Proteomics of Bacillus pumilus

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Stefan Handtke

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Dekan: Prof. Dr. Klaus Fesser

1. Gutachter: Prof. Dr. Michael Hecker

2. Gutachter: Prof. Dr. Karl-Erich Jaeger

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2. Introduction

2.1 *Bacillus pumilus*

Members of the species *Bacillus pumilus* are Gram-positive, aerobic, spore-forming bacteria with diverse life styles. The species is closely related to *B. subtilis* and *B. licheniformis* and belongs phylogenetically to the *B. subtilis* species complex (BSsc) [1]. *B. pumilus* cells are rod-shaped, 2 – 2.5 µm long and about 0.5 µm wide (Figure 1). *B. pumilus* strains are ubiquitary and have been isolated from soil and marine habitats [2], plant-associated samples [3,4], biofilms [2,5] and extreme habitats like acidic lakes [6], basalt rocks from the Sonoran desert [7] or spacecraft surfaces [8].

![Figure 1: Electron microscopy photography](image)

*Figure 1: Electron microscopy photography*

Scanning electron microscopy photograph of *B. pumilus* cells

*B. pumilus* is generally non-pathogenic in humans but in rare cases strains have been found in association with pathogenic events [9,10]. For *B. pumilus* strain SAFR-032, a complete genome sequence is available [11]. It has a size of 3.7 Mb and encodes about 3700 proteins. Therewith, the strain has a significantly smaller genome than other
members of the *B. subtilis* species complex, whose genomes encode more than 4000 proteins \[12,13\]. SAFR-032 was isolated from a cleanroom at the Jet Propulsion Laboratory spacecraft assembly facility in Pasadena, CA. The spores and vegetative cells of this strain exhibit elevated resistance to UV radiation and H\(_2\)O\(_2\) compared to other *Bacillus* species \[8\]. In addition, draft sequences of five *B. pumilus* strains, the type strain ATCC 7061, *B. pumilus* BA06 and *B. pumilus* S-1, as well as *B. pumilus* CCMA-560 and *B. pumilus* INR7 are available at NCBI.

### 2.2 Industrial relevance of the *B. subtilis* species complex

In our modern economy white biotechnology becomes more and more important. Microorganisms can be employed to produce a great variety of compounds that were produced chemically before or compounds that improve further production processes. Using microbes can lead not only to more effective but also to far more eco-friendly production methods. Microorganisms can produce under conditions that are less harmful and energy-consuming like low temperature, low pressure and with reduced or even without formation of toxic byproducts. As a result especially the usage of enzymes has become an important alternative to none or inorganically catalyzed production processes over the last decades. Enzymes even found their way into common consumer products such as washing and cleaning agents.

*Bacillus* strains are the dominant enzyme-producing microorganisms in applied and industrial microbiology. About 50\% of the worldwide industrial enzyme production uses members of the *B. subtilis* species complex as production hosts \[14\]. They are easily cultivable, fast-growing and they can produce and secrete large amounts of enzymes. Furthermore, they are generally harmless and non-pathogenic. *B. subtilis* is used as gram-positive model organism and in this role it is one of the best-studied organisms worldwide. The physiology of *B. subtilis* is well-known which facilitates optimizing cultivation and production conditions for industrial fermentation processes. Furthermore, the development of a variety of genetic tools to handle this organism enables easy DNA transfer to the cells. Thus, construction of industrial production host strains that can overproduce heterologous proteins of industrial interest is relatively simple. Furthermore, this provides opportunities to engineer production strains to optimize the process and the product.
Emerging markets for biocatalysts leading to an increasing demand of new and improved industrially available enzymes as well as the competition between producing companies demand a constant improvement of established products and production processes to assure economic viability. Therefore the development of tomorrow’s production hosts is already today a major challenge. *B. pumilus* is closely related to *B. subtilis* and *B. licheniformis*, which have been widely used for production processes for a long time. Therefore, *B. pumilus* is a consequential candidate to be analyzed for its potential as new industrial production host.

Several *Bacillus* strains can produce up to 20-25 g/L of extracellular enzymes [14,15]. Like their close relatives *B. subtilis* and *B. licheniformis*, *B. pumilus* strains show the potential to be used in industrial production of enzymes like proteases and lipases. The strain *B. pumilus* SG2 is known to secrete such enzymes in significant amounts [16]. Besides enzymes, *B. pumilus* is also able to produce small molecules like acetoin [17]. *B. pumilus* strain S-1 is capable of efficiently transforming isoeugenol to vanillin, the most important aromatic flavour compound used in food, beverages, perfumes, and pharmaceuticals [18]. The production of diverse bioactive compounds like pumilicin 4, a bacteriocine with anti-MRSA (methicillin-resistant *Staphylococcus aureus*) and anti-VRE (vancomycin-resistant *Enterococcus faecalis*) activity [19,20], phytotoxins and anti-biofilm compounds has been reported and investigations are running concerning the use of *B. pumilus* as biocontrol agent, for the biodegradation of sewage wastewater or in assisting phytoremediation [21,22].

### 2.3 Adaptation to stress conditions

In their natural habitat (soil) as well as during fermentation processes a variety of stresses (e.g. salt, heat and oxidative stress) can impair the fitness of an organism. During fermentation processes this can also influence the quality of the fermentation product [23-25]. *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 spacecraft [7,8]. This natural resistance 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 [23-25].

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 [25-27]. Increased ROS production that exceeds the cell defence capacity leads to oxidative stress in the cell and to the oxidation and damaging of nucleic acids, proteins and lipids [24,25,28-30].

In \( B. subtilis \), the cellular defence 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, LexA/RecA, Spx and OhrR, as previously described in detail [31-33]. The oxidative stress response of \( B. pumilus \) differs significantly from the response in \( B. subtilis \), as major oxidative stress responsive 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 question, which genes compensate the missing genes and are thus responsible for the high oxidative stress resistance of \( B. pumilus \). A mechanistic understanding of this phenomenon could help to improve the oxidative stress resistance of other production strains, which are already in use.

Catalases are key enzymes for the degradation of hydrogen peroxide. In many well-known \( Bacillus \) species KatA is the primary catalase expressed in vegetative cells [33,34]. The genome of \( B. pumilus \) does not encode any gene homolog of KatA [11]. Instead, it encodes two genes annotated as catalases KatX1 and KatX2. KatX1 shows a similarity of 83% to \( B. subtilis \) KatX, the spore catalase of this organism [12]. KatX2 shows only about 50% similarity to both, the KatX and the KatA amino acid sequence of \( B. subtilis \). Like KatA, KatX2 is an iron dependent catalase.

The presence of toxic compounds is not the only influence that can affect the cells and the product of a fermentation process. The absence of nutrients can also hamper cell growth and the efficiency of the process. It may induce special extracellular enzymes that occupy secretion capacity. On the other hand, the strong induction of several genes during nutrient limitation, e.g. genes involved in gluconeogenesis during glucose
starvation, makes such genes interesting for developing of new regulatory tools for industrial production processes.

Glucose is the main carbon and energy source of many bacteria especially of *Bacillus* species used in production processes. A lack of glucose has a severe impact on the cells. They need to significantly change their protein equipment to survive the starvation and to retain their fitness. The majority of changes in gene expression during glucose starvation are expected in the central carbon metabolism including glycolysis, TCA cycle and gluconeogenesis, as shown for the closely related gram-positive model organism *B. subtilis* [35-38]. Cells subjected to glucose starvation have to search for alternative carbon and energy sources. Most genes encoding proteins that are involved in the usage of alternative carbon sources are repressed by the global regulator CcpA in *B. subtilis*. [39-43]. As long as glucose is present, this carbon catabolite repression (CCR) ensures that proteins involved in usage of other potential carbon sources are not expressed or only expressed in really low amount so that the cells focus on metabolizing glucose as their main carbon source. In the absence or during limitation of glucose these proteins are no longer repressed and the cells can metabolize alternative carbon sources to fulfill their needs of energy and carbon compounds.

### 2.4 Proteomics

The proteome is described as the entity of all proteins expressed in a cell at a certain time point. In contrast to another, less popular description of the proteome as the entity of all proteins that can be expressed by the genome of a cell, the first definition shows the power of proteomic analysis to access physiological questions. The subset of proteins a cell contains at a certain point of time determines its physiological abilities. The genome of a cell is more or less stable whereas the proteome is far more flexible and can be adapted to a variety of conditions and influences the cell is exposed to. Transcriptome analysis revealed that there are huge differences between the expression levels of the individual genes encoded in the genome of an organism. Some genes are growth-phase dependent expressed on a high level, others are only expressed under specific conditions. However, the amount of an mRNA is not always determining the amount of its encoded functionally active protein that can be found in a cell. Regulation can also occur post-transcriptionally and at the level of translation. Furthermore,
different stability of proteins can lead to varying cellular protein amounts too. Therefore, investigating the proteome directly is a powerful tool to further analyse the physiology of an organism.

The development of the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as a tool to separate proteins from cellular extracts by Klose and O’Farrell was the first step in the analysis of proteomes [44,45]. Proteins are separated by their isoelectric point in the first dimension and by their mass in the second dimension. Soft ionization techniques like matrix-assisted laser-desorption/ionization (MALDI), developed by Karas et al. [46] in combination with mass spectrometric analysis enabled the identification of separated proteins using databases of already known proteins [47-49]. The upcoming genome sequencing enabled the identification of still unknown proteins and the assignment to their encoding genes by comparison of peptide masses with the masses determined by theoretically digestion based on the amino acid sequence (peptide mass fingerprinting) [50,51].

Besides the 2D-PAGE, techniques were developed that allow separation on peptide level instead of protein level [52,53]. This enabled the identification of proteins in highly complex protein mixtures. By using one-dimensional PAGE followed by liquid chromatography (LC) or solely LC it was possible to access proteins that can poorly be identified by 2D-PAGE approaches [54]. Among them are low abundant proteins, proteins with extreme isoelectric points or masses as well as hydrophobic proteins like those who contain hydrophobic membrane spanning domains. Protein modifications like oxidation, phosphorylation or acetylation can also be detected and localized using LC-MS/MS techniques.

2.5 Integration of data for process analysis

Analysis of metabolism and physiology of bacteria using omics-techniques could be used to identify key enzymes/ proteins/ pathways to engineer potential host strains for industrial production processes. The metabolic engineering of bacterial production strains is a challenging task. Only by combination of functional data can the physiology of the bacterium be elucidated. Ideally such data sets also include the qualitative and quantitative analysis of the low molecular metabolites in the bacterial cell. The
metabolome data can be used to further optimize the metabolism towards a more efficient production of enzymes and metabolites [55].
3. **Material and methods**

3.1 **Strains and growth conditions**

In this study we used mainly the strains *B. pumilus* Jo2 (DSM 14395) and *B. pumilus* SAFR-032. *B. subtilis* 168 and *B. licheniformis* DSM13 were used as reference strains. Fermentation was performed using sporulation deficient strain *B. pumilus* Jo2.1 (Jo2ΔyqfD) in two plasmid carrying variants: *B. pumilus* Jo2.1/pMM39 (non-overexpressing) and *B. pumilus* Jo2.1/pHP49 (protease-overexpressing).

For proteomic analysis of growing *B. pumilus* Jo2, cells were grown aerobically in shake flasks at 37°C and 180 rpm either in a complex medium containing 0.44 % w/v casitone and 0.89 % w/v tryptone supplemented with 57.4 mM K$_2$HPO$_4$, 47.8 mM MOPS, 4 mM MgSO$_4$ x 7 H$_2$O, 0.18 mM FeSO$_4$, 0.25 mM MnCl$_2$ x 4 H$_2$O, 6.8 mM CaCl$_2$ x 2 H$_2$O and 1.5 % w/v glucose or in a chemically defined medium (minimal medium) containing 15 mM (NH$_4$)$_2$SO$_4$, 8 mM MgSO$_4$ x 7 H$_2$O, 27 mM KCl, 7 mM Na citrate x 2 H$_2$O, 50 mM Tris-HCl (pH 7.5) supplemented with 1.8 mM KH$_2$PO$_4$, 2 mM CaCl$_2$, 1 µM FeSO$_4$ x 7 H$_2$O, 10 µM MnSO$_4$ x 4 H$_2$O, 4.5 mM glutamate, 0.2 % w/v glucose and 0.04 µM biotin.

The glucose starvation response of *B. pumilus* was analyzed growing *B. pumilus* SAFR-032 in a chemically defined medium as described above. Labeling was performed providing either 14N (NH$_4$)$_2$SO$_4$ or 15N (NH$_4$)$_2$SO$_4$. Growth on a non-glycolytic carbon source was performed by exchanging the glucose with an equal amount of ribose.

For the analysis of the oxidative stress response and catalase activity the cells were grown in a chemically defined medium as described above. Hydrogen peroxide was added in various concentrations as described for the specific experiments.

Fermentation processes were carried out in the lab at Henkel AG & Co. KGaA in Düsseldorf in stirred tank reactors (STR) with a total volume of 16 L (“NLF”, Bioengineering AG, Switzerland) in a complex medium in distilled water containing: glucose, 30 g/L; complex plant protein, 120 g/L; (NH$_4$)$_2$SO$_4$, 2.8 g/L; KH$_2$PO$_4$, 6.8 g/L; MgSO$_4$ x 7H$_2$O, 1.4 g/L; CaCl$_2$ x 2H$_2$O, 0.5 g/L; MnSO$_4$ x H$_2$O, 90 mg/L; Kanamycin 50 mg/L; at 39°C, aeration rate of 1.4 vvm. The baffled STR was equipped with pH, dissolved oxygen (DO) and temperature probes for online monitoring and regulation of
pH, temperature and DO by stirrer speed (N). The concentration of O$_2$ and CO$_2$ in the off-gas analysis was measured by gas analyzer Siemens Ultramat 23 (Siemens AG, Munich, Germany). Poly Propylene glycol with a Mr of 2000 (PPG 2000) was added on demand as antifoam agent. All experiments were carried out in three independent cultivations as biological replicates.

### 3.2 Sampling

Samples were taken during the exponential growth phase at an OD$_{600}$ of about 1.2-1.4 for the complex medium and an OD$_{500}$ of about 0.6-0.7 for the minimal medium. Cells used for analysis of glucose starvation were also harvested during transient phase at an OD$_{500}$ of about 0.9-1 and during stationary phase one hour after transition. Samples for RNA extraction were taken before (control) and 3 and 8 min after addition of H$_2$O$_2$.

During the fermentation processes, samples were taken during early exponential growth phase (4-6 h), late exponential growth phase (8-11 h), transient phase (12-15 h), stationary phase (32 h) and late stationary phase before the fermentation was finished (50 h). The first time points differ between overexpressing and non-overexpressing cells due to differences in growth rates.

### 3.3 Preparation of protein extracts

Bacteria were harvested by centrifugation (20000 x g, 4°C, 10 min). For preparation of cytosolic extracts cells were washed twice in 100 mM Tris-HCl buffer, pH 7.5, and resuspended in TE buffer (10 mM Tris, pH 7.5, 10 mM EDTA) containing 1.4 mM PMSF. Cell disruption was performed by sonication (1 min pulse, 1 min pause, 50 Watt) and disrupted cells were centrifuged twice (20000 x g, 4°C, 30 min) to remove the cell debris. The protein concentration of the supernatant was determined with RotiNanoquant (Roth).

For the preparation of the membrane protein fraction, cells were washed twice in 20 mM TEAB (triethylammonium bicarbonate) buffer, resuspended in 20 mM TEAB buffer containing Complete protease inhibitor (Roche Applied Science) and disrupted by sonication (see above). Samples were centrifuged twice (20000 x g, 4°C, 30 min).
Material and methods

The supernatant was subjected to ultracentrifugation (100000 x g, 60 min, 4°C). The pellet containing crude membranes was homogenized in 8 ml high-salt buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 M NaCl) and incubated for 30 min at 4°C on a rotary shaker and again subjected to ultracentrifugation as described above. The remaining pellet was homogenized in 100 mM Na₂CO₃-buffer (Na₂CO₃-HCl - pH 11, 10 mM EDTA, 100 mM NaCl), followed by another ultracentrifugation step as described above. The pellet was resuspended in 4 ml 20 mM TEAB-buffer and subjected to a fourth ultracentrifugation step. The resulting pellet was resuspended in 100 µl SDS sample buffer (11% Tris/HCl - pH 6.8, 18% glycerin, 4.5% 2-mercaptoethanol, 0.315 M SDS, a spatula tip of brom phenol blue) and used for separation via 1D-PAGE. To gain exoproteome samples the cells were removed by centrifugation (20000 x g, 4°C, 10 min). The proteins were precipitated from the supernatant using a final concentration of 10 % of TCA at 4 °C overnight. The proteins were collected by centrifugation (20000 x g, 4°C, 60 min). The pellet was washed six times with 98 % ethanol, two times with 70 % ethanol and another time with 98 % ethanol. The resulting pellet was dried using a vacuum concentrator and dissolved in a solution containing 8 M urea and 2 M thiourea. The protein concentration of the supernatant was determined with RotiNanoquant (Roth).

3.4 Protein separation

3.4.1 2D-SDS-PAGE

Isoelectric focusing (IEF) was done with commercially available IPG strips in the pH-ranges 4-7 for cytosolic and 3-10 for extracellular protein extracts (GE Healthcare or SERVA Electrophoresis). IPG strips were loaded with 500 µg (GE Healthcare strips for Coomassie staining), 300 µg (SERVA Electrophoresis strips for Coomassie staining) or 100 µg (Flamingo staining) protein adjusted to 360 µl with 2 M thiourea/8 M urea (GE Healthcare strips) respectively adjusted to 306 µl with 2 M thiourea/8 M urea (SERVA Electrophoresis strips). Aliquots of 1/10th of CHAPS solution (20 mM DTT, 1% w/v CHAPS, 0.5% v/v Pharmalyte, pH 4–7 or 3–10) were added. Isoelectric focusing was performed according to Büttner et al. [57] using programs as described in Table 1.
Table 1: Programs for isoelectric focusing

Programs used for isoelectric focusing of proteins using different kind of IPG strips

<table>
<thead>
<tr>
<th>GE-Healthcare strips - pH 4-7</th>
<th>500 Vh</th>
<th>500 Vh</th>
<th>2.500 Vh</th>
<th>500 Vh</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5 W</td>
<td>1 mA</td>
<td>500 Vh</td>
<td>0 → 500 V</td>
</tr>
<tr>
<td>II</td>
<td>5 W</td>
<td>1 mA</td>
<td>2.500 Vh</td>
<td>500 Vh</td>
</tr>
<tr>
<td>III</td>
<td>5 W</td>
<td>1 mA</td>
<td>10.000 Vh</td>
<td>500 → 3.500 V</td>
</tr>
<tr>
<td>IV</td>
<td>5 W</td>
<td>1 mA</td>
<td>35.000 Vh</td>
<td>3.500 V</td>
</tr>
<tr>
<td>SERVA Electrophoresis strips - pH 4-7</td>
<td>75 Vh</td>
<td>75 Vh</td>
<td>150 Vh</td>
<td>150 Vh</td>
</tr>
<tr>
<td>I</td>
<td>1 W</td>
<td>1 mA</td>
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<td>III</td>
<td>1 W</td>
<td>1 mA</td>
<td>600 Vh</td>
<td>600 V</td>
</tr>
<tr>
<td>IV</td>
<td>1 W</td>
<td>1 mA</td>
<td>1500 Vh</td>
<td>1.500 V</td>
</tr>
<tr>
<td>V</td>
<td>1 W</td>
<td>1 mA</td>
<td>57500 Vh</td>
<td>3000 V</td>
</tr>
<tr>
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</tr>
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<td>1 W</td>
<td>1 mA</td>
<td>150 Vh</td>
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</tr>
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<td>1 mA</td>
<td>250 Vh</td>
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<tr>
<td>IV</td>
<td>1 W</td>
<td>1 mA</td>
<td>1000 Vh</td>
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<tr>
<td>V</td>
<td>1 W</td>
<td>1 mA</td>
<td>6000 Vh</td>
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<tr>
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<td>150 Vh</td>
</tr>
<tr>
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<td>1 mA</td>
<td>75 Vh</td>
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<td>II</td>
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<td>III</td>
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<td>IV</td>
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<td>1 mA</td>
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<tr>
<td>V</td>
<td>1 W</td>
<td>1 mA</td>
<td>6000 Vh</td>
<td>6000 V</td>
</tr>
</tbody>
</table>
Material and methods

After IEF, strips were equilibrated in solutions containing DTT and iodacetamide, respectively, as described by Görg et al. [58]. Separation in the second dimension was done in gels of 12.5% acrylamide and 2.6% bisacrylamide. Separation was performed using the following parameters:

<table>
<thead>
<tr>
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<td>4 W per gel</td>
<td>1,5 W per gel</td>
</tr>
<tr>
<td>1 mA</td>
<td>1 mA</td>
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<tr>
<td>500 Vh</td>
<td>2.500 Vh</td>
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<tr>
<td>500 V</td>
<td>500 V</td>
</tr>
<tr>
<td>1 h</td>
<td>overnight</td>
</tr>
</tbody>
</table>

Gels were stained with Coomassie Brilliant Blue as described by Voigt et al. [59] or Flamingo Fluorescent Gel Stain (Bio-Rad Laboratories) according to the instructions of the manufacturer.

3.4.2 1D-SDS-PAGE

40 µg of the protein extracts was mixed with an equal volume of sample buffer (11% Tris/HCl - pH 6.8, 18% glycerin, 4,5% 2-mercaptoethanol, 0.126 M SDS, a spatula tip of brom phenol blue). Separation was performed in 12% acrylamide gels (150 W, 500 mA, 180 V, 45 min). Gels were stained with Coomassie Brilliant Blue as described above.

3.5 Visualization of radioactive labeling

Autoradiography of radioactively labeled gels was performed as previously described [56]. The gels were stained with Coomassie Brilliant Blue as described above. After drying on a heated vacuum dryer the gels were exposed to storage phosphor screens (Molecular Dynamics). Screens were scanned using a Typhoon Scanner (Amersham Biosciences).

3.6 Gel image analysis, spot digestion and identification of 2D-separated proteins

Gel images were analyzed with the Delta2D software version 4.1 (Decodon). Spot quantification was also performed with the Delta2D software as described by Wolf et al. [60]. Briefly, gel images from one medium (three replicates) were overlaid and a
Material and methods

Fusion gel was generated *in silico* using the image fusion function of the Delta2D software set to union fusion. The fused image was used to detect protein spots. After editing the spots, they were transferred to the single gels. This method prevented occurrence of missing spots. Spot quantities were calculated by the software as % volume representing the relative portion of an individual spot of the total protein present on the gel.

The protein spots were excised from stained 2D gels using a spot cutter (Proteome Works™, Biorad) with a picker head of 2 mm diameter with the help of pick lists created with the Delta2D software. Cut spots were transferred into 96 well micro titer plates. The tryptic digest with subsequent spotting on a MALDI-target was carried out automatically with the Ettan Spot Handling Workstation (Amersham Biosciences) using the following protocol:

The gel pieces were washed twice with 100 µl of a solution of 50% CH₃CN and 50% 50 mM NH₄HCO₃ for 30 min and once with 100 µl 75% CH₃CN for 10 min. After drying at 37°C for 17 min 10 µl trypsin solution containing 20 ng/µl trypsin (Promega) was added and incubated at 37°C for 120 min. For extraction gel pieces were covered with 60 µl 0.1% TFA in 50% CH₃CN and incubated for 30 min at 40°C. The peptide containing supernatant was transferred into a new micro titer plate and the extraction was repeated with 40 µl of the same solution. The supernatants were dried at 40°C for 220 min completely. The dry residue was dissolved in 2.2 µl of 0.5% TFA in 50% CH₃CN and 0.4 µl of this solution were directly spotted on the MALDI target. Then 0.4 µl of a saturated α-cyano-4-hydroxy cinnamic acid solution in 70% CH₃CN were added and mixed with the sample by aspirating the mixture five times. The samples were allowed to dry on the target 10 to 15 min before measurement in MALDI-TOF.

The MALDI-TOF-TOF measurements were carried out by Dr. Dirk Albrecht using the 4800 MALDI TOF/TOF Analyzer (Applied Biosystems). From the TOF-spectra, the three strongest peaks were measured. For one main spectrum 20 sub-spectra with 125 shots per sub-spectrum were accumulated using a random search pattern. The internal calibration was automatically performed as one-point-calibration with the mono-isotopic Arginine (M+H)+ m/z at 175.119 or Lysine (M+H)+ m/z at 147.107 reached a signal to noise ratio (S/N) of at least 5. The peak lists were created by using the script of the GPS Explorer™ Software Version 3.6. Settings were a mass range from 60 to
Precursor - 20 Da, a peak density of 5 peaks per 200 Da, a minimal area of 100 and maximal 20 peaks per precursor.

Peak lists were searched with the MASCOT search engine version 2.1.0.4 (Matrix Science) using a *B. pumilus* Jo2 in house database for proteins of *B. pumilus* Jo2 samples or the public *B. pumilus* SAFR-032 database for *B. pumilus* SAFR-032 samples. Search parameters were set as described in [60]: trypsin digestion with one missed cleavage permitted, variable modifications (oxidation of methionine and cabamidomethylation of cysteine), mass tolerance for MS data 50 ppm, and mass tolerance precursor ions 0.6 Da.

### 3.7 Digestion and identification of 1D-separated proteins

Ten bands were excised from the gel for each sample, destained and digested as described by Dreisbach *et al.* [61]. Gel pieces were destained twice using 200 mM NH₄HCO₃/30% acetonitril-ultrapure for 30 min at 37°C and dried in a vacuum centrifuge. The gel pieces were soaked with a trypsin (Promega) containing solution (10 ng/ml of 20 mM NH₄HCO₃) and incubated overnight at 37°C. Peptide extraction was performed by covering the gel pieces with ultra-pure water (prepared with a Sartorius Stedim unit) and 15 min incubation in an ultrasonic water bath.

Proteins were identified by members of the MS-group of Prof. Dörte Becher by LC-MS/MS with an LTQ OrbiTrap mass spectrometer (Thermo Fisher Scientific) as described by Otto *et al.* [38]. Database searches were conducted with the SEQUEST software v28 (rev.12, Thermo Fisher Scientific) against the previously described databases with settings as described by Otto *et al.* [38].

### 3.8 Denomination of proteins and reconstruction of metabolic pathways

Proteins were named according to their homologs of *B. subtilis*. Proteins with no homolog in *B. subtilis* were named with their accession number from the UniProt database for strain SAFR-032 (http://www.uniprot.org/) or their accession number from strain Jo2. The metabolic pathways were derived from KEGG

3.9 Prediction of signal peptides and transmembrane domains

The *B. pumilus* SAFR-32 sequence was used for predictions and localization was inferred by homology. Prediction of signal peptides for protein secretion was done with the SignalP 3.0 tool (http://www.cbs.dtu.dk/services/SignalP/) [62]. For prediction of hydrophobic transmembrane domains the TMHMM 2.0 [63] and the SOSUI 1.11 [64] algorithm were used. Prediction of Lipoproteins was done with the LipoP 1.0 tool (http://www.cbs.dtu.dk/services/LipoP/) [65].

3.10 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 [66] using the consensus sequence as described by Fuangthong *et al.* (TTATAATnATTATAA) [67].

3.11 Metabolome analysis

For intracellular metabolite analysis cells were harvested with the previously described fast filtration approach [68]. The modified sampling protocol is described below. For analysis of extracellular metabolites 2 ml of cell suspension were sterile filtered on ice by using a 0.45 µm pore size filter to obtain cell-free extracellular metabolite samples. The filtrates and the lyophilized samples were stored at -20 °C until measurement. Detection of cytosolic and extracellular metabolites via IP-LC/MS, GC-MS and ¹H-NMR were performed by Karen Methling of the metabolomics group of Prof. Michael Lalk.

Extracted intracellular metabolites were analyzed by IP-LC/MS after re-dissolving the dried extract in 100 µL of ultra-pure water. Chromatographic conditions and mass spectrometer parameters described by Donat *et al.* [69] were used with some modifications described. Intracellular amino acids were analyzed by a HILIC-LC-MS/MS-method. Additionally, the extracts of intracellular metabolites were analyzed by GC-MS after derivatization with MeOx (methoxyamine) and MSTFA (N-methyl-N-
trimethylsilyl-trifluoroacetamide). Details of derivatization and GC-MS conditions were described recently [70]. Data analysis was performed with the exported netCDF files (Chemstation Vers. E.02.00 Service Pack 2, Agilent) by ChromaTOF 4.41 (LECO). Peak areas of extracted ions were normalized to the internal standard ribitol. For analysis of extracellular metabolites by $^1$H-NMR measurement sample preparation was done according to Liebeke et al. [71]. $^1$H-NMR analysis and quantitative evaluation was carried out as described previously [72]

3.11.1 Modified protocol for processing of samples for metabolome analyses

28 ml of cell suspensions (OD$_{500nm}$ 0.7) were cooled for 15 s by displacing in liquid nitrogen. Then cells from suspensions were filtered with a 0.45 mm pore size filter on a vacuum filter system. Filters with cells were immediately quenched in 5 ml of cold extraction solution (60% w/v ethanol/water mixed with chloroform 3:2 v/v) and subsequently frozen in liquid nitrogen. For metabolite extraction solutions with filters were thawed, alternating shaked and vortexed ten times and then centrifugated (10 min, 8000 rpm, 4°C). The supernatants, containing intracellular metabolites, were transferred into new Falcon tubes. Afterwards filter and cell pellets were extracted with 5 ml of ice-cold water by shaking, vortexing and centrifugating as described above. The aqueous supernatants were combined with the respective organic from the same sample and used for lyophilization till complete dryness.

3.11.2 Modifications in processing the Metabolome analysis

The modified conditions of IP-LC-MS for the analysis of intracellular metabolites were as following: The mobile phase A (5% methanol and 95% water, containing 10mM tributylamine as ion-pairing reagent) was adjusted with acetic acid to pH 4.9. The gradient elution started with 100% A for 2 min, 0%–20% B in 6 min, 20%–31% B in 12 min, 31%–60% B in 19 min, 60%–100% B in 5 min, hold 100% B for 5 min, 100%–0% B in 1 min, and hold for 13 min at 0%B. The gradient flow rate was 0.3 ml/min. The flow was splitted after column in one part to MS and 4 parts to waste. Injection volume was 25 µL.
Mass spectral tags were extracted and integrated using Bruker DataAnalysis Vers. 3.4 (Bruker Daltonics) and QuantAnalysis Vers. 1.8 (Bruker Daltonics). All peak areas were normalized to peak area of the internal standard Camphersulfonic acid.

For amino acid analysis with LC-MS/MS an Agilent 1200 HPLC system (Agilent Technologies) coupled to a Triple quadrupole mass spectrometer 6460 (Agilent Technologies) was used. In the Jet stream ESI source (Agilent) nitrogen was used as nebulizing gas at 40 psi, as sheath gas at 10 l/min and 350 °C, and also as drying gas at 300 °C and a flow rate of 10 l/min. Capillary voltage was 2500 V in the positive mode. For each metabolite masses for quantifier and qualifier product ion were selected, and collision energy and fragmentor voltage were optimized for the transitions from precursor to product ion with the optimizer software (Agilent).

Separation of metabolites was done by a hydrophilic interaction chromatography method [73] modified as described below. For separations a Luna NH₂ column 150 mm x 3 mm, 3 μm (Phenomenex) at a flow rate of 0.3 ml/min was used, LC-solvents were 10 mM ammonium acetate and 10 mM ammonium hydroxide in 97.5:2.5 water:acetonitrile, pH 9.45 (solvent A) and 100% acetonitrile (solvent B). The gradient started with 70% B to 0% B in 15 min, hold 0% B for 1 min, returned to 70% B in 0.1 min und equilibrated with 70% B for 12.9 min.

3.11.3 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 [74]. 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. BS(mBBr)
Material and methods

(Monobromobimane-derivative of BSH) standards were synthesized as described previously [27,75]. For detection and quantification of LMW-thiols, ion pairing HPLC was performed as described before [76]. 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.

3.12 Microarray approach

Array analyses were performed by Rebecca Schroeter with the help of Dr. Sacha v. Hijum. Total RNA of B. pumilus was prepared by the acid phenol method [77] with the modifications described elsewhere [78]. The isolated RNA was treated with DNase (RNase-free DNase Set, Quiagen) and subsequently concentrated and cleaned (RNA cleanup and concentration Kit). Quantity of RNA was determined on a microscale spectrophotometer (Nanodrop ND-1000, Peqlab Biotechnologie GmbH) and RNA integrity was analyzed using a capillary electrophoresis system (Bioanalyzer 2100, Agilent Technologies). Synthesis and purification of fluorescently labeled cDNA was carried out according to Schroeter et al. [34] 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). 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 [79]. 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 wash step with acetonitrile (Carl Roth GmbH + Co. KG) 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). Data were extracted from scanned images using Agilent’s Feature Extraction Software (version 10.5.1.1) (Agilent Technologies) using default settings. Spot signals were normalized using Lowess as described earlier [80]. 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 [81] by means of multiple testing correction [80]. Differential regulation was defined as a two-fold or higher differential expression with a FDR cut-off value of 0.05 or lower.

3.13 Electron Microscopy

3.13.1 Scanning electron microscopy

Electron microscopic pictures were taken with the help of Dr. Rabea Schlueter. 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 overnight. 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 overnight, 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).

3.13.2 Transmission electron microscopy

Cells were fixed in 1 % glutaraldehyde, 4 % paraformaldehyde, 50 mM NaN_3 in 5 mM HEPES for 1 h at room temperature and then at 4°C overnight. 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), stained with uranyl acetate and lead citrate and analyzed with a transmission electron microscope LEO 906 (Carl Zeiss microscopy GmbH).
3.14 Fluorescence thiol modification assay and analysis of protein modifications

Proteins with reversibly oxidized cysteines were visualized using a protocol described by Hochgräfe et al. [82]. Cells were harvested directly on ice-cold trichloroacetic acid [TCA, final concentration 10% (w/v)] by centrifugation with 8900 g for 10 min at 4°C. The resulting pellet was washed twice with ice-cold deionized water (pH 1.5, adjusted with TCA) and resuspended in 450 μl denaturing buffer (8 M urea, 1% CHAPS, 1 mM EDTA, 200 mM Tris-HCl pH 8.0) supplemented with 50 mM IAM. Crude cell extracts were obtained by sonication and were then incubated for 30 min at room temperature to irreversibly alkylate accessible thiol groups. To terminate the blocking reaction, proteins were precipitated by addition of four parts ice-cold acetone with 1% (w/v) TCA and stored at −20°C for at least 1 h. Samples were then centrifuged with 21 900 g for 15 min at 20°C and washed twice with acetone. The resulting protein pellet was dried in a vacuum centrifuge. Samples were dissolved in 150 μl denaturating buffer and the protein concentration was determined. For the overall thiol labeling IAM was omitted from the denaturing buffer.

Reduction of reversibly modified thiol groups was achieved by using Tris(2-carboxyethyl)phosphin (TCEP). TCEP was resuspended in 500 mM Tris-HCl pH 8.0 to a final concentration of 100 mM. This solution was further diluted with the denaturing buffer to a final concentration of 20 nmol μl⁻¹. The TCEP working solution was added to protein samples in a ratio of 50 μg protein to 10 nmol TCEP and samples were incubated for 30 min at room temperature. For thiol labeling with BODIPY FL C1-IA (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl)iodoacetamide) (Invitrogen) the dye was dissolved to a final concentration of 15 nmol μl⁻¹ in acetonitrile. This labeling solution was added to the reduced protein extract in a ratio of 50 μg protein to 15 nmol dye. The proportion of acetonitrile in the reaction mixture is critical because of the high hydrophobicity of BODIPY FL C1-IA. The labeling mix was incubated for 20 min at room temperature. To stop the labeling reaction and to remove excess dye and salts, 75 μl of labeled extract was passed through a Micro Bio-Spin 6 Column (Bio-Rad, Hercules, CA, USA) equilibrated with rehydration buffer [8 M urea, 2 M thiourea, 20 mM DTT, 1% CHAPS (w/v)]. Protein extracts were loaded onto IPG-strips in the pH-range 4-7 (SERVA Electrophoresis). 2D-PAGE was performed as described above in the dark. Following fluorescence scanning of reversibly oxidized
proteins the gels were stained with Flamingo Fluorescent Gel Stain (Bio-Rad Laboratories). Spot quantification and MS-analyses were performed as described above. For the analysis of possible modifications protein spots were excised from the gels as described above, destained (0.2 M NH₄HCO₃, 30% acetonitrile) and double digested with trypsin and chymotrypsin (both Promega). Peptide extraction was performed by covering the gel pieces with ultra-pure water (prepared with a Sartorius Stedim unit) and 15 min incubation in an ultrasonic water bath. Peptides were detected by LC-MS/MS using an Orbitrap Elite (Thermo Fisher Scientific). Database searches were conducted with the SEQUEST software v28 (rev.12, Thermo Fisher Scientific) against a Bacillus subtilis 168 database (NCBI, version August 2014) with added amino acid sequence of the B. pumilus SAFR-032 KatX2 protein allowing sulfenic (+16), sulfinic (+32) and sulfonic (+48) acid formation as modifications. Data were analyzed using Scaffold proteome viewer version 4.0.5.

3.15 Analysis of fermentation process parameters

The number of viable cells (colony forming units, CFU) in the complex medium was determined according to aerobic plate count method as described in the bacterial analytical manual by the Food and Drug Adminstration (FDA) [21]. A set of serial dilutions of 100 µL sample to 900 µL saline solution to a dilution factor of 10⁹ were plated separately on LB-Agar plates and incubated for 24 h at 37°C. The number of viable cells in each sample was estimated based on the average cell count of the dilution rate showing 20 to 250 colonies on the respective agar plates.

Acetate and glucose analytics were carried out in three technical replicates by using the automated photometric analyzer system Arena 30 (Thermo Fisher Scientific GmbH). The commercially available assays Enzytec™ fluid D-Glucose and the Enzytec™ fluid Acetic acid (Thermo Fisher Scientific GmbH) were applied to measure the glucose and acetate concentration in culture supernatants.

Protease activity in the culture supernatant was determined by a protease assay described by van Raay et al. [22] based on the peptidolytic cleavage of casein. After a specific reaction time, the proteolysis of casein was stopped by adding tri-chloro acetic acid to precipitate the remaining protein which was subsequently separated by centrifugation. The absorbance of aromatic tryptophan side chains in the supernatant
Material and methods

was measured at 290 nm and corrected against a blank measurement: 0.5 abs $\Delta$ 10 U/ml. Protease activity of culture supernatants was thereby quantitatively determined and scaled to maximum value.
4. Results and discussion

4.1 Basic proteomic analysis of exponentially growing *B. pumilus* cells

4.1.1 Proteome data set – overview

In this study cells for analyzing the proteome of *B. pumilus* Jo2 were grown in two different media, a defined minimal medium and a complex medium containing tryptone and casitone. Furthermore, the complex medium provided a significantly higher amount of glucose (83 mM) than the minimal medium (11 mM).

The complex medium was used to study the proteome in a medium similar to media used in industrial fermentations. The minimal medium was used to gain a more comprehensive view on the metabolic capacity on the proteome level, especially for amino acid biosynthesis. As shown in Figure S1, *B. pumilus* cells grow about 2 times faster in the complex medium ($\mu_{LSJ-CT} = 2$, $\mu_{BMM} = 1$). The cells were harvested in the exponential growth phase at an OD$_{500}$ of 0.6 in minimal medium and at an OD$_{540}$ of about 1.2 in complex medium.

We identified a total of 1542 proteins in exponentially growing *B. pumilus* cells, 1313 in the minimal medium and 1285 in the complex medium employing a combination of 2D gel electrophoresis combined with MALDI-TOF-MS/MS and GeLC-MS/MS (Tables S1, S2, S3). 1505 of the proteins were detected by GeLC-MS/MS and 546 proteins were detected in 2D gels. In *B. subtilis* about two-thirds of all proteins are significantly expressed in exponentially growing cells [83]. Given that this number is similar in *B. pumilus*, our data set indicates a coverage of about 65% of the proteins expressed in growing cells of *B. pumilus*.

4.1.2 Cytosolic and membrane proteome

The combination of GeLC-MS/MS and 2D-SDS-PAGE and MALDI enabled us to identify 1176 cytosolic proteins and 281 proteins predicted to be intrinsic membrane proteins (Figure S1, S2, Table S1, S2). A protein was predicted to be cytosolic when it did not contain a signal peptide, a membrane spanning domain or a lipid anchor. The
identified proteins were classified into functional groups, based on their homology to known proteins (Figure 2, Table S1).

**Figure 2: Functional classification of cytosolic proteins**

Functional classification of cytosolic proteins identified in our proteome analysis. Proteins were classified according to the SubtiList database (http://genolist.pasteur.fr/SubtiList/help/function-codes.html). No discrimination depending on the growth medium was made.

With one-third the highest number of identified proteins is involved in metabolic processes such as glycolysis, TCA cycle, synthesis or degradation of amino acids, lipids and nucleotides (Figure 2). These proteins were assigned to metabolic pathways based on KEGG and SubtiPathways (Figure S3). About one-fourth of the proteins are active in information pathways, including synthesis and modification of DNA and RNA as well as protein synthesis, protein modification and folding. The number of proteins identified in most functional sub-classes of the information pathways is similar between both media – but more proteins involved in regulation of RNA synthesis in minimal medium were identified (63) than in complex medium (42, Table S1). Function of most of these additional regulators is unknown. 25% of the proteins have still unknown functions (Figure 1). As shown in previous studies of other bacilli, the percentage of identified proteins involved in metabolic pathways is significantly higher in the 2D gel-based approach than in the GeLC-MS/MS approach [59,83]. On the other hand, the amount of unknown proteins identified is significantly lower in the 2D gel-based approach.
Results and discussion

About one-third of the identified membrane proteins act as transporter components or permeases (Figure 3, Table S2).

**Figure 3: Functional classification of membrane proteins**

Functional classification of membrane proteins identified in our proteome analysis. Proteins were classified according to the SubtiList database (http://genolist.pasteur.fr/SubtiList/help/function-codes.html). No discrimination depending on the growth medium was made.

A high number of the identified membrane proteins is predicted to be involved in iron transport (8 proteins in minimal medium, 11 in complex medium, Table S2). The number of transport-related proteins among the identified membrane proteins of cells grown in minimal medium was a considerably higher when compared to cells grown in complex medium (102 to 60, Table S2). Especially some components of transporters for sugars and cobalt were found exclusively in cells grown in minimal medium. Another important function of membrane proteins is signal transduction. Many sensor kinases of two-component systems are anchored in the membrane [61]. 22 different sensor proteins were detected in this study. Some were sensor kinases of two-component systems whose response regulators were detected in the cytosolic fraction.

The 2D gel-based approach allowed relative quantification of proteins. For both media, the 40 most abundant cytosolic proteins are presented in Table 2.
## Table 2: Most abundant cytosolic proteins

The 40 most abundant cytosolic protein spots detected on 2D-gels of *B. pumilus* cells growing exponentially in minimal and complex medium. Protein quantification was performed by the Delta 2D software (Decodon) from 3 biological replicates. Spots without identified protein are marked with n.i.

<table>
<thead>
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<th>complex medium</th>
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<td></td>
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</tr>
<tr>
<td>Cnb</td>
<td>0.97</td>
</tr>
<tr>
<td>FusA</td>
<td>0.93</td>
</tr>
<tr>
<td>GroES</td>
<td>0.87</td>
</tr>
<tr>
<td>CspD</td>
<td>0.82</td>
</tr>
<tr>
<td>36260</td>
<td>0.78</td>
</tr>
<tr>
<td>GapA</td>
<td>0.75</td>
</tr>
<tr>
<td>09280, FumC</td>
<td>0.74</td>
</tr>
<tr>
<td>TenA</td>
<td>0.73</td>
</tr>
<tr>
<td>SufC</td>
<td>0.69</td>
</tr>
<tr>
<td>AtpA</td>
<td>0.68</td>
</tr>
<tr>
<td>PdxS</td>
<td>0.67</td>
</tr>
<tr>
<td>YdjL</td>
<td>0.67</td>
</tr>
<tr>
<td>CspD</td>
<td>0.67</td>
</tr>
<tr>
<td>YxG</td>
<td>0.64</td>
</tr>
<tr>
<td>Csb</td>
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</tr>
<tr>
<td>PdhD</td>
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<tr>
<td>SerA</td>
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<tr>
<td>n.i.</td>
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<tr>
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<td>0.60</td>
</tr>
<tr>
<td>CysK</td>
<td>0.60</td>
</tr>
<tr>
<td>GinA</td>
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<tr>
<td>DegU</td>
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</tr>
<tr>
<td>AtpD</td>
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</tr>
<tr>
<td>SufD, SufB</td>
<td>0.52</td>
</tr>
<tr>
<td>RpoB</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Elongation factor TufA was the most abundant protein in both media. As expected, other proteins involved in translation like the elongation factors FusA and Tsf as well as some ribosomal proteins - most of the ribosomal proteins are not located in the range between pH 4-7 so there are no quantitative data available - and proteins of main metabolic pathways were among the abundant proteins [57,59]. The flagellin protein, Hag, was highly abundant too. Flagellin was identified as abundant protein in other Bacillus species, but in the genome of B. pumilus four different Hag proteins are encoded. In our study we could see all four proteins expressed in both media. Proteins identified in the GeLC-approach could not be quantified, however, with very few exceptions all proteins identified in 2D gels were also found in the GeLC-approach (Table S1). In the 2D gels we could also see that in complex medium there are much more protein fragments than in minimal medium (e.g. TufA, Tig, PtsI, Figure S1, S2).

4.1.3 Carbohydrate metabolism

Most of the proteins involved in the central carbon metabolism including a PTS transport system for glucose were identified in exponentially growing cells in our study (Table S1, S2). In B. subtilis, the carbon catabolite repressor protein CcpA represses the expression of genes for the utilization of alternative carbon sources as long as sufficient amounts of glucose are present in the medium [41]. The identification of a CcpA protein indicates a similar glucose dependent repression of such genes in B. pumilus. As B. subtilis, and contrary to B. licheniformis, the B. pumilus genome lacks the genes that are necessary for a glyoxylate cycle which would enable the cells to grow on C2-compounds like acetate [11-13].

Many proteins employed in the central carbon metabolisms were expressed in both media at a quite equal level, but there were some differences, likely based on the higher growth rate in the complex medium (Figure S3) and the different nutrient composition of the media. The complex medium contained a much higher concentration of glucose than the minimal medium (83 mM versus 11 mM). Measured extracellular glucose at the time when the cells were harvested was also higher in the complex medium (38 mM complex medium, 8 mM minimal medium). In the minimal medium enzymes of the
Results and discussion

TCA cycle exhibited higher abundance than in complex medium, whereas in cells grown in complex medium enzymes of the glycolysis were more abundant. This crabtree-effect is based on the high glucose concentration in the complex medium. Intracellular amounts of metabolites of glycolysis were also higher (3-fold to 5-fold) in cells grown in glucose rich complex medium (Table 3). Comparable intracellular abundance was shown for metabolites of the TCA cycle with the exception of fumarate (5-fold higher amount in cells grown in complex medium) and malate (higher quantity in cells grown in minimal medium). 2-oxoglutarate as metabolite of the TCA cycle was secreted into the medium from cells grown in minimal medium (Figure 4).

Table 3: Intracellular glycolytic and citrate cycle metabolites

Relative quantification of intracellular glycolytic and citrate cycle metabolites from cells growing exponentially in minimal medium and complex medium (peak areas normalized to internal standard and OD) from 3 biological replicates. Metabolites not identified in a sample are marked with n.i.

<table>
<thead>
<tr>
<th></th>
<th>minimal medium</th>
<th>complex medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean value</td>
<td>SD</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>0.0197</td>
<td>0.0074</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>0.0030</td>
<td>0.0007</td>
</tr>
<tr>
<td>Dihydroxyacetone phosphate</td>
<td>0.0005</td>
<td>0.0001</td>
</tr>
<tr>
<td>2-Phosphoglycerate</td>
<td>n. i.</td>
<td>0.0001</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>0.0030</td>
<td>0.0020</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>0.0012</td>
<td>0.0002</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.0115</td>
<td>0.0032</td>
</tr>
<tr>
<td>cis-Aconitate</td>
<td>0.0012</td>
<td>0.0007</td>
</tr>
<tr>
<td>2-Oxoglutaric acid</td>
<td>0.0077</td>
<td>0.0032</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.0062</td>
<td>0.0009</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.0034</td>
<td>0.0007</td>
</tr>
<tr>
<td>Malic acid</td>
<td>0.0038</td>
<td>0.0005</td>
</tr>
</tbody>
</table>
Results and discussion

Figure 4: Secretion of 2-oxoglutarate
2-oxoglutarate concentration detected in extracellular metabolite samples of cells grown in minimal medium.

*B. subtilis* and *B. licheniformis* produce acetate as primary overflow metabolic product and also acetoin in lower amounts [84,85]. The same metabolites were found for *B. pumilus* in both media (Table S4). Based on the different glucose concentration, cells growing in complex media show a much higher degree of overflow metabolism than those growing in minimal medium. Strikingly, the primary overflow metabolite secreted by those cells is acetoin, which is secreted into the medium up to a concentration of about 60 mM (Figure 5). Cells growing in minimal medium nearly exclusively secrete acetate, but in significantly lower amounts (about 0.8 mM).

The proteins for the synthesis of these metabolites were expressed in *B. pumilus* during exponential growth (Table S1). Strikingly, *B. pumilus* cells grown in the complex medium synthesized a significant amount of the lactate dehydrogenase Ldh (Table S1, Figure S3). Analysis of extracellular metabolites confirmed secretion of significant amounts of lactate into the medium under these conditions (Figure 5).
Results and discussion

Figure 5: Secretion of overflow metabolites
Concentration of overflow metabolites of *B. pumilus* cells in the extracellular metabolome samples during growth in complex medium (A) and minimal medium (B).

Ldh expression is likely due to an oxygen limitation observed in the complex medium with an oxygen assay performed with the Senbit system (teleBITcom.). When cells were harvested (OD$_{500} = 0.6$ in minimal medium, OD$_{600} = 1$ in complex medium), there
was a partial pressure of about 80% oxygen in the minimal medium (starting conditions were set as 100%) whereas in the complex medium there was almost no oxygen left and the cells could only use the amount of oxygen which diffused into the medium by shaking (Figure 6).

**Figure 6: Oxygen consumption during growth**

Decreasing oxygen partial pressure during growth of *B. pumilus* cells in complex medium (A) and minimal medium (B). The measurements of oxygen partial pressure showed a standard derivation of about 1% at each time points.
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4.1.4 Other metabolic pathways

The most abundant enzymes required for amino acid synthesis of cells grown in minimal medium are IlvC (branched chain amino acids biosynthesis), GlyA (glycine biosynthesis) and MetE (methionine biosynthesis) (Table 2). In cells grown in minimal medium we could detect a protein (BPJ11110) which is annotated as methionine synthase and might be involved in methionine biosynthesis (Table S1). Enzymes of the methionine salvage pathway for recycling the polyamine synthesis product methylthioadenosine were found exclusively in cells grown in minimal medium (Table S1) [86]. In complex medium, cells expressed a considerably lower number of proteins acting in amino acid biosynthesis (Figure S3, Table S1) likely because of the content of oligopeptides and amino acids in this medium. Mainly proteins involved in synthesis of arginine, histidine, leucine and tryptophan were not found in cells grown in complex medium. In these cells, proteins used for degradation of amino acids like 1-pyrroline-5-carboxylate dehydrogenase (RocA) involved in arginine degradation were among the most abundant spots (Table 2).

Further proteins involved in amino acid degradation are found among the less abundant spots such as the arginase (RocF) and the aspartate ammonia-lyase (AnsB1, AnsB2) involved in aspartate degradation (Table S1). These data indicate that cells grown in minimal medium have to invest a considerable part of their translation capacity to synthesize enzymes for anabolic metabolism. On the other hand, cells grown in complex media focus on degradation of the provided substrates to cover their demands. However, most of the synthesis pathways were not completely repressed; some enzymes involved in these pathways were still present in the cells (Figure S3, Table S1).

4.1.5 Secretion system and extracellular proteome

Bacillus strains are known to produce and secrete extracellular enzymes like proteases or amylases in high quantities per liter [14]. This high secretion capability was also found in preliminary tests for the here introduced strain B. pumilus Jo2. In general, 5-10% of the chromosomally encoded bacterial proteins are secreted, using two main secretion pathways, the Sec-dependent and the Twin-arginine translocation (Tat-) pathway [87-91]. For each component of both secretion pathways corresponding genes
have been identified in the *B. pumilus* Jo2 genome data (Table 4). Thus both systems are apparently orthologous to the well described secretion systems of *B. subtilis* [89,91,92]. The Sec-secretory pathway translocates proteins in their unfolded state, the Tat-pathway translocates folded proteins and protein-complexes including posttranslational modified molecules.

**Table 4: Proteomic expression of B. pumilus Jo2 secretory proteins**

*B. pumilus* Jo2 proteome was analyzed using cells exponentially grown in minimal medium and cells grown in complex medium. Cytosolic, secreted and membrane fraction were analyzed separately. x indicates the presence under the respective media conditions. "/" represents an RNA and therefore cannot be detected in a proteome study.

<table>
<thead>
<tr>
<th>Locus-tag</th>
<th>gene</th>
<th>secretion pathway</th>
<th>proteome fraction</th>
<th>minimal medium</th>
<th>complex medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPJ05785</td>
<td>scr</td>
<td>sec pathway</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>BPJ20960</td>
<td>hps</td>
<td>sec pathway</td>
<td>cytosolic</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>BPJ15840</td>
<td>fhp</td>
<td>sec pathway</td>
<td>cytosolic</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>BPJ15820</td>
<td>fhsY</td>
<td>sec pathway</td>
<td>cytosolic, membrane</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>BPJ19120</td>
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<td>cytosolic</td>
<td>x</td>
<td>-</td>
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<td>x</td>
<td>x</td>
</tr>
<tr>
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<td>sec pathway</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>BPJ31240</td>
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<td>membrane</td>
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<td>x</td>
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<td>BPJ01280</td>
<td>secY</td>
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<td>membrane</td>
<td>x</td>
<td>-</td>
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<tr>
<td>BPJ24900</td>
<td>secDF</td>
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<td>membrane</td>
<td>x</td>
<td>x</td>
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<tr>
<td>BPJ24950</td>
<td>yajC</td>
<td>sec pathway</td>
<td>membrane</td>
<td>-</td>
<td>x</td>
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<tr>
<td>BPJ37910</td>
<td>spoIIIJ</td>
<td>membrane protein insertion</td>
<td>membrane</td>
<td>x</td>
<td>x</td>
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<tr>
<td>BPJ22120</td>
<td>yIDC</td>
<td>membrane protein insertion</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>BPJ09260</td>
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<td>membrane</td>
<td>x</td>
<td>x</td>
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<td>membrane</td>
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<td>x</td>
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<td>BPJ10200</td>
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<td>membrane</td>
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<td>tat pathway</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>BPJ13880</td>
<td>ykuE</td>
<td>tat translocated</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Since typical production enzymes are secreted via the Sec-pathway a special focus was set on the identification of all Sec-correlated genes. The signal peptides of Sec-dependent preproteins are recognized by the signal recognition particle (SRP) [93], an RNA-protein complex, consists of a RNA molecule sec and two proteins Ffh and FtsY [94]. The SRP is responsible for keeping the preprotein unfolded and delivering it to its membrane-bound receptor FtsY, before the target protein is secreted by the translocase membrane complex SecY-SecE-SecG. The chaperone CsaA shows partially overlapping binding characteristics with SecB, the export-specific chaperone of *Escherichia coli* and is therefore thought to act as secretion chaperone in the Sec-dependent translocation pathway [95]. The ATPase SecA provides the driving force for Sec-dependent preprotein translocation, and has also a role in chaperoning and targeting the secretory proteins to the translocase membrane complex and in mediation of translocation [89,96,97]. The preprotein translocase complex SecDF-YrbF is not essential for Sec-dependent preprotein secretion, but at least SecDF seems to be required to maintain high secretion capability [98,99]. SpoIIJ and YidC are paralogous proteins and *B. subtilis* requires at least one for viability [100-102]. They are thought to have a unique role in sporulation and membrane protein insertion [100]. In addition to the described proteins, *B. pumilus* Jo2 possesses homologues for three (SipT, SipV, SipW) of five *B. subtilis* signal peptidase 1 [103,104] and has one additional signal peptidase 1 (SipP) [105], as well as one signal peptidase 2 (LspA) [106,107], which is specialized for the cleavage of lipoprotein and also occurs in *B. subtilis*. PrsA assists in post-translocational folding of secretory proteins and is essential for growth, although most secretory proteins are PrsA-independent [108,109]. All described proteins, except LspA and SecE, could be detected during growth on minimal or complex medium (Table 4).

177 of the proteins encoded in the *B. pumilus* genome were predicted to contain signal peptides that would target them for Sec-dependent secretion. Further 35 proteins are predicted to be lipid-anchored. 70 secreted proteins could be identified in our study as well as 16 lipid-anchored proteins (Figure S4, S5, Table S3). Enzymes involved in the degradation of proteins and peptides form the largest group of secreted proteins (Figure 7). Proteins involved in metabolism of other compounds as well as proteins with functions related to the cell wall were also identified in the exoproteome of growing *B. pumilus*.
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Figure 7: Functional classification of secreted proteins

Functional classification of membrane proteins identified in our proteome analysis. Proteins were classified according to the SubtiList database (http://genolist.pasteur.fr/SubtiList/help/function-codes.html). No discrimination depending on the growth medium was made.

The most abundant protein secreted by cells exponentially grown in minimal medium is BPJ36330, a metalloendo-peptidase (Table 5). Two other peptidases (Vpr, BPJ17650) are among the highly abundant proteins in both media as well as several proteins with yet unknown functions like YlaE or BPJ37490. In the extracellular protein fraction of cells growing in complex medium a protein spot formed by Flagellin Hag4 was the one with the highest spot volume (41%). Altogether, the different Hag proteins form more than 50% of the total spot volume on these gels.

This correlates with results published for *B. licheniformis*. In this organism, Hag is the dominant protein in the extracellular protein fraction of exponentially growing cells, too [110]. Furthermore, flagellar basal-body rod protein (FlhO) and flagellar hook-associated protein (FlgK), also active in motility, are among the most abundant proteins secreted in complex medium. The amount of secreted proteins with functions in motility is therefore about 20-fold higher in complex medium than in minimal medium (about 60% in complex medium and 3% in minimal medium).
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One of the abundant proteins identified in the extracellular fraction from cells grown in complex medium is the cell wall associated protein WapA. It was shown for \textit{B. subtilis} and \textit{B. licheniformis} that cell wall-related proteins are present especially in the extracellular fraction of exponentially growing cells [110-112].

\textbf{Table 5: Most abundant extracellular proteins of exponentially growing cells}

The 15 most abundant extracellular proteins of \textit{B. pumilus} cells growing exponentially in minimal and complex medium. Protein quantification was performed by the Delta 2D software (Decodon) from 3 biological replicates. Spots without identified protein are marked with n.i.

<table>
<thead>
<tr>
<th>Name</th>
<th>% Vol.</th>
<th>SD</th>
<th>% Vol.</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metalloendopeptidase</td>
<td>18.49</td>
<td>1.75</td>
<td>44.60</td>
<td>3.97</td>
</tr>
<tr>
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<td>14.64</td>
<td>1.52</td>
<td>7.03</td>
<td>2.12</td>
</tr>
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<td>1.96</td>
<td>0.46</td>
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<tr>
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<td>0.41</td>
<td>1.43</td>
<td>0.24</td>
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<tr>
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<td>1.14</td>
<td>0.11</td>
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<tr>
<td>Endopeptidase</td>
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<td>1.00</td>
<td>0.17</td>
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<tr>
<td>Subtilisin</td>
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<tr>
<td>YwmC</td>
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<td>0.15</td>
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<td>n.i.</td>
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<td>0.18</td>
<td>0.67</td>
<td>0.11</td>
</tr>
<tr>
<td>YkuE</td>
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<td>36330</td>
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</tr>
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<td>Deoxyribonuclease I</td>
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<td>0.05</td>
<td>FlgK</td>
<td>0.33</td>
</tr>
<tr>
<td>Stress response</td>
<td>1.11</td>
<td>0.68</td>
<td>Motility</td>
<td>0.32</td>
</tr>
</tbody>
</table>

The Tat-system of most Gram-positive bacteria consists of a TatA and TatC component, and misses TatB [91,113], described for \textit{E. coli} and other Gram-negative bacteria. However, TatA from \textit{B. subtilis} was able to substitute for either TatA or TatB in \textit{E. coli} [114]. The genome of \textit{B. pumilus} Jo2 encodes a TatAC translocation system, but unlike \textit{B. subtilis} with two TatAC-systems and one additional TatA [114,115], \textit{B. pumilus} Jo2 has only one copy of the system (Table 4). A screening for the Tat-signal peptide motif SRRxFLK in \textit{B. pumilus} Jo2 identified the metallophosphoesterase YkuE as a Tat-signal peptide containing protein. This is consistent with \textit{B. subtilis} YkuE, being secreted via the Tat-translocation pathway [116]. The protein contains a metal-cofactor, which relates to its translocation pathway, since Tat-secretory proteins are fully assembled in the cytoplasm prior to translocation [116]. The identification of apparently...
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only a single protein predicted for secretion via the Tat-pathway is consistent with reports from other bacteria, for example *Staphylococcus aureus* possesses a TatAC system and only one substrate is predicted for Tat-secretion [114,117]. The components of the Tat-secretion system, as well as the putative substrate YkuE could not be detected in the proteome during growth in complex medium nor in minimal medium (Table 4).

4.1.6 Production-relevant features

Since *B. pumilus* Jo2 is a potential host for industrial enzyme production, safety is very important. Products that are used in food or detergent industry have to be nonhazardous. Potential synthesis of microbial antibiotics production and other secondary metabolites can add noxious or even toxic contamination to the fermentation product. Referring to this, non-ribosomal peptide synthases (NRPS) and polyketide synthases (PKS) are a matter of particular interest. NRPS and PKS are large multimodular enzymes involved in the production of oligopeptides respectively polyketides [118]. Some NRPS/PKS clusters are involved in the production of secondary metabolites such as antibiotics and fungicides, and are therefore of biotechnological relevance [14]. The genome of *B. pumilus* Jo2 harbors four NRPS/PKS clusters. Genes bpj03450, bpj03460 and bpj03470 of cluster A encode surfactin synthase-like proteins, and orthologs can be found in other *B. pumilus* strains as well as in strains of the BSsc (*B. subtilis* species complex). The genes bpj03480 and bpj03490 of cluster A encoding gramicidin-synthesis-like NRPS proteins can be found in the four other *B. pumilus* genomes (Table S5). Interestingly, gene products of bpj03450- bpj03470 and bpj03490 were found to be expressed in the cytosolic proteome fraction during growth in complex as well as minimal medium, whereas gene product of bpj03480 was found in the membrane fraction. Cluster B encodes four PKS proteins (BPJ07020, BPJ07040, BPJ07050 and BPJ07060) and one NRPS protein (BPJ07030). The PKS protein BPJ07020 is within this comparative study unique to *B. pumilus* Jo2, the remaining proteins have orthologs in *B. pumilus* SAFR-32 and *B. pumilus* ATCC7061. In addition, a single NRPS gene (bpj06940) is located close to cluster B. Since its gene product was found expressed in the proteome together with BPJ07030-BPJ07060 it might be part of cluster B. The unique PKS protein BPJ07020 was not detected in the proteome. Cluster C (bpj34820-bpj34870) encodes orthologs of proteins for the non-ribosomal biosynthesis of
bacilysin, a dipeptide antibiotic in *B. subtilis* (*bacBCDEFG/ywfH*) [119]. Corresponding orthologs have been found in other *Bacillus* strains, including *B. pumilus* strain ATCC7065 [120], and within this analysis in all remaining *B. pumilus* strains and in some strains of the *B. subtilis* species complex (BSsc). The *B. pumilus* Jo2 bacilysin operon misses *bacE* which is believed to mediate self-protection against bacilysin [120]. However, an MFS efflux transporter gene (*bpj34810*) is localized next to the *bacBCDFG/ywfH* operon of *B. pumilus* Jo2 and might function as *bacE* substitute. Cluster D (*bpj37510- bpj37550*) contains protein-encoding genes for the biosynthesis of the siderophore bacillibactin, for which homologues can be found in *B. pumilus* ATCC 7061, in members of the BSsc and BCsc (*B. cereus* species complex), but not in the remaining *B. pumilus* strains. Cluster C and D are not expressed during growth on complex and minimal medium.

Also remarkably, an iron uptake and transport system (*bpj35760- bpj35850*) was found in *B. pumilus* Jo2, which is also almost complete in all compared *B. pumilus* strains. The system was not found in the remaining BSsc members, but in members of the BCsc. The system is encoded within a genomic island and the proteome analysis of *B. pumilus* Jo2 showed that at least five proteins of this cluster are expressed in complex medium, but not in minimal medium. The supply with iron is essential for many microorganisms, since many bacteria live in iron limited habitats. Dobrindt *et al.* [121] have shown that pathogenic as well as environmental bacteria benefit by an island-encoded increase of their general adaptability through improved iron acquisition.

Furthermore, the genome of *B. pumilus* Jo2 harbors the chloramphenicol O-acetyltransferase-encoding gene *cat*. The putative resistance gene can also be found in the remaining *B. pumilus* strains. However, *Bpu* Jo2 does not grow on agar plates supplemented with chloramphenicol. The most striking genome feature to discuss here is a type 1 restriction modification system (*hsdM/hsdS/hsdR*) present in *B. pumilus* Jo2, but not in the remaining *B. pumilus* strains. The DNA specificity subunit-encoding gene *hsdS* shows only a low similarity to known systems. This subunit determines the specificity of the restriction modification system and was probably adapted to a *B. pumilus*-specific foreigner-DNA spectrum. Functional restriction modification systems represent a major barrier for evolution as well as for rational strain design. Waschkau *et al.* [122] showed in *B. licheniformis* that the deletion of such systems significantly increases the genetic susceptibility and the current investigation of ∆*hsdR*
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mutants of *B. pumilus* Jo2 also shows a clearly enhanced transformation efficiency after deletion of the restriction-modification system component [123].
4.2 The response of *B. pumilus* cells to glucose starvation

4.2.1 Growth of cells

The cells were grown in a chemically defined medium containing either only 14N nitrogen or 15N nitrogen to label the proteins. Glucose was provided as only carbon source. During the growth we took samples at three time points, in the exponential growth phase, at the transition from exponential to stationary phase and in the stationary phase (Figure 8). Analyzing the samples of these time points, we identified and quantified in total 1033 proteins in the cytosolic fraction of *B. pumilus* cells. The exoproteome was analyzed from cells grown in the same medium as used for the cytosolic samples with 14N nitrogen supply using 2D-PAGE.

![Figure 8: Growth of *B. pumilus* cells under glucose starvation conditions](image)

Cells were harvested at the marked time points in the exponential growth phase, at the transition into stationary phase and 60 min after entry into stationary phase.
4.2.2 Central carbon metabolism

Since glucose was the main carbon source of the cells, the major changes in the protein equipment were expected and observed for proteins involved in processes of carbon metabolism. As previously described in a similar study for *B. subtilis*, we observed no significant changes in the accumulation level of glycolytic proteins (Figure 9) [38]. Gluconeogenesis protein GapB and phosphoenolpyruvate carboxykinase PckA were strongly induced under glucose starvation conditions (Table S6). These two proteins are under control of CcpN in *B. subtilis* and also significantly induced under comparable conditions in this organism [38,124]. As previously shown for *B. subtilis*, we could not observe a significant change in the amount of another gluconeogenesis enzyme, Fructose-1,6-bisphosphatase YwjI, (annotated as GlpX in *B. subtilis*).

Strikingly, *B. pumilus* cells exposed to glucose starvation conditions did not show any significant changes in the accumulation pattern of TCA cycle enzymes. For *B. subtilis* and *B. licheniformis* a significant induction of the TCA cycle enzymes under comparable conditions has been reported [38,125]. For both organisms it was shown that the expression of TCA cycle enzymes is repressed by glucose and therefore they were upregulated when the cells entered glucose limited conditions [39,59].

As expected, one of the largest groups of proteins that appeared significantly upregulated in glucose starving cells was formed by proteins involved in different ways of acquiring and using alternative carbon sources and feeding the products into the central carbon metabolism (Figure 9). 21 proteins known to be related to such processes were detected to be accumulated in significantly higher amounts after the cells entered the stationary phase (Table S6). Many of these proteins are under control of CcpA in *B. subtilis*. The close relationship of the two organisms and the presence of a CcpA protein in the genome sequence of *B. pumilus* indicate a similar regulation in *B. pumilus*. Among the proteins accumulating to higher amounts during glucose starvation were components of ABC transporters for ribose (RbsB) and melibiose (MsmE) as well as ribokinase RbsK which phosphorylates the ribose to ribose-5-P and feeds it into the pentose phosphate pathway. Glycerol-3-phosphate dehydrogenase GlpD and glycerol kinase GlpK, also under control of CcpA in *B. subtilis*, were significantly induced too. They are involved in the usage of glycerol and its feeding into glycolysis.
Furthermore, several proteins which are not or not only under control of CcpA in \textit{B. subtilis} appeared to be induced in glucose starving \textit{B. pumilus} cells. A significant induction was observed for the N-acetylglucosamine-6-phosphate deacetylase NagA and the glucosamine-6-phosphate deaminase as well as for the associated PTS uptake system represented by the component NagP (Figure 9). These proteins are involved in transport and utilization of N-acetylglucosamine and are regulated by NagR in \textit{B. subtilis} [126]. A protein showing 60\% similarity to NagR is encoded in the genome of \textit{B. pumilus} (A8FHS7) but it could not be detected in this study.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{carbon_metabolism}
\caption{Carbon metabolism in glucose starving \textit{B. pumilus} cells}
\end{figure}

Changes of protein amounts in \textit{B. pumilus} cells under glucose starvation compared to exponential growth conditions.

Red: enzyme at least fivefold induced, orange: enzyme at least twofold induced, yellow: enzyme at least 1.5-fold induced, green: amount of the enzyme at least 1.5-fold reduced, black: no significant changes in the amount of the enzyme, grey: enzyme not detected in this study.
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As shown for *B. licheniformis*, *B. pumilus* cells did not secrete a decent amount of carbohydrate degrading enzymes into the medium [125]. Licheninase BglS is the only carbohydrate degrading enzyme detected in the exoproteome of glucose starving *B. pumilus* cells (Figure 10, Table S7). It is known that the genome of *B. pumilus* does not encode any amylases [11]. However, it includes several genes for degradation of carbohydrates as alternative carbon and energy sources such as pectate lyase *pelB* and arabinogalactan endo-1,4-beta-galactosidase *yvfO*. The expression of these genes might not be induced only by a limitation of glucose [110]. As described for *B. licheniformis*, the presence of an inducer may be necessary to activate these genes.

![Exoproteome of glucose starving *B. pumilus* cells](image)

**Figure 10: Exoproteome of glucose starving *B. pumilus* cells**

Exoproteome map of *B. pumilus* cells under glucose limitation (red) compared to exponentially growing cells (green). Proteins were separated in a pH gradient 3 (right) –10 (left) and from ca. 100 kDa (upper side) to ca. 10 kDa (lower side).
4.2.3 Amounts of glycolysis and TCA cycle enzymes in cells growing on a non-glycolytic carbon source

We aimed to further elucidate the regulation of the protein expression for enzymes of central carbon metabolism pathways. Using 2D gel electrophoresis we therefore compared the amounts of glycolysis and TCA cycle enzymes in *B. pumilus* cells growing on a carbon source metabolized not via glycolysis (ribose) with the amounts in cells growing on glucose. When *B. subtilis* cells are grown on the non-glycolytic carbon source, the cells accumulate a significantly higher amount of TCA cycle enzymes and on the other hand, a lower amount of glycolytic enzymes compared to cells grown on glucose [39]. In our study with *B. pumilus*, we detected a significantly lower amount of the putatively CggR regulated glycolytic enzymes TpiA, GapA, Pgk, GpmI and Eno in cells grown on ribose compared to cells grown on glucose (Table 6, Figure 11). As shown for glucose starving conditions and contrary to *B. subtilis*, in *B. pumilus* cells grown on a non-glycolytic carbon source, no increase of the amount of TCA cycle enzymes could be detected (Table 6, Figure 11). This indicates a different regulation of the TCA cycle enzymes in *B. pumilus* compared to the repression by glucose that is known in *B. subtilis*.

**Table 6: Comparison of glycolytic and non-glycolytic carbon source**

Relative quantification of proteins in cells grown on ribose as only carbon source compared to cells grown on glucose. Ratio compares the spot volumes of the protein spots on 2D gels. Quantification was performed using the Delta2D software (Decodon) from three biological replicates each.

<table>
<thead>
<tr>
<th></th>
<th>ribose/glucose ratio</th>
<th>standard derivation</th>
<th>ribose/glucose ratio</th>
<th>standard derivation</th>
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<tr>
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</tr>
</tbody>
</table>
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Figure 11: Comparison of glycolytic and non-glycolytic carbon source

Spots of proteins involved in glycolysis, TCA cycle and energy metabolism. Spots from gels of cells grown on glucose appear in green; spots of cells grown on ribose appear in red.

4.2.4 Fatty acid degradation

Fatty acids can be a valuable carbon source for cells that have no access to glucose or comparable carbohydrates. As shown for B. subtilis and B. licheniformis, we detected many proteins involved in degradation processes of fatty acids as well as proteins involved in the transfer of the generated metabolites into the central carbon metabolism [37,125]. The proteins LcfA1 (Long-chain-fatty-acid--CoA ligase), EtfA, EtfB (Electron transfer flavoprotein alpha and beta subunit) and YsiB (Enoyl-CoA hydratase) appeared significantly induced in glucose starving cells (Figure 9, Table S6). These proteins are parts of the beta-oxidation pathway of fatty acids. Acyl-CoA dehydrogenase A8FH77, acetyl-CoA C-acetyltransferase A8FH78 and 3-hydroxyacyl-CoA dehydrogenase A8FH79 were also induced under this condition. These proteins show significant similarity to B. subtilis proteins FadE (A8FH77), FadA (A8FH78) and FadN (A8FH79) and they have been shown to feed the products of beta-oxidation into central carbon metabolism by formation of acetyl-CoA and propionyl-CoA [37]. The latter is channeled into the TCA-cycle via the methylcitrate shunt [37]. In our study, the proteins involved in this pathway, citrate synthase MmgD, 2-methylcitrate dehydratase
PrpD and carboxyvinyl-carboxyphosphonate phosphorylmutase YqiQ were also induced under glucose starvation. Furthermore, extracellular triacylglycerol lipase Lip was induced in the cytosolic faction. In the extracellular faction, this protein could not be detected.

### 4.2.5 Metabolism of amino acids and peptides

*B. pumilus* is known to produce significant amounts of proteases and peptidases [16]. Therefore proteins and peptides are a logical alternative carbon and energy source for glucose starving *B. pumilus* cells. Our study revealed a significant induction of several proteases and peptidases in the exoproteome as well as in the cytosolic fraction of *B. pumilus* cells under glucose starvation (Figure 10, Table S6, S7). In the cytosolic fraction, intracellular serine protease IspA and seven peptidases (YsdC, YtjP, YtV, YqjN, YxeP, A8FH23 and A8F9Q4) were detected in higher amounts in starving cells (Table S6). In the exoproteome fraction of glucose starving cells, M6 family metalloendopeptidase A8FJ07, subtilisin Carlsberg and S8 family endopeptidase Vpr were among the highest accumulated proteins each with a share of about 4.5% of the total protein amount visible on the gels (Figure 10). Furthermore, subtilisin AprE1 and S1 family glutamyl endopeptidase Mpr were secreted in significant amounts.

In contrast, enzymes involved in amino acids synthesis were mostly detected in lower amounts in glucose starving cells compared to their exponentially growing counterparts. This was observed for the synthesis pathways of most amino acids with the exception of histidine and tryptophan synthesis proteins (Table S6, S8). A similar induction of tryptophan synthesis was observed for *B. licheniformis*, but not the induction of histidine synthesis [125].
4.2.6 Further upregulated proteins

Glucose starving *B. pumilus* cells also induced several proteins involved in the acquisition of specific nutrients except carbon sources. A significant upregulation was observed for the proteins CbiQ, YbaE and YbaF, which are involved in cobalt uptake. ABC superfamily ATP binding cassette transporter, YcdI was also upregulated (Table S6). In *B. subtilis*, it is involved in zinc uptake [127]. Furthermore, the manganese transport regulator MntR was significantly induced, too. With the exception of CbiQ, all these proteins could not be detected in exponentially growing cells.

An alternative way for *B. pumilus* cells to deal with carbon starvation is sporulation. Several proteins involved in the initiation of sporulation appeared in higher amounts in cells under glucose starvation compared to exponentially growing cells. Among them were sporulation sensor histidine kinase KinA, aspartate phosphatase response regulator RapA2 and stage 0 sporulation protein Spo0A (Table S6). Spo0A and RapA were also induced during glucose starvation in *B. subtilis* and *B. licheniformis* [38,125].

Another possible strategy for starving cells is to become competent and to get new genes via horizontal gene transfer. These new genes may empower the cells to use new resources. In our study, the proteins ATP-dependent helicase/nuclease subunit A (AddA) and ATP-dependent helicase/deoxyribonuclease subunit B (AddB) were induced (Table S6). These proteins are involved in DNA repair, recombination and genetic competence processes.

As shown for *B. licheniformis* and contrary to *B. subtilis*, glucose starvation did not induce a sigmaB-related general stress response in *B. pumilus* [38,125]. This missing induction might be explained by the absence of the genes *rsbP* and *rsbQ* in the *B. pumilus* genome [11]. *B. licheniformis* also misses the *rsbQP* operon [13,125]. In *B. subtilis*, RsbP and RsbQ form the energy signaling branch of the sigmaB regulon. These proteins are responsible for the induction of the sigmaB-regulon under nutrient starving conditions [128].

4.2.7 Downregulated processes

Starving cells have to economize their metabolism. That means they need to use the low amount of nutrients efficiently. In our study the purine synthesis proteins PurC, PurF
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and PurL as well as the pyrimidine synthesis proteins PyrB, PyrG and PyrH were detected in lower amounts in starving cells compared to exponentially growing cells (Table S8). Furthermore, nucleoside diphosphate kinase Ndk and guanylate kinase Gmk, involved in nucleotide and nucleotide triphosphate metabolism, were also present in significantly lower amounts in the cells under glucose starvation condition. The cells grow very slowly after entry into stationary phase and therefore DNA replication is reduced. This leads to a lower demand in nucleotides.

Furthermore, several NRPS and PKS proteins were detected in significantly lower amounts in the cells after entering glucose starvation (Table S8). The synthesis of antimicrobial compounds may not be “considered” essential by the cells and seemed to be shut down.

Even if the growth rate of the cells was diminished when they entered glucose limitation, a significant change in the amount of μ-regulated proteins like ribosomal proteins could not be detected.
4.3 *Bacillus pumilus* reveals a remarkably high resistance to hydrogen peroxide provoked oxidative stress

4.3.1 Effects of $\text{H}_2\text{O}_2$ on growth and cell morphology

Exponentially growing *B. pumilus* cells were treated with 2 mM hydrogen peroxide. Thus, the concentration of $\text{H}_2\text{O}_2$ 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* [33,34]. The highest peroxide concentrations allowing growth for *B. subtilis* and *B. licheniformis* were 4 and 1 mM, respectively (Table 7). *B. pumilus* is still able to grow with 20 mM hydrogen peroxide. This indicates a striking resistance of *B. pumilus* against peroxide stress. Compared to unstressed cells, growth was significantly impaired for a short time (approximately 15 min) after the $\text{H}_2\text{O}_2$ treatment (Figure 12). However, after that time, cells continued to grow for about one hour. An electron microscopy analysis indicated that after exposure to $\text{H}_2\text{O}_2$ most of the cells are morphologically intact, but some of the cells exhibited major damage of their envelope (Figure 13D). Furthermore, scanning electron microscopy revealed some atypically long cells (up to approximately 10-20% two hours after $\text{H}_2\text{O}_2$ treatment, Figure 13B, Figure 13E) indicating an impact of hydrogen peroxide on processes involved in cell division.

### Table 7: Minimal inhibition concentration of hydrogen peroxide

Determination of minimal inhibition concentration of hydrogen peroxide; "+" means growth, "-" means no growth

<table>
<thead>
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<td><em>B. subtilis</em></td>
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<td><em>B. licheniformis</em></td>
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Figure 12: Growth of *B. pumilus* before and after H$_2$O$_2$ treatment

Growth of *B. pumilus* under control conditions (filled squares) and stressed with 2 mM H$_2$O$_2$ at OD$_{500\text{nm}}$ 0.6 (empty squares).

Figure 13: Electron microscopy micrographs.

Scanning (A,B,E) and transmission (C,D) electron microscopy micrographs of *B. pumilus* cells under control conditions (A,C), 30 min (B,D) and 120 min after treatment with 2 mM H$_2$O$_2$ (E).
4.3.2 Global expression profile

To analyze the reaction of *B. pumilus* cells to oxidative stress caused by hydrogen peroxide, proteomic and transcriptomic studies were performed. The arrays were analyzed by Rebecca Schroeter. All values presented for up- and downregulation of genes or proteins are fold change values.

The analysis of the soluble intracellular proteome of *B. pumilus* revealed 54 significantly upregulated and 111 downregulated proteins 10 min after H$_2$O$_2$ treatment (with a threshold of 2-fold, Table S9, S10, Figure 14). 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 S9, S10, Figure 15). Transcriptome analysis revealed an at least 2-fold increased transcription of 181 genes 3 minutes after treatment with H$_2$O$_2$; 76 of them were more than 3-fold upregulated. Eight minutes after treatment, the transcription of 558 genes appeared at least 2-fold increased (307 genes with an at least 3-fold increased transcription). Three minutes after the stress, 266 genes were transcribed with an at least 3-fold lower rate than under control conditions, for 296 genes this decreased transcription rate has been shown 8 minutes after treatment.

To compare the physiological changes in H$_2$O$_2$ 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* [33,34]. 139 of the upregulated genes and proteins could be assigned to these putative regulons (Table S9). The thus classified genes and proteins identified in this study are summarized and discussed below.
Figure 14: Cytosolic proteome 10 min after H$_2$O$_2$ treatment

The cytosolic proteome of *B. pumilus* cells 10 min after H$_2$O$_2$ treatment. Cell samples were labeled with L-$[^{35}S]$-methionine during the exponential growth phase (OD$_{500	ext{ nm}}$ 0.6), and 10 min after H$_2$O$_2$ addition. Proteins were separated in a pH gradient 4 (right) – 7 (left).
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Figure 15: Cytosolic proteome 30 min after H$_2$O$_2$ treatment
The cytosolic proteome of B. pumilus cells 30 min after H$_2$O$_2$ treatment. Cell samples were labeled with L-$[^{35}$S]-methionine during the exponential growth phase (OD$_{500}$ nm 0.6), and 30 min after H$_2$O$_2$ addition. Proteins were separated in a pH gradient 4 (right) – 7 (left).
4.3.3 PerR regulon

The PerR regulon is known to be highly induced by oxidative stress caused by hydrogen peroxide and paraquat [33]. 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* [34]. Transcription of the *perR* gene was significantly increased immediately after stress (Table S9). This indicates a regulation mechanism of PerR in H₂O₂ treated *B. pumilus* cells that is similar to the de-repression model reported for *B. subtilis* [129].

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 S9).

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 [11]. 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 S9). Thereby, KatX2 was one of the proteins with the highest induction rates detected. *B. subtilis* and *B. licheniformis* subjected to hydrogen peroxide exhibit a more than 100-fold induction of KatA [33,34]. KatX2 comprises about 0.38% of the cytoplasmic protein present in the gel before addition of hydrogen peroxide. The values for *B. subtilis* and *B. licheniformis* are 0.13% in both strains (personal communication C. Scharf, B. Voigt). After addition of hydrogen peroxide KatX2 comprises about 3.8% of the cytoplasmic protein. This is comparable to the value of 3.6% for *B. licheniformis* (personal communication B. Voigt) but higher than for *B. subtilis* (1.2%, personal communication C. Scharf). These values indicate that in *B. pumilus* there is a higher synthesis of KatX2 already in unstressed cells compared to *B. subtilis* and
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*B. licheniformis* KatA explaining the lower induction rate. In *B. subtilis*, KatX is the major spore catalase and under control of SigB and SigF [130,131]. We detected a *B. subtilis* PerR consensus sequence [67] containing 2 mismatches about 90 bases in front of the start codon indicating a possible involvement of PerR in its regulation.

4.3.4 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 [67]. The regulator protein Fur of *B. subtilis* controls the expression of genes responsible for iron uptake [132]. Immediately after exposure to H$_2$O$_2$, cytosolic iron concentration is considerably reduced to prevent the formation of OH$^-$ by the Fenton reaction [33]. Upregulation of the Fur-controlled genes may be a reaction of the cells to optimize iron uptake in order to face the resulting iron limitation. Alternatively it might be that Fur is H$_2$O$_2$ sensitive as it is in *E. coli* [133].

Nine genes of a putative Fur regulon showed a significantly increased expression in *B. pumilus* cells after H$_2$O$_2$ treatment, including the ABC transporter system *fhuB1C1G1* (Table S9). The *fhuC* gene was induced by H$_2$O$_2$ in *B. subtilis* and *B. licheniformis*, too [33,34]. Further Fur regulon member genes known to be induced by H$_2$O$_2$ 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 8 minutes after treatment, these were among the highest upregulated genes in this putative regulon. The putative nitroreductase YfhC, also induced in H$_2$O$_2$ stressed *B. subtilis* cells, was the only member of the putative Fur regulon we observed to be upregulated at translational level.

The gene *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$_2$O$_2$ in this organism [12,33]. In *B. subtilis* and *B. licheniformis*, the siderophore biosynthesis complex encoded by *dhbACEBF* was strongly upregulated by H$_2$O$_2$. In our study, these genes showed no significant changes in their expression level. Other genes that exhibited higher transcription rates after H$_2$O$_2$ treatment were the iron ABC transporter protein encoding gene *feuA* and its upstream-located regulator *ybbB*.
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(renamed btr in B. subtilis) [134]. 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 fluG2 and fluD 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 S9). 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 [34]. The sufU gene was found to be only slightly upregulated at the mRNA level.

4.3.5 Spx regulon and bacillithiol

Another regulator protein assigned to the putative PerR regulon is SpxA, controlling the expression of the Spx regulon in B. subtilis [135,136]. This gene exhibited an about 4-fold increased transcription rate in H2O2 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 H2O2 treatment.

In our study we detected six genes of a putative Spx regulon to be induced following H2O2 treatment (Table S9). The proteins encoded by three of them, nitro/flavinreductase NfrA, putative NADPH-dependent butanol dehydrogenase YugJ and thioredoxin-disulfide reductase TrxB, were induced in H2O2 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 [137-139]. 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 [13,136,140].
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We noticed an increased transcription of *ypdA* and *yqiW* as well as an induction of the *yphP* gene product (Table S9). These genes co-occur with bacillithiol (Cys-GlcN-malate, BSH) synthesis genes [141]. However, only one gene encoding a protein involved in bacillithiol synthesis, *yojG* was transcribed at a slightly elevated level (Table S9). Bacillithiol is one of the major thiols in *B. subtilis* and known to be involved in resistance against organic peroxide stress and disulfide stress [27,142,143]. For further investigation, we analyzed the cytosolic metabolome of H$_2$O$_2$ treated *B. pumilus* cells concerning the concentration of thiol compounds. Our analysis revealed a bacillithiol level of 2.6 nmol per mg cell dry weight already under control conditions. Similar BSH concentrations have been detected in *B. subtilis* (0.6-2.2 nmol per mg) [27,141,144]. Ten minutes after H$_2$O$_2$ treatment, the cytosolic concentration of bacillithiol increased to 5 nmol per mg cell dry weight (Figure 16). The increase continued up to a concentration of about 6.2 nmol per mg cell dry weight 60 minutes after stress. Since only one bacillithiol synthesis gene (*yojG*, renamed bshB2 in *B. subtilis*) was slightly upregulated, increase of bacillithiol concentration in the cells might regulated allosterically, for example, by an oxidation of the BSH pool leading to a relief of feedback inhibition. [145,146].

![Figure 16: Concentration of thiol compounds in *B. pumilus* cells](image)

Cytosolic concentration of bacillithiol (BSH), CoA and cysteine (Cys) per mg cell dry weight (CDW) during the exponential growth phase (OD$_{500\text{nm}}$ 0.6 at 0 min) and 10, 30 and 60 min after H$_2$O$_2$ treatment.
4.3.6 SOS regulon

H$_2$O$_2$ treatment leads to the formation of OH’ by Fenton reaction, which exhibits a high DNA-damaging potential. Lowering the concentration of iron in the cells reduces this threat. As a result, *B. subtilis* and *B. licheniformis* cells subjected to oxidative stress caused by H$_2$O$_2$, induced the SOS regulon, regulated by the proteins RecA and LexA, responsible for repair of DNA [33,34,147,148].

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$_2$O$_2$ treatment (Table S9). 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$_2$O$_2$ and involved in suppression of cell division in *B. subtilis*, was also strongly induced in our study [33,149]. 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$_2$O$_2$ addition [150].

4.3.7 CtsR regulon

The CtsR regulon, mediating refolding and/or degradation of misfolded and damaged proteins, was induced by several oxidative stressors in *B. subtilis* and *B. licheniformis* [33,34,151]. 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$_2$O$_2$ on protein quality (Table S9). The operon *ctsR-mcsAB-clpC* was transcribed with significantly higher intensity after the addition of H$_2$O$_2$ as well as the genes *clpE* 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.
4.3.8 SigB regulon

Besides the induction of the above described putative regulons more or less directly associated to oxidative stress, H$_2$O$_2$ treated cells exhibited an upregulation of 47 genes known to be under control of the general stress sigma factor SigB in *B. subtilis* (Table S9) [152,153]. H$_2$O$_2$ is not a typical inducer of the sigmaB regulon in *B. subtilis*. Its genes are induced as a side-effect when the cells are exposed to higher concentrations of H$_2$O$_2$ [33,154]. The high concentration of H$_2$O$_2$ that was used in this study may be the reason for the induction of putatively sigmaB-regulated genes in *B. pumilus*.

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* [155].

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$_2$O$_2$ stressed *B. licheniformis* cells [34]. 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$_2$O$_2$ treated *B. pumilus* cells, too [156]. This protein may be able to substitute the missing MrgA.

4.3.9 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 [157]. 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$_2$O$_2$ [33]. Our proteome study showed a strong induction of three putatively CymR-regulated proteins. The adenyllyl-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 S9). 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 precrorrin2. An induction of the enzymes that continue the pathway from precrorrin2 to siroheme could not be detected.

Since bacillithiol contains cysteine, previously described bacillithiolation may consume a lot of cysteine, which can explain the induction of the putative CymR regulon.

4.3.10 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 [158]. Following H₂O₂ treatment, there was no induction of this gene observed in B. subtilis and B. licheniformis [33,34]. In our study, we observed a strongly induced expression of this gene at transcriptional and translational level indicating an involvement of this peroxiredoxin in the H₂O₂ resistance of B. pumilus (Table S9). OhrA can adopt the function of the missing AhpC protein. 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.

H₂O₂ treatment induced some additional regulator genes. One of them is fadR, encoding a regulator protein mediating fatty acid degradation in B. subtilis [159]. Two genes putatively controlled by FadR, etfAB - encoding the electron transfer flavoprotein alpha and beta subunit, were also induced (Table S9). Another regulator, AbrB1, controlling the expression of genes induced by transition from exponential to stationary growth in B. subtilis [160], 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$_2$O$_2$ treatment. Its function is also unknown. Several genes and proteins involved in transport processes were detected to be upregulated following H$_2$O$_2$ stress (Table S9). H$_2$O$_2$ treatment caused an upregulation of the sodium uptake system natAB and the mrpABCDEF 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* [161,162]. 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$_2$O$_2$ impacts osmotic homeostasis in *B. pumilus* cells [163]. Furthermore, it is worth to mention that H$_2$O$_2$ 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.

### 4.3.11 Downregulated genes/proteins

As shown for many other organisms, the adaptation mechanism of *B. pumilus* cells to oxidative stress includes also a downregulation of vegetative cellular functions. Most of the down-regulated genes encode proteins involved in main 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 S10) [33,34]. 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$_2$O$_2$ treatment. This repression might due to the iron sparing response described by Gaballa et al. [164]. Repression of enzymes involved in branched chain amino acid synthesis has been found during iron starvation in *B. subtilis* [132]. Furthermore, we observed a reduced expression of most of the aminoacyl-tRNA-synthetase genes, with the exception of tryptophanyl-tRNA-synthetase gene *trpS*, which matched the upregulation of the tryptophan operon.
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Strikingly, a stringent response, i.e. a downregulation of ribosomal proteins or elongation factors like \textit{fusA}, \textit{tsf} or \textit{tufA}, as described for other organisms (\textit{B. subtilis}, \textit{B. licheniformis}, \textit{E. coli}) could not be detected in \textit{B. pumilus} [33,34,165].

4.3.12 Modification of the KatX2 protein after peroxide stress

Unlike the KatA protein, KatX2 contains three cysteines. Under oxidative stress conditions cysteine residues can be oxidized [82,166]. This could irreversibly damage the protein, e.g. when oxidized to cysteine sulfenic and sulfonic acid as shown for the GapA protein of \textit{S. aureus} [167]. To prevent this, \textit{Bacillus} cells protect cysteine residues in proteins by reversibly oxidizing them using different low-molecular-weight thiol compounds such as bacillithiol [27,142]. Using the fluorescence thiol modification assay described by Hochgräfe \textit{et al}. [82] we analyzed reversible thiol-modifications in the \textit{B. pumilus} KatX2 protein. This procedure uses two different staining methods, one for protein accumulation and one for reversible thiol oxidations. Quantification of proteins is done using relative spot volumes (volume of a spot compared to the volumes of all spots visible on the 2D-gel). The ratio between the spot volumes of a protein spot in the thiol modification staining and the protein accumulation staining is an indicator for the amount of reversible oxidations of the cysteine residues in a protein. In exponentially growing cells, KatX2 cysteine residues were nearly completely reduced (Figure 17A). A hydrogen peroxide treatment caused a significant increase of reversible cysteine oxidation (Figure 17B). The ratio of thiol modification to protein accumulation increased from about 0.5 to 1.36.

Furthermore, LC-MS/MS analysis was performed to show irreversible oxidation of cysteine residues in the different catalase spots excised from the 2D-gels. This irreversible oxidation cannot be accessed by the fluorescence thiol modification assay. We found evidence for the oxidation of the thiol group of cysteine 461 in one of the catalase 2D-gel spots. A mass shift of +48 representing sulfonic (+48) acid formation was detected in \textit{B. pumilus} KatX2 (Figure 18). None of these peptides could be detected in exponentially growing cells without peroxide treatment.
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Figure 17: Reversible oxidation of KatX2
KatX2 protein spots in exponentially growing *B. pumilus* cells (A) and 20 min after the addition of 2 mM H$_2$O$_2$ (B). Protein accumulation is shown in green, reversible oxidized thiol-modifications stained with BODIPY fluorescent stain is shown in red.

Figure 18: Irreversible oxidation of KatX2
Spectrum of modified cystein-containing peptide of KatX2 detected in *B. pumilus*. 
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4.4  
*B. pumilus* in an industrial fermentation process – a proteomic comparison of enzyme overexpressing and wild type cells

4.4.1  
Production and reference strain

Our aim was to characterize the previously developed *B. pumilus* platform in a fermentation process on the proteome level in order to gain a better understanding of the strain under process conditions [168]. Therefore the previously described, subtilisin BL18 overexpressing strain Jo2.1/pHP49 was compared to a non-overexpressing Jo2.1 reference strain constructed by Tobias Küppers at Henkel AG & Co. KGaA. Since *B. pumilus* spores are highly resistant against oxidative as well as thermal stress and thereby cause significant difficulties in sterilization procedures [11,169], the endospore formation was knocked out in *B. pumilus* strain derivative Jo2.1 [123,168]. As previously described the subtilisin BL18 overexpression by Jo2.1 was achieved by introducing the production plasmid pHP49. The pBC16-derived plasmid encodes a protease expression cassette consisting of a protease promoter, a sec-pathway dependent signal peptide and the mature subtilisin BL18 protease gene with an upstream located propeptide, involved in the extracellular folding of the secreted protease [168]. Based on findings of the production strain blueprinting contribution, the strain Jo2.1/pHP49 was chosen for this characterization. As an appropriate reference for this application we considered a Jo2.1 strain harboring a predecessor of pHP49 lacking the encoded protease as well as the upstream signal- and propeptide. This plasmid was chosen in order to simulate the metabolic burden caused by the presence of a plasmid itself under process conditions. This reference plasmid, which possesses in contrast to pHP49 an empty expression cassette, was named pMM39 and already existed in the Henkel plasmid collection (Henkel AG & Co KGaA, Düsseldorf, Germany). To generate the reference strain Jo2.1/pMM39, plasmid DNA was purified, *in vitro* methylated and finally introduced into Jo2.1 cell by PEG mediated protoplast transformation as previously described [168].
4.4.2 Cultivation of *B. pumilus* Jo2.1/pHP49 and Jo2.1/pMM39

Based on the previously described fed batch process [168], the BL18-overexpressing strain Jo2.1/pHP49 and the non-overexpressing reference strain Jo2.1/pMM39 were cultivated in stirred tank reactors (STR), each in three replicates. The process analysis of both strains shows no significant differences in the processes of both strains (Figure 19).
Figure 19: Fed batch cultivation of the BL18-overexpressing (Jo2.1/pHP49) and the non-overexpressing (Jo2.1/pMM39) *B. pumilus* strains at pilot scale

Cultivations were based on a previously described lab scale fed batch process [168]. Colony forming Units (CFU) (blue triangle) [L-1], glucose concentration (orange diamond) [g/l], acetate concentration (green square) [g/l] and protease titer (gray square) were measured at line.
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The diauxie, indicated by the beginning of the degradation of the previously synthesized acetoin, is marked with the vertical dashed line. After this time point the glucose feed was started. Yielded enzyme activities were scaled to the maximum value of both strains, which was reached by Jo2.1/pHP49 at 48 h. Data points display the average with associated standard deviations of three cultivations. Sample points for proteome analysis are marked by black arrows pointing upwards at the abscissa.

4.4.3 Proteome analysis – overview

During the fermentation processes we took five samples for analysis of the cytosolic proteome and the exoproteome as well as the extracellular metabolome. Samples were taken at the exponential growth phase, after the diauxie and in early, middle and late stationary phase (Figure 19, Figure S6, S7). Due to differences in in cell growth and process speed, the time points for the first three samples differ slightly between both processes. We were able to identify 448 proteins in the cytosolic fraction of cells carrying the plasmid without protease. A 2D-gel-based proteomic approach using three biological replicates each was performed and 453 proteins were identified in the cytosolic fraction of cells carrying the protease overexpressing plasmid. 370 of these proteins were detected in both sample types. The identified proteins were relatively quantified according to their spot volume on the 2D gels using the Delta2D software (Decodon).

All changes in the amounts of proteins can be the result of a changed expression but differences in the stability of proteins may also influence the amount of protein present in the cells at a specific time point.

4.4.4 Individual proteomic analysis of both processes

4.4.4.1 Central carbon metabolism

Since glucose as carbon source is the limiting factor for exponential growth of the cells, significant changes in the proteome setup concerning the central carbon metabolism were expected.

We observed a significant downregulation of several glycolysis enzymes during the process in both strains beginning at the second time point. At this time the glucose,
Initially provided by the medium, was consumed. Beginning at this time point, glucose was fed to the process continuously, but in an amount that kept the cells in a limitation. The glycolytic enzymes glucose-6-phosphate isomerase Pgi, ATP-dependent 6-phosphofructokinase PfkA and fructose-bisphosphate aldolase FbaA did not show significant changes in their amount until the last time point. The accumulation of glyceraldehyde-3-phosphate dehydrogenase GapA, phosphoglycerate kinase Pgk and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase Pgm decreased significantly right after the exponential phase (Figure S6, S7). GapA, Pgk and Pgm are controlled by CggR in *B. subtilis*, a regulator protein that is also encoded in the genome of *B. pumilus* [170]. The other two proteins regulated by CggR in *B. subtilis* differed in their expression level. While the amount of enolase (Eno) was reduced considerably during stationary phase, triosephosphate isomerase (TpiA) even appeared to be induced in non-overexpressing but not in overexpressing cells during fermentation. Furthermore, glyceraldehyde-3-phosphate dehydrogenase GapB, an important gluconeogenesis-protein was upregulated in non-overexpressing cells at the end of exponential growth.

The TCA cycle enzymes did not exhibit a consistent accumulation pattern. The amount of the citrate synthase, CitZ, did not change during fermentation. The enzymes catalyzing the next four reactions of the cycle (CitB, Icd, OdhAB, SucCD) were present in lower amounts in both strains when the cells stop exponential growth (Figure S6, S7). However, in the overexpressing strain the amounts of these enzymes were much lower than in the non-overexpressing strain, especially after the diauxie. No significant change could be observed for the enzymes catalyzing the last three steps in the cycle (SdhA, FumC, Mdh,) (Figure S6, S7).

During both processes acetoin synthesis proteins (AlsD, AlsS) were present in significant amounts during the exponential growth phase before diauxie and showed decreased amounts in the following samples (Figure S6, S7). The amount of acetoin utilization proteins (AcoABC) significantly increased after the cells passed the diauxie. This demonstrated a similar overflow metabolism in both processes during exponential growth followed by reusing the overflow products later when the cells entered glucose limited conditions. Based on these data we assume that acetoin is the primary overflow product of *B. pumilus* cells growing in complex fermentation medium as shown in shake-flask experiments before (Figure 5).
After the cells went through the diauxie, in both strains several proteins were upregulated that are known to be induced by glucose limitation in *B. subtilis* [37,38,125] and that have also been detected to be induced in glucose limited *B. pumilus* cells as described previously (chapter 4.2).

We detected the induction of several proteins, which are involved in usage of alternative carbon sources. Beta-glucanase BglS was detected to be induced in the exoproteome (Figure 20). The 5-dehydro-4-deoxy-D-glucuronate isomerase KduI (galacturonic acid usage) was induced in both strains and the alpha-galactosidase MelA (melibiose usage) was detected and induced in non-overexpressing cells (Figure S6, S7). These proteins are known to be CcpA-regulated in *B. subtilis*. CcpA is the key regulator for usage of carbon sources [39].

### 4.4.4.2 Metabolism of proteins, peptides and amino acids

The medium provided the cells with a complex source of proteins and peptides that could be used as nitrogen sources and also as carbon sources after exhaustion of glucose. Because of this, a significant upregulation of proteins involved in using these compounds was expected during the fermentation process.

Analysis of the exoproteome revealed that the cells synthesized and secreted a significant amount of proteases like AprE, AprN, Bpr and Vpr into the medium (Figure 20).
Figure 20: Exoproteome of the overexpressing and the non-overexpressing process

2D gels of the exoproteome of the cells during the fermentation process of the non-overexpressing Jo2.1/pMM39 (A) and the L18-overexpressing Jo2.1/pHP49 (B). Carbon hydrate degrading enzymes are shown in green, capsule degrading enzymes are shown in blue, proteases are shown in orange (chromosomal proteases) and red (overexpressed protease) and motility related proteins are shown in yellow.

A PfpI family intracellular protease (BPJ28950) was significantly induced in both strains in the later stationary phase (Figure S6, S7). Several peptidases like M55 family D-aminopeptidase DppA and C56 family peptidase YfKM were detected to be upregulated up to 20-50-fold in the later phases compared to exponential growth (Figure S6, S7). Furthermore, 1-pyrroline-5-carboxylate dehydrogenase RocA, a protein involved in the utilization of arginine was strongly induced in both strains at all time points after the exponential growth phase which indicates an increased utilization of amino acids. The 2-amino-3-ketobutyrate coenzyme A ligase Kbl, involved in utilization of threonine, was strongly induced at the same time points in the overexpressing strain but could not be detected on gels of extracts of the non-overexpressing strain.

In both strains the amount of the majority of proteins involved in synthesis of amino acids decreased significantly during the fermentation process (Figure S6, S7). Among them were the histidine synthesis proteins HisCGZ, the branched chain amino acid
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synthesis proteins IlvABCD and tryptophan synthesis proteins TrpABCE. Although not all of these proteins could be detected in the overexpressing cells the changes in protein amount was similar. Overall, the upregulation of proteins involved in the catabolism of amino acids on the one hand and the reduced amount of proteins involved in amino acid synthesis on the other hand strongly indicated, that during the stationary phase the cells preferentially use amino acids from the medium.

4.4.4.3 Degradation of fatty acids

Fatty acids seemed to be an important carbon and energy source for the cells. Both strains showed a strong induction of enzymes involved in beta-oxidation of fatty acid and the channeling of the products into the methylcitrate cycle (FadA, FadE, FadN) starting during late exponential phase (Figure S6, S7). Via the methylcitrate pathway the arising products are channeled into the TCA cycle in B. subtilis [37]. The potential methylcitrate pathway proteins 2-methylcitrate synthase MmgD (detected only on gels of non-overexpressing cells) and 2-methylcitrate dehydratase MmgE (in both strains) showed a similar induction. This may even explain why the amounts of the TCA cycle enzymes catalyzing the last three reactions (Figure S6, S7) did not decrease as the other TCA cycle enzymes did during the process.

4.4.5 Comparison of the proteome of the two strains

Although the processes appeared to proceed quite similar, the proteome analysis also exhibited significant disparities. Compared to non-overexpressing cells the overexpressing strain showed an increased amount of the glycolytic enzymes Pgi, PfkA and FbaA and TpiA during the first three time points (Figure 21, Figure S6, S7). No significant difference was observed for the enzymes GapA, Pgk, Pgm and Eno. The TCA cycle enzymes were detected in significantly lower amounts in the overexpressing strain compared to the non-overexpressing cells, with the exception of the enzymes catalyzing the last three reactions of the cycle. Those enzymes were detected in equal amounts in both strains.
Figure 21: Accumulation of glycolysis and TCA-cycle enzymes
Comparison of the accumulation of proteins involved in glycolysis and TCA-cycle between the two strains. Proteins on gels of extracts of non-overexpressing cells appear in green whereas proteins on gels of extracts of overexpressing cells appear in red.

The protease-overexpression provides the cells with a powerful tool to degrade and use the components provided by the medium in a highly efficient way. The exoproteome of the overproducing cells was dominated by the produced protease (Figure 20). Therefore, these cells showed a significantly lower accumulation of many proteins that are involved in synthesis of amino acids (e.g. I1vABCD, LeuA2C) (Figure S6, S7) when
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compared to the non-overproducing strain. On the other side, proteins that are active in usage of these components were accumulated in significantly higher amounts. Among them were peptidases like YmfF and amino acid degrading enzymes like RocA.

A significantly increased amount of many proteins that are involved in the synthesis of nucleotides and nucleic acids was detected in the overexpressing cells compared to the control strain. Purine and pyrimidine synthesis pathways represented by PurB, PurC, PurE, PurF and PurH as well as PyrAA, PyrB, PyrC, PyrF, PyrG and PyrH seemed to be much more active in the overexpression processes.

Greater differences were observed comparing cells of both strains in the late stationary phase and at the end of the process. Late stationary overexpressing cells exhibited a dramatic change in their protein pattern. Most proteins that were expressed in exponentially growing cells disappeared completely (Figure 22). This may be a result of differences in protein synthesis, but differences in protein stability can also have an impact on the protein pattern. Only a few proteins appeared on these gels in significant amounts. Among them are transporter proteins like PstS for glucose uptake as well as the peptide transporter protein DppE. A further strongly upregulated peptide transporter protein, OppA, is also known to be related to sporulation processes [171]. Furthermore, the pyruvate dehydrogenase complex components PdhC and PdhD were present in high amounts on late stationary phase cells gels. Strikingly, the other components of this protein complex did not show an increased amount. Besides its main function in glycolytic processes, PdhD is also involved in degradation of leucine, isoleucine and valine, which may explain an induction in the late stationary cells which strongly focus on degrading amino acids. For PdhC there is no similar function known yet.
Figure 22: Cytosolic proteome of L18-overexpressing Jo2.1/pHP49 cells

2D gels of cytosolic proteins of overexpressing Jo2.1/pHP49 cells after 50 hours of fermentation (red) compared to exponentially growing cells after six hours of fermentation (green). Proteins were separated in a pH gradient 4 (right) – 7 (left) and from ca. 130 kDa (top) to ca. 10 kDa (bottom). Spots marked with (f) are fragments of the presented protein.
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Figure 23: Cytosolic proteome of non-overexpressing Jo2.1/pMM39 cells

2D gels of cytosolic proteins of non-overexpressing Jo2.1/pMM39 cells after 50 hours of fermentation (red) compared to exponentially growing cells after six hours of fermentation (green). Proteins were separated in a pH gradient 4 (right) – 7 (left) and from ca. 130 kDa (top) to ca. 10 kDa (bottom). Spots marked with (f) are fragments of the presented protein.
Non-overexpressing cells showed a significant but more moderate change of their protein setup. Comparing late stationary non-overexpressing cells with exponentially growing cells far less proteins which were no longer present were observed (Figure 23). Several proteins known to be induced by glucose starving *B. subtilis* or *B. licheniformis* cells were detected to be induced in late stationary non-overexpressing cells, too [37,38,125]. Among them were the fatty acid degrading enzymes FadE and FadN as well as the methylcitrate shunt enzymes MmgD and MmgE, which channel the products of beta-oxidation into the TCC. Interestingly, kanosamine antibiotic synthesis protein NtdA appeared strongly induced in late stationary non-overexpressing cells, but not in their overexpressing counterparts [172].

4.4.6 Stress response

During fermentation processes a variety of stress conditions can occur [173]. Especially in the later phases characterized by high cell densities and elevated synthesis and secretion of target proteins the cells may be exposed to suboptimal conditions.

In general, non-overexpressing cells did not exhibit a significant induction of stress-indicating proteins. The only protein significantly induced during the fermentation was peroxiredoxin OhrB, an indicator of organic peroxide stress and part of the sigmaB-regulated general stress response in *B. subtilis* [174].

In overexpressing cells several stress-related proteins were accumulated during the fermentation. The majority of these proteins are known to be induced under oxidative stress conditions. Oxidative stress is a condition often occurring in later fermentation phases, primarily indicated by an upregulation of main vegetative catalases like KatA in *B. licheniformis* [15,34,59]. In this study we detected an upregulation of catalase KatX2 in late-phase overexpressing cells (Figure 22, Figure S6, S7). As shown previously, this catalase is also upregulated in *B. pumilus* cells stressed with hydrogen peroxide (chapter 4.3). Putative iron storage protein/DNA-protecting protein Dps, methionine sulfoxide reductase MsrA and superoxide dismutase SodA showed a comparable regulation along the fermentation processes. They are also known to be involved in oxidative stress resistance in Bacilli [175-177]. The upregulation of these proteins in late-phase overexpressing cells compared to non-overexpressing cells in the same phases indicated
an involvement of the expression and secretion of large amounts of protein in the occurrence of oxidative stress during the fermentation processes. During all time points we detected an upregulation of the TerD family tellurium resistance proteins YceC and YceD in overexpressing compared to non-overexpressing cells. YceE, encoded in the same operon, shows a significant increased expression in overexpressing cells at the last two time points. These proteins are described to be involved in the detoxification of toxic anions [152,174].
5. Concluding remarks

Our study began with an organism that was barely described at that time. We used the power of combined modern omics-techniques to gain a comprehensive overview over the physiology of \textit{B. pumilus} cells.

2D-SDS-PAGE was employed to create master gels for cytosolic and secreted proteins of \textit{B. pumilus} cells grown in minimal medium and in complex medium. GeLC-MS/MS nearly tripled the number of identified proteins. Based on the identification of approximately 1500 proteins and numerous metabolites associated to various metabolic pathways, insights into the physiology of growing \textit{B. pumilus} cells could be gained, for example, cells growing in minimal medium display a higher expression of TCA-cycle enzymes (percentage spot volume in the 2D gel) than cells growing in complex medium. In these cells glucose excess induces a Crabtree effect that means upregulation of glycolysis combined with overflow metabolism and downregulation of the TCA-cycle. This is likely because cells in minimal medium were provided with a significantly lower amount of glucose compared to cells in complex medium. They also have to synthesize amino acids and other components whereas cells in complex medium can gain such components at least partially from the medium. This hypothesis is backed by the fact that cells growing in minimal medium express more proteins from the corresponding pathways. In both media proteins involved in overflow metabolism were identified, but only cells growing in complex medium exhibit real overflow metabolism. In the complex medium synthesis of lactate in significant amounts was observed, apparently caused by oxygen limitation. The Sec and TAT-secretory system of \textit{Bpu Jo2} are orthologous to the well-studied systems of \textit{Bacillus subtilis}, which is already used as expression platform. In addition, production-relevant features like NRPS/PKS clusters could be identified in the proteome of \textit{B. pumilus} Jo2.

A 1D LC-MS/MS approach was performed to quantify the proteins using an N14/N15 labeling and to analyze the changes in the protein equipment when \textit{B. pumilus} cells stop their exponential growth and become stationary due to limitation of glucose. 1033 proteins in the cytosolic fraction of \textit{B. pumilus} cells were quantified and 272 of them appeared to be upregulated when the cells experience glucose starvation. 2D-PAGE was
used to analyze the exoproteome of those cells. Glucose starving *B. pumilus* cells seemed to focus on usage of proteins and peptides as alternative carbon and energy sources instead of other carbohydrates. Especially the exoproteome of glucose starving cells is dominated by proteases and peptidases. Furthermore, cells used fatty acids as carbon source indicated by upregulation of enzymes involved in β-oxidation and the methylcitrate pathway.

131 proteins were detected in lower amounts in glucose starving cells. Among them are many enzymes involved in the synthesis of amino acids and nucleotides. A sigmaB induced general stress response was not observed in this study.

The combination of proteomics and transcriptomics revealed a specific adaptation of *B. pumilus* cells caused by the oxidative stress trigger H₂O₂. Although many of the induced genes and proteins could be assigned to well-known oxidative stress regulons like PerR, CtsR and Fur, there are particular mechanisms detectable which seem to be involved in the remarkable oxidative stress resistance of *B. pumilus*. The concentration of H₂O₂ that was used to trigger the stress in our study was about 40-fold higher than those used for comparable analysis of *B. subtilis* or *B. licheniformis*. Our study could enlighten several points at which the peroxide stress response of *B. pumilus* cells is different from its Gram-positive relatives. It is suggested that the catalase KatA is replaced by the catalase KatX2 in *B. pumilus*. 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 H₂O₂ 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.

Using the physiological knowledge gained during our studies, we analyzed samples taken during an industrial fermentation process. Five samples were taken during the
Concluding remarks

processes using a protease overexpressing *B. pumilus* strain and a non-overexpressing *B. pumilus* reference strain. 2D-PAGE was employed to analyze the samples. 448 proteins could be identified in the samples from the protease overexpressing stain as well as 453 proteins in the reference strain. The proteins were quantified relatively comparing the different growth phases of each strain as well as comparing the strains to each other. The physiological knowledge gained from the shake flask studies enabled us to interpret the findings. Both strains showed an induction of proteins involved in acquisition of alternative carbon sources and of proteins involved in degradation and usage of fatty acids, e.g. the methylcitrate pathway, when they stop exponential growth. This is comparable to the results gained from the analysis of *B. pumilus* cells under glucose limitation, indicating similar conditions during the processes. Especially in the late phases of the fermentation processes the cells were obviously exposed to severe stress conditions. Our results demonstrated that overexpressing cells showed a significantly stronger oxidative stress response at the end of the fermentation process compared to non-overexpressing cells, which indicates that not only the high cell densities but also the overproduction of the target protein might be responsible for these conditions.
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7. Eigenständigkeitserklärung

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.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.
8. Lebenslauf

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*Bacillus pumilus reveals a remarkably high resistance to hydrogen peroxide provoked oxidative stress*  
Stefan Handtke\(^a\), Rebecca Schroeter\(^a\), Britta Jürgen, Karen Methling, Rabea Schlüter, Dirk Albrecht, Sacha van Hijum, Johannes Bongaerts, Karl-Heinz Maurer, Michael Lalk, Thomas Schweder, Michael Hecker, Birgit Voigt  
# These authors contributed equally

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*Cell physiology of the biotechnological relevant bacterium Bacillus pumilus-an omics-based approach*  
Stefan Handtke\(^a\), Sonja Volland\(^a\), Karen Methling, Dirk Albrecht, Dörte Becher, Jenny Nehls, Johannes Bongaerts, Karl-Heinz Maurer, Michael Lalk, Heiko Liesegang, Birgit Voigt, Rolf Daniel, Michael Hecker  
# These authors contributed equally
In preparation

*Bacillus pumilus* KatX2, a highly efficient catalase – confers enhanced hydrogen peroxide resistance to a *Bacillus subtilis katA::katX2* mutant strain

Stefan Handtke, Dirk Albrecht, Daniela Zühlke, Andreas Otto, Dörte Becher, Thomas Schweder, Kathrin Riedel, Michael Hecker, Birgit Voigt
Danksagung

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