Chapter 5
Stress Responses of the Yeast *Saccharomyces cerevisiae* Under High Hydrostatic Pressure

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Abstract Effects of high hydrostatic pressure on biological systems have been mainly investigated from three perspectives: (1) structural understanding of macromolecules such as proteins and lipids and kinetic analysis of biochemical reactions, (2) adaptation of microbes such as mesophiles and piezophiles to high pressure, and (3) inactivation of food-spoiling microbes and application of nonthermal food processing. The yeast *Saccharomyces cerevisiae* has been an invaluable organism in establishing the molecular basis of cellular responses to high pressure as well as in applying a basic knowledge of the effects of pressure on industrial processes involving various microbes. In this chapter, the general effects of high pressure on biological systems and recent advances in research on the response of *S. cerevisiae* to high pressure with respect to intracellular pH homeostasis, significance of tryptophan uptake, ubiquitin-dependent degradation of tryptophan permeases, global analyses on transcription and gene functions, and attempts toward industrial applications are reviewed.

Keywords *DAN/TIR* family genes • Fermentation • Global functional analysis • Global transcriptional analysis • High hydrostatic pressure • High-pressure growth mutants • Hsp104 • Intracellular acidification • Rsp5 ubiquitin ligase • *Saccharomyces cerevisiae* • Tryptophan permeases Tat1 and Tat2 • Ubiquitination • Volume changes

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5.1 General Effects of High Hydrostatic Pressure on Biological Systems

The oceans occupy 70 % of the earth and have an average depth of 3,800 m. In terms of volume, the majority of the biosphere is made up of high-pressure environments. Therefore, organisms have to experience a broad range of hydrostatic pressures from 0.1 to 100 MPa (0.1 MPa = 1 bar = 0.9869 atm = 1.0197 kg of force/cm^2; for clarity, "MPa" is used throughout) in combination with varying temperatures. There is a growing interest in deep-sea microorganisms, namely "piezophiles," that prefer high hydrostatic pressure for growth (Yayanos 1995; Abe and Horikoshi 2001; Bartlett 2002; Oger and Jebbar 2010). However, the molecular basis of high-pressure adaptation has not yet been established in piezophiles occurring in natural environments because of the difficulty of cultivation and genetic manipulation (e.g., gene disruption, overexpression, or mutagenesis). Humans also experience high hydrostatic pressure. The knees are exposed to several dozen MPa of pressure during exercise (Muir 1995); the back teeth are also exposed to the same levels of pressure. Nonetheless, the mechanisms underlying the effects of high pressure on living systems are not yet sufficiently understood.

Inhibitory effects of high pressure on biological processes are listed in Table 5.1. In general, high hydrostatic pressures in the range of several dozen MPa do not readily kill microorganisms but exert adverse effects on the activities of organisms that are adapted to atmospheric pressure (Abe et al. 1999; Bartlett 2002). These effects depend not only on the magnitude but also on the duration of the applied pressure, temperature, pH, oxygen supply, and composition of culture media. Accordingly, the effects are very complex, thus making their interpretation difficult. Practically, oxygen supply is one of the limitations for a long-term culture of organisms in a closed hydrostatic chamber. The yeast *Saccharomyces cerevisiae* is a facultative anaerobe and thus a useful model organism in this experimental situation. The yeast genome encodes approximately 6,600 genes, including more than 4,800 nonessential genes. The recent, large-scale phenotypic screening of the *S. cerevisiae*

Table 5.1 Inhibitory effects of high pressure on biological processes

Cellular process/structure	Inhibitory pressure (MPa<)
Motility	10
Cell division	20
Nutrient uptake	15–20
Ethanol fermentation	25–50
Membrane protein function	25–50
Replication	50
Transcription	50–100
Protein synthesis	50
Protein oligomerization	50–100
Soluble enzyme function	100
Viability	100–200
Protein structure (monomer)	200
DNA structure (double helix)	1,000

gene-deletion library revealed numerous unexpected genes and metabolic pathways that are involved in the tolerance of environmental stress.

Application of pressure yields a fundamental physical parameter in any reaction, that is, volume change. The following two equations describe the effect of hydrostatic pressure on the equilibrium $A \leftrightarrow B$ and the reaction $A \rightarrow B$, respectively:

$$\left(\partial \ln K / \partial p\right)_T = -\Delta V / RT \tag{5.1}$$

$$\left(\partial \ln k / \partial p\right)_T = -\Delta V^{\neq} / RT, \tag{5.2}$$

where K is the equilibrium constant; k, rate constant; p, pressure (MPa); T, absolute temperature (K); R, gas constant (ml MPa K^{-1} mol^{-1}); ΔV, difference between the final and initial volumes in the entire system at equilibrium (reaction volume), including the solute and the surrounding solvent; and $\Delta V^{\neq}$, apparent volume change in activation (activation volume), representing the difference in volume between the reactants and the transition state. The direction and degree of pressure effects on any reaction are governed by the sign and magnitude of volume change. When a reaction is accompanied by volume increase, it is inhibited by increasing pressure. When a reaction is accompanied by volume decrease, it is facilitated by increasing pressure. A definite distinction between pressure and temperature is that temperature accelerates reactions, as defined by the Arrhenius equation, whereas pressure accelerates, inhibits, or does not affect reactions depending on the sign and magnitude of ΔV and $\Delta V^{\neq}$ values.

High hydrostatic pressure of approximately 100 MPa generally dissociates oligomeric proteins because hydration of charged groups, and exposure of nonpolar groups to water is usually accompanied by negative volume change (Gross and Jaenicke 1994; Meersman et al. 2006); therefore, high pressure prefers the dissociated forms of proteins in aqueous solution. Ribosome subunits are dissociated at pressures above 60 MPa in *Escherichia coli*, which could be the upper limit of pressure for growth of this bacterium (Gross and Jaenicke 1994). High pressure, above 200–300 MPa, causes unfolding of the protein monomer (Meersman et al. 2006; Fourme et al. 2012; Akasaka et al. 2013). During protein unfolding, water molecules penetrate into cavities within the proteins, and nonpolar groups are exposed to the solvent. Consequently, proteins form aggregates within the cell, which have harmful effects on cell viability.

Phase behavior of lipid bilayers is one of the most pressure-sensitive processes in biological systems. High pressure and low temperature increase the order of fatty acid acyl chains; consequently, the membrane becomes packed and its fluidity is decreased (Winter 2002; Matsuki et al. 2013). In artificial lipid bilayers such as dipalmitoylphosphatidylcholine, the temperature for the transition (T_m) from ripple gel ($P\beta'$) phase to a liquid crystalline ($L\alpha$) phase is 41.6 °C at atmospheric pressure; however, T_m increases to 66 °C at a pressure of 100 MPa (Ichimori et al. 1998). Although biological membranes do not exhibit a clear phase transition, high pressure inevitably stiffens the membranes, thereby affecting membrane proteins to a greater or lesser extent. In the following sections, the occurrence and responses of *S. cerevisiae* cells to high pressure are described (Table 5.2).

Table 5.2 Pressure-induced effects on yeast physiology and survival[a]

Pressure (MPa)	Effect/occurrence	Notes	References
0.1–100 MPa	Arrest of cell growth	Cell-cycle arrest in G_1 phase in Trp^- strains[b] (15–25 MPa and 24 °C)	Abe and Horikoshi (2000); Abe and Iida (2003)
		Trp^{+c} or *HPG* strains,[d] (50 MPa and 24 °C)	
	Metabolic change	Calorimetry	Tamura et al. (1999)
	Inhibition of amino acid uptake	Severity, Trp>Lys>His>Leu	Abe and Horikoshi (2000)
	Stress-inducible gene expression	During growth (25 MPa and 24 °C)	Abe (2007)
	Stress-inducible gene expression	During growth (30 MPa and 25 °C)	Iwahashi et al. (2005)
	Stress-inducible gene expression	After pressure release (40 MPa and 4 °C)	Iwahashi et al. (2003)
	Enhancement of esterase activity	Nonspecific esterases	Abe and Minegishi (2008)
	Inhibition of ethanol fermentation	Internal ATP level is unchanged.	Abe and Horikoshi (1997, 1998)
	Cytoplasmic and vacuolar acidification	Fluorescence analysis; internal pH decreases by 0.3–0.5 units	Abe and Horikoshi (1997, 1998)
	Ubiquitin-dependent degradation	Degradation of tryptophan permeases Tat1 and Tat2 (25 MPa and 25 °C)	Abe and Iida (2003)
	Growth defects	Nonessential gene disruptants (25 MPa and 24 °C)	Abe and Minegishi (2008)
	The role of unsaturated fatty acids	Increased viability, C18:3>C18:2>C18:1>C16:1	de Freitas et al. (2012)
100 MPa–	Reduction in viability	Colony-forming unit measurement	Iwahashi et al. (1991); Hamada et al. (1992); Kobori et al. (1995)
	Disruption of microtubules	Electron microscopy	Kobori et al. (1995); Sato et al. (1996)
	Depolymerization of F-actin	Electron microscopy	Sato et al. (1996, 1999)
	Nuclear membrane perturbation	Electron microscopy	Kobori et al. (1995); Sato et al. (1996, 1999)
	Acquired piezotolerance	Heat shock (42 °C); Hsp104, Hsc70, and trehalose have a role	Iwahashi et al. (1991, 1997, 2000)
	Acquired piezotolerance	Msn2/Msn4 transcription factors	Domitrovic et al. (2006)
	Stress-inducible gene expression	After pressure release (180 MPa and 4 °C)	Iwahashi et al. (2005)

(continued)

Table 5.2 (continued)

Pressure (MPa)	Effect/occurrence	Notes	References
200 MPa–	Acquired piezotolerance	H_2O_2, ethanol, or cold-shock treatment	Palhano et al. (2004)
	Induction of petite mutation	Strain- and growth phase dependent	Rosin and Zimmerman (1977)
	Induction of homozygous diploids	Dye-plate method	Hamada et al. (1992)
	Shrinkage of cells	Direct microscopic observation	Perrier-Cornet et al. (1995)
	Leakage of internal substrates	Amino acids and ions	Shimada et al. (1993)
	Stress-inducible gene expression	After pressure release (200 MPa, 30 min)	Fernandes et al. (2004)

[a]It should be noted that most effects are dependent on time, strain, growth phase, and analytical procedure
[b]*Trp*$^-$ tryptophan auxotrophic
[c]*Trp*$^+$ tryptophan prototrophic
[d]*HPG* high-pressure growth

5.2 High Pressure Induces Intracellular Acidification

High pressure affects chemical reactions of intracellular small molecules. During ethanol fermentation, large amounts of carbon dioxide (CO_2) are produced by yeast cells. At atmospheric pressure, more than 99 % of aqueous CO_2 exists as dissolved gas and less than 1 % exists as carbonic acid H_2CO_3, which partly dissociates to produce H^+, HCO_3^-, and CO_3^{2-}. The reaction volume (ΔV) of the equilibrium $H_2CO_3 \leftrightarrow H^+ + HCO_3^-$ is negative (−26.0 ml/mol), indicating that high pressure operates to shift the equilibrium considerably toward generating protons. The simple equilibrium shift occurs in the yeast cytoplasm during ethanol fermentation. Cytoplasmic pH and vacuolar pH were monitored in a hydrostatic chamber with transparent windows. Two pH-sensitive fluorescence probes, 5- (and 6-) carboxy SNARF-1 and 6-caroboxyfluorescein, were used for staining the cytoplasm and the vacuole, respectively (Abe and Horikoshi 1997, 1998). The labeled cells were placed in the chamber, and fluorescence was emitted through the window under various pressure conditions. High hydrostatic pressure in the range of 40–60 MPa acidified the cytoplasm, with a pH decrease by approximately 0.3 units; this resulted in the concomitant acidification of the vacuole (Abe and Horikoshi 1997). Because the key glycolytic enzyme phosphofructokinase is highly sensitive to acidic pH, high pressure will indirectly diminish ethanol fermentation in yeast. Neutral cytoplasmic pH is primarily maintained through proton extrusion by plasma membrane H^+-ATPase Pma1, whereas the acidic vacuolar pH is maintained by vacuolar H^+-ATPase. The pressure-induced internal acidification is attributed to the enhanced

generation of protons along with the equilibrium shift because it occurs only with fermentable sugars such as glucose or fructose, but not with nonfermentable carbohydrates such as ethanol or glycerol (Abe and Horikoshi 1998). The same levels of hydrostatic pressure activate nonspecific esterase in the cells (Abe 1998). Esterase activity influences the production of isoamyl alcohol and ethyl caproate, which are important flavor compounds of Japanese sake. Yeast cells undergo a very small fluctuation in hydrostatic pressure during circulation in a large fermenting vessel. Therefore, production of such flavor compounds may vary with the depth at which the yeast cells exist. Although the relationship between internal acidification and esterase activation is unclear, the chemical reactions of internal small molecules should be considered for elucidating the physiological effects of high hydrostatic pressure in living yeast cells.

5.3 Tryptophan Availability Is a Limiting Factor for Cell Growth Under High Pressure

Experimental *S. cerevisiae* strains such as YPH499 and W303 carry nutrient auxotrophic markers (e.g., *ade2*, *ura3*, *his3*, *lys3*, *leu2*, and *trp1*) for plasmid selection. Tryptophan auxotrophic strains appear to be highly sensitive to high pressure. Regardless of other auxotrophic markers, Trp^- strains exhibit growth defects at nonlethal hydrostatic pressures. When the cells are exposed to pressures of 15–25 MPa for 5–10 h, the cell cycle is arrested in the G_1 phase without any loss of viability (Abe and Horikoshi 2000). Trp^- strains also exhibit growth defects at low temperature, 10–15 °C. The sensitivity of Trp^- cells to high pressure and low temperature is thought to have originated from the following two specific properties of tryptophan uptake mediated by tryptophan permeases Tat1 and Tat2: (1) Tat1 and Tat2 activity is readily compromised by stiffening the membrane either by high pressure or low temperature, and (2) Tat1 and Tat2 undergo ubiquitination-dependent degradation in response to high pressure (see following). Trp^+ strains, including distillers' yeasts, are capable of growing at 15–25 MPa; however, the growth rate is decreased. Addition of excess amounts of tryptophan in the growth medium or overexpression of *TAT1* or *TAT2* in a multicopy plasmid confers Trp^- strains the ability to grow at 25 MPa (Abe and Horikoshi 2000; Abe and Iida 2003). Accordingly, *S. cerevisiae* cells are capable of growing at pressures up to 25 MPa if tryptophan availability is ensured.

5.4 Ubiquitin-Dependent Degradation of Tryptophan Permeases in Response to High Pressure

Even at nonlethal levels, hydrostatic pressure results in the structural perturbation of biological membranes, thus directly or indirectly affecting membrane proteins. Denatured membrane proteins are likely to undergo ubiquitination-dependent

degradation (Hershko and Ciechanover 1998). Ubiquitination was initially described to promote proteasomal degradation of proteins and has since been shown to regulate other processes, including DNA repair, signaling, endocytosis, or membrane trafficking (Lauwers et al. 2010). The ubiquitin ligase (E3) Rsp5 complex plays an essential role in the ubiquitination and subsequent endocytosis of yeast plasma membrane proteins by vacuole for degradation (Belgareh-Touze et al. 2008; Rotin and Kumar 2009). The Rsp5 complex is also required for the ubiquitination of endosomal proteins. The primary structure of Rsp5, including the N-terminal C2 domain, three central WW domains, and C-terminal HECT (homologous to E6-AP C-terminus) domain, is similar to that of Nedd4 family proteins. Ubiquitination of Tat2 was initially reported as a starvation response of yeast, in which covalent binding of ubiquitin occurs on one or more of the five lysine residues within the N-terminal cytoplasmic domain (Beck et al. 1999). Then, ubiquitin-bound Tat2 undergoes endocytosis, followed by vacuolar degradation. Upon exposure of the cells to high pressure, Tat2 also undergo ubiquitination-dependent degradation.

High-pressure growth (*HPG*) mutants, which are capable of growing at 25 MPa, were isolated from Trp^- strain YPH499 and were classified into four semidominant complementation groups, namely, *HPG1* to *HPG4* (Abe and Iida 2003). *HPG1* mutation sites were found within the catalytic HECT domain of Rsp5. Because of the reduction in Rsp5 activity, Tat2 becomes undegraded, leading to a substantial accumulation in the plasma membrane (Abe and Iida 2003). Given that the accumulated Tat2 proteins retain some tryptophan import activity, *HPG1* cells become endowed with the ability to grow at high pressure. *HPG2* mutation sites were found within the N- and C-terminal cytoplasmic domains of Tat2 (E27F, D563N, or E570K) (Nagayama et al. 2004). All three *HPG2* mutations result in the loss of a negative charge within the cytoplasmic tails. Accordingly, negatively charged residues at certain positions in the cytoplasmic tails might be required for recruiting the Rsp5 complex through ionic interactions. The Rsp5 WW domains are responsible for binding to proteins that have a PPxY motif (Gupta et al. 2007). However, most substrate proteins do not have a canonical PPxY motif. In this situation, several Rsp5 adaptors are characterized, acting on ubiquitination. Bul1 is a PPxY motif-containing protein that was initially identified as an Rsp5-binding protein (Yashiroda et al. 1996). Loss of Bul1 and its close homologue Bul2 results in a considerable accumulation of Tat2. Eventually, the *bul1*Δ*bul2*Δ mutant becomes capable of growing under high pressure (Abe and Iida 2003). The W > G mutation of the WW3 domain also results in Tat2 accumulation (Hiraki and Abe 2010a). Therefore, Bul1 and/or Bul2 are likely to interact with Rsp5 on the WW3 domain to ubiquitinate Tat2. Other PPxY motif-containing proteins Ssh1, Ear1, and Sna3 also contribute to Rsp5-Tat2 interaction because overexpression of one of these three proteins inhibits Tat2 degradation (Hiraki and Abe 2010b). Thus, Ssh1, Ear1, and Sna3 impinge on Tat2 in the direction opposite to Bul1 and Bul2. Although Tat2 is a short-lived protein, the other tryptophan permease Tat1 is a long-lived protein with a half-life of more than 3 h. High pressure of 25 MPa dramatically accelerates Tat1 degradation in an Rsp5-dependent manner (Suzuki et al. 2013). In this process, multiple adaptors, including Bul1/Bul2 and arrestin-like proteins ARTs, have a redundant role in

mediating Rsp5 and Tat1. However, how the denatured states of Tat1 and Tat2 are defined in terms of structural alterations and how denatured proteins are recognized by the Rsp5 complex remains unclear.

5.5 Global Screening of Genes Responsible for Growth Under High Pressure

As already mentioned, tryptophan availability is the primal requirement for yeast to grow under high pressure. However, it has been shown that many genes other than those involved in tryptophan biosynthesis or uptake are responsible for growth under high pressure. Tryptophan prototrophic strain BY4742 is capable of growing at pressures up to 30 MPa. By using BY4742 as a parental strain, systematic gene deletion has been performed using PCR-generated deletion strategy (http://www-sequence.stanford.edu/group/yeast_deletion_project/project_desc.html#intro) (Giaever et al. 2002). Of the 6,600 genes encoded by the yeast genome, single deletions in approximately 4,800 genes do not cause lethality under normal culture conditions. These genes are assumed to be necessary for particular cellular functions such as stress resistance or sporulation, although some of them are functionally redundant. Genetic defects that result in sensitivity to high pressure (25 MPa and 24 °C) or low temperature (0.1 MPa and 15 °C) were elucidated using the deletion library in which 1 of the 4,800 nonessential genes was disrupted: This procedure revealed 80 genes, of which 71 were required for growth under high pressure and 56 were required for growth under low temperature (Abe and Minegishi 2008). Given the significant overlap of 47 genes, a number of shared biological processes support growth under both high pressure and low temperature. The 80 genes are involved in a broad range of cellular functions such as amino acid biosynthesis, mitochondrial function, actin cytoskeleton formation, membrane trafficking, transcription, ribosome biogenesis, chromatin structure, and some unknown functions (Abe and Minegishi 2008). It should be noted that the 80 deletion mutants display a broad range of sensitivities to high pressure and low temperature. Genes involved in the biosynthesis of aromatic amino acids, such as *ARO1*, *ARO2*, *TRP1*, *TRP2*, *TRP4*, and *TRP5*, are highly important for growth under high pressure and low temperature, which is consistent with the defective growth observed in Trp^- strains as previously described. Deletions in *HOM3*, *THR4*, and *SER1* result in auxotrophy for methionine (*hom3*Δ), threonine (*thr4*Δ), and serine (*ser1*Δ); thus, the growth of yeast cells harboring these deletions depends on the corresponding amino acid permeases. Similarly, deletions in two mitochondrial protein genes, *ACO1* and *CAF1*, result in auxotrophy for glutamine or glutamate. Therefore, multiple amino acid permeases are the likely targets of high pressure and low temperature. Genes encoding mitochondrial ribosomal subunits (*MRPL22*, *MRP51*, and *MRPL38*) or a protein involved in the folding of mitochondrially synthesized proteins (*MDJ1*) are also required for growth under the conditions.

Genes that give rise to remarkable growth sensitivity under high pressure and low temperature upon deletion are those encoding for components of the EGO complex (*EGO1*, *EGO3*, *GTR1*, and *GTR2*) (Dubouloz et al. 2005; Abe and Minegishi 2008). The EGO complex is a vacuolar membrane-associated protein complex comprising Ego1 (also known as Meh1 and Gse2), Ego3 (also known as Slm4, Nir1 and Gse1), and Gtr2 (Dubouloz et al. 2005). Cells lacking one of the EGO components normally show growth arrest after the addition of the immunosuppressive drug rapamycin to culture medium and do not revert to the growth phase upon the release of the rapamycin block. The EGO complex interacts with and activates the target of rapamycin complex 1 (TORC1) in an amino acid-sensitive manner (Binda et al. 2009). Taken together, high pressure and low temperature are likely to compromise biological processes that are involved in amino acid signaling in yeast cells. Requirement of TORC1 and EGO complex for growth under high pressure and low temperature could be a common feature in eukaryotes because of the substantial conservation in the primary structures.

Numerous genes involved in membrane trafficking (*VID24*, *VPS34*, *SEC22*, *PEP3*, *CHC1*, *PEP5*, *VPS45*, *ERG24*, *VPS54*, *AKR1*, and *SAC1*) are responsible for growth under high pressure and low temperature (Abe and Minegishi 2008). The products of these genes might facilitate the delivery of newly synthesized proteins to appropriate locations, such as the bud neck, cell surface, or cell wall, under high pressure and low temperature. The appropriate actin network is a prerequisite for bud formation and polarized cell growth in yeast. Several genes involved in these processes (*LTE1*, *HOF1*, *SLM3*, *CLA4*, *CDC50*, and *SLM6*) are required for growth under high pressure and low temperature. Of these, *CDC50* encodes a protein that interacts with Drs2, a P-type ATPase of the aminophospholipid translocase (Kishimoto et al. 2005). Drs2 is required for the organization of the actin cytoskeleton and trafficking of proteins between the Golgi complex and the endosome/vacuole (Chen et al. 2006). The lack of *DRS2* also confers high-pressure and low-temperature sensitivity. Therefore, the Drs2–Cdc50 complex is likely to modulate protein trafficking to establish appropriate actin network formation under high pressure and low temperature. Ergosterol is a major constituent of the plasma membrane and has multiple functions such as maintenance of plasma membrane rigidity and solute impermeability, resistance to alkaline cations, and trafficking of membrane proteins. Deletion mutants for one of the enzymes that catalyze the later steps of ergosterol biosynthesis, such as *ERG24*, *ERG2*, *ERG6*, and *ERG3*, accumulate structurally abnormal sterols in the cells. Such mutants exhibit marked high-pressure and low-temperature sensitivity. Therefore, ergosterol plays a role in maintaining an appropriate membrane property that opposes the stiffening effect of high pressure and low temperature.

Genes involved in transcription and mRNA degradation (*SNF6*, *MOT2*, *POP2*, *SHE3*, *CDC73*, *RPB4*, *HFI1*, *PAF1*, *ELF1*, *SNF1*, *SRB5*, *TAF14*, *CCR4*, and *SAP155*) comprise a major class of essential genes for growth under high pressure and low temperature (Abe and Minegishi 2008). Among them, the loss of a constituent of the Ccr4–Not complex (Pop2, Not4, or Cdc73) results in marked sensitivity. This finding raises the possibility that the Ccr4–Not complex might fail

to assemble in cells under high pressure and low temperature if Pop2, Not4, or Cdc73 is lost. Eventually, several genes under its control will not be transcribed. Twelve genes without any functional annotation were obtained in the same screening. The products of these genes might have an overlapping function with any proteins with functional identification.

5.6 Resistance of Cells to Lethal Levels of High Pressure

High hydrostatic pressure, greater than 100 MPa, is considered to be lethal. This fact is put to practical application for sterilization of foodstuffs without heat treatment (Reineke et al. 2013). A pressure of 100 MPa increases the occurrence of cytoplasmic petite mutants that are characterized by a small colony size and respiration deficiency of cells, reflecting high-pressure sensitivity of the mitochondrial function (Rosin and Zimmerman 1977). Pressures of 100–150 MPa disrupt the spindle pole bodies and microtubules (Kobori et al. 1995), and those of 200–250 MPa induce tetraploids and homozygous diploids, potentially providing industrial uses for strains with increased growth rates (Hamada et al. 1992). It is well known that a mild heat treatment (e.g., 42 °C for 30 min) dramatically increases the viability of cells against a subsequent severe heat shock treatment (e.g., 50 °C for 10 min). Among the numerous heat-shock proteins (Hsps), the molecular chaperone Hsp104 is essential in this acquired heat tolerance by unfolding denatured intracellular proteins in an ATP-dependent manner (Glover and Lindquist 1998). Such a mild heat treatment also allows the cells to survive high pressures of 140–180 MPa, with a 100- to 1,000-fold increase in viability (Iwahashi et al. 1991). After high-pressure treatment at 140 MPa, Hsp104 associates with an insoluble protein fraction, suggesting that Hsp104 unfolds high pressure-induced denatured intracellular proteins (Iwahashi et al. 1997, 2000). Indeed, the *hsp104*Δ mutant is unable to acquire heat-inducible high-pressure tolerance (hereafter, piezotolerance; (Iwahashi et al. 1997). Cold shock and moderate concentration of H_2O_2 or ethanol also increase piezotolerance of the cells (Palhano et al. 2004). Similarly, a pressure treatment at a nonlethal level (50 MPa for 1 h) increases the viability of the cells at 200 MPa (Domitrovic et al. 2006). The acquired piezotolerance by moderate pressure treatment is governed by two transcription factors, Msn2 and Msn4, that are induced by various types of stress. The loss of both *MSN2* and *MSN4* genes results in susceptibility to high pressure (Domitrovic et al. 2006). However, how high pressure regulates Msn2 and Msn4 after the transcription of their downstream genes remains to be clarified. Trehalose is a nonreducing disaccharide known to protect proteins, membranes, and other macromolecules against various stresses. This disaccharide plays a role in the piezotolerance of yeast by preventing the formation of protein aggregates and promoting the refolding of Hsp104. Although yeast mutants defective in the accumulation of trehalose are susceptible to increasing pressure (Iwahashi et al. 1997), a mutant lacking neutral trehalase Nth1, which

catalyzes the hydrolysis of one trehalose molecule to two glucose molecules, shows susceptibility to high pressure (Iwahashi et al. 2000). Neutral trehalase is required for recovery after high-pressure treatment but is not required during the treatment. Accordingly, glucose is likely to be required as an energy source for recovery from pressure-induced damage at atmospheric pressure.

5.7 Transcriptional Regulation Under High Pressure

Global transcriptional analysis under high pressure has been performed using DNA microarray hybridization in several laboratories, but the results vary with strains and experimental conditions. Under a pressure of 30 MPa and 25 °C, which allows the growth of tryptophan prototrophic strains, 366 genes were upregulated by more than twofold and 253 genes were downregulated by more than twofold (Iwahashi et al. 2005). According to the functional categories of the Munich Information Center for Protein Sequences (http://mips.gsf.de/), the highly upregulated genes were categorized to cell cycle and DNA processing, cell rescue, defense and virulence, and metabolism. It is rationalized that heat shock-responsible genes such as *HSP12*, *HSP150*, *HSP26*, *SSE2*, and *HSP104* are induced by high pressure in terms of the acquired piezotolerance (Iwahashi et al. 2005). However, the result does not fully coincide with another report showing that *HSP104*, *HSP10*, *HSP78*, *HSP30*, *HSP42*, and *HSP82* are upregulated by more than twofold but others are not (Abe 2007). This difference is possibly the result of differences in strains (S288C versus BY4742), culture medium (YPD versus SC medium), and culture conditions (30 MPa and 25 °C for 16 h versus 25 MPa and 24 °C for 5 h). In addition, deletion of *HSP104*, *HSP78*, *HSP30*, *HSP42*, *HSP82*, *HSP150*, *HSP12*, or *HSP26* resulted in a fourfold increase in the survival rate at 125 MPa from 1 % to 4 %, suggesting that the cellular defensive system against high pressure could be strengthened upon the loss of *HSP* genes (Miura et al. 2006). The function of Hsp104 is seemingly contradictory, but these observations indicate that cell death at 140–180 MPa is mainly caused by the misfolding or denaturation of cellular proteins whereas cell death at 125 MPa could occur for other reasons.

High pressure and low temperature also upregulate the transcription of *DAN/TIR* family mannoprotein genes, which are optimally expressed under hypoxia (Abramova et al. 2001). In support of the role of mannoproteins in cell wall integrity, cells preexposed to high pressure or low temperature acquire tolerance against treatment with low concentrations of sodium dodecyl sulfate (SDS) and zymolyase or at lethal levels of high pressure (125 MPa for 1 h) (Abe 2007). However, how seemingly unrelated environmental factors induce the *DAN/TIR* family genes remains to be addressed. High pressure and low temperature increase the order of fatty acid acyl chains in the membrane, thus decreasing membrane fluidity. Hypoxia potentially exerts a decrease in membrane fluidity because acyl chain unsaturation, which requires molecular oxygen, could be compromised under hypoxia.

In this sense, membrane sensor(s) likely exist in the membrane and might transduce the changes in membrane fluidity into an intracellular signal that stimulates the transcription of *DAN/TIR* family genes.

Global transcriptional analysis was performed under pressures that did not allow yeast cell growth at 180 MPa and 4 °C or 40 MPa and 4 °C. During recovery after the treatments, transcription of genes required for energy metabolism, cell defense, and protein metabolism was highly upregulated (Iwahashi et al. 2003). According to a hierarchical clustering analysis, transcriptional patterns at growth-nonpermissive pressures are analogous to the treatment of cells with detergents such as SDS or sodium *n*-dodecyl benzene sulfonate (LAS). Hence, membrane perturbation by high hydrostatic pressure or detergents might have a direct effect on gene expression on a genome-wide level (Iwahashi et al. 2003). Fatty acid composition affects cell survival under lethal levels of pressure at 150–200 MPa. A strain lacking Ole1, a membrane-bound Δ9 desaturase, was cultured in a medium supplemented with various fatty acids such as palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), or linolenic acid (C18:3). Subsequently, the cells were subjected to pressures of 150–200 MPa for 30 min. The order of promoting survival by fatty acids was linolenic acid > linoleic acid > oleic acid > palmitoleic acid, indicating that a higher proportion of unsaturated fatty acids would help maintain a favorable membrane fluidity under high pressure and that reduced membrane fluidity was fatal under high pressure (de Freitas et al. 2012). However, reduced membrane fluidity is not the sole reason for high pressure-induced cell death because yeast cells are still alive at low temperatures of 0–4 °C.

Pretreatment with a nonlethal pressure level increases ethanol production in a yeast strain occurring in Brazilian spirit. After 4 h of fermentation, ethanol concentration reached 0.3 % in cells treated with a pressure of 50 MPa for 30 min whereas cells treated with no pressure produced negligible levels of ethanol (Bravim et al. 2013). After extended time periods, the difference in ethanol production between the two treatments became small. Global transcriptional analysis of this naturally occurring strain to identify the genes responsible for pressure-enhanced ethanol fermentation showed that genes associated with stress response (*CTT1*, *SOD2*, *STF1*, *GAC1*, *HSP12*, *HSP26*, *HSP30*, *HSP104*, *SSE2*, and *SYM1*), methionine biosynthesis and transport (*SAM3*, *MET14*, *MET16*, *MUP3*, *MET2*, and *MXR1*), glutamate transformation (*GAD1*, *UGA1*, and *UGA2*), or glycogen and trehalose metabolism (*TPS1*, *GSY2*, *NTH1*, and *NTH2*) were upregulated during recovery from pressure treatment (Bravim et al. 2013). Among the ten genes responsible for stress response, *SYM1* conferred resistance of 12–15 % and enhanced the production of ethanol upon its overexpression. Sym1 is a mitochondrial matrix protein, and its overexpression might have a general defect in NADH oxidation. To compensate for the defect, NADH could be oxidized by ethanol production in the cytoplasm, leading to enhanced ethanol production. In this way, application of high hydrostatic pressure offers an opportunity to modulate gene expression associated with the fermentation capacity of yeast in industrial production.

5.8 Conclusion

Hydrostatic pressure is a thermodynamic parameter that merely affects equilibria or the rate constant of chemical reactions without introducing any components into the experimental system. The system returns to its original state after the release of pressure. Differing from heat treatment, hydrostatic pressure uniformly transmits through the system in a moment. In this sense, hydrostatic pressure is a unique tool to modulate cellular metabolism or material production with saving energy. However, we have not completely understood the effects of high pressure on living systems or how to handle the complex cellular responses caused by high pressure. Evidently, a mechanistic understanding of the effects of high pressure on proteins, membranes, or intracellular small compounds is necessary to elucidate the complete picture of cellular responses to high pressure. Furthermore, advanced studies at levels of the transcriptome, proteome, and metabolome should converge on the analysis in a unified way of using the same yeast strains, media, temperature, or holding time of pressure in worldwide research.

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