High pressure-sensitive gene expression in *Lactobacillus sanfranciscensis*

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Abstract

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Lactobacillus sanfranciscensis is a Gram-positive lactic acid bacterium used in food biotechnology. It is necessary to investigate many aspects of a model organism to elucidate mechanisms of stress response, to facilitate preparation, application and performance in food fermentation, to understand mechanisms of inactivation, and to identify novel tools for high pressure biotechnology. To investigate the mechanisms of the complex bacterial response to high pressure we have analyzed changes in the proteome and transcriptome by 2-D electrophoresis, and by microarrays and real time PCR, respectively. More than 16 proteins were found to be differentially expressed upon high pressure stress and were compared to those sensitive to other stresses. Except for one apparently high pressure-specific stress protein, no pressure-specific stress proteins were found, and the proteome response to pressure was found to differ from that induced by other stresses. Selected pressure-sensitive proteins were partially sequenced and their genes were identified by reverse genetics. In a transcriptome analysis of a redundancy cleared shot gun library, about 7% of the genes investigated were found to be affected. Most of them appeared to be up-regulated 2- to 4-fold and these results were confirmed by real time PCR. Gene induction was shown for some genes up-regulated at the proteome level (*clp*L/*gro*EL/*rbs*K), while the response of others to high hydrostatic pressure at the transcriptome level seemed to differ from that observed at the proteome level. The up-regulation of selected genes supports the view that the cell tries to compensate for pressure-induced impairment of translation and membrane transport.

Introduction

High hydrostatic pressure (HHP) exerts manifold effects on cells and microorganisms, leading to adaptation, stress response and cell death. The possibility of HHP-inducible cell death has stimulated research by food scientists aiming at the improvement of the hygienic safety of minimally processed foods, with the retention of many of their natural properties such as color, vitamin content and "freshness". The behavior of vegetative cells and bacterial endospores has been studied in HHP treatment of material ranging from simple cell suspensions to complex food matrices. Whereas vegetative cells

can be inactivated by HHP, the effective inactivation of bacterial endospores is only achieved in combined pressure/temperature treatments (1-5).

The inactivation kinetics of microorganisms by HHP follows a constantly declining curve which may end in a "resistant" fraction. It is currently unclear whether this should be referred to a sub-population. The survival of vegetative cells is strongly dependent on the food matrix (4,6-9). Sublethally injured cells may loose their resistance to adverse environmental conditions, e.g., low pH or the presence of osmolites (6,7,10-12).

More recently, efforts have been made to elucidate the molecular mechanisms of HHP-induced cellular effects. It has been shown that HHP affects all levels of cellular physiology targeting cellular organization, transcription, translation, protein conformation, enzyme activity, and membrane function (12-15). This knowledge has permitted preliminary interpretations of the mechanisms of HHP-induced cell death and stress response and has raised the possibility of using the high pressure response of cells and microorganisms as a novel approach to study biological systems and use cells as factories in HHP biotechnology.

In recent investigations, we have used lactic acid bacteria as Gram-positive model organisms applied to food biotechnology to study HHP-induced inactivation kinetics, mechanisms of stress response, and sublethal injury followed by cell death (16).

It was shown that above a threshold level the cell membrane changes its fluidity and permeability and most likely its hydration (9). The proton gradient is no longer maintained and membrane proteins lose their function (11). As a result, the intracellular pH adopts the extracellular level and cells may die as a result of adverse environmental conditions. Furthermore, solvents showed protective as well as antagonistic effects with respect to the barophysiology of the membrane.

In the present study, proteome and transcriptome analyses were used to gain insight into the global cellular mechanisms of the response to HHP stress.

Material and Methods

Proteome analyses

For the proteome analyses *Lactobacillus sanfranciscensis* DSM 20451^T was grown at 30ºC in mMRS medium, pH 6.1 (17), and cells were harvested from the exponential growth phase. HHP treatment was carried out in fresh medium for 60 min at 30ºC and at pressures of 80 MPa at 30 MPa/min. Under these conditions, the maximal growth rate was reduced to 1/10. Protein extraction and 2-D electrophoresis were carried out as described by Drews et al. (18) using pH gradients of pH 3-10, 4-7, 4.5-5.5, and 5.5-6.5.

Protein analyses and sequencing were done by MALDI-MS and N-terminal sequencing by TopLab (Munich, Germany). Genes were identified by reversed genetics using degenerated primers deduced from the peptide fragments.

Transcriptome analyses and real-time PCR

L. sanfranciscensis genomic DNA was isolated by the method of Lewington et al. (19). All of the DNA manipulations in this study were performed according to standard procedures (20). For the transcriptome analyses shot-gun libraries from genomic DNA of *L. sanfranciscensis* DSM 20451 were prepared using *E. coli* DH5α, *E. cloni*™ and the plasmid psmart-HCAmp (Lucigen, Middleton, UK) with partially digested (*Bsu*RI, *Alu*I) or sonicated DNA of *L. sanfranciscensis* DSM 20451^T. Redundancy of the libraries was checked by random sequencing of 100 inserts and the inserts of 2000 clones were amplified by PCR and spotted onto silylated microscope slides to form the array. Array

hybridizations were performed as described by Huang et al. (21) with cDNAs labeled with Alexa Flour®555 or Alexa Flour®647 from untreated and pressurized cells, respectively. The pressure treatment was carried out for 30 min at 45 MPa at 30ºC at 200 MPa/min. All experiments were done in triplicate. A more than two-fold change in signal intensity was considered to be significant. Selected pressure-sensitive genes were (partially) sequenced and their expression was verified by real-time PCR in the Light Cycler Instrument (Roche Molecular Biochemicals, Mannheim, Germany) with the QuantiTect™ SYBR® Green kit (Qiagen, Hilden, Germany). Real-time PCRs were performed according to the instructions of the SYBR® Green manufacturer. All expression ratios were normalized against the reference gene, phosphoketolase.

Results

Proteome analysis

Five independent experiments repeatedly showed that the synthesis of 16 proteins (P1-P16) was affected by high pressure treatment (Figure 1). The expression of 9 of the 16 proteins showed an increase of 2- to 12-fold while the expression of 7 proteins decreased by more than 50%. The sequences of four internal peptides of these proteins could be determined (data not shown). Comparison of these sequences with the BLAST databases at the National Center for Biotechnology Information revealed that the two most increased proteins P1 and P2 showed strong homology to ribokinase and an ATP-dependent protease, ClpL. Figure 2 shows the differential expression of these proteins as determined by 2-D gel electrophoresis. The peptide sequences of proteins P9, P13 and P16 showed homologies to the general stress protein GroEL, also known as HSP60, to elongation factor Tu, and to glyceraldehyde-3-phosphate dehydrogenase, respec-

tively. The genes of these proteins were characterized by reversed genetics, and their function was confirmed by strong homologies to the respective genes present in databases. Protein P1 consists of 540 amino acids encoded by 1620 bp with a deduced molecular weight of 58 kDa and is therefore much larger than any other known ribokinase. Further database searches revealed that the first 308 amino acids show homology to ribokinase and specific conserved regions, whereas the latter 232 amino acids show strong homology to ribose-5-phosphate-isomerase. Thus, this enzyme appears to
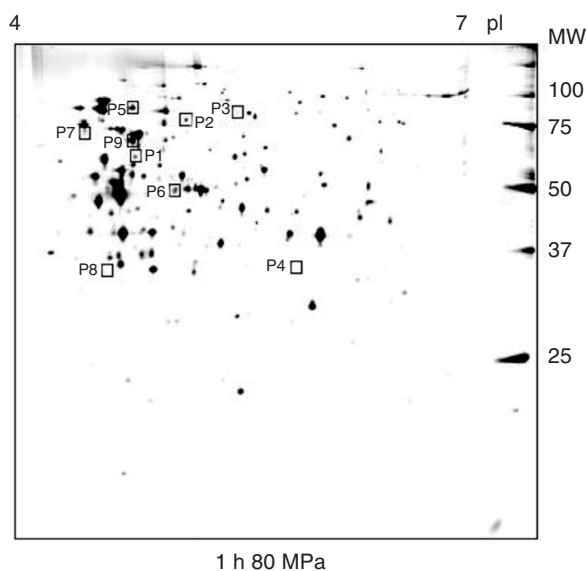

1 h 80 MPa

Figure 1. High pressure-inducible proteins in *Lactobacillus sanfranciscensis* as determined by 2-D gel electrophoresis. Cells were treated for 1 h at 80 MPa and 30ºC. The squares and numbers indicate upregulated proteins. MW = molecular weight.
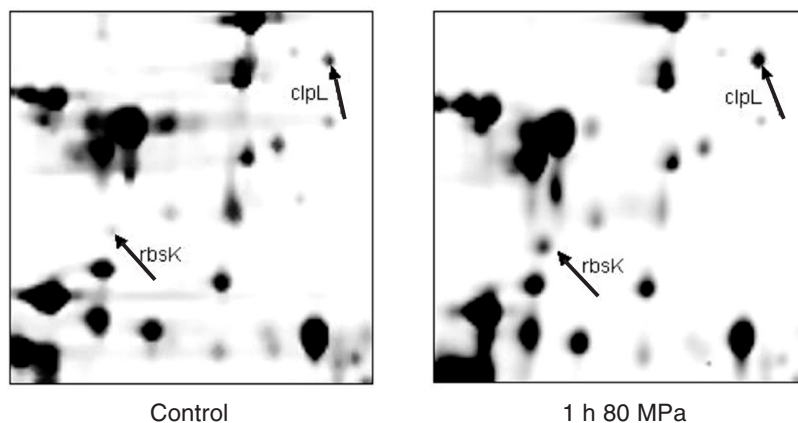

Control

1 h 80 MPa

Figure 2. High pressure induction of rbsK/rbki and clpL. Cells were treated for 1 h at 80 MPa and 30ºC.

combine the function of two enzymes in its two domains.

Transcriptome analysis

Changes in the transcriptional profile upon high pressure treatment were determined using DNA microarrays. Among the 750 spots that passed spot quality analysis, the mRNA level of genes or operons was affected in 48. About 6% of the genes evaluated were more than two-fold induced, while 1% were more than two-fold repressed. The gene encoding phosphoketolase (*xpk*) was not affected by high pressure and was therefore used as reference in the real-time verification of the transcriptome analyses. Real-time PCR results clearly supported the microarray data. A total of 48 high pressure-affected genes were classified with respect to their cellular function. As listed in Table 1, high pressure-affected genes of various cellular functions, namely protein synthesis and fate, stress response and cellular transport. Still, the function of 16 of the identified high pressure-sensitive genes remains unknown.

The most upregulated gene was an uncharacterized ORF with a conserved DEAD-ATP-dependent helicase motif (3.9-fold induced). DEAD helicases play overlapping roles in several processes including remodeling of RNA structures, biogenesis of the ribosome, and stabilization of mRNA (22,23). This gene was followed by *gro*EL involved in heat shock, and a GTPase of unknown function (2.9-fold induced). An application-based array with 76 selected target genes including those involved in translation was designed to control the expression ratio in more detail. The translation elongation factors *tuf* and *tsf*, both responsible for binding of aminoacyl-tRNA to the ribosomal A site, were induced, while translation factors involved in later steps of protein synthesis were either downregulated (*fusA* and *prfB*) or not affected (*prfC*). In addition, transcription of genes whose products modify tRNA-synthetases (*trmA*, *gidA* and *thd)* and some ribosomal proteins was induced. tRNA-synthetases were hardly affected.

Discussion

In contrast to piezophilic microorganisms, bacteria adapted to atmospheric pressure do not have a specific HHP response. This is mainly due to the fact that HHP does not cause stress to piezophilic bacteria at the level present in their deep sea environment and in the experiments in which they have been studied so far. While HHP stress is probably unknown to lactobacilli, the cellular response appears to be targeted rather than confused, aiming at the compensation of pressure-induced defects. Some of these major defects appear to result from the general effects of high pressure, which at a level of up to 80 MPa promotes dissociation and conformation of macromolecules (24). The results of both the proteome and transcriptome analyses of the present study support this view.

The HHP response of *L. sanfranciscensis* differs from that observed at the proteome level but still overlaps with it. In the pres-

Table 1. Functional classification of high pressure (45 MPa/30 min)-induced and repressed genes, respectively.

Cellular function	Number of high pressure-affected genes
Unknown function	16
Protein synthesis and fate	11
Central intermediary metabolism	5
Transport	4
Cellular responses	3
Energy metabolism	3
Cell envelope	2
DNA and purine/pyrimidine metabolism	2
Amino acid biosynthesis	1
Fatty acid biosynthesis	1

The classification is based on the COGs functional annotation database available at (http://www.ncbi.nlm.nih.gov/COG/old/palox.cgi?fun=all). COGs = clusters of orthologous groups of proteins.

ence of chloramphenicol no changes in the barotolerance and cross-resistance to other stresses were observed upon pressure treatment, indicating that *de novo* protein biosynthesis takes place in cells under sublethal pressure stress. The relative amount of mRNA of numerous genes is subject to change upon high pressure stress, which may result from selective transcription and also from mRNA stability. Typical HHP stress overlaps between proteome and transcriptome include induction of some general stress proteins (GroEL or ClpL) which may act as proteases or as molecular chaperones (25).

A more general interpretation of the cellular HHP response may be obtained from the transcriptome rather than the proteome analyses. This is due to the fact that the proteome analysis hardly includes low abundance regulatory proteins whose expression is rather determined by the highly sensitive real-time PCR. Assuming that the cell tries to compensate for pressure-induced inhibition or impairment of vital functions, differential expression of genes permits reaching conclusions about the affected functions *in vivo*. From these analyses it may be concluded that high pressure counteracts membrane damage and transport as well as translation to the ribosome. These conclusions are supported by the demonstration that translation is highly pressure sensitive *in vitro* and *in vivo* as a result of ribosome dissociation (26) and decreased binding of aminoacyl t-RNAs (27) due to changes in ribosome conformation (24,28), respectively.

These results support the idea that reduced ribosomal function may occupy a central position in the response to high pressure. A ribosomal sensor model implies that the physical state of the ribosome is a signal linking the stimulus (in this case high pressure) and the increased expression of a certain set of stress genes. In comparison, changes in translational capacity have also been reported to elicit cold and heat shock responses (29). Thus, such a model would explain the "links" between the response to various stresses. The strong response of a GTPase of unknown function further supports this model. GTPases are considered to be necessary for ribosome function and for transmission of information from the ribosome to specific targets to trigger specific cellular responses (30).

Apart from the effects triggered via changes in ribosomal structure and function, it has also been shown that pressure strongly affects membrane fluidity and transport (9). In the transcriptome this appears to be reflected by increased levels of proteins involved in transport.

In lactic acid bacteria the HHP stress response at first glance appears to be different from that observed with *Escherichia coli*, in which it is similar to heat shock (31) and may induce an SOS response (32). At the proteome level HHP stress was compared to other stresses including pH, temperature, cold shock and stationary phase, and overlaps were found with these stress responses (data not shown). On the basis of the results obtained with other organisms, it can be assumed that this is the case at the transcriptome level as well (33,34). This may be due to the lack in lactic acid bacteria of alternative sigma factors as they are found as global regulators in *E. coli* and some gram positives. While organisms employing global regulators are prone to react to stress in a stereotypical manner, the lack of these regulators may permit lactic acid bacteria to adapt to a variety of stresses in different environments.

References

1. Heinz V & Knorr D (1996). High pressure inactivation kinetics of *Bacillus subtilis* cells by a three-state-model considering distributed resistance mechanisms. *Food Biotechnology*, 10: 149-161.

2. Margosch D, Gänzle M, Ehrmann MA et al. (2004). Pressure inactivation of *Bacillus* endospores. *Applied and Environmental Microbiology*, 70: 7321-7328.

3. Reddy NR, Solomon HM, Fingerhut GA et al. (1999). Inactivation of *Clostridium botulinum* type E spores by high pressure processing. *Journal of Food Safety*, 19: 277-288.

4. San Martin MF, Barbosa-Cánovas GV & Swanson BG (2002). Food processing by high hydrostatic pressure. *CRC Critical Reviews in Food Science and Nutrition*, 42: 627-645.

5. Wuytack EY, Boven S & Michiels CW (1998). Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. *Applied and Environmental Microbiology*, 64: 3220-3224.

6. Garcia-Graells C, Hauben KJA & Michiels CS (1998). High pressure inactivation and sublethal injury of pressure-resistant *Escherichia coli* mutants in fruit juices. *Applied and Environmental Microbiology*, 64: 1566-1568.

7. Smelt JPPM, Hellemons JC, Wouters PC et al. (2002). Physiological and mathematical aspects in setting criteria for decontamination of foods by physical means. *International Journal of Food Microbiology*, 78: 57-77.

8. Ulmer HM, Gänzle MG & Vogel RF (2000). Effects of high pressure on survival and metabolic activity of *Lactobacillus plantarum. Applied and Environmental Microbiology*, 66: 3966-3973.

9. Ulmer HM, Herberhold H, Fahsel S et al. (2002). Effects of pressure induced membrane phase transitions on HorA inactivation in *Lactobacillus plantarum. Applied and Environmental Microbiology*, 68: 1088-1095.

10. Molina-Gutierrez A, Rademacher B, Gänzle MG et al. (2002). Effect of sucrose and sodium chloride on the survival and metabolic activity of *Lactococcus lactis* under high-pressure conditions. In: Hayashi R (Editor), *Trends in High Pressure Bioscience and Biotechnology*. Elsevier Science B.V*., Amsterdam, The Netherlands, 295-302.

11. Molina-Gutierrez A, Stippl V, Delgado A et al. (2002). Effect of pH on pressure inactivation and intracellular pH of *Lactococcus lactis* and *Lactobacillus plantarum. Applied and Environmental Microbiology*, 68: 4399-4406.

12. Wouters PC, Glaasker E & Smelt JPPM (1998). Effects of high pressure on inactivation kinetics and events related to proton efflux in *Lactobacillus plantarum. Applied and Environmental Microbiology*, 64: 509-514.

13. Kato C, Nakasone K, Qureshi MH et al. (2000). How do deep-sea microorganisms respond to the environmental pressure? In: Storey KB & Storey JM (Editors), *Cell and Molecular Response to Stress*. Vol. 1. *Environmental Stressors and Gene Responses.* Elsevier Science B.V., Amsterdam, The Netherlands, 277-291.

14. Abe F, Kato C & Horikoshi K (1999). Pressure-regulated metabolism in microorganisms. *Trends in Microbiology*, 7: 447-453.

15. Elo MA, Karjalainen HM, Sironen RK et al. (2004). High hydrostatic pressure inhibits the biosynthesis of eukaryotic elongation factor-2. *Journal of Cellular Biochemistry*, 94: 497-507.

16. Scheyhing CH, Hörmann S, Ehrmann MA et al. (2004). Barotolerance is inducible by preincubation under hydrostatic pressure, cold-, osmotic- and acid-stress conditions in *Lactobacillus sanfranciscensis* DSM 20451^T. *Journal of Applied Microbiology*, 39: 284-289.

17. Stolz P, Böcker G, Hammes WP et al. (1995). Utilization of electron acceptors by lactobacilli isolated from sourdough. I. *Lactobacillus sanfrancisco. Zeitschrift für Lebensmittel Untersuchung und Forschung*, 201: 91-96.

18. Drews O, Weiss W, Reil G et al. (2002). High pressure effects stepwise altered protein expression in *Lactobacillus sanfranciscensis*. *Proteomics*, 2: 765-774.

19. Lewington J, Greenaway SP & Spillane GJ (1987). Rapid small scale preparation of bacterial genomic DNA, suitable for cloning and hybridization analysis. *Letters in Applied Microbiology*, 5: 51-53.

20. Sambrook J, Fritsch EF & Maniatis T (1989). *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

21. Huang X, von Rad U & Durner J (2002). Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in *Arabidopsis* suspension cells. *Planta*, 215: 914-923.

22. de la Cruz J, Kressler D & Linder P (1999). Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box proteins and related families. *Trends in Biochemical Sciences*, 24: 192-198.

23. Tanner NK & Linder P (2001). DEAD/H box RNA helicases: from generic motors to specific dissociation functions. *Molecular Cell*, 8: 251-262.

24. Niven GW, Miles CA & Mackey BM (1999). The effects of hydrostatic pressure on ribosome conformation in *Escherichia coli*: an *in vivo* study using differential scanning calorimetry. *Microbiology*, 145: 419-425.

25. Beltramo C, Grandvalet C, Pierre F et al. (2004). Evidence for multiple levels of regulation of *Oenococcus oeni clpP-clpL* locus expression in response to stress. *Journal of Bacteriology*, 186: 2200-2205.

26. Schulz E, Lüdemann H-D & Jaenicke R (1976). High pressure equilibrium studies on the dissociation-association of *E. coli* ribosomes. *FEBS Letters*, 64: 40-42.

27. Schwarz JR & Landau JV (1972). Inhibition of cell-free protein synthesis by hydrostatic pressure. *Journal of Bacteriology*, 112: 1222-1227.

28. Gross M & Jaenicke R (1990). Pressure-induced dissociation of tight couple ribosomes. *FEBS Letters*, 267: 239-241.

29. VanBogelen RA & Neidhardt FC (1990). Ribosomes as sensors of heat and cold shock in *Escherichia coli. Proceedings of the National Academy of Sciences, USA*, 87: 5589-5593.

30. Caldon CE, Yoong P & March PE (2001). Evolution of a molecular switch: universal bacterial GTPases regulate ribosome function. *Molecular Microbiology*, 41: 289-297.

31. Aertsen A, Vanoirbeek K, De Spiegeleer P et al. (2004). Heat shock protein-mediated resistance to high hydrostatic pressure in *Escherichia coli. Applied and Environmental Microbiology*, 70: 2660-2666.

32. Aertsen A, Van Houdt R, Vanoirbeek K et al. (2004). An SOS response induced by high pressure in *Escherichia coli. Journal of Bacteriology*, 186: 6133-6141.

33. Van de Guchte M, Serror P, Chervaux C et al. (2002). Stress response in lactic acid bacteria. *Antonie van Leeuwenhoek*, 82: 187-216.

34. Duwat P, Ehrlich SD & Gruss A (1999). Effects of metabolic flux on stress response pathways in *Lactococcus lactis. Molecular Microbiology*, 31: 845-858.