Characterisation of a Piezotolerant Mutant of *Lactobacillus sanfranciscensis*

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*Dedicated to Professor Gérard Demazeau on the occasion of his 65*th *birthday*

Incubation under sublethal high pressure (50 MPa) allowed the isolation of a piezotolerant mutant of *Lactobacillus sanfranciscensis*. Compared to the wild type this strain showed faster growth at 50 MPa and an altered temperature-dependent growth at ambient pressure. Additionally, an altered antibiotic resistance pattern was detected. To address the molecular basis of the mutation the genotypic characterisation was focused on alterations of ribosomal components. Northern analysis using *ssrA* (transfer mRNA) as probe revealed a constitutive overexpression in the mutant. A 2.2 fold induction after pressure shock and increased pressure sensitivity of a *ssrA*-insertional mutant of *L. sanfranciscensis* indicate the tmRNA as genetic determinant of a piezotolerance response in the wild type. 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 piezotolerance.

Key words: High Pressure, Piezotolerance, *Lactobacillus sanfranciscensis*

Introduction

Hydrostatic pressure affects growth and metabolism of living organisms. Due to its high potential in pasteurising food, research is focused on identifying proper conditions for inactivation of microorganisms and investigation of pressure mediated effects on cellular and subcellular levels [1, 2]. Increased practical applications justify the question whether bacteria can become adapted to high-pressure (HP) conditions in a similar way as it occurs in acquired tolerance against low pH and other stresses [3, 4].

Several authors reported on HP treatments leading to selection of mutants, that showed enhanced ability to grow under HP [5 – 7] and/or were more resistant to HP inactivation [8 – 12]. A regulatory defect in the dihydrolase system of *Streptococcus faecalis* (now: *Enterococcus faecalis*) has been suggested to cause both, acid tolerance and piezotolerance [7]. Hauben *et al.* [9] reported, that the behaviour of some pressure-resistant mutants of *E. coli* partly overlaps with an increase in heat resistance.

Karatzas *et al.* [10, 11] isolated a spontaneous high hydrostatic pressure tolerant mutant of *Listeria monocytogenes* and suggested that codon deletion in the gene of the global class III stress regulator CtsR could be linked to higher pressure resistance of *Listeria monocytogenes*.

Recently, we described the pressure stress response of *Lactobacillus sanfranciscensis*, a Gram-positive bacterium employed in food biotechnology using a DNA array approach [13]. The majority of HP affected genes were found to encode translation factors (EF-G, EF-TU), ribosomal proteins (S2, L6, L11], as well as certain molecular chaperones (GroEL, ClpL). These findings indicate that translation is one of the primary targets of HP. Consequently, alterations within components involved in translation may result in pressure-adapted mutants. On the other hand, mutants in the ribosome and translational machinery are described to exhibit resistance to various antibiotics [14].

A first hint to relate HP to antibiotic resistance came from McMahon and Landau [15]. They proved that a streptomycin resistant mutant of *E. coli* had a more pressure-resistant protein synthesis than the streptomycin sensitive wild type. The rationale for that may be that the molecular mechanism underlying growth inhibition by HP and antibiotics are both acting

on ribosomes. Despite the importance of a pressure-inducible antibiotic resistance one study has dealt with this topic in more detail, recently [16]. Karatzas *et al.* [16] showed that an increased resistance to high hydrostatic pressure coincides with reduced antibiotic resistance in *Staphylococcus aureus*.

Generally, this information can be used to define further molecular targets and mechanisms to optimise HP pasteurisation processes towards hurdle and multitarget preservation technologies. Moreover, the development of piezotolerance and altered antibiotic susceptibility should be considered in high-pressure food processing.

Experimental Section

Bacterial strains and growth conditions

Lactobacillus sanfranciscensis DSM 20451^T was grown at 30 °C in mMRS4 (composition per L: 10 g maltose, 10 g fructose, 10 g peptone from casein, 5 g meat extract, 5 g yeast extract, 4.0 g KH_2PO_4, 2.6 g $K_2HPO_4 \cdot 3H_2O$, 3.0 g NH_4Cl, 1 mL Tween 80, 0.5 g L-Cystein-HCl, pH = 6.2) at 30 °C. 1 mL of separately autoclaved Mg-Mn-stock solution (100 g L^{-1} $MgSO_4 \cdot H_2O$, 50 g L^{-1} $MnSO_4 \cdot 4H_2O$) was added to 1 L of mMRS4 unless otherwise mentioned. *Escherichia coli* DH5α was grown at 37 °C in LB with agitation. Ampicillin (100 mg L^{-1}) was used to select cells harbouring the plasmid pstBlue-1 (Novagen, Darmstadt, Germany) or pBAD*Myc*His (Invitrogen, Karlsruhe, Germany). Tetracycline (15 mg L^{-1}) was used to select for those harbouring p3TET [17].

Construction of an ssrA insertional mutant

A 333bp internal gene fragment of *ssrA* from *L. sanfranciscensis* was amplified using primers ptm_V (TATATGTCGACCTACTTATGCTTTTGGTG) and ptm_R (TATATGTCGAGCTATGTCGCCACCTG), digested with Pst*I* and cloned into the insertional vector p3TET. The resulting plasmid, obtained after transformation of *E. coli* DH5α, was sequenced and introduced into *L. sanfranciscensis* DSM 20451^T [18]. Integrations by single-crossover recombinations within *ssrA* in tetracycline-resistant colonies (20 μg mL^{-1}) were verified by PCR and Southern Blot hybridisation (data not shown).

High-pressure treatment

For adaptation of *L. sanfranciscensis*, an overnight culture was grown at ambient pressure at 30 °C to OD_{590nm} 0.3, resuspended in mMRS4, and subjected to 50 MPa at 30 °C. After growth to OD_{590nm} 1.3 – 1.4 cultures were diluted in fresh medium to an OD_{590nm} 0.3 and incubated again at 50 MPa. After 25 growth cycles (one growth cycle is defined as growth from OD_{590nm} 0.3 to OD_{590nm} 1.3 – 1.4) under HP *L. sanfranciscensis* was plated out on mMRS4 to obtain single colonies from which glycerol stocks were prepared. One of these colonies was used for further characterisation and subcultured (0.1 MPa/30 °C) at least twice before each experiment, to ensure that observed differences in the behaviour of the mutant were due to mutations and not adaptations.

For HP inactivation studies *L. sanfranciscensis* was grown to OD_{590nm} 0.5. 2.5 mL were harvested by centrifugation, resuspended in the same volume of mMRS4 and subjected to a pressure between 0.1 and 300 MPa for 30 min at 30 °C. Compression and decompression rates were 200 MPa min^{-1}. Cell counts were determined on mMRS4. The appropriate dilution was plated using a spiral plater (IUL, Germany), and plates were incubated at 30 °C for 48 h under a controlled atmosphere (76 % N_2, 20 % CO_2, 4 % O_2).

For determination of pressure dependent growth *L. sanfranciscensis* was grown to OD_{590nm} 0.5 – 0.7 and washed with mMRS4. OD_{590nm} of either was adjusted to 0.2. Cultures were grown at 30 °C and 0.1 or 50 MPa for 15 h. Cell counts were determined before and after HP treatment.

Antibiotic susceptibility testing

Differences of antibiotic susceptibilities were determined by disk-diffusion assays. *L. sanfranciscensis* wild type and HP mutant were grown overnight to stationary phase, harvested and resuspended in mMRS4 to an optical density of 1.0. 40 μL of the respective cell suspension was plated out on mMRS4 agar containing 20 mL mMRS4 per plate. Disks containing 500 μg spectinomycin or polymyxin, 400 μg kasugamycin, 200 μg streptomycin, 116.2 μg paromomycin, 100 μg puromycin, 50 μg ampicillin, 30 μg amikazin, erythromycin, kanamycin, neomycin, tetracycline, or tobramycin, 25 μg spiramycin or 10 μg gentamycin were placed on the plates, followed by an incubation at 30 °C for 48 h. Antibiotic susceptibility testing was repeated three to five times for each culture. Diameter of inhibition zones and occurrence of spontaneous mutants were monitored.

Sequencing of ssrA and probe-labelling

Universal primers L11 and L12 for PCR amplification of *ssrA* were previously deduced by Schönhuber *et al.* [19]. PCR was performed in a 100 μL reaction containing 1.5 mM $MgCl_2$, 10 μM dNTP, 50 pM primers L11 (ACG GAT TCG ACA GG) and L12 (GGG AGT CGA ACC C) and 2 U Taq polymerase. PCR conditions were 2 min at 94 °C, followed by 32 cycles at 94 °C for 45 s, 43 °C for 45 s and 72 °C for 30 s. PCR products were run on agarose gel, the fragment of corresponding size (390 bp) was cut from the gel, purified using the E.Z.N.A. gel extraction kit (Peqlab Biotechnologie GmbH, Erlangen, Germany). A part of the purified PCR-product was sequenced, the other part was lig-

ated into pstBlue-1 (pSTBlue-1 AccepTor^{TM} Vector Kit, Novagen) and transformed into *E. coli* DH5α. *SsrA*-insert containing plasmids were isolated (E.Z.N.A. Plasmid Miniprep Kit I, Peqlab). This plasmid was used for directly labelling the tmRNA probe by PCR amplification with DIG-dUTPs in the reaction according to the manufacturers instructions (Roche, Mannheim). Labelling of the *xpk* gene with DIG-dUTP and primers xpk_{for} (TCACCAAGATCCAGGTCT) and xpk_{rev} (TTATTTGTTAAGTGGTTCC) was carried out directly from genomic DNA.

RNA isolation and Northern Blot

An overnight culture of *L. sanfranciscensis* was grown to OD_{590nm} 0.35 – 0.4 and subjected to 0.1 or 50 MPa for 30 min. Total RNA was isolated using RNAprotect and the RNeasy Mini kit from Qiagen (Hilden, Germany) according to the manufacturers instructions. The concentration and purity of total RNA were determined by GeneQuant RNA/DNA calculator (Pharmacia/Amersham, Freiburg, Germany). RNAs were adjusted to the same concentrations and checked by agarose gel electrophoresis. Northern Blot analysis was performed with DIG-labelled probes specific for *xpk* and *ssrA*, respectively. Quantification of hybridisation signals was performed by scanning the X-ray film using a calibrated flatbed scanner with a transparency unit. Image Master II (Amersham Biosciences) was utilised for the computer aided quantification of hybridisation signals. The level of *ssrA* expression was normalised against the constitutively expressed reference gene *xpk* [13].

Sequencing of 16S rRNA and other ribosomal genes

L. sanfranciscensis contains at least six operons encoding ribosomal RNAs [20]. A major part of divergencies between the 16S rRNAs of the wild type is known from a shot-gun genome sequencing approach [13]. 16S rDNAs of the HP mutant were amplified with primers 616V and 630R [21]. Pfu-DNA-Polymerase (Promega) was used for amplification according to the manufactorers instructions. One part of the PCR-products was sequenced directly (16S rDNA amplicon), the other one was cloned in *E. coli*. The 16S rDNA inserts of 43 randomly selected clones were partially analysed (position 931-1542, *E. coli* numbering system). This approach ensured a probability of 90 % that 16S rDNA sequences from each rRNA operon were represented at least once. Additionally, the genes encoding for ribosomal proteins S4, S5 and S12 were sequenced from both, the wild type and the HP mutant.

Database accession numbers

The sequences reported in this paper have been deposited in GenBank under accession numbers AJ586560 (*xpk*) and ACDQ089687 (*ssrA*).

Results

Pressure- and temperature-dependent growth of Lactobacillus sanfranciscensis

Lactobacillus sanfranciscensis was subjected to 50 MPa for about 1000 h involving 25 growth cycles. Assuming an average growth rate during these cycles at least 150 doublings would have taken place. Finally, a single colony was isolated for physiological and genetic characterisation (note: At first several single colonies were tested concerning growth at 50 MPa, all

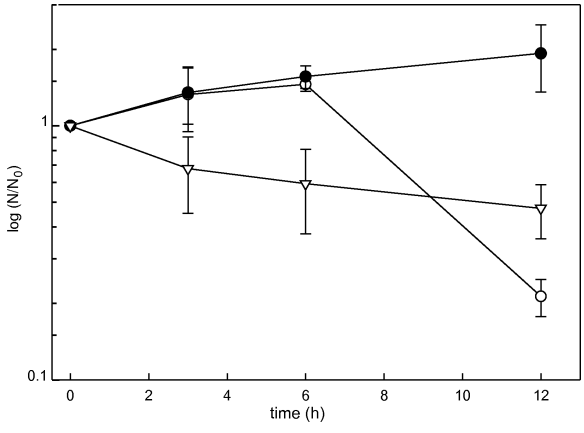

Fig. 1. Growth of *Lactobacillus sanfranciscensis* wild type (○), the high-pressure adapted (●) and the *ssrA*-knockout- (▽) mutant at 50 MPa and 30 °C. Initial cell counts were $1.0 \cdot 10^8 \pm 0.19 \cdot 10^8$. Data show the mean and standard deviations of at least three independent experiments.

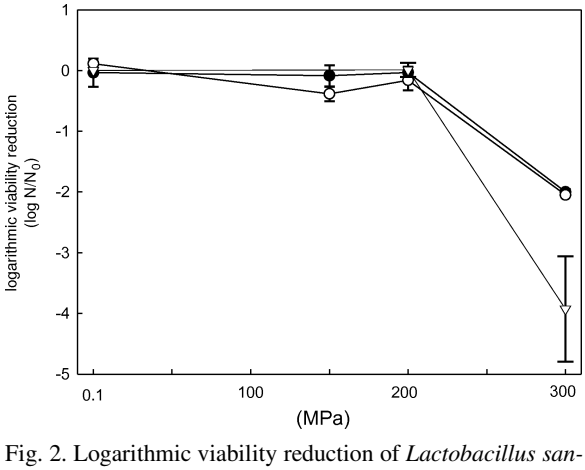

Fig. 2. Logarithmic viability reduction of *Lactobacillus sanfranciscensis* wild type (○), the pressure-adapted (●) and the *ssrA*-knockout- (▽) mutant in mMRS4 after 30 min pressure treatment at 30 °C. Initial cell count was $5.6 \cdot 10^7$ to $1.2 \cdot 10^8$. Data show the mean and standard deviations of at least three independent experiments.

Table 1. Susceptibilities of *Lactobacillus sanfranciscensis* wild type and the pressure-adapted mutant to various ribosomal antibiotics. Data show the mean and standard deviations of at least three independent experiments.

	Zone diameter (mm)[a]		
Antibiotic	Wild type	Mutant	Function affected
decrease in resistance			
Kasugamycin	6.7 ± 0.8	14 ± 0.7	Initiation (fMet-tRNA-binding)
Tetracycline	20.2 ± 0.3	26.2 ± 0.3	aa-tRNA-EF-TU-GTP-binding
Puromycin	20.2 ± 0.3	25.5 ± 1.0	Elongation
Erythromycin	41.0 ± 0.7	46.8 ± 0.9	Elongation
increase in resistance			
Kanamycin	13.0 ± 0.0	11.5 ± 1.0[b]	Translation accuracy
Paromomycin	8.4 ± 0.8	11.9 ± 0.2[b]	Translation accuracy
Tobramycin	15.7 ± 0.4	16.9 ± 1.0[b]	Translation accuracy
Spectinomycin	15.0 ± 0.7	11.4 ± 0.5	Translocation (EF-G-GTP-interaction)
Spiramycin	24.8 ± 0.4	20.5 ± 1.0	Peptide bond formation
Streptomycin	27.8 ± 0.4[c]	25.2 ± 0.4	Translation accuracy
no effect			
Amikacin	20.4 ± 0.5	20.4 ± 0.5	Translation accuracy
Gentamycin	14.0 ± 0.0	15.3 ± 0.6	Translation accuracy
Neomycin	15.0 ± 0.0	15.6 ± 0.5	Translation accuracy

[a] Disk diameter is 6 mm. A value of 6 mm indicates no zone of inhibition; [b] spontaneous resistant colonies occur in the zone of inhibition of the mutant in contrast to that of the wild type; [c] spontaneous resistant colonies occur in the zone of inhibition of the wild type in contrast to that of the mutant.

of them were more piezotolerant than the wild type). After 12 h of incubation at 50 MPa the HP mutant showed enhanced ability to grow at elevated pressures (Fig. 1). Pressure resistance was investigated at pressures ranging from 0.1 to 300 MPa at 30 °C, and inactivation over time was monitored (Fig. 2). Both cultures showed no significant differences in their pressure tolerance in mMRS4.

Altered antibiotic susceptibility pattern

Since pressure is known to affect ribosomal processes, the enhanced piezotolerant growth of the *L. sanfranciscensis* mutant may be due to mutations or differences in regulations of ribosomal processes. Consequently, changes were observed in susceptibilities of the mutant to ribosomal antibiotics (Table 1). Wild type and mutant had different abilities to form spontaneous resistant mutants in case of the aminoglycosides kana-, paromo-, tobra- and streptomycin (Table 1, Fig. 3), for instance.

To ensure that the ancestor *L. sanfranciscensis* population did not contain a subpopulation that already had different antibiotic susceptibilities combined with an enhanced ability to grow under pressure, antibiotic susceptibility testing was also carried out with the *L. sanfranciscensis* after the first HP growth cycle. No differences in antibiotic susceptibilities were observed (data not shown).

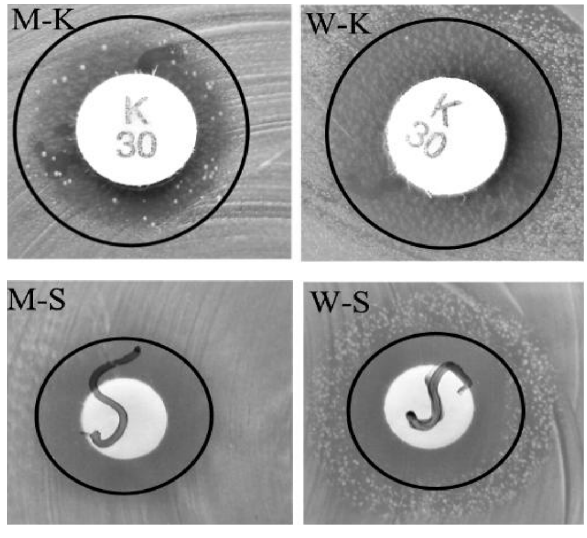

Fig. 3. Kanamycin (M-K and W-K) and streptomycin (M-S and W-S) susceptibilities of *L. sanfranciscensis* wild type (W-K, W-S) and high-pressure mutant (M-K, M-S) on mMRS4-agar. Circles surrounding inhibition zones have identical diameters for the respective antibiotic.

16S rRNA Sequencing

As nucleotide changes in 16S rRNA are described as one of the major reasons for changes in antibiotic susceptibilities, 16S rDNA of the HP mutant was sequenced using two different approaches. Sequence analysis of a 16S rDNA amplicon did not reveal any

mutations when compared to the wild type 16S sequence. As *L. sanfranciscensis* contains at least six rRNA operons [20], a mutation occurring only in one of these operons might not be detectable with this approach. Therefore, the 16S rDNA amplicon was cloned and a representative number of clones were sequenced from base pair 931 to 1542 (*E. coli* numbering system). This fragment corresponds to that part of the 16S rRNA where most nucleotide changes resulting in changed antibiotic resistances was reported up to now [22 – 25]. No differences were observed between sequences of the wild type and the HP mutant (data not shown).

ssrA Expression and characterisation of an ssrA-insertional mutant

As ribosomal processes seem to play a major part in the HP stress response of *L. sanfranciscensis* [13], and no changes in sequences of rRNAs and ribosomal proteins described above were observed, we started to examine expression of genes involved in ribosomal processes on transcriptional level. As deletion of *ssrA* causes changes in susceptibilities for ribosomal antibiotics in other bacterial species [26, 27] and the HP mutant also displayed those changes, *ssrA* expression was analysed.

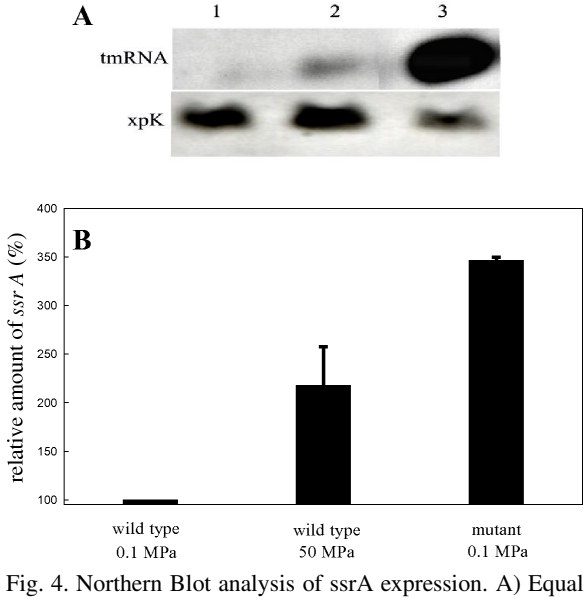

Fig. 4. Northern Blot analysis of ssrA expression. A) Equal amounts of RNA were loaded on the gel, and Northern Blot hybridisations were carried out with tmRNA and *xpk* as probes, lane 1) wild type: 0.1 MPa; lane 2) 50 MPa/30 min; lane 3) high-pressure mutant: 0.1 MPa; B) quantification of *ssrA* expression in relation to *xpk* expression from Northern Blots with Image Master II. Data show the mean of two independent experiments, error bars represent standard errors. The level of *ssrA* expression was normalised against the constitutively expressed reference gene *xpk.*

As shown in Fig. 4, *ssrA* expression was induced (2.2 fold) under HP for the wild type. The mutant showed a more than 3.5 fold increase in *ssrA* expression at atmospheric pressure compared to the wild type at 50 MPa. To estimate whether tmRNA is important for the growth at elevated pressures, an *ssrA* insertional knockout mutant was constructed. This strain showed decreased ability for growth at 50 MPa (Fig. 1) and increased inactivation at 300 MPa (Fig. 2) in comparison to the wild type.

Discussion

The characterisation of the HP mutant revealed differences to the wild type with respect to high-pressure dependent growth, susceptibility to certain antibiotics and regulation of tmRNA expression. These differences can be interpreted as closely interrelated and could be referred to changes in the translational apparatus of the cell. The antibiotic sensitivity of the HP mutant changed against 2/3 of the tested antibiotics described to affect ribosomal functions. The acquired capacity of the HP mutant to form resistant colonies against the aminoglycosides Kana-, Tobra- and Paromomycin points out that HP treatment leads to a mutation that renders the possibility for a second mutation leading to these isolated resistant colonies. Otherwise spontaneous mutants of the wild type occurring within the inhibition zone of streptomycin were detected in the HP mutant that showed *per se* an increased tolerance to streptomycin. The occurrence of these rare additional mutations in the HP mutant in the presence of streptomycin hints to a different type of mutations causing similar effects. A relation of antibiotic susceptibility and piezotolerance remains to be proven.

In the literature five mechanisms resulting in antibiotic resistances are described: a) transport processes, b) modifying enzymes, c) methylation of rRNA and d) nucleotide changes in genes for rRNAs or ribosomal proteins, and e) tmRNA expression [27]. In the case of the HP-mutant, the observed alterations in antibiotic susceptibilities could not be referred to nucleotide changes in 16S rRNA or genes of ribosomal proteins S4, S5 and S12, often involved in *e. g.* streptomycin resistance. To what extent overexpression of *ssrA* was

responsible for changes in antibiotic susceptibilities of the HP mutant could only be shown clearly by overexpression of *ssrA* in the *L. sanfranciscensis* wild type, which failed up to now.

Reports on the occurrence of mutants with enhanced ability to grow under elevated pressure [6 – 7] or to survive HP inactivation [8 – 12] have been published sporadically until now. Only in a few cases their molecular background was characterised. Some mutants showed altered expression of heat shock proteins DnaK, GroEL, GroES, GrpE, ClpB in *E. coli* MG1615, or they overexpressed global regulatory elements, *e. g.* the master stress regulator sigma^S encoded by *rpoS* in *E. coli* O157 : H7 or CtsR in *Listeria monocytogenes* [10, 12]. Thus, overproduction of chaperones and proteases as well as upregulation of their regulators discloses accumulation of truncated and misfolded proteins as the general problem cells are facing during growth under HP. As mature (successfully translated) proteins and enzymes sustain significant damage under moderate pressures (below 150 MPa) the origin of proteinaceous "waste" is rather residing in their incomplete synthesis *via* impaired ribosomal translation. TmRNA may form a connection between both, ribosomal processes and degradation of proteins by ATP-dependent proteases [28].

TmRNA (also known as SsrA or 10Sa RNA) is a small, highly-structured RNA that has been found in bacteria and in chloroplasts and mitochondria of eukaryotes [29]. It intervenes in selected translation reactions to release ribosomes from the mRNAs and targets the nascent polypeptides for proteolysis [29]. It provides a quality control function for translation by recognising stalled ribosomes and targeting the incomplete proteins for degradation before they are released into the cell [28, 30]. *SsrA* is found to be inducible by sublethal HP in the wild type (Fig. 3). By using a proteome and a transcriptome approach to characterise the HP response of *L. sanfranciscensis* remarkable increases were found in ClpL and ClpX [13, 31]. Therefore, 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 at higher pressures and make proteolysis more efficient. Thus, the finding of a tmRNA overproducing piezotolerant HP mutant and the increased HP sensitivity of the *ssrA* insertional mutant agree well with a picture of ribosomal sensing of a HP stress response in *L. sanfranciscensis*, because the tmRNA-directed tag targets the unfinished proteins for proteolysis *via* the Clp-protease-system [32]. However, increased piezosensitivity of the *ssrA* insertional mutant clearly points out that tmRNA is involved in growth at moderately increased pressures and survival at lethal pressures (Figs. 2 and 3).

One reason for the altered susceptibility to ribosomal-acting antibiotics may also be the increased amounts of tmRNA in the mutant. A deletion of the tmRNA gene in a strain of *Synechocystis* sp. leads to changed susceptibilities to various ribosomal antibiotics as compared to the wild type [27]. Additionally, the sensitivities to aminoglycoside antibiotics in other bacteria increased when *ssrA* was deleted [26, 33]. Hence, continuous overexpression of tmRNA should result in increased aminoglycoside resistance, as it is shown for the HP-adapted mutant in case of kana-, tobra-, strepo- and paromomycin (Table 1). Overexpression of tmRNA could also explain increased resistance of spira- and spectinomycin referring to the mode of action of tmRNA as already discussed by Vioque and de la Cruz [33].

The occurrence of mutations in the HP mutant in other genes or regulations except those for tmRNA still has to be examined. However, a connection between high-pressure tolerance and antibiotic resistances was shown. Recently, Karatzas *et al.* isolated high-pressure resistant variants of *S. aureus* after a single high-pressure treatment at 400 MPa for 30 min and showed a connection between increased piezoresistance and decreased antibiotic susceptibility [16]. However, they did not detect any changes at the molecular level that could explain one of the observed changes in the high-pressure variants. The involvement of tmRNA in pressure tolerance as shown for *L. sanfranciscensis* might be the first step in solving the connection between high-pressure stress resistance and antibiotic susceptibility.

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[1] A. Aertsen, C. W. Michiels, *Crit. Rev. Microbiol.* **2004**, *30*, 263 – 273.
[2] D. H. Bartlett, *Biochim. Biophys. Acta* **2002**, *1595*, 367 – 381.
[3] M. De Angelis, L. Bini, V. Pallini, P. S. Cocconcelli, M. Gobbetti, *Microbiology* **2001**, *147*, 1863 – 1873.
[4] Y. Wang, J. Delettre, A. Guillot, G. Corrieu, C. Beal, *Cryobiology* **2005**, *50*, 294 – 307.
[5] F. Abe, K. Horikoshi, *Mol. Cell. Biol.* **2000**, *20*, 8093 – 8102.
[6] K. A. Bidle, D. H. Bartlett, *J. Bacteriol.* **1999**, *181*, 2330 – 2337.
[7] R. E. Marquis, G. R. Bender, *Can. J. Microbiol.* **1980**, *26*, 371 – 376.
[8] S. Funaoka, I. Takagi, Y. Furuyama, S. Morishita, *Okajimas Folia Anat. Jpn.* **1965**, *40*, 833 – 837.
[9] K. J. Hauben, D. H. Bartlett, C. C. Soontjens, K. Cornelis, E. Y. Wuytack, C. W. Michiels, *Appl. Env. Microbiol.* **1997**, *63*, 945 – 950.
[10] K. A Karatzas, H. Bennik, *Appl. Env. Microbiol.* **2002**, *68*, 3183 – 3189.
[11] K. A. Karatzas, J. A. Wouters, C. G. Gahan, C. Hill, T. Abee, H. Bennik, *Mol. Microbiol.* **2003**, *49*, 1227 – 1238.
[12] M. Robey, A. Benito, R. H. Hutson, C. Pascual, S. F. Park, B. M. Mackey, *Appl. Env. Microbiol.* **2001**, *67*, 4901 – 4907.
[13] M. Pavlovic, S. Hörmann, R. F. Vogel, M. A. Ehrmann, *Arch. Microbiol.* **2005**, *184*, 11 – 17.
[14] A. Dessen, A. M. Di Guilmi, T. Vernet, O. Dideberg, *Curr. Drug Targets Infect Disord* **2001**, *1*, 63 – 77.
[15] G. McMahon, J. V. Landau, *J. Bacteriol.* **1982**, *151*, 516 – 520.
[16] K. A. Karatzas, A. Zervos, C. C. Tassou, C. G. Mallidis, T. J. Humphrey, *Appl. Env. Microbiol.* **2007**, *73*, 1873 – 1881.
[17] L. E. Hancock, M. Perego, *J. Bacteriol.* **2004**, *186*, 7951 – 7958.
[18] S. Hörmann, R. F. Vogel, M. Ehrmann, *Appl. Gen. Mol. Biotechnol.* **2006**, *70*, 690 – 697.
[19] W Schönhuber, G. Le Bourhis, J. Tremblay, R. Amann, S. Kulakauskas, *BMC Microbiol.* **2001**, *1*, 20 – 27.
[20] M. A. Ehrmann, R. F. Vogel, *Syst. Appl. Microbiol.* **2001**, *24*, 443 – 450.
[21] M. A. Ehrmann, M. R. A. Müller, R. F. Vogel, *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 7 – 13.
[22] K. L. Triman, A. Peister, R. A. Goel, Expanded versions of the 16S and 23S ribosomal RNA mutation databases (16SMDBexp and 23SMDBexp). *Nucleic Acids Res.* **1998**, *26*, 280 – 284.
[23] K. L. Triman, B. J. Adams, Expansion of the 16S and 23S ribosomal RNA mutation databases (16SMDB and 23SMDB). *Nucleic Acids Res.* **1997**, *25*, 188 – 191.
[24] K. L. Triman, The 16S ribosomal RNA mutation database (16SMDB). *Nucleic Acids Res.* **1996**, *24*, 166 – 168.
[25] K. L. Triman, The 16S ribosomal RNA mutation database. *Nucleic Acids Res.* **1994**, *22*, 3563 – 3565.
[26] T. Abo, K. Ueda, T. Sunohara, K. Ogawa, H. Aiba, *Genes to Cells*, **2002**, *7*, 629 – 638.
[27] J. de la Cruz, A. Vioque, *RNA* **2001**, *7*, 1708 – 1716.
[28] J. Withey, D. Friedman, *J. Bacteriol*, **1999**, *181*, 2148 – 2157.
[29] K. P. Williams, *Nucleic Acids Res.* **2002**, *30*, 179 – 182.
[30] K. C. Keiler, P. R. Waller, R. T. Sauer, *Science* **1996**, *271*, 990 – 993.
[31] S. Hörmann, C. Scheyhing, J. Behr, M. Pavlovic, M. Ehrmann, R. F. Vogel, *Proteomics* **2006**, *6*, 1875 – 1885.
[32] P. W. Haebel, S. Gutmann, N. Ban, *Curr. Opin. Struct. Biol.* **2004**, *14*, 58 – 65.
[33] A. Vioque, J. de la Cruz, *FEMS Microbiol. Lett.* **2003**, *218*, 9 – 14.