Genomic expression pattern in *Saccharomyces cerevisiae* cells in response to high hydrostatic pressure

Patricia M.B. Fernandes[a,*], Tatiana Domitrovic[b], Camilla M. Kao[c], Eleonora Kurtenbach[b]

[a] *Departamento de Ciências Fisiológicas, CBM, UFES, 29090-040, Vitória, ES, Brazil*
[b] *Departamento de Bioquímica Médica, ICB/CCS, UFRJ, Rio de Janeiro, RJ, Brazil*
[c] *Department of Chemical Engineering, Stanford University, Stanford, CA, USA*

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Abstract Gene expression patterns in response to hydrostatic pressure were determined by whole genome microarray hybridization. Functional classification of the 274 genes affected by pressure treatment of 200 MPa for 30 min revealed a stress response expression profile. The majority of the >2-fold upregulated genes were involved in stress defense and carbohydrate metabolism while most of the repressed ones were in cell cycle progression and protein synthesis categories. Furthermore, uncharacterized genes were among the 10 highest expressed sequences and represented 45% of the total upregulated genes. The results of this study revealed a hydrostatic pressure-specific stress response pattern and suggested interesting information about the mechanisms involved in adaptation of cells to a high-pressure environment.

Key words: Yeast; Stress; Pressure; Microarray; Gene expression; *Saccharomyces cerevisiae*

1. Introduction

Hydrostatic pressure is a thermodynamic variable in the biosphere that ranges from 0.1 to more than 110 MPa in ocean depths. Surprisingly, some living organisms are able to withstand such high-pressure environments despite the strong effect of hydrostatic pressure on cell structures and their functions [1].

High pressure challenges life because it forces a decrease in volume. In this situation, several cellular components suffer structural modifications favoring a more compact form. Besides the structural alterations in biomolecules, pressure also disturbs the equilibrium of chemical reactions towards volume reduction [2].

Hydrostatic pressure also interferes with cellular membrane structure increasing the order of lipid molecules, especially in the vicinity of proteins. This phenomenon is driven by the smaller volume associated with a more ordered, tighter packing. The consequence is a decrease in cell membrane fluidity followed by an increase in thickness [3–5]. Proteins are also severely affected by pressure. Even though pressures over approximately 500 MPa are required to cause denaturation, lower pressures around 200 MPa can generate important conformational alterations that lead to modifications in enzymatic reactions as well as protein interactions and functionality [6]. In this way, even modest pressures such as 20–40 MPa are sufficient to induce microtubule dissociation, both in vitro (isolated microtubules) and in vivo (cellular mitotic spindle) [7–9].

DNA–protein interactions are also disturbed by hydrostatic pressure. For example, Lynch et al. [10] showed that at 30 MPa the restriction enzyme *Bam*HI loses affinity for its site due to a pressure-induced protein structural modification. On the other hand, the association of the repressor factor LexA with its DNA binding sequence was greatly stabilized under hydrostatic pressure [11] showing that high-pressure environments can interfere with transcription regulation processes, altering gene expression.

In addition to structural alterations, ionization constants of charged molecules will be affected by pressure. Elevated hydrostatic pressure tends to shift equilibria towards the production of ionized species because charged molecules lead to a decrease of the reaction volume by condensing water molecules [12–14]. Indeed, it has been demonstrated that *Saccharomyces cerevisiae* cells submitted to hydrostatic pressure undergo intracellular acidification, most likely caused by the dissociation of protons from H_2CO_3 and sugar phophoesters [15,16].

Therefore, with respect to biophysical experimentation, industrial applications and the understanding of new life forms, pressure can be considered as an additional variable to those more classically investigated, such as temperature, pH, osmolarity and concentration of chemical substances.

The yeast *S. cerevisiae* has long been used as a model for the study of cell stress responses. When environmental conditions change abruptly, yeast cells must rapidly adjust their genomic expression program to adapt to the new conditions. A nearly universal response of organisms, including *S. cerevisiae*, to an increase in temperature or other stresses is the induction of a set of proteins referred to as heat shock or stress proteins (Hsps). The function of these stress-induced proteins is varied. Some of them, like Hsp104 and Hsp60 have proved to be chaperonins, while others work as antioxidants or participate in the synthesis of trehalose, a membrane stabilizer [17]. However the action of many Hsps remains unknown.

The new pattern of gene expression induced by a mild temperature allows the cells to achieve tolerance to high temperature, osmotic pressure, dehydration and cryotreatment [18]. Moreover, the state of protection is also achieved when yeast

*Corresponding author. Fax: (55)-27-335 7330.
E-mail address: pat@npd.ufes.br (P.M.B. Fernandes).

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cells reach stationary phase [19]. Indeed, several authors have already shown that stationary phase *S. cerevisiae* cells are more resistant than proliferating ones not only to heat shock [20] but also to hydrostatic pressure [21]. This phenomenon of cross-protection is evidence of the existence of a general stress response. Nevertheless, this is not true for all situations, as cells exposed to high osmolarity do not acquire resistance against heat stress. Furthermore, the protection induced by a different stress form is usually not as efficient as a mild pretreatment with the same kind of stress. This indicates that, despite the general response, each stress situation leads to a particular gene expression profile [17]. This assumption was confirmed by the recent results of *S. cerevisiae* microarray analysis performed under various stress conditions. Even though there are some common genes that respond to a variety of stresses, there is not a uniform response for all kinds of stress situations [22].

The barotolerance induced by heat pretreatment leads to the notion that hydrostatic pressure is comparable to temperature in the damage it causes to yeast cells [23]. However, we have shown that the very heat-sensitive trehalose synthase mutant cells, unable to accumulate the well-established protectant disaccharide trehalose, can achieve some barotolerance when at stationary phase or after a mild heat shock [21]. These results suggest that the key elements for cell adaptation to temperature are not the same as for pressure and also that other unknown stress-induced elements are important for hydrostatic pressure survival.

Therefore, in this work we employed yeast microarray analysis, a powerful tool for understanding the mechanisms of cell stress responses, to provide information about the changes in gene transcription after hydrostatic pressure treatment. The functional analysis of genomic expression patterns revealed the ways by which a given stress form affects the cell and also provided important clues about the mechanisms involved in the adaptation and survival of organisms under adverse environmental conditions.

2. Materials and methods

2.1. Yeast strains and growth conditions

S. cerevisiae Y440 Mat *a leu2* was grown in YEPD (2% glucose, 1% yeast extract, 2% peptone) at 28°C with aeration to exponential growth phase.

2.2. Hydrostatic pressure treatment

Yeast cells were subjected to a hydrostatic pressure of 50 and 200 MPa for 30 min at room temperature. Samples were pressurized in the absence of air bubbles. The experiments were performed as previously described [21].

2.3. RNA isolation

Total RNA was extracted using phenol/chloroform and further precipitated with 3 M sodium acetate/absolute ethanol. Extracted RNA samples were treated for 10 min with 0.5 U of RNase-free DNase I/μg RNA at 37°C to remove any residual genomic DNA. RNA pellets were washed in 70% ethanol and resuspended in diethyl pyrocarbonate (DEPC)-treated water.

2.4. Probe preparation, microarray hybridization, and data acquisition

Probe preparation and microarray construction were performed as previously described [22]. Arrays were scanned using a commercially available scanning laser microscope (GenePix 4000) from Axon Instruments (Foster City, CA, USA). Full details on using the GenePix 4000 can be obtained form Axon. All assays were analyzed using the program ScanAnalyze (available from http://rana.stanford.edu). Briefly, the data obtained were normalized (mean value) applying a linear regression method. Two unpressurized reference samples were first labeled with Cy3 and Cy5 for microarray analysis to confirm identical labeling efficiency of transcripts between these dyes. As the ratio of fluorescent intensities was within 2-fold for the majority of the cDNA spots on the microarray, this value was established as the cutoff. Hybridization of pressurized samples against unpressurized cDNA was done in duplicate. Data files were entered into the Stanford Microarray database (http://genome-www5.stanford.edu) by the name 'Transcript expression in *S. cerevisiae* at high hydrostatic pressure'.

2.5. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

First-strand synthesis of cDNA was performed in a 25 μl reaction volume containing 1 μg of RNA, 0.2 μg/μl random hexadeoxynucleotide pd(N)$_6$ primer, 0.34 mM of each deoxyribonucleoside triphosphate (dNTP), 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 6.8 mM dithiothreitol (DTT) and 60 U M-MLV reverse transcriptase (Life Technologies) for 60 min at 37°C. The resulting cDNA was amplified in 25 μl of PCR reaction medium, containing 2.5 μl of the first-strand reaction, 50 pmol of specific oligonucleotides, 200 μM dNTP, 2.5 U of Taq polymerase (Promega), enzyme buffer (10 mM Tris–HCl, 50 mM KCl and 0.1% Triton® X-100) and 1.5 mM $MgCl_2$. The primers used were as follows: HSP12f, 5′-CCAGACT CTCAAAAGTCATA-3′; HSP12r, 5′-CATGTAATCTCTAGCTTG-GT-3′; HSP30f, ′-TTGACTAGATATGCCTTAGC-3′; HSP30r, 5′-GTGTAATAACCCCACTTGTA; ACT1f, 5′-TACGTTTCCATCC-AAGCC GTT-3′; ACTr, 5′-AACATACGCGCACAAAAGCAGA-3′; YER067Wf, 5′-ATGACAAAGAAGGATAAGAAGGAAGTA-AAAGTTCAAACG; YER067Wr, 5′-TTGGATCCACGCGGAAC-CAGATTTGCGCCTACAGGA TGT-3′. The PCR program for amplification of both the 377 bp *HSP30* fragment and the 192 bp *HSP12* fragment was 94°C for 1 min, 47°C for 1 min and 72°C for 1 min, followed by incubation at 72°C for 5 min. *ACT1* (755 bp) and *YER067W* (504 bp) fragments were amplified by the same program except for the annealing temperatures of 53 and 56°C respectively. PCR samples were taken after every four cycles from cycle 24 to 44 and analyzed on a 2% agarose gel stained with SYBR® green I (Sigma). The gel images were generated by Storm 860 phosphorimager/fluorimager scanner and analyzed for peak intensity using QuantiScan version 1.25 software. Data were expressed as intensity of the gel bands corresponding to the *HSP12*, *HSP30* and *YER067W* relative to the band intensity of the PCR product corresponding to the internal control yeast actin gene. Experiment was done in triplicate.

3. Results and discussion

3.1. Global microarray analysis

Yeast cells at mid-log phase were submitted to 200 MPa for 30 min and changes in the expression program were analyzed by whole genome microarray hybridization. Among the 6200 known or predicted genes that had been identified at the time of our analysis, approximately 5% were affected by hydrostatic pressure treatment. 131 genes were >2-fold induced while 143 suffered a <2-fold downregulation. Table 1 lists the upregulated open reading frames (ORFs) that had already been characterized by molecular or biochemical methods. Hydrostatic pressure-responsive genes were classified in distinct categories (metabolism and energy; cell cycle, DNA processing and cell fate; transcription; protein synthesis and fate; cellular transport and transport mechanisms; cell rescue, defense and regulation of/interaction with cellular environment; control of cellular organization; transport and facilitation; unknown) according to the MIPS functional database (http:// mips.gsf.de/proj/yeast/CYGD/db/index.html). A schematic representation of the global gene expression profile in functional categories is shown in Fig. 1.

The analysis revealed that most of the pressure-regulated mRNAs corresponded to uncharacterized ORFs and even

Table 1
Upregulated characterized genes after 30 min of high hydrostatic pressure of 200 MPa

Accession	Gene	Description	Fold induction
Stress response (cell rescue, defense and regulation of/interaction with cellular environment)			
YCR021C	HSP30	Hsp	6.062
YFL014W	HSP12	Hsp	5.775
YLR178C	TFS1	lipid binding, cdc25-dependent nutrient and ammonia response cell cycle regulator	3.397
YGR088W	CTT1	catalase T, cytosolic	3.208
YOL052C-A	DDR2	Hsp, DNA repair	3.135
YBR072W	HSP26	Hsp	2.373
YBR054W	YRO2	strong similarity to HSP30 Hsp	2.305
YHR053C	CUP1-1	copper binding	2.213
YHR055C	CUP1-2	copper binding, metallothionein	2.123
YHR008C	SOD2	manganese superoxide dismutase	2.102
YMR251W-A	HOR7	hyperosmolarity-responsive protein	2.085
YKL150W	MCR1	cytochrome b_5 reductase, response to oxidative stress	2.002
Metabolism and energy			
YHR092C	HXT4	moderate- to low-affinity glucose transporter	3.378
YGR008C	STF2	ATP synthesis coupled proton transport	3.247
YDR342C	HXT7	high-affinity hexose transporter	3.154
YDR343C	HXT6	high-affinity hexose transporter	3.026
YGR060W	ERG25	ergosterol biosynthesis, C-4 methyl sterol oxidase	2.713
YDL021W	GPM2	phosphoglycerate mutase, gluconeogenesis	2.687
YHR215W	PHO12	acid phosphatase	2.668
YOR374W	ALD4	ethanol metabolism aldehyde dehydrogenase (NAD+)	2.645
YFR053C	HXK1	hexokinase	2.615
YOR178C	GAC1	protein phosphatase type 1	2.605
YMR169C	ALD3	aldehyde dehydrogenase	2.521
YCL040W	GLK1	glucokinase, carbohydrate metabolism	2.517
YPR184W	GDB1	4-alpha-glucanotransferase, glycogen catabolism	2.511
YLR258W	GSY2	glycogen (starch) synthase	2.467
YDR516C	EMI2	strong similarity to glucokinase	2.422
YGL055W	OLE1	stearoyl-CoA desaturase	2.398
YPL093W	NOG1	GTPase	2.39
YBR183W	YPC1	ceramidase	2.387
YML054C	CYB2	L-lactate dehydrogenase (cytochrome)	2.304
YPL087W	YDC1	alkaline dihydroceramidase	2.213
YMR250W	GAD1	glutamate decarboxylase, amino acid metabolism	2.202
YJL052W	TDH1	glyceraldehyde 3-phosphate dehydrogenase (phosphorylating), gluconeogenesis	2.148
YIL070C	MAM33	aerobic respiration	2.141
YDR343C	HXT6	high-affinity hexose transporter	2.116
YMR105C	PGM2	phosphoglucomutase, glycogen metabolism	2.112
YER183C	FAU1	folic acid and derivative biosynthesis	2.106
YIL155C	GUT2	glycerol-3-phosphate dehydrogenase, carbohydrate metabolism	2.098
YIL045W	PIG2	protein phosphatase regulator	2.081
YHR044C	DOG1	2-deoxyglucose-6-phosphatase, glucose metabolism	2.048
YMR271C	URA10	orotate phosphoribosyltransferase, pyrimidine base biosynthesis	2.044
YBL088C	TEL1	inositol/phosphatidylinositol kinase	2.021
YDL138W	RGT2	glucose transporter	2.013
Cell cycle			
YIL066C	RNR3	ribonucleoside diphosphate reductase, DNA replication	2.826
YGR248W	SOL4	strong similarity to Sol3p	2.595
YAR014C	BUD14	bud site selection	2.277
YHR079C	SAE3	meiotic recombination	2.201
YMR117C	SPC24	chromosome segregation, spindle pole body protein	2.135
YCR018C	SRD1	rRNA processing	2.026
Transcription			
YDR463W	STP1	specific RNA polymerase II transcription factor, positive regulation	2.656
YHR006W	STP2	specific RNA polymerase II transcription factor	2.178
YPL089C	RLM1	transcription factor of the MADS box family	2.115
YGL141W	HUL5	ubiquitin protein ligase, polyubiquitination	2.099
Protein synthesis and fate			
YDL077C	VAM6	vacuolar carboxypeptidase	3.177
YMR174C	PAI3	endopeptidase inhibitor	2.354
YMR101C	SRT1	protein amino acid glycosylation	2.131
YDL093W	PMT5	dolichyl-phosphate-mannose protein mannosyltransferase	2.015
YDL075W	RPL31A	structural constituent of ribosome	2.007
Unknown			
YDL204W	RTN2	biological process unknown	7.899
YER150W	SPI1	biological process unknown	5.149
YML128C	MSC1	molecular function unknown	3.156
YGL121C	GPG1	molecular function unknown	2.606
YJL151C	SNA3	molecular function unknown	2.416
YJL047C	RTT101	molecular function unknown	2.155
YGL259W	YPS5	biological process unknown	2.063
YBR214W	SDS24	molecular function unknown	2.036
YAR027W	UIP3	molecular function unknown	2.03

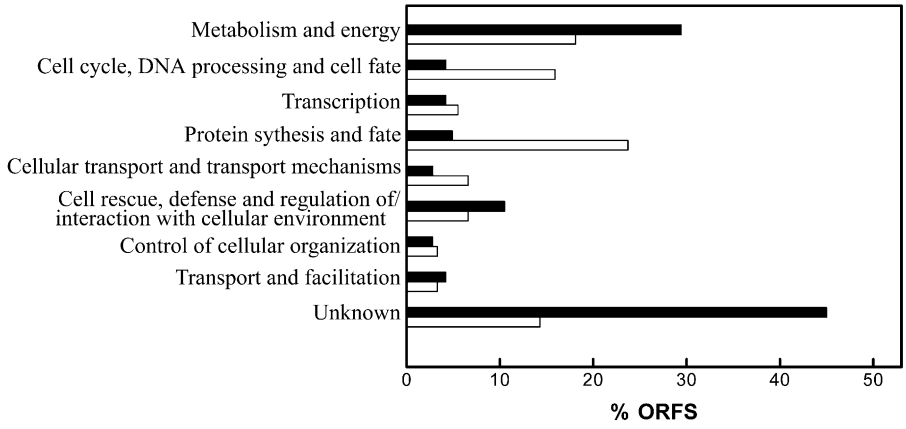

Fig. 1. Global gene expression profile in functional categories. Black bars and white bars represent the percentage of induced and repressed genes, respectively. The classification is based on the MIPS database available on the web.

among the characterized induced genes, almost half remain with unknown function. In fact, one of the top most upregulated genes was an uncharacterized ORF, *YER067W* (data not shown), followed by two genes that code for the small Hsps: *HSP30* and *HSP12* (Table 1).

In order to confirm the microarray data, we performed a semiquantitative RT-PCR analysis for the *YER067W*, *HSP12* and *HSP30* genes (Fig. 2). After hydrostatic pressure treatment of 200 MPa for 30 min, the corresponding mRNAs were detected in earlier PCR cycles (24 cycles for *YER067W*, *HSP30* and *HSP12*) when compared to control cells (36 cycles for *YER067W* and 28 cycles for *HSP12* and *HSP30*). A comparable response was detected after 50 MPa for 30 min (Fig. 2). Results from semiquantitative RT-PCR clearly supported the microarray data, even though the differences between the techniques sensitivity reflected in different induction levels measured by the two methods. Quantification of the specific PCR products revealed distinct patterns of expression of *YER067W* and Hsp messages during pressure treatment. Compared with control samples, there was a gradual increase of about 3- and 5-fold for *YER067W* when yeast cells were submitted to 50 and 200 MPa respectively. These results show that while very few copies of *YER067W* mRNA are present in non-stressed cells, the pressure treatments were able to cause a strong upregulation of this gene. In this case, the induction observed after the milder treatment with 50 MPa was slight slower than that promoted by 200 MPa. Meanwhile, Hsp messages presented a smaller and similar constant increase (approximately 2-fold) for both pressure conditions. Therefore, even with some differences in the induction levels, a mild pressure of 50 MPa is sufficient to promote the upregulation of the most upregulated genes detected by the microarrays.

Further functional analysis revealed some interesting features (Fig. 1). Categories such as transport facilitation, control of cellular organization and transcription had approximately the same number of genes induced and repressed; conversely, other classes showed a distinct up- or downregulation. Most of the known pressure-induced genes were classified in metabolism and stress defense categories, resembling a typical stress response pattern [22,24]. In addition genes related to protein synthesis and fate (folding, modification and destination) together with genes involved in cell cycle progression were strongly repressed, leading to the characteristic yeast growth arrest caused by stressful conditions [17].

Besides the common environmental stress response (ESR), microarray studies have demonstrated that the gene expression modifications caused by different environmental changes are specific to individual stress conditions [22]. Therefore, in the following sections we turn to a more detailed analysis of hydrostatic pressure-regulated genes within the mentioned categories.

3.2. Genes involved in stress response

Induced genes classified in stress response category (CR) were specified in Table 1. Two genes that encode for Hsps, *HSP12* and *HSP30*, were strongly induced by hydrostatic pressure as confirmed by RT-PCR (Fig. 2).

Studies with *S. cerevisiae* proved that the *HSP12* gene is regulated by Msn2/Msn4p trans-activators that bind to STRE (stress-responsive element). STRE was activated not only by heat shock but also by a diverse range of other stress conditions including osmotic stress, oxidative stress, nitrogen starvation, exposure to weak organic acids, low external pH and ethanol [25].

On the other hand, *HSP30* is more specifically regulated since it is activated by some, but not all, STRE-inducing stresses. Its highest induction level was observed in the following situations: organic acid stress, high ethanol concentration, entry to stationary phase and under conditions where glucose becomes a limiting factor. Even though the controlling system for *HSP30* transcription is still unidentified, it has already been demonstrated that *HSP30* activation is not related to Msn2/Msn4p or other classical transcription stress factors such as Hsf1p [26]. It is worth noting that hydrostatic pressure is a particular condition in which *HSP12* and *HSP30* genes were both induced at high levels. Interestingly, the majority of the Hsps are downregulated by cold shock, whereas only *HSP12* and *HSP26* are upregulated [27]. Furthermore, genes coding for high molecular weight Hsps, such as *HSP104* or *HSP60*, did not suffer hydrostatic pressure regulation. A recent work presented a gene expression profile of yeast cells submitted to high pressure at low temperature of 4°C [28]. Due to the differences in experimental conditions and in the time points analyzed it was difficult to address a direct comparison with the present work. However, some interesting features must be noted. Corroborating our results, the induc-

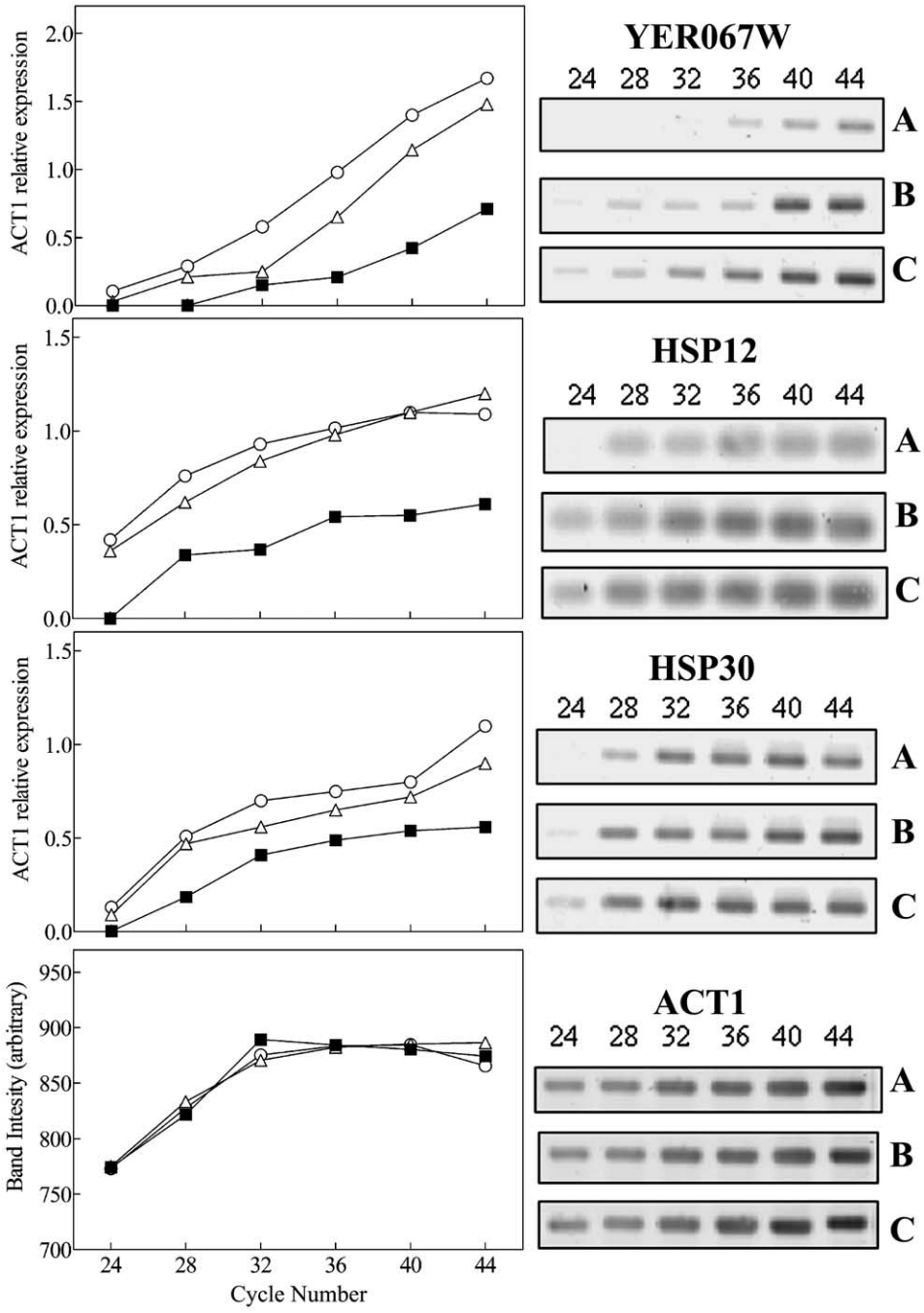

Fig. 2. Semiquantitative RT-PCR analysis of the pressure-induced genes *YER067W*, *HSP12* and *HSP30*. Pictures show agarose gel of RT-PCR products from cycles 24 to 44 using as the template, cDNA of the total RNA extracted from cells grown at atmospheric pressure, 0.1 MPa (A) and also, from pressure-treated yeast at 50 MPa (B) or 200 MPa (C) during 30 min. Graphs demonstrate band intensities plotted against PCR cycle number registered for the bands shown in the pictures, as quantified by Quantiscan software. *HSP30*, *HSP12* and YER067W band intensities were normalized by ACT1, the internal standard. (○) 200 MPa, (△) 50 MPa and (■) atmospheric pressure growing cells. This is a representative result derived from three independent experiments.

tion of *HSP30*, *HSP26* and *HSP12* was also seen after a hydrostatic pressure treatment at low temperature. On the other hand, the *HSP104* gene, which did not suffer upregulation by pressure or by cold stress individually [27], was upregulated by the combined stress when cells were assayed 1 h after the treatment [28]. Hsp104p is mainly involved in rescue of proteins from insoluble aggregates after heat stress. The overexpression of this gene has been implicated in thermotolerance and cell resistance at stationary phase [17,19]. So it seems to be that *HSP104* is not immediately regulated by pressure or cold, but it is induced as a late response to the cellular damages caused by those stresses.

High hydrostatic pressures, as well as other kinds of stresses promote a cytoplasm acidification of yeast cells. The drop in intracellular pH leads to an increase in the activity of H^+-adenosine triphosphatase (ATPase) when yeast cells are sub-

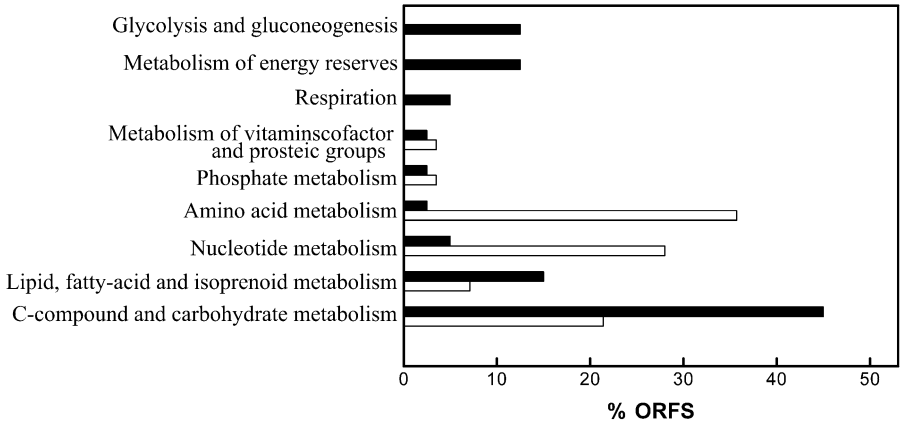

Fig. 3. Classification of hydrostatic pressure-regulated genes within metabolism and energy category, according to the MIPS database. Black bars and white bars represent the percentage of induced and repressed genes, respectively.

mitted to stresses such as heat shock, ethanol exposure and osmostress [17]. This situation provokes energy depletion during the stress period. Hsp30 then acts downregulating stress-stimulated H^+-ATPase activity, playing a role in energy conservation during stress conditions [26]. Additionally, *YRO2*, a gene that shows a strong similarity to *HSP30*, was also upregulated by the pressure treatment used in this work (Table 1).

The function of Hsp12 and Hsp26 proteins, whose genes are also controlled by Msn2/Msn4p, is less well understood in stress conditions. However, the induction of both genes has been related to stresses that cause severe membrane damage, especially osmotic and ethanol stress [24,27]. Hydrostatic pressure also induced dramatic alterations in membrane structure [9,29,30], suggesting that the induction of these small Hsps could be related to membrane destabilization.

Among other common stress-induced genes were those that encode for enzymes involved in oxidative stress repairing such as catalase (*CTT1*), mitochondrial superoxide dismutase (*SOD2*), and metallothionein (*CUP1-1*) (Table 1). This observation suggests that hydrostatic pressure also promotes an important oxidative stress.

3.3. Metabolism

Yeast cells submitted to 200 MPa suffered strong alterations in their metabolism and energy gene expression program (Table 1). Regardless of the large number of induced and repressed genes in those two categories, a further classification revealed that high pressure caused a specific modulation within distinct metabolism pathways (Fig. 3). Most of the down-regulated genes were involved in amino acid or nucleotide anabolism. This observation was in agreement with the repression acquired for genes coding for protein synthesis processes. On the other hand, energetic pathways had various important components overexpressed by pressure.

Hydrostatic pressure induced both synthetic (*GSY2*) and catabolic enzymes (*PGM2*, *GDB1*) of glycogen metabolism. In addition, enzymes involved in carbon flux, such as the stress inducible high-affinity hexose transporters *HXK4*, *7*, *6* and *1*, as well as glycolytic pathway genes (*HXK1*) were upregulated together with ones related to gluconeogenesis, such as *GPM2* and *TDH1* (Table 1). The apparent paradox of simultaneous induction of genes involved in glycolysis, gluconeogenesis and glycogen metabolism (anabolism and catabolism) may be explained by the need of the stressed cell for a circular flux of carbon to rapidly buffer and manage energy and osmotic stability, as proposed by Yale and Bohnert [24].

Interestingly, trehalose metabolism genes were neither induced nor repressed under hydrostatic pressure. It is well known that heat stress leads to trehalose accumulation in yeast cells [31]. Furthermore, microarray analysis showed that the gene *TPS1* coding for trehalose 6-P synthase, is up-regulated, not only by high temperature [22], but also by osmotic [24], cold [28] and ethanol [32] stress. The lack of *TPS1* induction was an intriguing feature of pressure on yeast cells suggesting that the signaling pathways that control the expression of that gene may be different for each kind of stress. In fact, regulation of *TPS1* seems to be dependent on multiple factors. This assumption is supported by the fact that, even though the gene *TPS1* possesses STREs in its promoter region, it is not enough for that gene to be induced in all stressful conditions, demonstrating the existence of further controlling mechanisms [33]. Despite the important role of trehalose in protecting the cell membrane during heat stress it is probably not the main membrane protector during pressure, given that *tps1 S. cerevisiae* mutant can acquire barotolerance when at stationary phase or after a mild temperature pretreatment [21].

It was demonstrated that other elements were also important for membrane protection. Ergosterol plays an important role in ethanol tolerance and in thermotolerance. Experiments involving *S. cerevisiae* mutants in the ergosterol biosynthesis showed that trehalose failed to confer the same level of protection against the mentioned stresses [34]. As already stated, hydrostatic pressure, as well as low temperature force packing of lipids resulting in a decrease in membrane fluidity. It has been found that membranes richer in cholesterol were more resistant to high pressures without acquiring rigidity than those poorer in this molecule [6]. Ergosterol is a molecule similar to cholesterol with an analogous function in yeast cells. The sterol biosynthetic pathway in yeast has two major portions, from acetate to farnesyl pyrophosphate and then to ergosterol. After farnesyl pyrophosphate synthesis, the induction of any gene that encodes an enzyme involved in the pathway leads to an increase in ergosterol amounts [35]. Our microarray analysis showed that at least one gene (*ERG25*) implicated in ergosterol biosynthesis after farnesyl pyrophosphate was upregulated, suggesting that this molecule may be

an important protector of cell membranes under hydrostatic pressure (Table 1).

Another interesting feature was the induction of the *OLE1* gene, which encodes for a Δ9-desaturase in yeast. There was considerable evidence that an increased proportion of unsaturated fatty acids in membrane lipids was strongly correlated with bacterial resistance under high pressure as well as at low temperature [27]. A higher proportion of unsaturated fatty acids would help to maintain favorable fluidity and viscosity of biological membranes under high pressure or at low temperature. In fact the cell membranes of at least two extremely piezophilic bacteria – *Shewanella* sp. strain DB21MT-5 and *Moritella* sp. strain DB21MT-5 – present a higher proportion of unsaturated lipids when compared with normal atmospheric pressure-adapted strains [36]. Furthermore, microarray analysis performed with *S. cerevisiae* submitted to low temperatures also demonstrated the induction of the *OLE1* gene [27], indicating that the cell can sense the fluidity state of the membrane and also possesses mechanisms to compensate for the deleterious effects of environmental conditions.

3.4. Unknown genes

Hydrostatic pressure treatment caused the induction of 64 ORFs with no further characterization. Interestingly, 87.5% of these unknown genes coded for proteins that present putative transmembrane domains. Among the unknown genes encoding proteins with molecular mass of less than 20 kDa, this proportion rises to 98.3%. An increase in the expression of small membrane binding proteins could be a protection mechanism against membrane damage. Actually, a fuller understanding of the function of these unknown genes would bring valuable knowledge about new stress defense mechanisms not only for hydrostatic pressure but also for other stress situations. In fact, this is the present focus of our research. A careful analysis of the *S. cerevisiae* genome database (http://www.yeastgenome.org) provided interesting insights into the role of some overexpressed ORFs on the pressure stress response. The level of expression of the unknown genes was screened in whole genome microarray profiles of *S. cerevisiae* cells exposed to diverse stress situations. Some genes were commonly induced under a wide range of stress conditions while others, such as *YNL266C*, *YDL121C* and *YNL198C*, revealed a quite specific response to pressure.

The information about possible interactions of each ORF with other known genes suggested some functional clues to us. It was shown by two hybrid experiments that *YNL266W*, *YPR096C* and *YOL101C* genes interact with *JSN1* [37], a gene that, when superexpressed, proved to be a phenotype suppressor of *tub2-150* mutation that causes an increase in the stability of microtubules. This observation showed that *JSN1* might be implicated in the process of turnover and elongation of microtubule structures [38]. Many other ORFs, for example *YAL064W*, *YER067W* [37] and *YDR070C* [39], were found to be associated with proteins related to actin and cellular wall synthesis. As hydrostatic pressure severely affects the cytoskeleton components [8,9], a cellular mechanism for cytoskeleton repair and structural integrity preservation must be crucial for cell survival under high pressure. The gene *YER067W* was also induced by hydrostatic pressure and low temperatures and in addition, strains lacking those genes were revealed to be more sensitive to hydrostatic pressure [28].

The analysis of pressure-regulated genes revealed some stress defense mechanisms of yeast cells for overcoming the damage caused by hydrostatic pressure. High hydrostatic pressure has gained a great biotechnological importance in the last decade, with studies on food decontamination, vaccine production, among others [1,6]. Therefore, the results presented in this work are interesting not only for the comprehension of the physiological adaptations of piezophiles and piezoytolerant organisms but also to shed light on changes imposed by pressure on microorganisms.

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References

[1] Rothschild, L.J. and Mancinelli, R.L. (2001) Nature 409, 1092–1101.
[2] Mentre, P., Hamraoui, L., Hui Bom Hoa, G. and Debey, P. (1999) Cell Mol. Biol. 45, 353–362.
[3] Braganza, L.F. and Worcester, D.L. (1986) Biochemistry 25, 7484–7488.
[4] Hubner, W., Wong, P.T. and Mantsch, H.H. (1990) Biochim. Biophys. Acta 1027, 229–237.
[5] Reyes, M.C., Tauc, P. and Brochon, J.C. (1993) Biophys. J. 65, 2248–2260.
[6] Molina-Garcia, A.D. (2002) Biotechnol. Genet. Eng. Rev. 19, 3–53.
[7] Robinson, C.R. and Engelborghs, Y. (1982) J. Biol. Chem. 257, 5367–5371.
[8] Salmon, E.D. (1975) Science 189, 884–886.
[9] Fernandes, P.M.B., Farina, M. and Kurtenbach, E. (2001) Lett. Appl. Microbiol. 32, 42–46.
[10] Lynch, T.W. and Sligar, S.G. (2002) Biochim. Biophys. Acta 1595, 277–282.
[11] Foguel, D. and Silva, J.L. (1994) Proc. Natl. Acad. Sci. USA 91, 8244–8247.
[12] van Eldik, R., Asano, T. and Le Noble, W.J. (1989) Chem. Rev. 89, 549–688.
[13] Kitamura, Y. and Itoh, T. (1987) J. Sol. Chem. 16, 715–725.
[14] Gross, M. and Janicke, R. (1994) Eur. J. Biochem. 221, 617–630.
[15] Abe, F. and Horikoshi, K. (1995) FEMS Microbiol. Lett. 130, 307–312.
[16] Abe, F. and Horikoshi, K. (1997) Extremophiles 1, 89–93.
[17] Hohman, S. and Mager, W.H. (1997) Yeast Stress Responses, R.G. Landes Company, Austin, TX.
[18] Varela, J.C.S. and Mager, W.H. (1996) Microbiology 142, 721–731.
[19] Craig, E.A. (1992) The Molecular and Cellular Biology of The Yeast Saccharomyces Gene Expression Vol 2. Cold Spring Harbour Laboratory Pressc Cold Spring Harbor, NY.
[20] Paris, S. and Pringle, J.R. (1983) Ann. Microbiol. 134, 379–385.
[21] Fernandes, P.M.B., Panek, A.D. and Kurtenbach, E. (1997) FEMS Microbiol. Lett. 152, 17–21.
[22] Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisein, M.B., Storz, G., Botstein, D. and Brown, P.O. (2000) Mol. Biol. Cell 11, 4241–4257.
[23] Iwahashi, H., Fujii, S., Kaouru, O., Kaul, S.C., Sato, A. and Komatsu, Y. (1993) FEMS Microbiol. Lett. 108, 53–58.
[24] Yale, J. and Bohnert, H.J. (2000) J. Biol. Chem. 276, 15996–16007.
[25] Estruch, F. (2000) FEMS Microbiol. Rev. 24, 469–486.
[26] Seymour, I.J. and Piper, P.W. (1999) Microbiology 145, 231–239.
[27] Sahara, T., Goda, T. and Ohgiva, S. (2002) J. Biol. Chem. 277, 50015–50021.
[28] Iwahashi, H., Shimizu, H., Odani, M. and Komatsu, Y. (2003) Extremophiles 7, 291–298.
[29] Shimada, S., Andou, M., Naito, N., Yamada, N., Osumi, M. and Hayashi, R. (1993) Appl. Microbiol. Biotechnol. 40, 123–131.

[30] Bourns, B., Franklin, S., Cassimeris, L. and Salmon, E.D. (1988) Cell Motil. Cytoskeleton 10, 380–390.
[31] Panek, A.D. (1995) Braz. J. Med. Biol. Res. 28, 169–181.
[32] Alexandre, H., Ansanay-Galeote, S., Dequin, B. and Blodin, B. (2001) FEBS Lett. 498, 98–113.
[33] Alexandre, H., Plourde, L., Charpentier, C. and François, J. (1998) Microbiology 144, 1103–1111.
[34] Watson, K. and Swan, T.M. (1998) FEMS Microbiol. Lett. 169, 191–197.
[35] Daum, G., Lees, N.D., Bard, M. and Dickson, R. (1998) Yeast 14, 1471–1510.
[36] Abe, F., Kato, C. and Horikoshi, K. (1999) Trends Microbiol. 7, 447–453.
[37] Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M. and Sakaki, Y. (2001) Proc. Natl. Acad. Sci. USA 10, 4569–4574.
[38] Machin, N.A., Lee, J.M. and Barners, G. (1995) Mol. Biol. Cell 6, 1241–1259.
[39] Uetz, P., Giot, L., Cagney, G., Mansfield, T.A., Judson, R.S., Knight, J.R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., Qureshi-Emili, A., Li, Y., Godwin, B., Conover, D., Kalbfleisch, T., Vijayadamodar, G., Yang, M., Johnston, M., Fields, S. and Rothberg, J.M. (2000) Nature 10, 623–627.