Insights from lactic acid bacteria into mechanisms of high pressure adaptation

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Abstract
The high hydrostatic pressure (HHP) response and adaptation was studied of Lactobacillus sanfranciscensis used in food biotechnology. Changes were characterized in the membrane physiology with fluorescent techniques, the proteome with 2-D electrophoresis and the transcriptome with microarrays and real time PCR. The up-regulated proteins and genes included representatives of heat and cold shock corroborating the hypothesis that the cell tries to compensate for pressure induced impairing of membrane transport and translation. Overexpression of ssrA (transfer mRNA) in a barotolerant mutant suggests a role of the ribosome as primary thermodynamic HHP sensor determining the adaptive capacity of the cell. Thus, we propose trans-translation and peptide tagging, processes that promote recycling of stalled ribosomes and prevent accumulation of abortively synthesised polypeptides, to be involved in combating high pressure damage and conferring moderate barotolerance.

High hydrostatic pressure, Lactobacillus, ribosomal sensing

1. Introduction

Lactic acid bacteria (LAB) harbor a large variety of species with their broadest diversity within the lactobacilli [1]. They are widely used for food fermentations and can also cause food spoilage [2]. Selected strains of Lactobacillus are widely used as probiotics, primarily in dairy products and dietary supplements. Yet, the use of many metabolically interesting strains is hampered by the lack of knowledge on their stress response and tolerance. Among these are strains from cereal fermentations, namely Lactobacillus sanfranciscensis, which therefore were a focus in the work described here for lactic acid bacteria. The consumers demand for freshness and minimally processed foods along with a prolonged shelf life has driven the development of novel technologies in food preservation with high hydrostatic pressure (HHP) being the most promising. Therefore, the response to HHP of lactic acid bacteria in their ambivalent role as factories in biotechnology and in biomedical applications versus food spoiling organisms is most interesting.

2. Cellular responses to HHP

2.1 Sublethal injury and cell death

Lactic acid bacteria are inactivated at pressures between 200 – 600 MPa at holding times of 5 – 60 min. with the decrease of colony forming units (CFU) following asymmetric sigmoid declines. Before the cells die off, they suffer from sublethal injury. Such cells can be differentiated from fully active survivors by their inability to recover on stress media containing 4% NaCl.
2.2 Metabolism and membrane integrity

The sublethal damage of HHP treated cells was referred to membrane damage and metabolic inactivation [3-5]. The membrane loses its barrier function to ions and larger molecules. Therefore, the cells are unable to maintain a pH gradient and proton motive force across the membrane if the pressure exceeds 200 MPa. In the lower pressure range which is also observed in the deep sea mesophilic organisms can survive and grow, whenever their growth rate is strongly reduced. This offers the possibility to use mesophilic bacteria to study HHP adaptation. As lactic acid bacteria lack major routes of stress responses found in other bacteria, e.g., use of alternative sigma factors or SOS response, they are prime candidates to investigate primary “thermodynamic” HHP sensation. Fig. 1 demonstrates for two strains of *L. sanfranciscensis* that studies on cellular responses induced by high pressure are best performed within a pressure range of 30 – 100 MPa. In this range, cells are able to grow and metabolize maltose.

![Fig 1. Survival and metabolic activity upon HHP treatment of *L. sanfranciscensis*. Maltose consumption goes down before the cells die, indicating their sublethal injury.](image)

2.3. HHP responses reflected in the proteome

Many aspects of the cellular response to HHP including metabolic changes, cell membrane composition, transport mechanisms, and acquired stress tolerance and cross resistance to other stresses indicate that changes in the proteome are induced by HHP. It has also been shown for *L. sanfranciscensis* and *L. rhamnosus*, that cross resistance is not inducible in the presence of protein biosynthesis inhibitors [6,7]. The changes in the proteome can be referred to altered and impaired protein biosynthesis on the one side and stress response and adaptation on the other. On the other hand, the definition of HHP specific stress on the proteome level is most obvious upon comparison with other stresses in differential proteomics. Therefore, the stress response of *L. sanfranciscensis* was investigated during starvation in the stationary phase and at various stresses normalized to levels of 10% of its maximum growth rate including HHP (80 MPa), cold (12.5°C), heat (43°C), acid (pH=3.7), salt (1.9%). Fig. 2 provides an overview of pressure sensitive proteins observed in this study.
Nine of them were upregulated (P1 to P9), whereas seven (P10 to P16) appeared to be repressed under these conditions. Their identity was determined by N-terminal sequencing and mass spectrometry. *L. sanfranciscensis* ribokinase (P1) is one of the strongest increased enzymes after exposure to the different stress conditions and thus appears to be useful for the cell to cope with stress. Through the formation of ribose-5-P ribokinase plays a central role in the initiating steps of synthesis of purine and pyrimidine nucleotides and also for the amino acids histidine and tryptophan. Most of the other upregulated proteins were identified as chaperones and proteases, which are found in a variety of other stress responses. The comparison with the proteomes from cells subjected to the other stresses revealed a Clp homolog as the only HHP specific inducible protein. At first glance it appears nearly a contradiction that the overlap of the HHP response with cold- and NaCl-stressed (11 of 16 proteins) and heat stressed cells (10 of 16 proteins) is at the same level. Thus, the HHP specific stress response consists of distinctive subsets of other stress responses.

### 2.3. HHP responses reflected in the transcriptome

The limits of this proteome approach can be seen in the limited number of membrane proteins, which can be solubilized and included in the analysis and in the detection limit which usually does not allow to detect low copy regulatory proteins. Pavlovic et al. [7] performed a transcriptome analysis with a redundancy cleared shot-gun-microarray of *L. sanfranciscensis* allowing the reliable readout of 750 spots. Upon HHP treatment with 45 MPa for 30 min. the intensity of 42 spots were increased, and 6 were decreased. The expression of the most sensitive genes was quantified with real time PCR to confirm the array data. An overview of the identified HHP sensitive genes is given in Table 1.
Table 1. High pressure (45 MPa/30 min) sensitive genes determined by array hybridisation and/or Real-Time-PCR. The induction of Real-Time-PCR analyses of the investigated genes was normalised against the expression of phosphoketolase (AJ586560).

<table>
<thead>
<tr>
<th>gene</th>
<th>Protein</th>
<th>similarity to known genes</th>
<th>induction/repression</th>
<th>accession</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>similarity to known genes</td>
<td>MA 45°</td>
<td>RT 45°</td>
<td>RT 80°</td>
</tr>
<tr>
<td>stress response</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsp60</td>
<td>GroEL</td>
<td>ACQ93GQI (75%)</td>
<td>2.9</td>
<td>2.9±0.8</td>
</tr>
<tr>
<td>ClipL</td>
<td>ATP-dependent Clp Protease</td>
<td>AAD 34338 (55%)</td>
<td>2.1</td>
<td>4.2±0.5</td>
</tr>
<tr>
<td>GaoA</td>
<td>GMP-synthetase</td>
<td>AC0851E2 (66%)</td>
<td>3.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>GyrA</td>
<td>DNA -gyrase, A subunit</td>
<td>ACQ890K3 (65%)</td>
<td>2.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>PpK</td>
<td>Polyphosphokinase</td>
<td>ACQ88YD2 (99%)</td>
<td>2.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ppx</td>
<td>Exopolypophatase</td>
<td>ACQ88YD1 (98%)</td>
<td>2.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>ORF1</td>
<td>hypothetical protein (similar DEAD ATP-dependent RNA-Helicase)</td>
<td>ACQ88Z45 (73%)</td>
<td>3.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>PepO</td>
<td>endopeptidase</td>
<td>AL935254 (60%)</td>
<td>2.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>translation factors and ribosomal proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RyfK</td>
<td>ribosomal protein L11</td>
<td>ACQ88YX0 (82%)</td>
<td>2.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>RyfL</td>
<td>ribosomal protein L6</td>
<td>ACQ88XJ1 (66%)</td>
<td>2.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>rpsB/tsx</td>
<td>and translation elongation factor tsf</td>
<td>ACQ88YJ4 (86%)</td>
<td>2.2</td>
<td>n.d.</td>
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<tr>
<td>Tuf</td>
<td>elongation factor TU</td>
<td>ACQ88MK6 (83%)</td>
<td>2.2</td>
<td>1.9±0.2</td>
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<tr>
<td>FtsA</td>
<td>elongation factor G</td>
<td>ACQ88XJ8 (80%)</td>
<td>-2.5</td>
<td>-3.4±0.0</td>
</tr>
<tr>
<td>LepA</td>
<td>translation elongation factor</td>
<td>NP_785542.1 (65%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PrfB</td>
<td>peptide chain release factor 2</td>
<td>AE037203 (70%)</td>
<td>-2.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>PrfC</td>
<td>peptide chain release factor 3</td>
<td>ACQ880023 (65%)</td>
<td>1.0</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>InfB</td>
<td>translation initiation factor 2</td>
<td>ACQ880023 (334)</td>
<td>n.d.</td>
<td>4.5±3.85</td>
</tr>
<tr>
<td>Hiss</td>
<td>histidyl-tRNA-synthetase</td>
<td>ACQ88VQ7 (45%)</td>
<td>2.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>ORF2</td>
<td>GTPase with unknown function</td>
<td>ACQ86WT7 (96%)</td>
<td>2.9</td>
<td>3.3±0.3</td>
</tr>
<tr>
<td>TRNA-modifying enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TrmA</td>
<td>tRNA-methyl transferase, trmA family</td>
<td>ACQ73E15 (55%)</td>
<td>2.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>GidA</td>
<td>glucose inhibited cell division protein</td>
<td>ACQ88RX6 (80%)</td>
<td>2.3</td>
<td>2.5±0.7</td>
</tr>
<tr>
<td>thf/thrE</td>
<td>tRNA modifying GTPase</td>
<td>ACQ88RX5 (61%)</td>
<td>n.d.</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>TrmA</td>
<td>tRNA-methyl transferase, trmA family</td>
<td>ACQ73E15 (55%)</td>
<td>2.0</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

| a MA: x-fold induction data from microarray assay (45 MPa, 30 min) |
| b RT 45: x-fold induction data from Real-Time-PCR analyses (45 MPa, 30 min) |
| c RT 80: x-fold induction data from Real-Time-PCR analyses (80 MPa, 30 min) |
| d n.d.: not determined |
| e Real-Time-PCRs for infB were poorly reproducible and varied between 2.0-8.3 fold induction |

A significant overlap with the proteome data is observed which helps the interpretation of the data. More than 20% of the HHP sensitive genes were found to encode either translation factors (EF-G, EF-TU), ribosomal proteins (S2, L6, L11), or genes changing translational accuracy or molecular chaperones (GroEL, CplP). This provides strong in vivo evidence that the translational machinery is a major target for HHP and the cell tries to counteract the decrease in translational capacity by i) regulating translational factors, ii) regulation of genes changing translational accuracy and - referring to the ribosome sensor model - iii) inducing stress proteins. The view of the ribosome as a major HHP target observed in this transcriptome analysis with *L. sanfranciscensis* is supported by in vitro data provided by Schwarz and Landau [8,9] and Smith et al. [10] obtained with ribosomes of *E. coli*. Following their results, the inhibition of translation by HHP takes place at aa-tRNA-binding or translocation. A common principle of cold shock and high pressure residing in the decrease of the translational capacity was repeatedly suggested [11-13] and may explain the
apparent paradox of upregulation of heat and cold shock genes upon HHP shock. The picture of the ribosome as a sensor and mediator of the HHP stress response in *L. sanfranciscensis* receives another convincing addition through the overexpression of ssrA encoding tmRNA [14], a molecule which recycles stalled ribosomes and tags truncated proteins. This gene was detected in the analysis of a HHP tolerant mutant.

3. Analysis of a HHP tolerant mutant

HHP can also introduce permanent changes in the genome, which are handed down to the progeny. As with many other cellular functions, the analysis of mutants provides insight into the mechanisms of HHP tolerance and stress response. We have obtained HHP tolerant mutants of *L. sanfranciscensis* upon growth for 25 cycles (including approx 100 generations) at 50 MPa. Compared to the wildtype this strain showed a 2-fold increase in growth when incubated under 50 MPa for 15 hours. An increase was observed in resistance of two log units against lethal pressure conditions (150 MPa). Interestingly, an altered behaviour against antibiotics was recorded primarily for those influencing ribosomal action. In the mutant the basic expression of tmRNA was 3.5 fold higher than in the wild type. A mutation in the regulation of the tmRNA gene leading to increased amounts of tmRNA might help to prevent accumulation of truncated, potentially harmful proteins and making proteolysis more efficient. Thus, the finding of a tmRNA overproducing, barotolerant mutant fits well with a picture of ribosomal sensing of a high pressure stress response in *L. sanfranciscensis* because the tmRNA-directed tag targets the unfinished proteins for proteolysis via the Clp-protease-system [15]. This system was induces upon HHP in on the transcriptome and proteome level.

4. Conclusion

We propose trans-translation and peptide tagging, processes that promote recycling of stalled ribosomes and prevent accumulation of abortively synthesised polypeptides, to be involved in combating HHP damage and conferring barotolerance between 30 – 100 MPa (Fig. 1). In such a model, the ribosome would be the primary target and “sensing” thermodynamic changes induced by pressure, while the expression of specific enzymes, chaperones and stress proteins appear as a secondary step. This is in accordance with the present inability to identify common “pressure sensitive” motifs in “pressure inducible” promoters. A closer look at these mechanisms in deep sea bacteria may help to understand their adaptation to this environment.

Acknowledgements
This work was funded by the Deutsche Forschungsgemeinschaft (DFG) in grants no. VO582/2-5 and VO582/3-1.

5. References


