Biotechnol. Bioprocess Eng. 2000, 5: 400-406

Multiple-end-point Bioassays Using Microorganisms

Hitoshi Iwahashi

National Institute of Bioscience and Human-Technology, Higashi 1-1, Tsukuba, Ibaraki 305-8566, Japan

Abstract Since the 1950s, the numbers of species and chemicals produced have significantly increased. Despite the fact that industrial chemicals have given us numerous benefits, there is no doubt that they have damaged the environment. The chemicals being dispersed on the earth should be carefully controlled to prevent adverse effects. Bioassay is one of the methods to assess chemical safety. In bioassay systems, chemical safety is estimated by monitoring biological responses to environmental pollutants and newly synthesized chemicals. This report introduces multiple-end-point bioassay systems that are based on chemical sensitivities of microorganisms, responses of one kind of organism, and micro-array technology. Multiple-end-point bioassays enable the prediction of chemicals in the environment and the understanding of toxicities of newly synthesized chemicals.

Keywords: bioassay, micro-array, chemicals, pollutants, yeast

INTRODUCTION

At present, more than 17 million materials are registered in the Chemical Abstract database, and it is estimated that more than 10,000 synthetic chemicals are accumulating and increasing in the environment every year. Despite the fact that these industrial chemicals have given us numerous benefits, there is no doubt that they have damaged the environment. The chemicals being dispersed on the earth should be carefully controlled to prevent their adverse effects. In fact, many chemicals can be detected from environmental samples; however, only 10% of those chemicals can be identified by current technology [1]. Ten percent is an inadequate number to protect the environment. Thus, new methods must be developed to assess the safety of chemicals in our environment.

Bioassays are one of the methods used to assess chemical safety. Bioassays are used for the assessment of environmental pollution and risk assessment of chemicals [2]. In bioassay systems, chemical safety is estimated by monitoring biological responses to environmental pollutants (the estimation of environmental pollutants) and newly synthesized chemicals (risk assessment). One of these studies is the multicenter evaluation of *in vitro* cytotoxicity (MEIC) programme, organized by the Scandinavian Society for Cell Toxicology [2]. The investigators compared LD50 obtained *in vivo* (whole organism) and IC50 obtained *in vitro* ("Bioassay"). They found a correlation between the LD50 *in vivo* and the IC50 *in vitro*, and they suggested "basal cytotoxicity". "Basal cytotoxicity" can be understood as the toxic effect to cellular components, functions and biosynthesis that are universal to all cell lines. The Ames test is one of the most powerful methods used for monitoring the mutagenicity of environmental samples [3]. In this system, mutants of *Salmonella typhimurium* are grown in a medium made from environmental samples, and mutagenicity is estimated according to the frequency of back mutation. As the frequency of back mutation is depend on the damages to the chromosome, we can estimate mutagen in the medium containing chemicals or environmental samples. From this system, mutagenicity of environmental samples or newly synthesized chemicals can be predicted.

So far, there are decades of bioassay systems that include tests by Ames, Microtox, Umu, and others [2]. Each system can be used for estimating toxicity in environmental samples; however, the information that can be estimated is limited to the degree of toxicity or mutagenicity. Information concerning the nature of the pollutants remains unavailable. In addition, bioassay systems sometimes mistakenly identify natural products as the toxic substance (data not shown). Although it is important to quantify toxicity in the environment, information concerning the nature of chemicals is essential for the assessment and treatment of environmental pollutants. Bioassay systems are required that can be used for predicting the behavior of chemicals in the environment and that, furthermore, can be used to understand the toxicity found in newly synthesized chemicals.

Rieger *et al.* [4,5] analyzed chemotyping of yeast mutants using robotics. They observed the growth abilities of the mutants under the presence of 300 kinds of chemicals. These mutants were constructed as the mutants that have the deletion in the genes functionally not characterized. They succeed to characterize the

*Corresponding author
Tel: +81-298-61-6059 Fax: +81-298-61-6009
e-mail: iwahashi@nibh.go.jp

growth ability of the each mutant using robotics. Their approach is not for bioassay to chemicals but to the role of genes, however, their approach proves that the chemical sensitivity can be the end points of bioassay and the robotics are useful.

Recently, the DNA micro-array technology enables genome-wide analyses of cellular responses at transcriptional level [6,7]. On the yeast micro-array, approximately 6,000 open reading frames (ORFs) are printed and fixed on one sheet of a slide. Fixed DNA can be used as probes for estimating the expression level of mRNA, thus we can monitor almost all gene features in one micro-array. This technology has a great opportunity for bioassay of risk assessment and environmental assessment, because it has the ability to overview thousands of genes at the same time and shed light on the way to investigate toxicological problems. Nuwaysir *et al.*[6] and Afshari *et al.* [7] focused on the possibility of micro-array for the field of toxicology. They concluded that micro-array should be used in bioassay for risk assessment and environmental assessment. We also tried to apply micro-array system to multiple-end-point bioassay.

This report introduces multiple-end-point bioassay systems that can predict chemicals in the environment and lead us to an understanding of the toxicity of newly synthesized chemicals.

MATERIALS AND METHODS

Reference Chemicals

Chemicals used in this work are listed in Table 1. The organic chemicals were dissolved in DMSO. These chemicals were selected because of their toxicity and presence in the environment. Reference chemicals are constituted with some classes of chemicals, such as heavy metals, mutagens, and endocrine disrupters.

Strains and Growing Conditions

Chemical sensitive strains shown in Fig. 1 were selected from more than 400 strains derived from the type culture collections. The chemical sensitivities of the microorganisms were characterized using the 32 chemicals listed in Table 1. According to the sensitivities, several strains were selected as indicator strains that are sensitive to specific chemicals and resistant to others. Microorganisms were grown in the medium containing 1% polypeptone and 0.5% yeast extract.

Saccharomyces cerevisiae of IFO-0224 (Diploid, Wild Type), W303-1A β-gal (*ade2, his3, ura3, leu2, trp1, CIFI), 1A-H19[PSI+] (ade2-1(UAA), leu2-3,112 SUQ5; [PSI+])*, and IS110-18A [psi-] *(aro7-1 leu2-2(UGA) ilu1-2 his4-116 lys2-101 met8-1 trp5-48 ura4-1)* were used as the indicator strains. *S. cerevisiae* grown overnight (10^8 cells/ mL) in a YPD (2% polypeptone, 1% yeast extract, and 2% glucose) medium or an SD medium (0.67% yeast nitrogen base without amino acid, 2% glucose) [8] was inoculated (200X dilution) into fresh medium or sterilized water that contained 32 kinds of model chemicals with various concentrations and was incubated at 25°C. These experiments were carried out in 96 well microplates. CFU was estimated on YPD agar (2%) plate [8]. Petite colony formation was estimated on YPG agar (2% polypeptone, 1% yeast extract, 0.1% glucose, 2% glycerol, 2% agar) plates [8].

For micro-array analysis, yeast cells were grown to the degree that the absorbance at 660 nm is 1.0. To this condition of culture, chemicals were added and further incubated for 2 h (1 in Fig. 2).

Table 1. List of reference chemicals for bioassays and concentrations for storage

2-Aminoanthracene (100 mM), Benzo(a)pyrene (10 mM), Bisphenol-A (100 mM), Di-2-ethylhexyl phthalate (25 mM), 2,5-Dichlorophenol (100 mM), 2,4-Dichlorophenoxy acid (200 mM), Formaldehyde (50 mM), Methylmercury chloride (20 mM), 4-Nitroquinoline-N-oxide (0.5 mM), *p*-Nonylphenol (20 mM), Pentachlorophenol (20 mM), Sodium arsenite (10 mM), Thiuram (10 mM), Tributyltin chloride (2 mM), 2,4,5-Trichlorophenol (100 mM), Trp-P-2(Acetate) (10 mM), Paraquat (30 mM), Cadmium chloride (10 mM), Lindane (100 mM), Malathion (20 mM), Maneb (10 mM), Nickel chloride (100 mM), Potassium dichlomate (1 mM), Triphenyltin chloride (0.1 mM), Phenol (500 mM), Benthiocarb (200 mM), Hexachlorophene (20 mM), Triclosan (10 mM), Mercuric chloride (20 mM), Cupric sulphate (100 mM), Potassium cyanide (500 mM), DMSO (100%)

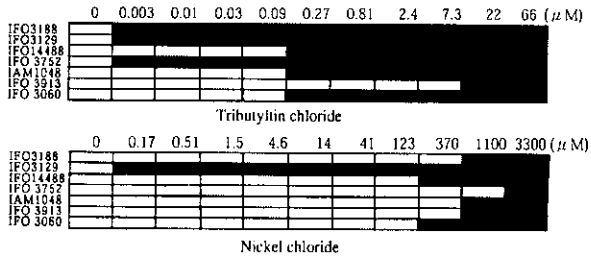

Fig. 1. Multiple-end-point bioassays based on chemical sensitivities of microorganisms. The seven indicator strains were grown in the medium containing nickel chloride and tributyltin chloride with the concentration indicated in the figure. Growth was monitored as the absorbance at 650 nm. Closed box shows that the indicator microorganisms were affected by chemicals.

Bioassays Using Yeast Cells

Growth inhibition was monitored at A_{650} of IFO-0224 in 96 well microplates. Each well contained a 200 μL YPD medium containing chemicals of various concentrations.

Viability was estimated from the CFU after the yeast cells (IFO-0224) were treated in a water solution of chemicals for 24 h.

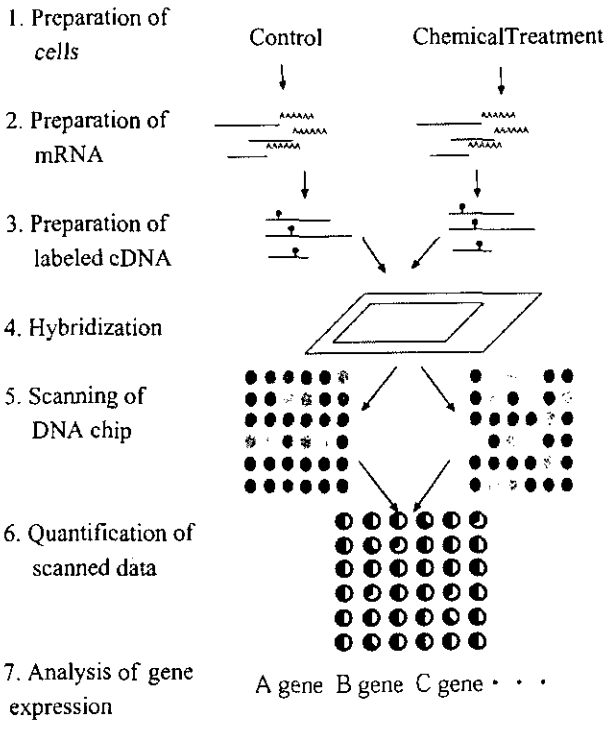

Fig. 2. Procedures for micro-array analysis in Cellular System Laboratory was summarized. Details were described in the text.

Stress induction was monitored as a β-galactosidase activity in which β-galactosidase structure gene was connected after the Hsp104 promoter region (W303-1A β-gal) [9]. Thus, the levels of β-galactosidase activities reflect the induction levels of Hsp104.

Prion curing mutagenecity were estimated from the number of red colonies due to an adenine requirement after colony formation [10] on the YPD plates. The strain of 1A-H19 has a nonsense mutation (UAA) in the ADE2 gene and has a prion of [psi]. As the prion of [psi] suppresses this nonsense mutation, the 1AH19 does not require adenine. Whereas once this strain lost a prion, the strain required adenine and formed a red colony due to the adenine requirement [10].

Cytoplasmic mutagenecity was estimated as the frequency of petite colony formation on YPG plates after growing the strain of IFO-0224 for 24 h in a YPD medium containing chemicals. The strains that form petite colonies on YPG plates cannot utilize glycerol as a carbon source [11] because of its deletion in mitochondrial DNA by chemicals.

Chromosomal mutagenecity was estimated as the frequency of reversion of a leucine requirement after growing the IS110-18A strain for 24 h in a YPD medium containing chemicals. The strain of IS110-18A has the nonsense mutation (UGA) in LEU2 gene [12]. As more than ten kinds of suppressor mutations to the UGA nonsense mutation were reported [12], the frequency of reversion could be more than ten times higher than the *LEU2 gene reversion itself.*

RNA Preparation, Hybridization to Micro-array, and Characterization of Induced Genes

Total RNA was isolated by a hot-phenol method (16). Poly (A)+RNA was purified from total RNA with Oligotex-dT30 mRNA purification kit (Takara, Kyoto, Japan) (2 in Fig. 2). Fluorescently labeled cDNA was synthesized by oligo dT-primed polymerization using PowerScriptTM reverse transcriptase (Clontech, California, USA). cDNA made from poly(A)+ RNA of control was fluorescently labeled with Cy3 and that of cadmium treated sample was labeled with Cy5. 2-4 μg of poly(A)+ RNA was used for each labeling and the same amount of each poly(A)+ RNA was used in one slide (3 in Fig. 2). The two labeled cDNA pools were mixed and hybridized for 24-36 h at 65°C (4 in Fig. 2). After hybridization, the labeled micro-arrays were washed and dried. Subsequently labeled micro-arrays were scanned using a conforcal laser ScanArray 4000 (GSI Lumonics, MA, USA) system (5 in Fig. 2). Resulting image data were quantitated using QuantArray (GSI Lumonics, MA, USA) (6 in Fig. 2). The fluorescence intensity of each spot on images was subtracted each background and the ratios of intensity Cy5/Cy3 were calculated and normalized using *ACT1* as positive control using GeneSpring (Silicon Genetics, CA, USA) (7 in Fig. 2).

RESULTS AND DISCUSSION

Multiple-end-point Bioassays

To develop bioassay systems for predicting chemicals and for understanding their toxicity, author suggests using multiple-end-point bioassay systems. In multiple-end-point bioassay systems, we use a variety of end points, which provide information that enables us to classify environmental pollutants and newly synthesized chemicals. Cellular growth, protein induction, gene expression, and accumulation of metabolite can be the end points; however, the end points must be affected by chemicals. An end point may be understood as the target of chemicals. Once ten kinds of independent end points are obtained, the chemicals can be theoretically classified into approximately 1,000 groups. According to the group, the chemicals may be predicted and their toxicity understood.

We are studying two multiple-end-point bioassay systems. One is based on plural microorganisms that are sensitive to chemicals. There is a variety of microorganisms in the world and their sensitivity to chemicals must be variety. Once microorganisms are prepared that are sensitive to specific chemicals, they can be classified according to the sensitivities of the microorganisms. Various sensitivities of microorganisms to chemi-

cals are used as the end points. The other system is based on the plural responses of one organism. Chemicals that cause toxicity injure cells, and the injured cells begin to repair the damage or take steps to decrease toxicity. If these chemical responses are monitored, toxicity can be estimated and the chemicals classified according to the responses. Here, various cellular responses to chemicals are used as end points.

Multiple-end-point Bioassays Based on Chemical Sensitivities of Microorganisms

The variety of existing microorganisms and their chemical sensitivities differ between strains [4,5] and species. Thus, there are various sensitivities for multiple-end-point bioassays. Once a microorganism that is sensitive to a specific chemical is isolated, it can be used as an indicator strain for a specific chemical. By collecting these microorganisms, the strains can be used as indicator strains for predicting specific chemicals in the environment. In addition, the information gained by studying and accumulating the reasons that indicator strains are sensitive to specific chemicals may be used for risk assessment for newly synthesized chemicals.

When indicator strains were screened, microorganisms were isolated from soils or obtained from culture collections. The chemical sensitivities of the microorganisms were characterized using the 32 chemicals listed in Table 1. According to the sensitivities, several strains were selected as indicator strains that are sensitive to specific chemicals and resistant to others. In this report, we showed the results obtained from the strains derived from the type culture collections. The strains that we selected were IFO3188 (*Acetobacter pasteurianus*), IFO3129 (*Acetobacter pasteurianus*), IFO14488 (*Clysomyces rutagersensis*), IFO3752 (*Aureobacterium esteraromatium*), IAM1048 (*Comamonas Testosteroni*), IFO3913 (*Pseudomonas synxantha*), and IFO3060 (*Staphylococcus aureus*).

These indicator strains enabled us to characterize approximately 20 out of 32 chemicals [13] by merely using growth abilities in the media containing the chemicals. In Fig. 1, growth responses of the indicator strains to nickel chloride and tributyltin chloride using seven strains are summarized. In these spectra, we understand that tributyltin chloride inhibits the growth of the strains IFO3188, IFO3129, and IFO3752 and that nickel chloride inhibits the strain of IFO3129. Ttributyltin chloride and nickel chloride may be distinguished according to the growth of strains IFO3188 and IFO3752. In the reverse, the chemicals that reduce the growth of strains IFO3188, IFO3129, and IFO3752 may be considered to be tributyltin chloride or related compounds. Seven strains are insufficient to classify 10,000 chemicals, but by accumulating chemically sensitive strains, chemicals could be predicted. As this system can be carried out in 96-well microplates (theoretically combination can be $2^{96} > 10^{28}$) or 384-well microplates, 10,000 chemicals could be classified in one microplate.

Multiple-end-point Bioassays Based on Responses of One Organism

When organisms are exposed to chemicals, they respond to toxicity so that they can survive in their environment. These responses can be specific to the damages caused by chemicals. If the responses are detected, the toxicity could be understood, which would enable us to predict the specific chemicals that caused the responses. Following is a description of the possibilities for the classification of chemicals according to the responses of the yeast *S. cerevisiae*.

Growth inhibition, viability, stress induction, prion curing mutagenicity, cytoplasmic mutagenicity, and chromosomal mutagenicity were selected as response [14]. Growth inhibition was monitored at A650 in 96-well microplates. Viability was estimated from the CFU after the yeast cells were treated in a water solution of chemicals for 24 h. Stress induction was monitored as a β-galactosidase activity in which *lacz* gene was connected after the *Hsp104* [15] promoter region [9]. Prion curing mutagenicity was estimated from the number of red colonies due to an adenine requirement after colony formation on the YPD plates [10]. Cytoplasmic mutagenicity was estimated as the frequency of petite colony formation on YPG plates after growing the yeast cells for 24 h in a YPD medium containing chemicals [16]. Chromosomal mutagenicity was estimated as the frequency of reversion of a leucine requirement after growing the yeast cells having the nonsense mutation (UGA) in *LEU2* gene [12] for 24 h in a YPD medium containing chemicals. The responses are summarized in Table 2.

From the multiple-end-point yeast bioassay system, chemicals can be classified into seven groups (Table 3). In Group 1 are the chemicals that cause chromosomal mutation, mitochondrial mutation, prion mutation, and stress induction. 4-Nitroquinoline-*N*-oxide belongs to this group, and we recognize this group as the most harmful to organisms. In Group 2 are the chemicals that cause mitochondrial mutation, prion mutation, and stress induction. Lindane belongs to this group. In Group 3 are the chemicals that cause mitochondrial mutation. They include 2,4,5-trichlorophenol, benzo(a)pyrene, and di-2-ethylhexyl phthalate. This group has low or no induction of Hsp104. In Group 4 are the chemicals that strongly induce stress proteins. Benthiocarb, triphenyltin chloride, malathion, sodium arsenite, and methylmercury chloride belong to this group. In Group 5 are the chemicals that show much lower LD50 values than IC50 values. These phenomena could be understood as the results of scavenging ability or excluding ability of yeast cells under rich medium. Paraquat, cadmium chloride, tributyltin chloride, nickel chloride, mercuric chloride, and cupric sulphate belong to this group. Except for paraquat, these chemicals are metal-related compounds. 2-Aminoanthracene, 2,5-dichlorohenol, formaldehyde, *p*-nonylphenol, thiuram, maneb, potassium dichlomate, hexachlorophene, triclosan, and DMSO are not characterized by the yeast

Table 2. Overview of the values obtained by yeast multiple-end-point bioassays

Chemicals	IC50	LD50	Stress Induction		Prion*	Mit.*	Chrom.*
			Optimum*	Level*			
2-Aminoanthracene	1.0 mM	8 mM	2.7 mM	63			
Benzo(a)pyrene	1.8 mM	0.84 mM	ND			3.3 mM	
Bis-phenol-A	1.2 mM	0.68 mM	ND				
Di-2-ethylhexyl phthalate	10 mM	> 2.5 mM	ND			8.3 mM	
2,5-Dichlorophenol	0.21 mM	> 10 mM	0.89 mM	67			
2,4-Dichlorophenoxy acid	8.4 mM	0.13 mM	0.2 mM	18			
Formaldehyde	1.6 mM	> 5 mM	1.3 mM	75			
Methylmercury chloride	2.9 μM	0.8 μM	0.73 μM	100			
4-Nitroquinoline-N-oxide	1.3 μM	0.3 μM	0.61 μM	40	1.6 μM	0.62 μM	1.9 μM
p-Nonylphenol	6.7 μM	4.5 μM	20 μM	14			(0.1%)*
Pentachlorophenol	36 μM	13 μM	ND				
Sodium Arsenite	0.59 mM	> 1.0 mM	89 μM	100			
Thiuram	59 μM	4.1 μM	9.9 μM	33			
Tributyltin chloride	1.1 mM	3.9 μM	2.0 μM	12			
2,4,5-Trichlorophenol	12 μM	0.26 mM	11 μM	3.8		14 μM	
Trp-P-2(Acetate)	0.12 mM	0.19 mM	0.26 mM	85			
Paraquat	22 mM	58 μM	0.8 mM	55			
Cadmium chloride	2.9 mM	1.4 μM	3.3 μM	76			
Lindane	38 mM	> 20 mM	5.8 mM	18	5 mM	22 mM	
Malathion	1.6 mM	> 2.0 mM	1.6 mM	96			
Maneb	67 μM	7.9 μM	88 μM	18			
Nickel chloride	3.0 mM	18 μM	99 μM	15			
Potassium dichlomate	> 0.1 mM	> 0.1 mM	0.08 mM	48			
Triphenyltin chloride	3.7 μM	0.45 μM	2.7 μM	170			
Phenol	4.3 mM	33 mM	13 mM	74			
Benthiocarb	0.71 mM	6 mM	5.3 mM	107			
Hexachlorophene	1.6 μM	0.39 μM	0.74 μM	36			
Triclosan	1.8 μM	8.5 μM	3.3 μM	28			
Mercuric chlori	52 μM	2.7 μM	2.2 μM	25			
Cupric Sulphate	3.3 mM	1.5 μM	11 μM	41			
Potassium cyanide	1.7 mM	> 50 mM	55 μM	31			
DMSO	-9.10%	> 10%	8%	40			

*, Prion., Prion mutation; Mit., Mitchondrial mutation; Chrom., Chromosomal mutation; Optimum, Conditions for the highest activity of β-galactosidase; Level, β-galactosidase activity(%) relative to that of Methylmercury chloride.

system and thus belong to Group 6. Pentachlorophenol and bis-phenol-A can be in Group 7 as this chemical showed no stress induction, and this characteristic is different from Group 6.

Although seven groups are not enough for predicting chemicals, these groupings may be used as preliminary assessments of environmental samples. For example, if an environmental sample belongs to Group 3, the sample would be understood to contain mutagens that cause mitochondrial mutation such as 2,4,5-trichlorophenol, benzo(a)pyrene, or di-2-ethylhexyl phthalate. This information helps to identify and manage the chemicals in the sample. The multiple-end-point yeast bioassay system may also be used for risk assessment. For example, 4-nitroquinoline-*N*-oxide inhibited yeast growth at a low concentration (IC50=1.3 μM, Table 2). From this information alone, it is possible to predict that this chemical will be very harmful to organisms. In addition, in yeast it had been shown that 4-nitroquinoline-*N*-oxide may induce stress conditions in organisms and may cause damage such as prion-like, cytoplasmic, and chromosomal inheritance. This information informs us as to how carefully this chemical must be handled, and this is one of the advantages of multiple-end-point bioassays.

Multiple-end-point Bioassays Based on Micro-array Technology

The above section illustrates the possibilities for multiple-end-point bioassay based on the responses of one organism to chemicals; however, the number of responses are insufficient for the classification of 10,000 chemicals. In order to increase the number of responses, micro-array technology was used. This technology provides the information of expression levels of concern to thousands of ORFs (open reading frames). Recently, the DNA chip Laboratory, Inc., started a production yeast micro-array (the Kuhara chip). On the Kuhara chip, approximately 6,000 ORFs are printed and fixed on one

Table 3. Classification of chemicals according to yeast multiple-end-point bioassay systems.

	CM*	MM*	PM*	SI*	LL*	Chemicals
Group 1	O*	O	O	O		4-Nitroquinoline-N-oxide
Group 2	●*	O	O	O		Lindane
Group 3	●	O	●	●		2,4,5-Trichlorophenol, Benzo(a)pyrene, Di-2-ethylhexyl phthalate
Group 4	●	●	●	OO*		Benthiocarb, Triphenyltin chloride, Malathion, Sodium Arsenite, Methylmercury chloride
Group 5	●	●	●		O	Paraquat, Cadmium chloride, Tributyltin chloride, Nickel chloride, Mercuric chloride, Cupric sulphate
Group 6	●	●	●	O		2-Aminoanthracene, 2,5-Dichloropheno Formaldehyde, p-Nonylphenol, Thiuram, Maneb, Potassium dichlomate, Hexachlorophene, Triclosan, DMSO
Group 7	●	●	●	●		Bis-phenol-A, Pentachlorophenol

* CM, Chromosomal Mutation; MM, Mitochondorial Mutation; PM, Prion Mutation; SI, Stress induction; LL, Lower LD50 than IC50; O, Positive; ●, Negative or Trace; OO, Strongly Positive

sheet of a slide. Fixed DNA can be used as probes for estimating the expression level of mRNA. Thus, we used this chip to examine the utility of multiple-end-point bioassays [17,18].

The procedure is shown in Fig. 2 and described in Material and Methods: 1) The yeast cells treated by chemicals and untreated control were prepared, 2) mRNA was extracted from each condition, 3) The mRNA was labeled with two kinds of fluorescent dye as cDNA, 4) The labeled cDNA from two tubes was mixed, mounted on a chip, and hybridized in a water bath, 5) Fluorescence was scanned by a Scan Laser 4000 (BM Instrument, USA), 6) Scanned data were quantified, and 7) Gene expression was analyzed by using the program of GeneSpring.

Gene induction spectra by nickel chloride and tributyltin chloride are shown in Fig. 3. In this experiment , the control cells were before the additions of nickel chlride or addition of DMSO with the same amount to that of tributyltin chloride. Thus, we may ignore the effect of solvent. In Fig. 3, approximately 300 types of genes induced by heavy metals from the top are alphabetically enumerated, and the top of the 50 genes induced by nickel chloride and tributyltin chloride is shown as a multiple of the expression level for the control by the length of the straight line. Nickel chloride and tributyltin chloride were described and distinguished like mass spectra [17].

Analyzing responses by micro-arrays need several days and high cost; therefore, this system may be inconvenient for environmental toxicity evaluation. To overcome this inconvenience, we are constructing indicator strains and convenient micro-arrays that are specific to toxicity evaluation. Because the specific genes induced by nickel chloride and tributyltin chloride can be seen in Fig. 3, 6,000 genes do not have to be analyzed. The information relative to these specific genes is sufficient for the evaluation. The indicator strains have a marker protein under the control of the promoter region of those specific genes. The expression levels of the specific genes can be detected by monitoring the marker

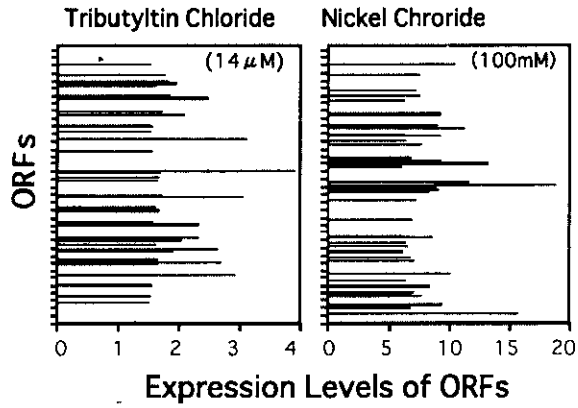

Fig. 3. Gene induction spectra by nickel chloride and tributyltin chloride. Approximately 300 types of genes induced by heavy metals from the top are alphabetically enumerated, and the top of the 50 genes induced by nickel chloride and tributyltin chloride is shown as a multiple of the expression level for the control by the length of the straight line.

proteins. It has been confirmed that these kinds of strains are useful for evaluation as a multiple-channel continuous-bioassay system [19]. So far we can not describe which genes are useful, because of the amount of data is limited. We are analyzing the responses by other chemicals and also trying to make micro-arrays arrayed by specific gene probes. In order to increase the number of groups, the number of end points in Table 3 should be increased by using indicator strains or convenient chips.

As mentioned above, multiple-end-point bioassays by micro-arrays are expensive and time-consuming. However, there are two purposes to the bioassay. One is environmental toxicity evaluation, and the other is the risk assessment of newly synthesized chemicals. For the risk assessment of newly synthesized chemicals we should use micro-arrays from now for protecting the human health and the environment.

CONCLUSION

There must be explanations for chemically sensitive strains being inhibited by specific chemicals, for specific phenotypes being transformed, and for specific genes being induced. These explanations must be reflected by the toxicity of chemicals. Multiple-end-point bioassays are based on this consideration. Recently, advanced technology has enabled us to extract a huge amount of information from organisms. For example, a 384-microplate reader informs us of the growth ability of 384 strains at once, and micro-arrays inform us of the expression levels of all the ORFs. By accumulating and selecting a huge amount of information relative to growth abilities, gene expression levels, protein expression profiles, and so on, we should only develop multiple-end-point bioassays.

This report has demonstrated the potenial of multiple-end-point bioassays. It is still necessary to isolate more sensitive strains and to analyze the responses of more chemicals with micro-arrays. It is particularly important that multiple-end-point bioassays be applied to the assessment of environmental pollution and the risk assessment of new chemicals; however, these tasks cannot be carried out by one laboratory alone. A worldwide collaboration would be welcomed, with a special invitation to Korea.

Acknowledgements Author thanks Drs. K. Fujita, Y. Momose, Y. Ishioka, S. Kameo, M. Arita, K. Kano for their contributions to the Cellular System Laboratory.

REFERENCES

[1] Suzuki, M. and H. Utsumi (1998) Bioassay for the control of chemicals, pp. III-IV Koudannsya Press Tokyo, Japan.
[2] Celemedson C, et al. (44Scientists). (1996) MEIC Evaluation of acute systemic toxicology. *ALTA* 24: 252-272.
[3] Reifferscheid. G. and J. Heil (1996) Validation of the SOS/umu test using test results of 486 chemicals and comparison with the Ames test and carcinogenicity data. *Mutat. Res.* 369: 129-145.
[4] Rieger K. J., A. Kaniak, J. Y. Coppee, G. Aljinovic, A. Baudin-Baillieu, G. Orlowska, R. Gromadka, O. Groudinsk, Di Rago, and J. P. Slonimski (1997) Large-scale phenotypic analysis-the pilot project on yeast chromosome III. *Yeast* 13: 1547-1562.
[5] Rieger K. J., M. El-Alama, G. Stein, C. Bradshaw, P. P. Slonimski, and K. Maundrell (1999) Chemotyping of yeast mutants using robotics. *Yeast* 15: 973-986.
[6] Nuwaysir, E. F., M. Bittner, M. Trent, J. Barrett, and C. A. Afshari (1999) Microarrays and toxicology. *Mol. Carcinog.* 24: 153-159.
[7] Afshari, A. C., E. F. Nuwaysir, and J. C. Barrett (1999) Application of complementary DNA microarray technology to carcinogen identification, toxicology, and drug safety evaluation. *Cancer Res.* 59: 4759-4760.
[8] Iwahashi H., W. Yang, and R. M. Tanguay (1995) Detection and expression of the 70 kDa heat shock protein ssb1p at different temperatures in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Com.* 213: 484-548.
[9] Fujita K., H. Iwahashi, R. Kawai, and Y. Komatsu (1998) Hsp104 expression and morophorogical changes associated with disinfectants in environmental bioassay using stress response. *Water Sci. Technol.* 38: 237- 243.
[10] Kochneva-Pervukhova N., S. V. Paushkin, V. V. Kushnirov, B. S. Cox, M. F Tuite, and T. M. D. Avanesyan (1998) Mechanism of inhibition of [psi] prion determinant propagation by a mutation of the *N*-terminus of the yeast Sup35 protein. *EMBO J.* 17: 5805-5810.
[11] Fox T. D., L. S. Folley, J. J. Mulero, T. W. McMullin, P. E. Thorsness, L. O. Hedin, and M. C. Costanzo (1991) Analysis and manipulation of yeast mitochondrial genes. *Methods Enzymol.* 194: 149-165.
[12] Ono B. I., R. Fujimoto, Y. Ohno, N. Maeda, Y. Tsuchiya, T. Usui, and Y. Ishino-Arao (1988) UGA suppressors in *Saccharomyces cerevisiae*: allelism, action spectra and map positions. *Genetics* 118: 41-47.
[13] Iwahashi, H., K. Fujita, Y. Takahashi, S. Kameo, and Y. Matsuyama (2000) Identification of chemicals using Microorganisms. *Japan Patent* 2000-52470.
[14] Iwahashi, H., K. Fujita, and Y. Takahashi (2000) Bioassay for chemical toxicity using yeast *Saccharomyces cerevisiae*. *Water Sci. Technol.* (in press).
[15] Piper P. W. (1993) Molecular events associated with acquisition of heat tolerance by the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 11: 339-356.
[16] Fox, T. D., L. S. Folley, J. J. Mulero, T. W. McMullin, P. E. Thorsness, L. O. Hedin, and M. C. Costanzo (1991) Analysis and manipulation of yeast mitochondrial genes. *Methods Enzymol.* 194: 149-165.
[17] Iwahashi, H. (2000) Bioassay using DNA chip technology. (2000) *Biosci. Bioind.* 58: 27-30.
[18] Iwahashi, H., Y. Momose, S. Kawai, and M. Matumoto (2000) Identification of chemicals. *Japan Patent* 2000-105907.
[19] Gu, M. B. (2000) Toxicity monitoring of endocrine-disrupting chemicals (EDCs) using freeze-dried recombinant bioluminescent bacteria. *Biotechnol. Bioprocess. Eng.* 5: 395-399.

[Received July 11, 2000; accepted October 28, 2000]