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The biological effects of high-pressure gas on the yeast transcriptome

H. Matsuoka[1,2], Y. Suzuki[1], H. Iwahashi[1], T. Arao[2], Y. Suzuki[2] and K. Tamura[2]

[1]National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, Japan
[2]Department of Chemical Science and Technology, Faculty of Engineering, The University of Tokushima, Minamijosanjima-cho, Tokushima, Japan

Correspondence
H. Matsuoka
National Institute of Advanced Industrial Science and Technology
Central 6, Higashi 1-1-1
Tsukuba, Ibaraki 305-8566
Japan
E-mail: hitoshi.iwahashi@aist.go.jp

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Abstract

The aim of the present study was to examine the feasibility of DNA microarray technology in an attempt to construct an evaluation system for determining gas toxicity using high-pressure conditions, as it is well known that pressure increases the concentration of a gas. As a first step, we used yeast (*Saccharomyces cerevisiae*) as the indicator organism and analyzed the mRNA expression profiles after exposure of yeast cells to nitrogen gas. Nitrogen gas was selected as a negative control since this gas has low toxicity. Yeast DNA microarray analysis revealed induction of genes whose products were localized to the membranes, and of genes that are involved in or contribute to energy production. Furthermore, we found that nitrogen gas significantly affected the transport system in the cells. Interestingly, nitrogen gas also resulted in induction of cold-shock responsive genes. These results suggest the possibility of applying yeast DNA microarray to gas bioassays up to 40 MPa. We therefore think that "bioassays" are ideal for use in environmental control and protection studies.

Key words
- DNA microarray
- Yeast
- Hydrostatic pressure
- Gas pressure
- Nitrogen gas

Introduction

While seeking a comfortable living environment, the human race has been continuously releasing large quantities of pollutants into the environment, consequently causing various problems such as air, water, and soil pollution. Although emission of pollutants is being gradually reduced by the recent tighter control of emissions of environmental pollutants, we are still polluting our environment. The problem is further compounded by the fact that we are not dealing with a single pollutant, but with a mixture of pollutants in the environment, and therefore it is rather difficult to recognize their effects on organisms. Methods that can determine their effect primarily include physiochemical analyses and bioassays. When pollution is supposed to be present to some extent, we can use physicochemical analyses as a qualitative and quantitative tool for a single substance; however, this is not useful for the assessment of the impact of tens of millions of chemical substances on the environment. Although we cannot determine the concentration of each chemical substance by bioassay, it is possible to do rapid toxicity and genetic toxicity analysis using animal organisms (mammals such as rodents, fish and shellfish, algae, single cells, etc.) because it focuses attention on the effect on organisms.

We have studied a bioassay for the hydrosphere using a yeast DNA microarray. In the present study, drawing on our previous experience, we attempted to develop a bioas-

say system for polluted air monitoring. By using yeast (*Saccharomyces cerevisiae* S288C), suitable for rapid toxicity assessment, we can monitor genome-wide gene expression profiles by DNA microarray after exposing yeast cells to high-pressure gas. Thus, we believe it will be possible to assess the impact of polluted gases within short periods of time.

In the present report, we focus mainly on nitrogen gas, a component of our life-supporting air in the environment. Our results revealed significant changes in gene expression profiles, such as those involved in the membrane system, energy, cellular transport, and even cold-response. Since we were able to detect and characterize the response, we conclude that we may use high-pressure gas conditions for gas bioassay up to 40 MPa.

Material and Methods

Strain and growth conditions

Saccharomyces cerevisiae S288C (*suc2 mal gal2 CUP1*) cultured in yeast extract/peptone/dextrose medium (YPD) (2% polypeptone, 1% yeast extract, 2% glucose) were inoculated into fresh YPD in two Erlenmeyer flasks. Yeast cells were grown to an absorbance (A_{660}) of 0.9 to 1.0 at 660 nm at 30ºC before pressurization.

Pressure treatment

The cultures were placed in two nylon bags and the holes were sealed with cotton wool, which were transferred to a high-pressure vessel (30-11HF4, High Pressure Equipment Co. Ltd., Elie, PA, USA). The sample was pressurized with nitrogen gas at 40 MPa and a control was kept under atmospheric pressure of 0.1 MPa. Both sample and control were incubated in a thermostated bath for 2 h at 30ºC. Thirty minutes were needed to bring the pressure up to 40 MPa, followed by another 30 min for decompression. Yeast cells were then incubated for 2 h at 40 MPa, thus being exposed to high pressure for a total of 3 h. The control was incubated for 3 h under the same conditions as the experimental sample, except that the pressure was 0.1 MPa.

DNA microarray analysis

Total RNA was isolated by the hot phenol method (1). Poly(A)+RNA was purified from total RNA with an Oligotex-dT30 mRNA purification kit (Takara, Otsu, Japan). Fluorescently labeled cDNA was synthesized by oligo dT-primer polymerization using PowerScript™ reverse transcriptase. cDNA obtained from control poly(A)+RNA was fluorescently labeled with Cy3 and cDNA obtained from the poly(A)+RNA of the sample treated with nitrogen gas was labeled with Cy5. Two to four micrograms poly(A)+RNA was used for each labeling and the same amount of each poly(A)+RNA was used on one slide. The two labeled cDNA pools were mixed and hybridized with a yeast DNA chip (DNA Chip Research, Inc., Yokohama, Japan) for 24-36 h at 65ºC. Open reading frames of 200- to 8,000-bp DNA (0.1-0.5 ng) were spotted on this microarray, and 5880 genes could be analyzed under these conditions. After hybridization, the labeled microarrays were washed and dried and scanned with a confocal laser Scanarray 4000 system (GSI Lunomics, Billeria, MA, USA). The resulting imaging data were quantified using the Quantarray system (GSI Lunomics). The fluorescence intensity of each spot on the image was subtracted from the background, and the Cy5/Cy3 intensity ratios were calculated and normalized using the median value of the GeneSpring software (Silicon Genetis, Red Wood, CA, USA) as positive control. The DNA microarray data in this report were obtained from two sheets of DNA microarray images. The details of the microarray procedure have been described previously (2).

Cluster analysis

To extract meaningful information from the large amount of data generated by the DNA microarray, we applied a bioinformatics tool called hierarchical cluster analysis which permits one to recognize changes in the transcription patterns following exposure to nitrogen gas. Hierarchical clustering was carried out using GeneSpring (3). The settings for the calculations were as follows: similarity was measured by standard correlation, the separation ratio value was 1.0, and the minimum distance value was 0.001. The genes used for this analysis have been described previously (4).

Results and Discussion

Conditions for treatment with pressurized gas

In DNA microarray analysis, the most important factor corresponds to the conditions for stress treatment. A weak stress cannot be expected to significantly affect cell physiology, whereas a strong stress may cause cell death. Generally, we can expect sufficient stress responses under a treatment that causes about 50% growth inhibition (5-11). After the pressure treatment, colony-forming units were monitored to select the appropriate conditions of pressure treatment. We found that 40 MPa of nitrogen gas caused 50% growth inhibition and consequently selected this condition for the pressure treatment.

Clustering analysis of hydrostatic pressure and gas pressure

Hierarchical cluster analysis was performed using the GeneSpring version 4.2.1 software (2). The clustering algorithm arranges conditions according to their similarity in expression profiles across all conditions, in such a way that conditions with similar patterns are clustered together as in a taxonomic tree (Figures 1 and 2). Cluster analysis of high-pressure conditions (hydrostatic pressure and gas pressure), surface

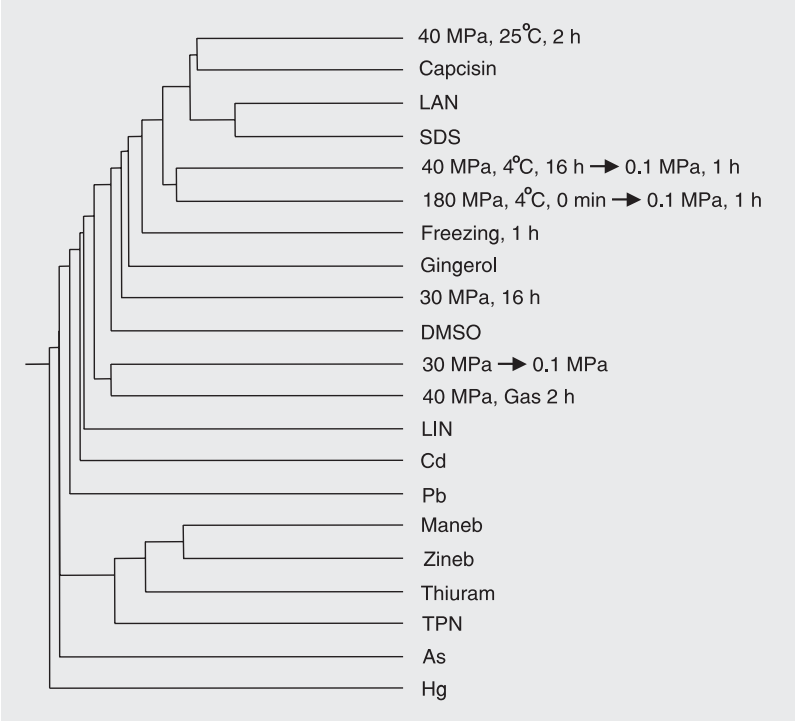

Figure 1. Cluster analysis of expression profiles following treatment of yeast with compressed gas pressures, heavy metals, surface active agent, freezing, and pesticides. Expression profiles based on 6335 spots were used for the calculation.

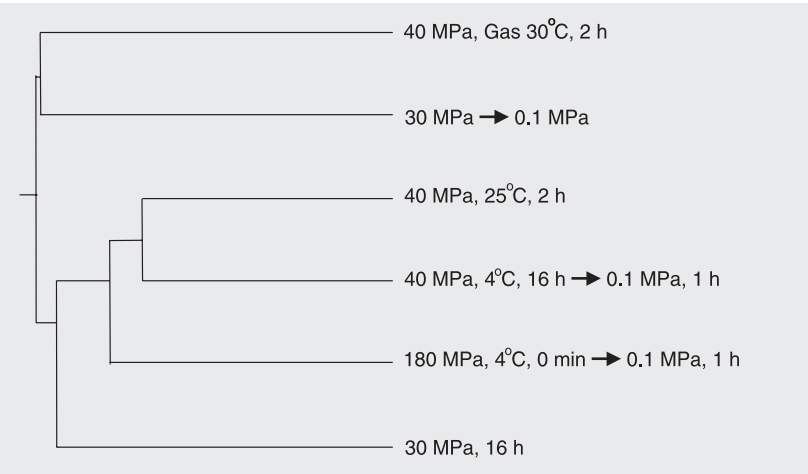

Figure 2. Cluster analysis of expression profiles following treatment of yeast with six different compressed gas pressures. A dendrogram was constructed to indicate the relationship among the effects of each compressed pressure. Expression profiles based on 6335 spots were used for the calculation.

active agent, heavy metals, freezing, and pesticide chemicals is shown in Figure 1. We found that high pressure (hydrostatic pressure and gas pressure), surface active agent, and freezing were grouped together, and were separate from pesticide chemicals and heavy metals. We also compared nitrogen gas pressure conditions with other pressure conditions (Figure 2). An interesting observation was that a 40-MPa pressure of nitrogen gas at 30ºC for 2 h did not form a cluster with hydrostatic pressure of 40 MPa at 25ºC for 2 h.

Characterization of induced genes by their functions

It was possible to detect 6335 spots. After global normalization, we removed the spots with a Cy5 intensity of less than 4000 because of the doubtful results of lower intensity. Given that more than 99% of the control spots without DNA had intensities lower than 1000 for Cy3, the cutoff line for precluding negative results was taken as a scanned intensity below 2000 for Cy3 (which corresponded to an intensity of 4000 for Cy5) (12). Results between 1000 and 2000 of intensity for Cy3 were considered doubtful, and therefore discarded. Finally, we selected a total of 5164 genes from 6335 spots and from these 5164 genes we selected 226 as the induced genes and 210 as the repressed genes, by pressure treatment. The 226 induced genes were selected as the genes having a Cy5 intensity two-fold higher than the Cy3 intensity. The 210 repressed genes were also selected as the genes having a Cy5 intensity 0.5 times lower than the Cy3 intensity.

We characterized induced genes using the functional category of MIPS (http://mips.gsf.de/) from two microarray data sets. MIPS is a database in which all yeast genes are assigned to 18 functional categories and more than 100 subcategories. Using this database we are able to determine the functions of the genes. Table 1 shows the functional

Table 1. List of functional categories and subcategories activated by nitrogen gas pressure.

Functional categories and functional subcategories	%	No. of genes	Total number
Energy	**17.9**	**45**	**252**
Respiration	23.9		
Transport facilitation	**8.6**	**27**	**313**
Transport mechanism	16.2		
Regulation of/interaction with the cellular environment	**8.0**	**16**	**199**
Ionic homeostasis	11.0		
Metabolism	5.9	63	1066
Cell rescue, defense and virulence	5.4	15	278
Cellular transport and transport mechanisms	4.8	24	495
Subcellular localization	4.5	11	2258
Classification not yet fully determined	4.3	5	115
Protein synthesis	3.6	13	359
Control of cellular organization	2.9	6	209
Protein fate (folding, modification, destination)	2.5	15	595
Unclassified proteins	2.1	51	2399
Cell fate	1.9	8	427
Cell cycle and DNA processing	1.8	11	628
Transcription	1.6	12	771
Cellular communication/signal protein activity regulation	0	0	59
Transduction mechanism	0	0	13
Transposable elements, viral and plasmid proteins	0	0	116

The functional categories and the proportion of induced genes in categorized genes are listed. The extensively activated categories are shown in bold.

characterization of 226 genes that were considered to be the induced genes. The functional categories that were significantly induced were "Energy (17%)", "Transport facilitation (8.6%)", and "Regulation of/interaction with cellular environment (8.0%)". Furthermore, the "Energy" category was characterized by the "Respiration" subcategory (Table 1), which in turn was further characterized with genes whose products were localized in mitochondria, such as cytochrome c oxidases. The categories of "Transport facilitation" and "Regulation of/interaction with cellular environment" were characterized by the subcategories of "Transport mechanism" and "Ionic homeostasis" (Table 1). These two subcategories belong to different categories which, however, consist of transporter genes. For example, the ABC transporter and Na^+ pump and ATP synthase belong to these subcategories. Table 2 lists the top 25 highly induced genes treated with nitrogen gas under 40 MPa at 30ºC for 2 h. The genes related to the transport system

Table 2. Top 25 induced open reading frames under pressure of 40 MPa at 30ºC for 2 h.

Name	Ratio	Description
BTN2	20.5	Gene/protein whose expression is elevated in a btn1
YLR162W	15.2	Hypothetical protein
SCW4	14.3	Soluble cell wall protein can be released from SDS-extracted cell walls under reducing conditions
THI22	10.7	Similarity to *Bacillus subtilis* transcriptional activator tenA
JEN1	10.7	Carboxylic acid transporter protein homolog
AGP1	8.8	Amino acid permease
ENA1	8.1	Plasma membrane P-type ATPase involved in Na^+ and Li^+ efflux
TIR1	8.1	Cold-shock-induced protein of the Srp1p, Tip1p family of serine-alanine-rich proteins
MSD1	7.7	Aspartyl-tRNA synthetase, mitochondrial
ENA5	7.3	Plasma membrane P-type ATPase involved in Na^+ and Li^+ efflux
CTR1	6.6	Plasma membrane copper transport protein
CAT8	6.5	Zinc-cluster protein involved in activating gluconeogenic genes related to Gal4p
FIT2	6.3	Mannoprotein that is incorporated into the cell wall via aglycosylphosphatidylinositol anchor, involved in the retention of siderophore iron in the cell wall
ENA2	6.0	Plasma membrane P-type ATPase involved in Na^+ and Li^+ efflux
DED1	5.9	ATP-dependent RNA helicase of the DEAD box family
COX3	5.6	Cytochrome c oxidase subunit III, mitochondrially coded
RPL4A	5.1	Ribosomal protein L4A (L2A) (rp2) (YL2)
YLR201C	5.0	Hypothetical protein
HXT5	4.9	Hexose transporter
COX2	4.8	Subunit II of cytochrome c oxidase
ATP1	4.7	Mitochondrial F1F0-ATPase alpha subunit
TIR2	4.6	Induced by cold shock
TY1A	4.4	TyA gag protein. Gag processing produces capsid proteins
SPI1	4.4	Similar to Sed1 highly expressed in the stationary phase
YFR044C	4.3	Similarity to the hypothetical protein YBR281c

The genes related to the transport system are underlined and the genes related to cold shock are indicated by broken lines.

are underlined and account for about 50% of the top 25 genes. Thus, it may be suggested that 40 MPa nitrogen gas affects cellular transport. These characteristics listed in Table 2 are different from those of hydrostatic pressure treatment because hydrostatic pressure induces genes linked to membrane metabolism such as *INO1*, *OPI3*, and *RTA1* (data not shown). High-pressure nitrogen gas is considered to be anaerobic and thus the effect on mitochondria only reflects the effect of gas pressure on transport in the mitochondria.

It has been reported that hydrostatic pressure induces genes which respond to heat shock proteins (13), but 40 MPa nitrogen gas pressure did not induce heat shock proteins. In contrast, nitrogen gas pressure induced genes which respond to cold shock, such as *TIR1* and *TIR2*. These genes are shown in Table 2 and are indicated by broken lines. It is interesting to note that hydrostatic pressure apparently increased temperature, whereas gas pressure apparently decreased temperature.

We could observe living cells with 50% colony-forming units and we could detect the responses after nitrogen gas treatment under 40 MPa. This suggests that, up to 40 MPa, we can expect a response and we can characterize it. Generally other gases have a more toxic potential than nitrogen gas. The gas bioassay under high pressure can be applied to other gases to characterize the toxicity that causes damage to east cells.

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