Impact of high hydrostatic pressure on bacterial proteostasis

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Abstract

High hydrostatic pressure (HHP) is an important factor that limits microbial growth in deep-sea ecosystems to specifically adapted piezophiles. Furthermore, HHP treatment is used as a novel food preservation technique because of its ability to inactivate pathogenic and spoilage bacteria while minimizing the loss of food quality. Disruption of protein homeostasis (i.e. proteostasis) as a result of HHP-induced conformational changes in ribosomes and proteins has been considered as one of the limiting factors for both microbial growth and survival under HHP conditions. This work therefore reviews the effects of sublethal (≤ 100 MPa) and lethal (> 100 MPa) pressures on protein synthesis, structure, and functionality in bacteria. Furthermore, current understanding on the mechanisms adopted by piezophiles to maintain proteostasis in HHP environments and responses developed by atmospheric-adapted bacteria to protect or restore proteostasis after HHP exposure are discussed.

Keywords: high hydrostatic pressure; *E. coli*; piezophiles; protein translation; protein aggregates; resistance development

1. Introduction

Hydrostatic pressure is an important parameter in the biosphere. Deep-sea environments, incurring hydrostatic pressures up to 110 MPa, are populated by a high diversity of microorganisms, known as piezophiles, which often require these high pressures for optimal growth [1]. By contrast, growth of atmospherically adopted microorganisms, such as the mesophile *Escherichia coli*, progressively becomes compromised with increasing pressure and completely ceases around 50 MPa [2-3]. At exposure to pressures exceeding 100 MPa, microorganisms start suffering lethal injuries, a phenomenon currently exploited in modern food preservation. In fact, high hydrostatic pressure (HHP) processing is considered a promising non-thermal pasteurization approach that inactivates foodborne pathogens and spoilage microorganisms without compromising the nutritional and sensorial properties of the food [4]. Unfortunately, foodborne pathogens such as *E. coli* O157:H7 and *Listeria monocytogenes* can acquire extensive levels of HHP resistance, indicating that pressure adaptation is a readily evolvable trait [5-6].

The influence of HHP on biomolecules is essentially described by the thermodynamic principle of Le Châtelier and Braun, which states that a molecular system will counteract an increase of pressure by occupying a smaller volume [7-8]. In proteins, disruption of hydrophobic and electrostatic interactions, which play a major role in maintaining the quaternary and tertiary structure, are known as the main driving forces in the reduction of protein volume [8-9]. In general, pressures < 200 MPa result in dissociation of oligomeric proteins, while higher pressures cause unfolding of monomeric proteins because of water penetration in internal cavities, typically reaching irreversible unfolding at pressures > 400 MPa [9-10]. In contrast, double stranded

structures of nucleic acids are stabilized under pressure because enhanced stacking of hydrophobic bases [11]. Pressure also induces better packing of the acyl chains within the phospholipid bilayer resulting in lateral shrinking and increased thickness, which causes a fluidity transition from liquid-crystalline to the gel phase [7, 12].

At the integrated level of the growing and living cell, however, the manifold of HHP effects on biomolecules inevitably culminates into pleiotropic cellular defects and phenotypes, compromising DNA replication, transcription, translation, protein functionality and membrane integrity. Since the cellular perception and impact of HHP are to an important extent driven by the effect of HHP on proteins, this review is focused on the effect of sublethal (≤ 100 MPa) and lethal (> 100 MPa) pressures on the synthesis, structure, functionality, and management of proteins in both mesophilic and piezophilic bacteria.

2. Effect of sublethal HHP on bacterial growth

2.1. Cellular impact of sublethal HHP on proteostasis

Dissociation of protein complexes at pressures under 100 MPa may play a decisive role in growth inhibition of mesophilic bacteria, as many of these complexes are involved in essential cellular processes such as replication, transcription, and translation (Table 1). In this context, DNA replication has been shown to be one of the most pressure sensitive processes of macromolecule synthesis [2]. A detailed *in vivo* study on the effect of HHP on DNA, RNA, and protein synthesis was performed by Yayanos and Pollard [2] in *E. coli*, whose cell division is inhibited at 50 MPa accompanied by a characteristic filamentous growth phenotype. These authors showed

that intracellular incorporation of radioactively ^{14}C-labelled thymine at pressures between 50 MPa and 81 MPa only occurred for an initial period and then ceased, suggesting that initiated DNA replication rounds were completed but initiation of new DNA replication rounds was compromised. Although it currently remains unclear how these pressures molecularly preclude the initiation of DNA replication, it was recently shown that destabilization of the clamp loading complex (by deleting the χ or HolC subunit) or disruption of the replication restart primosome (by deleting DnaT or PriA) inhibited growth of *E. coli* already at 30 MPa [3]. Aside from the replisome, divisome functionality has been shown to become compromised as well. In fact, Isshi et al. [13] demonstrated that the septum forming FtsZ polymers of *E. coli* undergo dissociation *in vitro* at 50 MPa, while *E. coli* cells displaying filamentous growth at 50 MPa lacked an FtsZ ring.

Exposure of mesophilic microorganisms to sublethal pressures also progressively impairs the process of protein production at both the transcriptional and translational level. According to the incorporation rate of ^{14}C-labelled uracyl in *E. coli* under pressure, Yayanos and Pollard [2] showed that RNA synthesis became impaired at 21 MPa and was completely inhibited at 77 MPa. In line with these findings, an *in vitro* study on *E. coli* RNA polymerase (RNAP) activity under pressure showed that transcriptional elongation was progressively delayed from 20 MPa upwards and halted at pressures exceeding 80 MPa, although the atmospheric rate of elongation could be completely recovered after decompression [14]. Interestingly, RNAP complexes actively involved in elongation were even able to resume transcription after exposure to 150 MPa for 30 min, whereas 80 % of free RNAP complexes were irreversibly inactivated at such pressure. Aside from structural RNAP effects, however, HHP can also affect gene expression in a regulatory fashion due to its impact on the interaction

between DNA and activator or repressor proteins [15]. As such, the activity of the *E. coli lac* operon promoter was shown to increase 78-fold at 30 MPa in the absence of the inducer isopropyl-beta-D-thiogalactopyranoside (IPTG) [16], presumably because of the dissociation of the tetrameric LacI repressor protein from the *lac* operator [17-18].

In turn, protein translation is completely inhibited in *E. coli* at pressures of 60–70 MPa, although it can be rapidly resumed after decompression [2, 19]. This inhibition has been linked *in vitro* and *in vivo* to ribosome subunit disassembly upon exposure to sublethal pressures [19-20]. Under high pressure, the association-dissociation equilibrium of the voluminous multimeric 70S complex shifts to the corresponding 30S and 50S constituents [21]. Furthermore, an *in vitro* study has suggested that the post-translocational complex is the most HHP sensitive intermediate of the translation elongation cycle [20].

A number of membrane proteins have been found to suffer from < 100 MPa exposure as well, although it remains unclear to which extent this can be attributed to HHP-mediated changes in lipid bilayer fluidity [22-23]. For instance, the proton translocating activity of the F0F1-ATPase of *Streptococcus faecalis* is arrested at 50 MPa [24], which may lead to ATP depletion and disruption of pH homeostasis [25-26]. Furthermore, the dissociation of the transmembrane dimer ToxR, which initiates the signalling cascade for transcription of *Vibrio cholerae* toxins, is induced at 20-50 MPa [27], likely because of conformational changes in the ToxR protein itself and independently of the lipid membrane state [23]. In this context, it is also noteworthy that the ToxR homologue of the deep-sea bacterium *Photobacterium profundum* is involved in the signalling of the pressure adaptation response [28].

In contrast to the previous effects that mainly stem from a compromised protein functionality, the activity and specificity of some enzymes can be directly modulated by pressures low enough to maintain their stability. Enzymatic activity under pressure can be favoured by a negative volume change associated with the chemical reaction and by partial substrate unfolding allowing a better enzymatic access [29]. For example, an *in vitro* assay demonstrated that the hydrolysis rate of β-lactoglobulin by the thermolysin protease produced by *Bacillus thermoproteolyticus* increased by 22-fold with pressure upshift from 0.1 MPa to 200 MPa, while it decreased at pressures above 300 MPa because of enzyme conformational changes [29-30]. Furthermore, thermolysin activity on non-specific protein substrates, such as alcohol dehydrogenase and haemoglobin, was accelerated by pressure increase up to 200 MPa, which was attributed not only to the pressure effect on the reaction equilibrium but also to partial substrate unfolding [29, 31].

Enhanced enzymatic activity upon pressurization has also been reported *in vivo* in the case of the enigmatic endogenous Type IV restriction endonuclease Mrr of *E. coli* K-12. Mrr-mediated restriction of the host chromosome and concomitant induction of the DNA damage (SOS) response was originally observed upon the heterologous expression of certain foreign methyl transferases in *E. coli* K-12 [32-33]. Strangely, however, exposure of this strain to a HHP shock of approximately 100 MPa appears to trigger Mrr activity even in the absence of foreign methyl transferases, in turn generating double strand breaks in the host chromosome that result in a RecBCD-dependent activation of RecA and the concomitant derepression of the SOS response [34]. Indeed, in contrast to the role of FtsZ ring dissociation in filamentous growth at 50 MPa [13], filamentation of *E. coli* K-12 after transient exposure to 75–100 MPa has been attributed to Mrr activation [35-36], since SOS-dependent SulA expression inhibits

FtsZ ring formation. Study of the cellular localization dynamics of Mrr translationally fused to the green fluorescent protein (GFP; yielding GFP-Mrr) in *E. coli* K-12 showed that under normal growth conditions (at atmospheric pressure), GFP-Mrr appears to be organized in several nucleoid associated foci without causing DNA damage [37]. Upon a short (15 min) exposure to 100 MPa, however, GFP-Mrr restriction activity causes nucleoid condensation with the transient assembly of Mrr foci at the middle of the nucleoid. Subsequently, ca. 30–60 min after the HHP shock, the centrally assembled GFP-Mrr progressively disperses in the cytoplasm, followed by loss of cellular integrity.

2.2. Adaptation of mesophilic bacteria to sublethal HHP

Transcriptomic and proteomic studies have evidenced that ribosome disruption and inhibition of translation is a critical aspect of growth arrest and survival of mesophilic bacteria under pressure. *E. coli* and *Lactobacillus sanfranciscensis* have shown to primarily respond to sublethal HHP shock by strong upregulation of rRNA genes, ribosomal proteins, and translation-associated proteins [38-39]. Moreover, growing of *L. sanfranciscensis* at 50 MPa for 25 generations allowed for the isolation of a mutant strain with enhanced ability to grow under pressure and cross-resistance to ribosome-targeting antibiotics, which presented upregulated expression of SsrA in comparison to its parent [40]. SsrA (also known as tmRNA) is a small, highly-structured RNA that controls protein synthesis and recycles stalled ribosomes [41], and disruption of the *ssrA* gene impaired growth (50 MPa) and even survival (300 MPa) under pressure [40].

In addition, exposure of *E. coli* and *L. sanfranciscensis* to sublethal HHP shock strongly upregulates the expression of various heat shock proteins (HSPs) [38-39, 42]. This set of proteins consist of chaperones (such as DnaK/J, GroEL/S, and IbpA/B) that assist in the correct folding and refolding of unfolded proteins, and proteases (such as Lon, FtsH, and Clp protease family) that degrade misfolded proteins [reviewed in 43-44]. While basal expression of HSPs serves as a protein quality control system under physiological conditions, proteotoxic stresses tend to increase HSP expression in order to maintain protein homeostasis [43-44]. In *E. coli* W3110, the largest expression of HSPs is reached approximately 60 to 90 min after pressure upshift from 0.1 MPa to 55 MPa and then it diminishes with time, although DnaK levels maintain elevated after 180 to 210 min exposures [38]. However, transcription of heat shock genes tends to decrease in cells growing at 30 MPa and 50 MPa in early (3.5 h and 5 h of incubation, respectively) and late exponential phase (9 h and 11 h of incubation, respectively) compared to those growing at atmospheric pressure, suggesting that heat shock response is re-adjusted upon long-term HHP exposure [45]. Recently, Sato et al. [46] reported that mRNA levels of HSP genes (*groES, dnaK, dnaJ, grpE, lon*, and their positive transcriptional factor *rpoH*) after 12 h of incubation were lower in cells grown at 30 MPa and 50 MPa than those grown at atmospheric pressure, while HSP protein levels did not change. The mechanisms underlying the induction of the heat shock response after sublethal HHP exposure remains unclear, but it has been suggested that stalled ribosomes and the subsequent formation of truncated proteins could already function as a trigger for this response, since a pressure of 50 MPa might be too low to cause protein unfolding [42].

2.3. Adaptation of piezophilic microorganisms to sublethal HHP

As piezophiles depend on functional proteins and protein complexes for their growth at HHP, these organisms have found ways to stabilize protein structure and functionality at their optimum growth pressure. While the replisome and divisome of piezophiles have hardly been studied, transcriptional activity of piezophiles was documented to be more pressure resistant than that of mesophilic bacteria. Indeed, while the cell-free RNAP of *E. coli* loses ca. 60 % of its atmospheric activity after exposure to 100 MPa (30 min), that of the deep-sea bacterium *Shewanella violacea* fully retains its activity [47]. Moreover, electrophoresis under HHP conditions (140 MPa) causes dissociation of the *E. coli* but not the *S. violacea* RNAP complex. In contrast, the activity of *S. violacea* RNAP seems to be more heat sensitive (50ºC) than that of *E. coli* [48].

Protein synthesis is suggested as one of the most limiting factors for deep-sea living, and piezophiles have adopted different strategies to maintain an appropriate number of functional ribosomes. In general, bacterial piezophiles contain a higher copy number of rRNA operons per chromosome than mesophiles. For instance, the genome of *Photobacterium profundum* SS9 includes 15 ribosomal operons [49], the maximum number so far reported in a bacterial genome, while *E. coli* and *Salmonella enterica* serovar Typhimurium each have seven copies per genome [50]. However, increasing the number of ribosomal subunits cannot be sufficient to maintain protein translation under conditions where ribosome functionally is compromised by HHP, and therefore piezophiles have evolved structural adaptations in their ribosomes as well [51]. As such, the appearance of elongated helices in 16S rRNA sequences when compared to the reference *E. coli* structure seems to be specific for piezofilic bacteria, and these

structures are believed to reinforce interactions among ribosome constituents [52]. The number and type of elongations present in the 16S subunits varies among rRNA operons within each species. In *P. profundum* SS9, five 16S ribotypes are identified, which are all constitutively expressed regardless of the pressure at which cells grow.

Although insights into the structural adaptations of piezophilic proteins are still rather scarce, genomic-based comparisons conducted so far in the amino acid content between orthologous proteins from piezophilic microorganisms and their mesophilic counterparts (i.e. with the same range of optimal growth temperature) indicate that piezophilic proteins tend to contain an increased number of amino acids with higher polarity and smaller size and a decreased number of hydrophobic residues [53-54]. Furthermore, studies *in vitro* focusing on the structural and functional differences in certain proteins isolated from piezophiles and their mesophilic homologues have reported the same tendency in amino acid changes. As such, it was demonstrated that the increasing optimum pressure for the activity of the single-stranded-DNA-binding protein (SSB) in piezosensitive, piezotolerant, and obligately piezophilic species of *Shewanella* correlates with a lower volume change upon pressurization and a reduction in glycine and proline content in the central part of the protein [55].

It should be noted that several proteins of piezophiles are not *per se* intrinsically piezotolerant, and the preservation of their functionality under HHP may not only depend on structural adaptations, but also or even exclusively on the cytoplasmic environment created by the cell, which might be complemented with chaperones or osmolytes [56]. For instance, no evident structural or functional adaptation to pressure was found *in vitro* in the dihydrofolate reductase (DHFR) isolated from *Moritella profunda* in comparison to that of *E. coli* [57].

The intracellular accumulation of specific osmolytes in response to pressure upshift (also referred to as "piezolytes") has been regarded as a strategy of piezophiles to protect protein structure [58]. *P. profundum* accumulates a higher concentration of intracellular solutes, preferentially β-hydroxybutyrate and its oligomers, when growing under HHP (28 MPa) than at atmospheric pressure [58]. Furthermore, *Desulfovibrio hydrothermalis* increases the intracellular concentration of glutamate when pressure increases (from 0.1 to 26 MPa), suggesting a potential piezolyte function [59]. The piezoprotective effect of these organic solutes likely stems from the reorganization of water molecules favoring protein folding and stability [60].

Piezophiles also display other stress responses related to proteostasis. As such, both pressure down and upshift from their optimal growth value result in changes in piezophile HSP expression. Transcriptome analysis of *P. profundum* cells growing at their optimum pressure (28 MPa) and at 0.1 MPa showed that DnaK, DnaJ, and GroEL were upregulated at atmospheric pressure [49], although mass spectrometry-based label-free quantitative proteomics revealed that GroEL and DnaK were instead downregulated at atmospheric pressure [61]. This discrepancy between proteomic and transcriptomic data might result from the intervention of post-transcriptional regulation factors and/or protein turnover [61]. In contrast, transcription and translation of HSPs in *S. violacea* increased when growing optimally at 30 MPa and even more at 50 MPa compared to atmospheric pressure, suggesting this stress response to be critical for pressure adaptation in this microorganism [46]. Interestingly, one of the major substrates of the chaperonine GroEL/GroES in *S. violacea* was the 50S ribosome subunit.

3. Effect of lethal HHP shock on bacteria

3.1. Cellular impact of lethal HHP on proteostasis

From ca. 200 MPa onwards, even short exposures to such HHP intensities tend to impose lethal injuries on microorganisms, and this lethal impact has mostly been studied in the context of HHP processing as a non-thermal food preservation approach to inactivate foodborne pathogens and spoilage microorganisms [4]. The cellular impact of a lethal HHP shock is likely to be pleiotropic (Table1), and cells within a HHP-treated population that manage to survive are typically sublethally injured and display long and heterogeneous lag phases [62-64]. In this context, inactivation of the transmembrane protein F0F1-ATPase by mild pressure (200-300 MPa) has been linked to the inability of lactic bacteria to maintain intracellular pH and loss of viability in acid environments [25-65]. Similarly, a 300 MPa shock irreversibly disrupted the pH homeostasis in *E. coli*; however, the incubation of pressurized cells at pH 4.0 in the presence of glutamate improved cell recovery, suggesting that the glutamate decarboxylase system involved in proton consumption for acid adaptation may still be functional after pressurization [66].

Evidence on ribosomal subunit dissociation *in vivo* after exposure to HHP treatment (50-500 MPa) has been reported by differential scanning calorimetry in several bacterial species [67-69]. Furthermore, a correlation between ribosome-associated enthalpy and loss of cell viability as a function of the intensity of HHP treatment has been shown in *E. coli* (50-250 MPa, 20 min [67]) and *Leuconostoc mesenteroides* (250-500 MPa, 5 min [68]). In view of these results, irreversible ribosomal damage has been proposed as one of the limiting factors for cell survival after mild HHP exposure [67-68], although a true causal relationship remains questionable. For instance, a correlation between ribosome-associated enthalpy, accumulation of

osmolytes, and survival of *E. coli* AW1.7 was found for heat treatment but not for HHP treatment [70-71]. In addition, exposure of *Enterobacter aerogenes* to few consecutive cycles of 200-270 MPa shock led to ribosome dissociation after membrane disruption [72]. Membrane permeabilization and consequent leakage of cytoplasmic components (such as Mg^{2+}, which is well known to stabilize ribosome structure and activity *in vivo* and *in vitro* [20, 67]) may favour ribosome dissociation as a post-mortem rather than a causative effect.

Exposures to pressures > 200 MPa cause accumulation of misfolded proteins or folding intermediates, which may assemble through non-functional intermolecular interactions after decompression, leading to the formation of insoluble protein aggregates (PAs) [9, 73]. In fact, the formation of cytosolic aggregated clumps, likely corresponding to aggregated proteins and ribosomes, after lethal HHP shock has been shown using transmission electron microscopy (TEM) in several bacteria such as *E. coli*, *Listeria monocytogenes*, and *Leuconostoc mesenteroides* [68, 74-75]. These aggregates may compromise cell survival because of the loss of a myriad of functional proteins, aberrant interactions with other molecules, and possible even cytoplasmic membrane permeabilization [76-77]. Some authors have proposed protein unfolding as one of the major targets for HHP inactivation because of the correspondence in pressure dependence of bacterial viability and protein denaturation (and not with membrane or nucleic acids stability) [12, 78] and the commonly observed induction of the heat shock response after pressurization [79]. However, other authors have not found a causal relationship between cell inactivation and TEM-visual aggregates [80], suggesting that protein damage may not be the only target for HHP inactivation of microorganisms.

The effect of HHP on the management and disaggregation of pre-existing PAs in *E. coli* has been reported at the single cell level, using a yellow fluorescent protein-labelled small heat shock protein (IbpA-YFP) for PA visualization [64, 81]. It has been proposed that PAs accumulate in growing cells, even at optimal conditions, as a consequence of errors occurring during transcription, translation, and protein folding [82-83]. HHP exposure was shown to result in the dispersion of existing polar PAs throughout the cytoplasm into distinct smaller foci (after 200-300 MPa shock) or even into more diffusely spread molecules (after 400 MPa shock), presumably due to pressure-mediated disruption of the hydrophobic interactions that tend to hold PAs together (Fig. 1) [64, 81]. Interestingly, the level of PA dispersal after mild pressure exposure (200 MPa) appeared to correlate negatively with the probability of cellular resuscitation and, independently of the degree of PA dispersal after HHP treatment, all monitored surviving cells largely reassembled their PAs before resuscitation. This observation might suggest that PA dispersion imposes an additional threat to pressure-treated cells, and that PA reassembly might be important to minimize PA-associated cytotoxic effects and proceed to resuscitation after HHP exposure. In addition, aside the chaperone- and protease-based protein quality control mechanisms that aim to resolubilize PAs for subsequent refolding or degradation of the trapped proteins, the reassembly and sequestration of dispersed PA material to the cell pole and the subsequent asymmetric segregation of these structures among daughter cells might provide a straightforward way to clear PAs from the cytoplasm of most of the progeny cells [82-83].

Using aggregates of phage P22 tailspike proteins, Foguel et al. [84] demonstrated *in vitro* that HHP treatment could reverse protein aggregation and increase the native refolding of proteins after decompression. Since then, the ability of

HHP to dissociate protein aggregates has been implemented for various biotechnological applications aimed at optimizing recombinant protein yield. The disaggregation and refolding of proteins from inclusion bodies (IBs) is one of the main challenges in the production of recombinant proteins in prokaryotes [85-86]. The traditional method for IB processing consists in first solubilizing PAs in a concentrated chaotrope solution and then refolding the denatured proteins by dialysis, diafiltration, or dilution. HHP alone or in combination with low amounts of chaotropes can simultaneously solubilize IBs and correctly refold the protein of interest in a more efficient and time saving way as it better preserves the secondary structure of proteins. Up to date, more than a hundred recombinant proteins have been refolded by commercial scale HHP processing [85-86].

3.2. Microbial adaptation to lethal HHP treatment

Upregulation of HSPs has been reported in *E. coli* after a 75-150 MPa treatment, although genome wide expression analysis has revealed many other genes to be affected by HHP shock as well [79, 87]. The protective effect of HSPs, however, is further underscored by the observation that exposure of *E. coli* MG1655 to a sublethal heat shock (40°C) provided protection to a subsequent lethal HHP shock [79], while disruption of its *dnaK* or *dnaJ* genes decreased its pressure resistance [81]. Another example of the significance of HSP-mediated protection against pressure is the fact that HHP-resistant mutants of *L. monocytogenes* (strains LO28 and ScottA) harbouring a compromised *ctsR* gene, which encodes the class III heat shock repressor, can be isolated with relatively high frequency after exposure of the parent strain to a severe HHP treatment [6, 88]. In addition, also some HHP-resistant mutants of *E. coli* MG1655

(i.e. LMM1010 and LMM1030; obtained by directed evolution) display increased basal expression of various HSPs [79].

In addition to the heat shock response, the RpoS-mediated general stress response also plays a critical role in HHP resistance of *E. coli*. In fact, deletion of *rpoS* in *E. coli* (strain BW25113) has been shown to increase HHP sensitivity (at 600 MPa for 8 min) ca. 1,000-fold compared to the wild-type strain [89]. In the same vein, disruption of RssB (the anti-sigma factor that quenches intracellular RpoS activity [90]) in *E. coli* O157:H7 (strain ATCC 43888) increased resistance to both HHP (300 MPa for 15 min) and heat (56ºC for 15 min) ca. 10,000-fold [91]. Furthermore, the large intrinsic variability in HHP resistance observed among natural enterohemorrhagic *E. coli* isolates has been related to variations in cellular RpoS activity [92-93], and the modulation of the RpoS response has proven to be an important evolutionary strategy in *E. coli* O157:H7 to improve stress tolerance [5, 94]. Indeed, exposure of *E. coli* O157:H7 (ATCC 43888) to a limited number of progressively intensifying HHP shocks with intermittent resuscitation rapidly selected for HHP-resistant (and heat cross-resistant) mutants that displayed clear signs of increased RpoS activity [5]. Interestingly, a similar long-term directed evolution experiment allowed to obtain *E. coli* MG1655 mutants able to survive HHP shocks in the GPa range [95].

Osmoregulation also has an important role in the protection of mesophilic bacteria to HHP treatment. The presence of high concentrations of sugars or inorganic salts in the treatment medium increases microbial resistance to pressure due to the cellular uptake or synthesis of disaccharides or compatible solutes [96-97]. For example, treatment of *L. monocytogenes* in a high osmolarity medium in the presence of both betaine and L-carnitine increased survival to 400 MPa by 15-fold, and a mutant

lacking the primary transporters of these compounds (i.e. BetL, OpuC, and Gbu) failed to develop HHP resistance [98]. Disruption of the osmotically-induced outer membrane lipoprotein OsmB and the trehalose synthesis genes (by deletion of *ostA* and *ostB*, encoding the trehalose-6-phosphate synthase and the trehalose-6-phosphate phosphatase, respectively) sensitized *E. coli* to HHP treatment [87, 89]. Interestingly, the synthesis of trehalose, which stabilizes cell envelope and protein structure from thermal denaturation [60], is induced by the RpoS sigma factor and may contribute to the increased HHP resistance of stationary phase cells [89].

Finally, the screening of random transposon-mediated gene disruptions for their effect on the HHP resistance of *E. coli* O157:H7 (ATCC 43888) revealed that loss of the cAMP/CRP system, which regulates the preferential carbon source usage, improved the HHP resistance of this strain as well [91]. However, since cAMP/CRP regulation can affect RpoS activity [99], it currently remains unclear whether this mechanism of HHP resistance is truly RpoS independent.

4. Future perspectives

Piezophilic microorganisms have adopted different strategies to maintain proteostasis in HHP environments. Although much research has been focused on molecular and physiological adaptations of piezophiles, the current knowledge is limited to few species because difficulties in isolation and growth of these microorganisms and the limited number of genome sequences available. Another extra difficulty is that deep-sea microorganisms tend to incur psychrophilic or thermophilic adaptations on top of their piezophilic adaptations because the wide range of

temperatures that can be encountered in the ocean (from -2 ºC in the polar sea to more than 80ºC in hydrothermal vents). Therefore, further isolation and characterization of extremophile proteins is essential to improve our knowledge on specific protein adaptation to HHP. Due to the HHP-mediated enhancement of certain enzymatic reactions, isolation and/or engineering of HHP-resistant enzymes can have a great potential for biotechnological applications.

Deeper insights into the mechanism of HHP inactivation can help to improve the efficiency of HHP-based food preservation, for instance by allowing the design of intelligent hurdle approaches that operate synergistically with HHP to inactivate foodborne pathogens. In this context, exploitation of novel fluorescence-based live cell biology tools may help to dissect and understand the nature and dynamics of injury and stress responses in HHP stressed cells with high resolution. Furthermore, identification and scrutiny of the HHP resistance conferring mutations acquired by extremely HHP resistant mesophiles can shed more light on the main cellular HHP targets, and can help to better anticipate and counteract HHP resistance development in foodborne pathogens.

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742 **Table 1.** Effect of sublethal (< 100 MPa) and lethal (> 100 MPa) HHP on bacterial
743 proteostatis. Data were obtained in *E. coli* except where indicated.

Phenotype	Pressure (MPa)	Reference
Effect of sublethal HHP on bacterial growth (< 100 MPa)		
Growth inhibition	50	[2-3]
Cell filamentation		
Depolymerization of FtsZ and inhibition of septum formation	50	[13]
Mrr-triggered DNA-damage response leading to SulA-mediated FtsZ inhibition	75-100	[35-36]
Inhibition of DNA replication	50-81	[2]
Inhibition of RNA synthesis	77-80	[2, 14]
Alterations in the regulation of gene expression	30-70	[16-18]
Inhibition of protein translation	60-70	[2, 19]
Alteration of transmembrane proteins		
Transient inhibition of proton transfer ATPase (*S. faecalis*)	50	[24]
Dimerization of ToxR (*V. cholerae*)	20-50	[27]
Effect of lethal HHP shock on bacterial survival (> 100 MPa)		
Loss of membrane integrity and leakage of intracellular proteins	100-600	[89, 100]
Irreversible ribosomal damage (*E. coli*, *L. monocytogenes*, *L. mesenteroides*)	100-500	[67-69]
Emergence of cytosolic aggregates (*E. coli*, *L. monocytogenes*, *L. mesenteroides*)	300-600	[68, 74-75]
Inactivation of transmembrane proteins and disruption of pH homeostasis		
F0F1-ATPase (*L. plantarum*, *Lactococcus lactis*)	250	[25, 65]
Arginine and glucose pH homeostasis transmembrane proteins	300	[66]
Disaggregation of pre-existing protein aggregates	200-400	[64, 81]

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Figure legends:

Figure 1. HHP-induced disassembly and reassembly of PAs in *E. coli* LMM1010 IbpA-YFP. Representative phase contrast and YFP epifluorescence (reporting IbpA concentration and localization) images of (A) unstressed control cells and (B) HHP-stressed cells (300 MPa, 15 min). For HHP-stressed cells, phase contrast and YFP images in combination with cell outlines are shown at the indicated times after HHP exposure illustrating the reassembly of dispersed PAs in surviving cells into larger polar PAs and subsequent outgrowth of the cells. The scale bar corresponds to 5 µm.

Figure 1