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Sensing of Heavy Metals Using *Caenorhabditis elegans* DNA Microarray

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Abstract—Previously we reported that the possibility of sensing of chemical substances using gene expression patterns analyzed by DNA microarray using *Caenorhabditis elegans* (*C. elegans*) as a sensor (Matsuno *et al*., 2002, 2006). In this study, we evaluated sensitivity and selectivity of above system using a customized DNA microarray for heavy metal responded genes. The results showed that this system showed high sensitivity and selectivity for heavy metals (cadmium chloride, mercury chloride and arsenate). The threshold value for heavy metals is shown to be less than 1 nM. The accumulation of gene expression patterns and responded gene information by this assay system enable us to provide not only the quantities and/or qualities of known chemicals but also those of unknown chemicals in environment samples. It is possible that this system can evaluate total biological and physiological effect level in pollution, since gene expressions reflect composite effects of sources in pollution.

Keywords: *Caenorhabditis elegans*, DNA microarray, heavy metal, gene expression pattern

INTRODUCTION

Caenorhabditis elegans (*C. elegans*) is free living nematode which is commonly used as laboratory model for genetics, molecular biology, and development biology (Brenner, 1974; Wood, 1988; Riddle *et al*., 1997; Baugh *et al*., 2003; Harris *et al*., 2006). Over the last decades, *C. elegans* has been also suitable organism for laboratory toxicity testing (Sochova *et al*., 2006) and the toxicity studies have been focused not only on mortality but also on numerous sublethal parameters including biomarkers (Roh *et al*., 2006, 2007). Recently the Omics technologies are developed rapidly and become potent tool to evaluate biological

and physiological effects of many of stressors and diseases for human and many organisms. Furthermore gene expression analysis can be performed as ecotoxicogenomic approach (Custodia *et al.*, 2001; Matsuno *et al.*, 2002, 2006; Reichert and Menzel, 2005).

A huge number of chemicals exist in surround our environment. These chemicals affect several physiological systems, such as maturation, growth, reproduction and behavior of human and wild life. So we want to evaluate environmental pollutants not only for concentration of individual chemical but also biological effects for organisms. However environmental analysis is developed from individual chemical analysis. Therefore, at present, popular environmental analysis is always chemical analysis based on instrumental analysis. These instrumental analyses can not estimate chemical effect on organisms. If we estimate the effect of chemicals on organisms, we should adopt an appropriate bioassay to evaluate the objective effect. However *in vivo* bioassay is always used with an individual organism, and experimental faults are sometimes occur, and we usually need a long bleeding and exposure period. For these reasons, bioassay has not become common in the environmental analysis field.

Previously, we showed that the effects of chemical substances on *C. elegans* gene expression were measured using the cDNA microarray. Then the hierarchical clustering method was applied to analyze the response patterns. We found that they were classified according to similarity of their responses and each cluster should, which was a group of similar response patterns, should correspond to one kind of chemical substance (Matsuno *et al.*, 2002, 2006). In this paper, we examine the potency of *in vivo* bioassay using *C. elegans* as a model organism and customized DNA microarray which made from selected chemical specific genes as a chemical sensing system for heavy metals.

MATERIALS AND METHODS

Animals and culture conditions

The wild-type N2 strain of *C. elegans* was maintained and handled as described by Brenner (1974). Briefly, five to ten worms were grown on a Nematode Growth Medium (NGM) agar plate seeded with *Escherichia coli* (*E. coli*) as a food source, and incubated at 20°C. Every four or five days, the worms were sub-cultured to new plates.

Synchronized Cultures of C. elegans

Worms were washed out from the NGM agar plates using M9 buffer and were subsequently washed twice with this buffer. They were then synchronized by the alkali-bleaching method. An appropriate number of eggs was transferred on a agar plate without food and incubated at 20°C for 16 hr. Over 90% of these eggs hatched, and *C. elegans* larvae were synchronized at the L1 larval stage. Then *E. coli* was added and cultured for another 24 hr. One hour before chemical exposure, larvae were collected and starved to remove and digest *E. coli*. About

Table 1. Expression level increasing genes by heavy metals following dose-response manner.

cDNA clone	Product name	Gene name	Gene annotation
yk77h11	F21D12.1a	nhr-21.a	nuclear Hormone Receptor (44.9 kD) (nhr-21)
yk509h8	M01H9.3		
yk223e5			
yk499a1	C06G1.4		putative nuclear protein, with a coiled coil-4 domain (XR231)
yk308d9	R03E9.1	mdl-1	yeast Mitosis Arrest DeFicient-Like, Helix Loop Helix containing protein (30.7 kD) (mdl-1)
yk130f5	C41D11.3		putative protein (1E998)
yk337g2			
yk368g1			
yk379f9	F41E6.9		HSPC177 (24.6 kD) (5J86)
yk403e5	D2096.2		interacting protein (21.0 kD) (4J145)
yk340d11			
yk368b9	Y44A6D.2		putative protein (7.4 kD) (5V259)
yk442g3	F59B1.2		putative secreted or extracellular protein precursor, with a coiled coil-4 domain (17.8 kD) (5E113)
yk139h8			
yk252e4	F59A7.2		putative secreted or extracellular protein family member precursor (12.6 kD) (5C494)
yk302b4	C01G10.7		citrate lyase (5P567)
yk335b3	Y57G11C.12		nadh dehydrogenase 1 alpha subcomplex 6 (15.0 kD) (4P560)
yk108f9			
yk364c6	K11G9.6	mtl-1	metallothionein, expressed in pharynx and induced by cadmium or heat shock in intestine (8.0 kD) (mtl-1)
yk290a9	ZK742.4		NADH oxidase family member (5I290)
yk476d2			

200,000 worms were used for one dose of chemical exposure. Worms were resuspended in S-basal medium containing chemicals and incubated for 5 hr. After chemical exposure, worms were harvested and frozen quickly by using liquid nitrogen.

Measurement of gene expression patterns

Total RNAs, including mRNAs reflecting the expression of corresponding genes, were extracted from the larvae with or without chemical exposure by using TRIZOL reagent (Invitrogen, Tokyo) following the manufacturer's manual. Then poly(A) + RNA were purified from total RNAs using oligo(dT) resin (GE healthcare bioscience, Tokyo). Cy-dye labeled cDNA probes were generated by reverse transcription reaction at the presence of Ct-3 or Cy-5 conjugated dUTP in the reaction mixture for control or chemical exposure, respectively. The labeled cDNA probes were mixed together prior to hybridization to a DNA microarray. The mixed labeled cDNA probes hybridized to the microarray. Then the microarray was washed thoroughly and scanned using a laser confocal

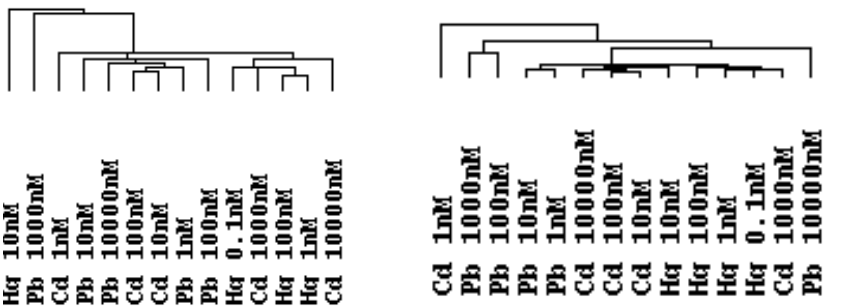
Table 2. Expression levels decreasing genes by heavy metal exposure.

cDNA clone	Product name	Gene name	Gene annotation
yk239c3	F57F4.4		
yk407d10	ZK270.2a	frm-1	FERM domain (protein 4.1-ezrin-radixin-moesin) (frm-1)
yk392b1	Y39G8C.b		
yk255f11	K09H9.6	lpd-6	drosophila Peter Pan homolog, LiPid Depleted LPD-6 (lpd-6)
yk590e10			
yk338g10	Y37A1B.1		DNA-binding SAP containing protein (4O841)
yk61h8	F56D12.5		
yk239g7	C44H9.4		regulator of nonsense transcripts family member (5N319)
yk205f3	K10B3.10	spc-1	spectrin (spc-1)
yk113d3			
yk364b11	K07F5.11	ssq-1	Sperm-Specific family, class Q SSQ-1, putative protein (24.4 kD) (ssq-1)
yk499c4	Y116A8C.35		auiliary factor (30.9 kD) (4S62)
yk198f10	C48D1.2	ced-3	cell death protein, Caspase, ICE-like apoptotic protease., CEll Death abnormality CED-3 (56.6 kD) (ced-3)
yk192a11	ZK617.1a	unc-22	UNCoordinated locomotion UNC-22, immunoglobulin-like and fibronectin, type III and protein kinase family member (unc-22)
yk562c8			
yk476a3	Y48B6A.3		5′-3′ exoribonuclease 2 (101.3 kD) (2O455)
yk608b11	F10D11.6		bactericidal Permeability Increasing Protein like family member (1J22)
yk60d10	Y51H4A.3	rho-1	small GTP-binding protein RHO RHO-1, small GTP-binding protein RHO, guanine nucleotide regulatory protein with prenylation domain (21.6 kD) (rho-1)
yk529f3	ZK1151.2b	vab-10	VAB-10A protein, Variable ABnormal morphology VAB-10 (vab-10)
yk534g7	C08B11.5	sap-49	Spliceosome-Associated Protein, and actin related protein (47.5 kD) (sap-49C)
yk596e6	R144.4		

scanner Scan Array (GSI Luminonics, USA). The coefficient from the Cy-3 and Cy-5 intensities was calculated to determine the relative amounts of particular gene transcript in the two samples. We observe an expression pattern of deviation from the normal (no exposure) condition.

DNA microarray

We used two types of DNA microarray. One was a cDNA microarray which including about 8,000 kinds of individual *C. elegans* cDNA spots. The other was a custom synthetic oligo DNA microarray which included customized synthetic long oligo DNA probes for selected *C. elegans* genes on glass slide. The latter is explained briefly as follows, about 100 heavy metal specific responsive genes were selected from cDNA microarray data and reference (actin and GAPDH) genes also selected. Then specific long oligo DNA probes 60–70 mers for each



(a) cDNAmicroarray (b) Customized DNA microarray

Fig. 1. Cluster analysis of exposed conditions by gene expression data from DNA microarray.

gene were constructed and synthesized. Synthesized oligo DNA were spotted on the glass slide.

Cluster analysis of gene expression patterns

The obtained data of the gene expression patterns under various exposure conditions were analyzed using the hierarchical clustering method, where software for the analysis written by Eisen *et al*. (1998) was used. We used the method known as average linkage clustering. The cluster analysis was performed with respect to both exposure conditions and genes. In the clustering with respect to exposure conditions, similar expression patterns were arranged in neighboring positions in the dendrogram.

RESULTS AND DISCUSSION

The measurements were performed under 14 different conditions, which were 4 (0.1 to 100 nM) exposures to mercury chloride, 5 (1 to 10000 nM) exposures to cadmium chloride, 5 (1 to 10000 nM) exposures to lead acetate and no exposure as control. First we used cDNA microarray for screening response gene. Out of 8000 genes, many genes showed interesting response pattern against heavy metal exposures. For example, some genes showed that expression level increased following dose-response manner by specific heavy metal exposure (Table 1). On the other hand, some genes expression levels were decreased significantly by any heavy metal (Table 2). Then we selected heavy metal response gene for the customized DNA microarray. 100 genes were selected from increased or decreased significant genes groups against all three heavy metals or individual heavy metal exposure. Looking over selected gene group, we could find some genes which already reported as heavy metal, stress or chemical response gene. For example, *mtl-1* product is methallothionine, which is well-known as a heavy metal and stress defense protein (Liao *et al*., 2002). These

results indicated that probability that these selected genes responded to actual heavy metals was comparatively high.

The dendrograms representing the similarity structure of expression pattern for heavy metal exposure made from cDNA and customized microarray are shown in Figs. 1(a) and (b), respectively. Previously we indicated that hierarchical clustering method was applied to analyze the pattern of natural and synthetic steroid stimuli. We found the each cluster, which was a group of similar response patterns, corresponds to one kind of chemical substance. This means that the *C. elegans* DNA microarray can be utilized as a chemical sensing system. In this paper, same method was applied to heavy metal sensing, and each heavy metal exposure also made one cluster (Fig. 1). However, when dendrograms were compared with each other, the scattering of dendrogram made from cDNA microarray (Fig. 1(a)) was bigger than from customized DNA microarray (Fig. 1(b)). Since cDNA microarray showed many nonspecific genes against heavy metals, response specificity was diluted by these gene responses. Then the synthetic oligo DNA probes for customized DNA microarray were designed successful, and we found that fluorescent intensity was comparatively higher than cDNA microarray. On the other hand, the threshold value for heavy metals is shown to be less than 1 nM. This sensitivity is enough to compete with instrument analyses.

Moreover, the accumulation of gene expression patterns and responded gene information by this assay system enables us to provide not only the quantities and/or qualities of known chemicals but also those of unknown chemicals in environment samples. It is possible that this system can evaluate total biological and physiological effect level in pollution, since gene expressions reflect composite effects of sources in pollution. From the genomic study, *C. elegans* conserves many common genes between from bacteria to human. These results indicated that we can estimate the ecotoxicological effects of specific chemicals which are present in environment on many ecological animals from independent gene expression data obtained by *C. elegans in vivo* bioassay.

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