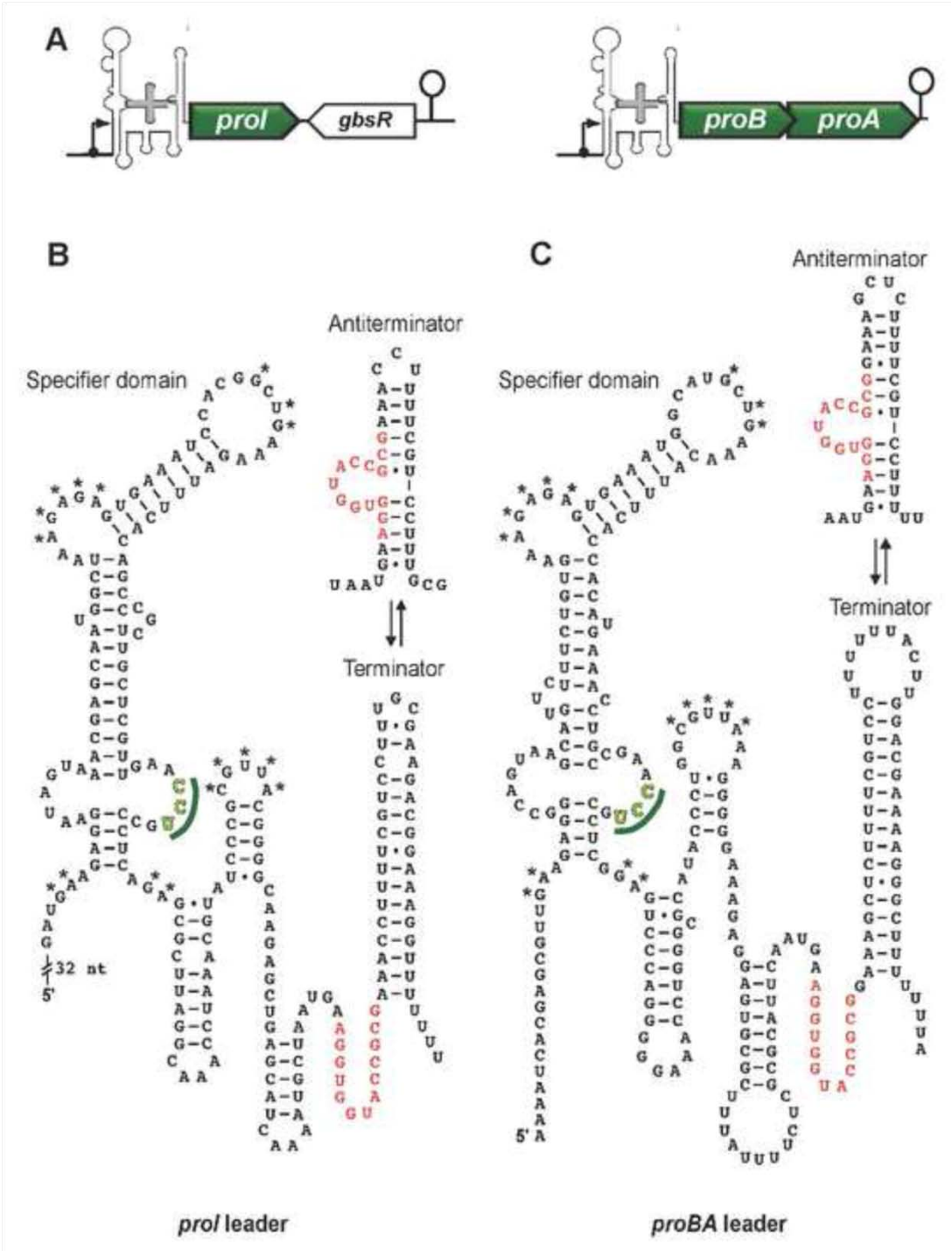

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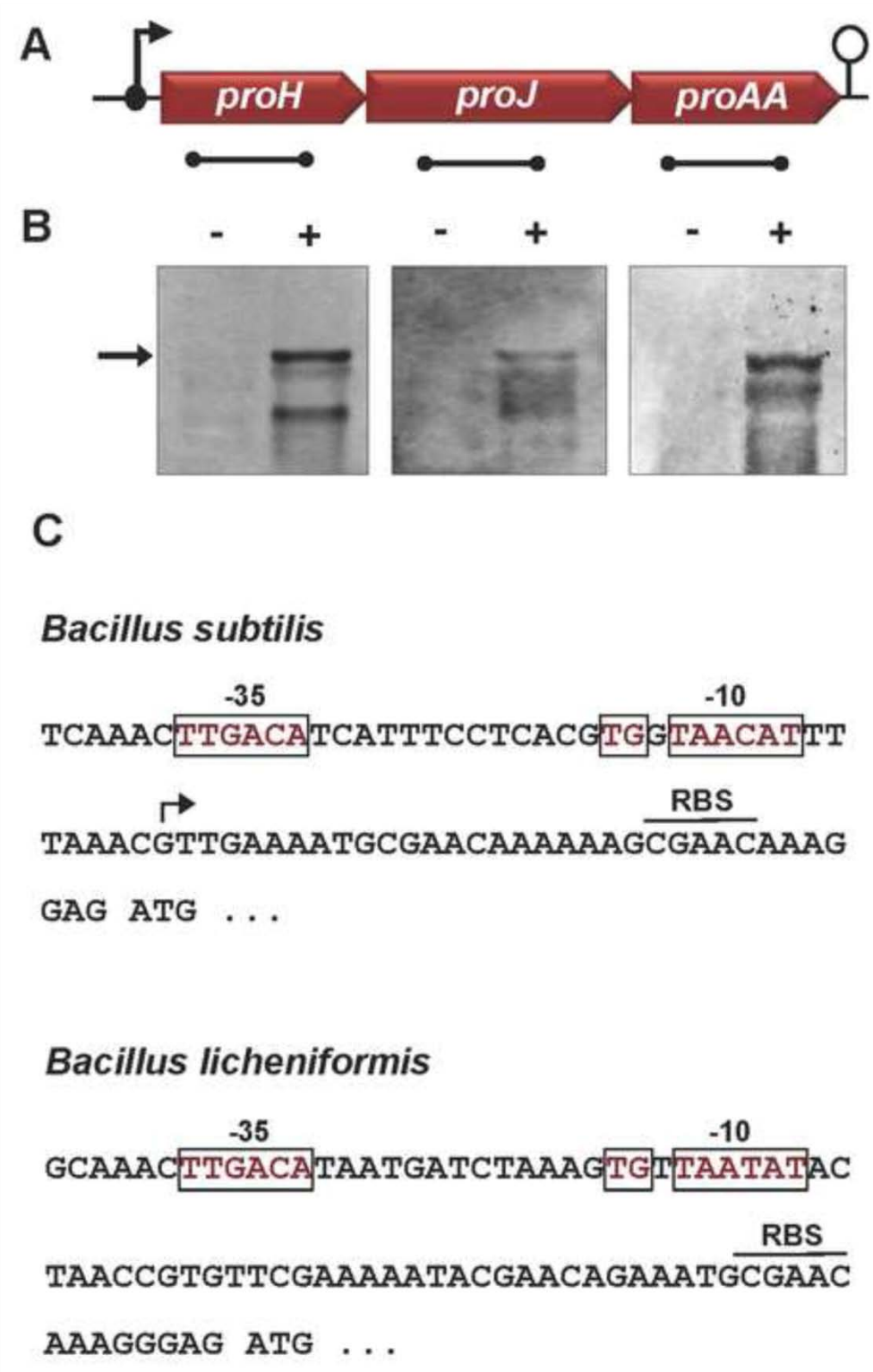

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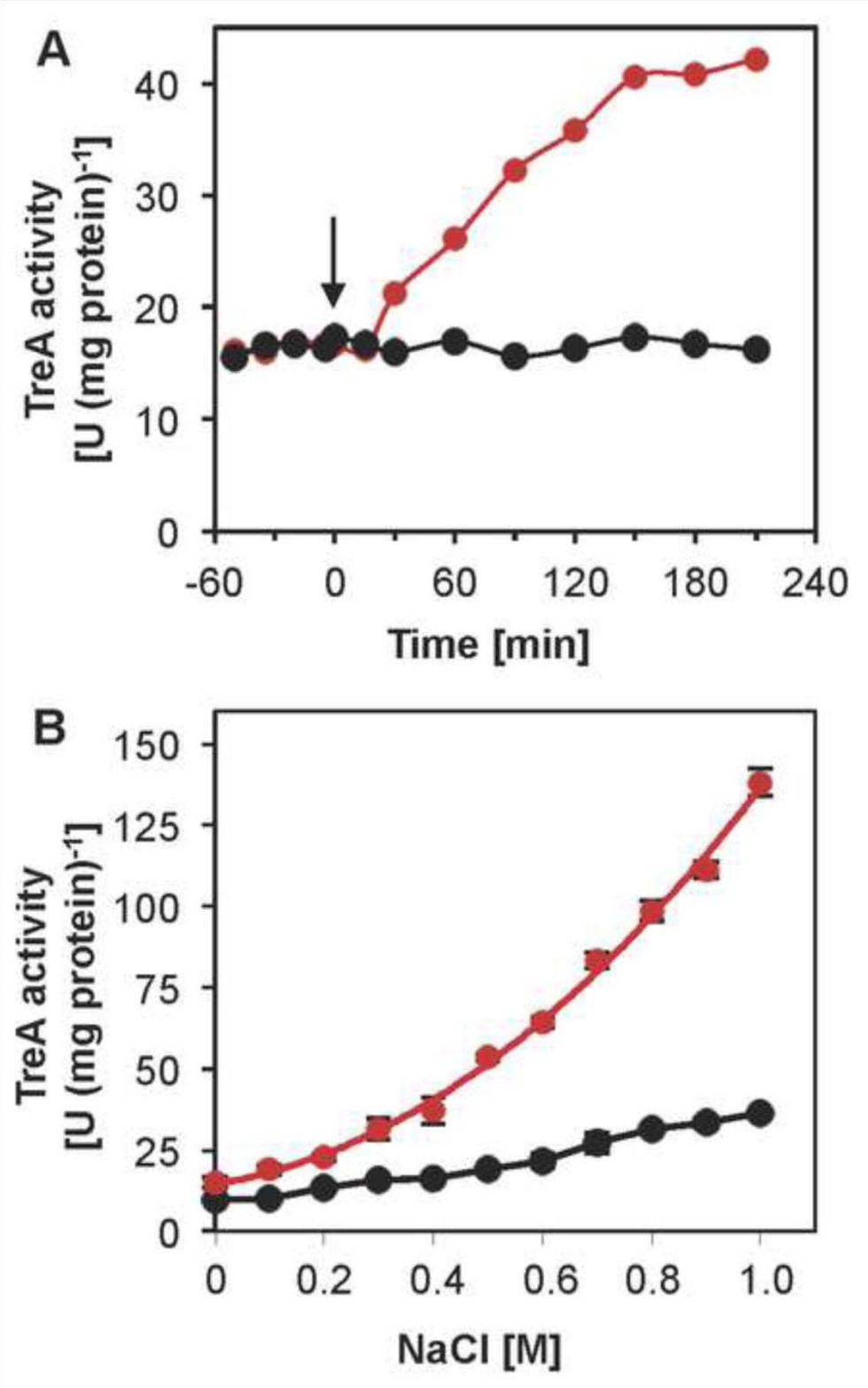

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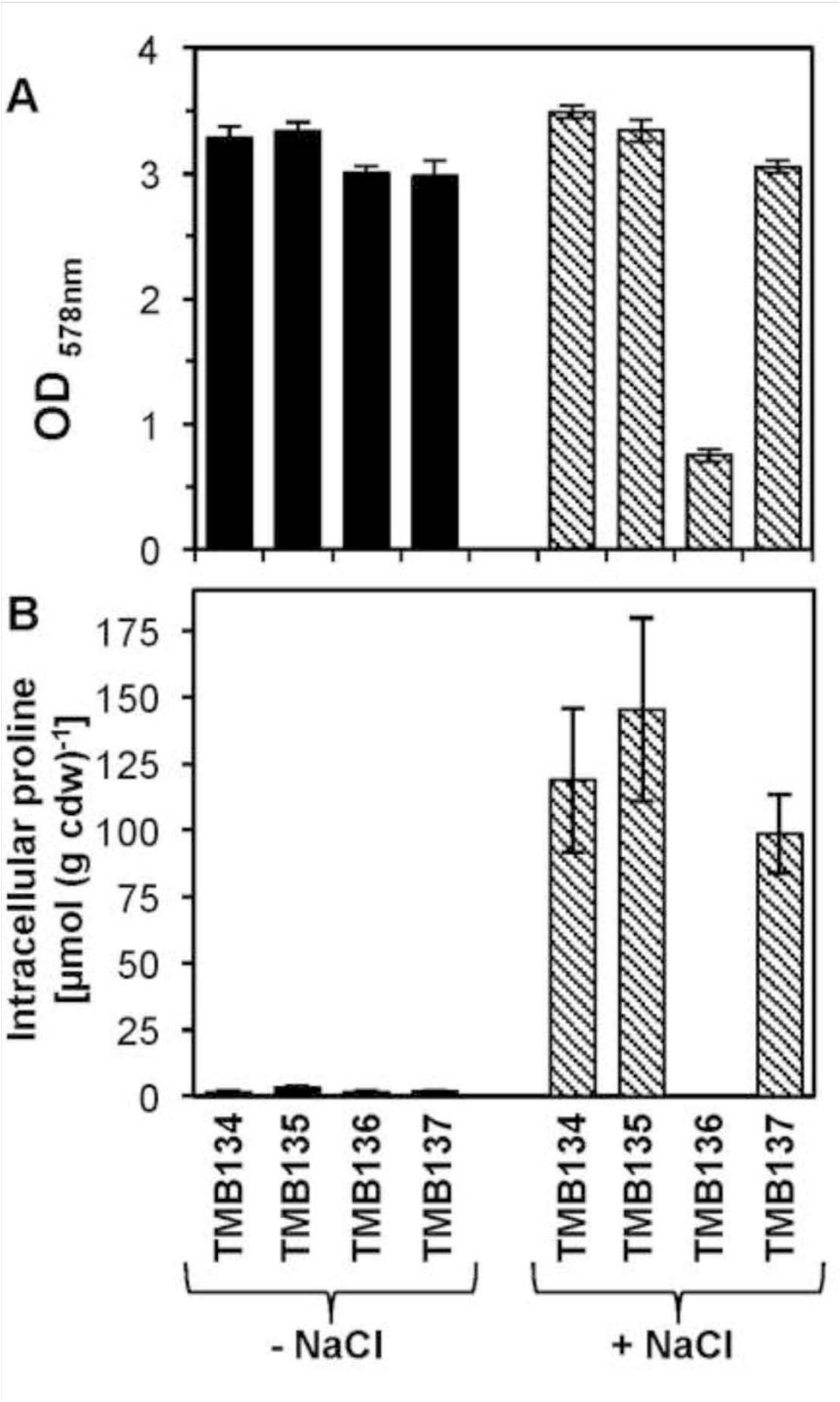

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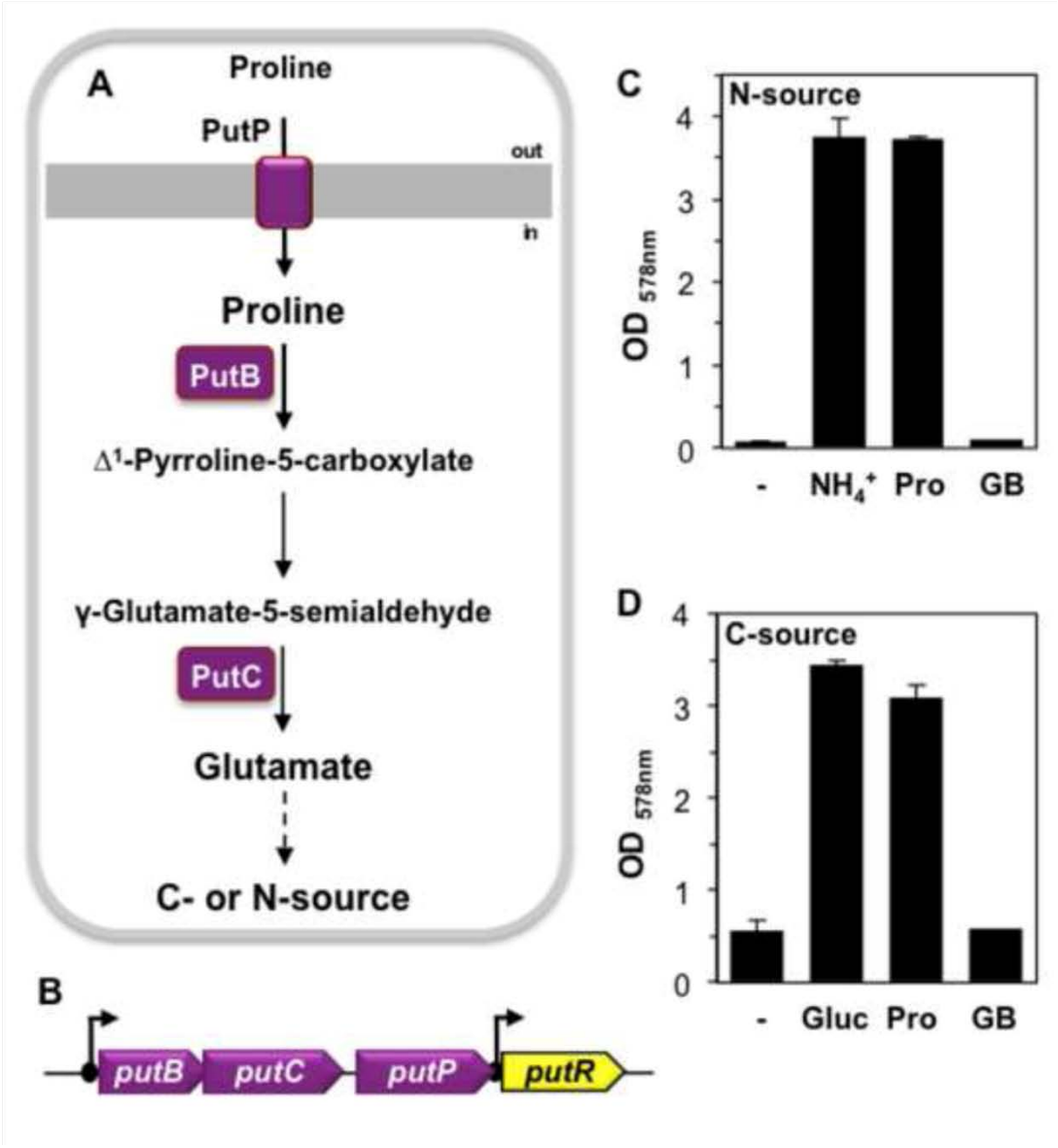

LIST OF PUBLICATIONS

Schroeter R., Voigt B., Jürgen B., Methling K., Pöther D.C., Schäfer H., Albrecht D., Mostertz J., Mäder U., Evers S., Maurer K.H., Lalk M., Mascher T., Hecker M., Schweder T. (2011). „The peroxide stress response of *Bacillus licheniformis*."

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Voigt B.* , **Schroeter R.***, Jürgen B., Albrecht D., Evers S., Bongaerts J., Maurer K.H., Schweder T., Hecker M. "The response of *Bacillus licheniformis* to heat and ethanol stress and the role of the SigB regulon"

Proteomics 2013 Jul;13(14):2140-61. doi: 10.1002/pmic.201200297

Handtke, S.*, **Schroeter, R.***, Methling, K., Albrecht D., Evers, S., Maurer K.H., Voigt, B., Lalk, M., Schweder, T., Hecker M. "*Bacillus pumilus* reveals a remarkably high resistance to hydrogen peroxide provoked oxidative stress"

Submitted to PLOS ONE

Schroeter, R., Hoffmann T., Voigt B., Meyer H., Bleisteiner M., Muntel J., Jürgen B., Albrecht D., Becher D., Lalk M., Evers S., Bongaerts J., Maurer K.H., Putzer H., Hecker M., Schweder T., Bremer E. „The salt-stress response of the industrial workhorse *Bacillus licheniformis*."

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* = Both authors contributed equally to the work

EIDESSTATTLICHE ERKLÄRUNG (AFFIDATIV)

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.
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Rebecca Schroeter

ANTEILE AN GEMEINSCHAFTSARBEITEN (AUTHOR'S CONTRIBUTION)

Die auf Seiten 47, 64, 87 und 117 aufgeführten Anteile an Gemeinschaftsarbeiten treffen zu.

Rebecca Schroeter

Prof. Dr. Thomas Schweder

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