EFFECTS OF MERCURY ON REPRODUCTION, AVOIDANCE, AND HEAT SHOCK
PROTEIN GENE EXPRESSION OF THE SOIL SPRINGTAIL *FOLSOMIA CANDIDA*YU-RONG LIU,^{†,‡} YUAN-MING ZHENG,[†] LI-MEI ZHANG,[†] YUN-XIA LUAN,[§] and JI-ZHENG HE^{*†}[†]State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China[‡]Graduate University, Chinese Academy of Sciences, Beijing 100049, China[§]Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai 200032, China

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Abstract—Based on the Cambisols of Beijing (used as agricultural soils), toxicity tests were conducted to investigate the effects of mercury (Hg) on reproduction and avoidance of *Folsomia candida* (Hexapoda: Collembola), as well as the transcriptional responses of the hsp70 gene, under different Hg concentrations and at different exposure times. Results showed that the hsp70 gene of the springtail was the most sensitive parameter to soil Hg stress, with a half-maximal effective concentration (EC50) of 0.42 mg/kg. The EC50 values based on reproduction and avoidance tests were 9.29 and 3.88 mg/kg, respectively. The expression level of the hsp70 gene was significantly up-regulated when soil Hg concentration was over 0.25 mg/kg (lowest-observed-effect concentration [LOEC]). In addition, responses of this gene expression were strongly induced after 48 h exposure under 1 mg/kg soil Hg, which probably was due to the fast and sensitive response of the gene transcription to Hg stress. Thus, the results suggested that the responses of the hsp70 gene and individual-level effects (reproduction and avoidance) could be integrated to provide helpful information for environmental monitoring and assessment of contaminated soils. Environ. Toxicol. Chem. 2010;29:654–659. © 2009 SETAC

Keywords—*Folsomia candida* Avoidance hsp70 gene Mercury Soil risk assessment

INTRODUCTION

Soil mercury (Hg) pollution is a worldwide problem that could threaten the sustainability of essential soil functions and the safety of human health [1], and the evaluation of Hg toxicity and bioavailability has been of widespread concern [2,3]. Biological approaches are useful for investigating the effects of bioavailable pollutants on organisms and their interaction. The springtail *Folsomia candida* is one of the important model organisms, which has been used in ecotoxicological tests of pollutants [4]. Some endpoints, such as survival, growth, and reproduction, have been commonly used in contaminated soil diagnosis and risk assessments because of sensitive responses of *F. candida* to environmental pollution [5–11]. In addition, the avoidance of *F. candida* in response to heavy metal stress has been used to evaluate the toxicity of pollutants to *F. candida* [12–14], although little information is available regarding the avoidance response of *F. candida* to soil Hg stress.

Many toxicological bioassays have shown that both molecular and whole-individual methods play important roles in determining biological responses to stressors [15]. Thus, some functional gene expressions of springtails following contaminated soil exposure have been used as endpoints, for example, the variation of metallothionein gene expression in the springtails *Orchesella cincta* exposed to Cd [16,17]. With the development of genomic techniques, springtail ecotoxicoge-

nomic studies have been used to investigate the variation of all gene expressions under different stress, which further improved our knowledge of the mechanisms responsive to stress in *F. candida* [18,19]. However, the applications of gene expression as a biomarker in environmental diagnosis or assessment are still very limited.

Biomarkers are used in early environmental diagnostics as predictive molecular-genetic, biochemical, cellular, or physiological responses to sublethal toxicant stress [20,21]. The heat shock protein (hsp) gene, despite debates and limitations, has been widely used in several organisms as a potential biomarker for monitoring environmental pollution [22–24]. The stress-inducible hsp70 is the most highly conserved and largest gene in all hsp families, which is caused by a more pronounced need for chaperones in order to maintain optimal cell function and homeostasis [25,26]. This inducible protein-coding gene can be expressed at extremely low levels of pollutant under normal conditions, and its transcription and translation will increase rapidly in response to various stressors [27]. Studies of the soil invertebrates *Lumbricus terrestris* and *Eisenia fetida* showed that the hsp70 gene was up-regulated after metal exposure [28,29]. Recently, it was also reported that there was a strong correlation between hsp70 expression in the springtail *O. cincta* and its thermal resistance [30]. The expression of the hsp70 gene in *F. candida* exposed to Hg together with cold or heat stress was also studied in aquatic environments [31,32]. However, little information was available on the effect of Hg pollution on the hsp70 gene expression in the soil environment.

The aim of the present study was to compare the sensitivity of different endpoints in *F. candida* to soil Hg stress from individual to molecular levels. The effects of Hg on reproduc-

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tion, avoidance, and hsp70 biomarker were measured with exposure to different Hg concentrations. Results based on these tests could be useful for the early and fast diagnosis of soil contamination and environmental risk assessment.

MATERIALS AND METHODS

Test soil and chemical analyses

The soil used in the study was collected from an agricultural field in the suburbs of Beijing, China. It was atypical fluvoaquic sandy loam soil (Cambisol, 9.0% clay, 21.8% silt, 69.2% sand). More information on soil characteristics is given in Table 1, in which a slightly high Zn concentration was considered to be of little impact on the test according to pre-experiments. Soil samples were air dried and ground to a size finer than 2 mm for incubation experiments and 0.149 mm for chemical analyses. Soil pH was determined at a soil:water ratio of 1:2.5, and soil organic matter was determined by the $K_2Cr_2O_7$ oxidation method [33]. For general element analysis, soil was digested with HNO_3 and H_2O_2 , and for Hg analysis the soil was digested with $HNO_3 + HCl$ (10 ml, 1:1 v/v) [34]. Manganese, Fe, Cu, Zn, and Cr were analyzed by inductively coupled plasma optical emission spectroscopy (PerkinElmer), and Hg was analyzed by atomic fluorescence spectroscopy (AFS; AFS-2202E Haiguang Analytical Instrument). Two standard reference soils, GBW-07401 (GSS-1) and GBW-07405 (GSS-5), obtained from the Center for National Standard Reference Materials of China, were included in the analytical process for quality assurance/quality control.

Test *F. candida*

The strain of *F. candida* was provided by the Institute of Plant Physiology and Ecology, Chinese Academy of Sciences. The individuals were cultured at $20 \pm 1^\circ C$ with a 12:12-h light:dark cycle. The springtails were kept in Petri dishes containing a mixture of water-saturated charcoal and plaster of Paris (1:8) and fed with granulated baker's dry yeast. Water was added once per week to maintain humidity. Adult animals were transferred to fresh containers, in which the springtails oviposited for 48 h, and then were removed. After 7 to 10 d, the eggs hatched and the juveniles were bred synchronously to ensure that all individuals were of approximately the same age at the beginning of the experiments.

General experimental design

Effects of Hg on reproduction. Chronic toxicity tests with *F. candida* were conducted following the International Organization for Standardization 11267 method [35]. The unpolluted soil was spiked with Hg, i.e., 2, 5, 10, or 20 mg/kg, by adding a solution of $HgCl_2$. The solutions were mixed thoroughly with the soil to give a moisture content of 55% of the water-holding capacity. One week after spiking, 10 springtails (10–12 d old)

were exposed in each experimental vessel containing 30 g of spiked soil or control soil. Granulated dry yeast was spread every 2 weeks on the soil surface as food. Five replicates were used for each concentration. The test vessels were kept at $20 \pm 1^\circ C$ with a 12:12-h light:dark cycle for 28 d. At the end of exposure, juveniles and adults were counted after extraction by flooding with water.

Avoidance tests. Unpolluted soil was spiked with Hg, i.e., 0, 0.5, 1, 2, 5, 10, or 30 mg/kg. Cylindrical plastic containers (7 cm in diameter and 6 cm in depth), divided into two sections by a cardboard divider inserted transversally, were used in the avoidance tests with *F. candida*. The two sections of the test container were filled with 30 g of control and spiked soil (dry wt), respectively [12]. Then, the divider was removed and 20 springtails were placed carefully onto the midline of each test container. Each treatment consisted of five replicates. The test containers were kept at $20 \pm 1^\circ C$ with a 12:12-h light:dark cycle for 48 h. At the end of exposure, the divider was reinserted. Then, the contents of two sections of the container were emptied separately into small vessels and flooded with water. A dual control test was also performed (both sections filled with soil with the same Hg level).

Transcriptional responses of hsp70 gene to Hg concentration. Based on results of the reproduction test, the soil was spiked with a maximum Hg concentration of 8 mg/kg (lower than the EC50 concentration of 9.29 mg/kg), i.e., 0.25, 0.5, 1, 2, 4, and 8 mg/kg. Four replicates were designed for both control and spiked soils. The exposure time was 4 d. To make the springtails eat food that originally existed in the soil, no other food was added during the experiments. Thirty springtails were placed carefully into each test container, and four replicates were designed for every treatment. Other general conditions during the exposure were the same as in the reproduction assay. When the exposure was ended, springtails were extracted from the spiked soil and carefully moved to Petri dishes containing a mixture of charcoal and plaster of Paris to remove surplus water. They were then put in sterile tubes, in which they were snap frozen in liquid nitrogen and kept at $-80^\circ C$ for RNA extraction. The whole harvesting procedure took approximately 4 min per sample.

Transcriptional responses of hsp70 gene to exposure time. The soil was spiked with a final concentration of 1 mg Hg/kg soil, which was designed based on results of pre-experiments. The tested number of individuals and all other procedures were the same as described above. After exposure, springtails were collected after different exposure times, i.e., 6, 12, 24, 48, 96, and 240 h.

Real-time quantitative polymerase chain reaction

RNA extraction and reverse transcription. All expression analyses were conducted on total RNA extracted from exposed springtails, using Trizol reagent (Molecular Research Center,

Table 1. Selected basic chemical characteristics of the tested soil

pH (H ₂ O)	OM ^a (%)	Total N (g/kg)	Available P (mg/kg)	Total metal concentration in the soil (mg/kg)					
				Mn	Fe	Cu	Zn	Cr	Hg
7.14	2.05	0.83	9.51	458	24525	2.36	89.23	4.53	0.05

^aOrganic matter.

Table 2. Polymerase chain reaction primer pairs

Gene	Amplicon length (bp)	Code	Sequence 5'-3'	Reference
28S	534	28S-F 28S-R	GACCCGTCTTGAAGCACG CCCACAGCGCCAGTTCTGCTTACC	[43]
β -Actin	111	ACT-1F ACT-1R	GCGACGTTGATATCCGAAAAG GCGACGTTGATATCCGAAAAG	This study
hsp70	243	H7-1F H7-1R	GCGACGTTGATATCCGAAAAG GCGACGTTGATATCCGAAAAG	This study

Invitrogen) according to the manufacturer's instructions. RNA purity and integrity were checked by ensuring that the absorbance ratio (A260/280) was between 1.8 and 2.0 and by agarose gel electrophoresis (2%). For cDNA synthesis, 2 μ l total RNA ($\sim$ 350 ng/ μ l) was reverse transcribed. The first-strand cDNA was synthesized using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) and oligod(T)₁₅ according to the manufacturer's instructions.

Cloning and sequencing. A 28S gene polymerase chain reaction (PCR) was conducted to check initially the results of reverse transcription. The β -actin gene was chosen as an internal control for hsp70 gene quantification [17]. Two pairs of primers were developed according to the nucleotide database from GenBank, as shown in Table 2. Polymerase chain reactions were done with Taq polymerase (Takara), following 35 cycles of 20 s at 94°C, 30 s at 58°C, and 30 s at 72°C. The PCR products were cloned into the pGEM-T Easy Vector (Promega). The recombinant clones were purified using a MiniBEST Plasmid Purification Kit (Takara). Two or three clones for each gene were sequenced, and the sequence analysis was performed by comparison with the GenBank database.

Two standard curves for real-time PCR. The two plasmid DNA concentrations were determined on a Nanodrop[®] ND-1000 spectrophotometer (NanoDrop Technologies), and the copy numbers of hsp70 and β -actin gene were calculated directly from the concentration of extracted plasmid DNA. Tenfold serial dilutions of a known copy number of plasmid DNA were subjected to real-time PCR assay in triplicate to generate standard curves for quantifying the two genes of interest.

Relative quantification of gene expression levels. Real-time PCR assays were performed on an iCycler iQ 5 thermocycler (Bio-Rad) for both target and internal control genes. Real-time PCRs (qPCR) were prepared for 25- μ l mixtures: 2 μ l cDNA, 0.5 μ M each β -actin or hsp70 gene primer, 9.5 μ l diethylenetriamine (DEPC) water, and 12.5 μ l SYBR[®] Premix Ex Taq[™] (TaKaRa). The following PCR conditions of the two genes were used: denaturation step (95°C, 1 min), amplification and quantification step repeated 40 times (95°C for 20 s, 57°C for 30 s, 72°C for 10 s), melting curve step (55–95°C with a heat rate of 0.5°C per s and continuous fluorescence measurement), and a cooling step at 40°C. Analyses of melting curves allowed optimization of annealing temperatures for amplification products. Quantifications of both hsp70 and β -actin genes were conducted on the same plate to reduce systematic error. For each mRNA, the absence of contamination (genomic DNA) was checked using a no-RT control with β -actin and 28S primer.

Statistical analyses

The data from the three toxicity tests were fitted to suitable regression models [36,37]: reproduction results with a hormesis model ($r^2 = 0.99$, $p < 0.01$), avoidance results with a logistic model ($r^2 = 0.99$, $p < 0.01$), and hsp70 response with a logistic model ($r^2 = 0.94$, $p < 0.05$). The EC50 values were calculated in the statistical package SigmaPlot 10.0.

No-observed-effect concentrations (NOEC) and low-observed-effect concentrations (LOEC) were determined by comparing reproduction rates, avoidance percentages, and relative expression levels of the hsp70 gene with corresponding control using Mann-Whitney U tests. Statistical significance was considered at $p < 0.05$. Gene expression was calculated by quantification of the hsp70 gene compared with quantification of the β -actin gene, which were measured by the iCycler iQ 5, and then the control treatment was normalized as a value of 1. One-way analysis of variance was used to assess differences between treatments, and all results are presented as means with standard errors. Statistical significance was assessed by the software SPSS[®] 11.5.

RESULTS

Reproduction and mortality

The over 80% survival rates and more than 100 juveniles produced in controls proved that the test was valid. The effects of soil Hg on the reproduction of springtails are shown in Figure 1. There was a decreasing trend when soil Hg concentration was higher than 5 mg/kg. The EC50 value based on

Fig. 1. Reproduction (bars; left y axis) and adult survival (curve; right y axis) of the springtail *Folsomia candida* exposed for 28 d to Hg-contaminated soils at different Hg concentrations. Error bars indicate standard deviation (SD). *Significantly different from control ($p < 0.05$).

Table 3. Effect concentration with 50% effect (EC50), no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) per mg Hg/kg soil for *Folsomia candida* after exposure to Hg-spiked soil

	Reproduction	Avoidance	hsp70 Gene expression
EC50	9.29	3.88	0.42
NOEC	5	2	—
LOEC	10	5	0.25

reproduction was 9.29 mg/kg ($p < 0.05$; shown in Table 3). A significant decrease in the reproduction rate was found at the concentration of 10 mg/kg compared with the control soil, and no juvenile was found at the concentration of 20 mg/kg. No significant effect of soil Hg on the mortality of *F. candida* was observed, although mortality was increased at the highest Hg concentration.

Avoidance tests

An average survival rate of $99 \pm 1\%$ was determined for all tested samples. Dual-control tests showed a random distribution of individuals in both sections of the test containers (data not presented) in all treatments. Testing soils with different Hg concentrations showed that there were significantly lower percentages of individuals in sections with soil polluted with more than 2 mg/kg compared with control soil (Fig. 2). An increasing trend of avoidance was found with ascending Hg concentration in the soil above 1 mg/kg.

Cloning and sequencing

Gene sequencing showed that the two partial fragments were both the genes of interest. Each sequence was identified using BLAST in the National Centre for Biotechnology Information. Sequence data were submitted to GenBank (accession Nos. FJ476077 for the hsp70 gene and FJ476078 for the β -actin gene).

Transcriptional responses of the hsp70 gene

Different soil Hg concentrations. Mercury exposure had significant effects on relative expression of the hsp70 gene

Fig. 2. Avoidance behavior of *Folsomia candida* in tests with soils spiked at different Hg concentrations. Values are numbers of individuals (mean ± SD) in the control side of test containers. Columns within each concentration not sharing the same lowercase letter are significantly different ($p < 0.05$).

Fig. 3. Expression of the hsp70 gene in *Folsomia candida* exposed to a control and to increasing test concentrations of soil Hg. Bars are mean of four relative expressions ± standard error (SE). Columns within each concentration not sharing the same lowercase letter are significantly different ($p < 0.05$).

compared with the control (Fig. 3). A significantly higher expression level of the hsp70 gene was found when the Hg concentration was greater than or equal to 0.25 mg/kg ($p < 0.05$). There was an increasing trend of expression with the rise in soil Hg concentration, and the highest expression level of the hsp70 gene was observed at 8 mg Hg/kg soil.

Exposure time following soil Hg stress. No significantly different expression levels of the hsp70 gene were observed from 0 to 24 h exposure to soil Hg (Fig. 4). Afterward, the hsp70 gene expression increased significantly up to about three times that of the control ($p < 0.05$). Then, there was some fluctuation in the expression levels, which remained higher than the control. Overall relative expression levels of 2-d, 4-d, and 10-d exposure times were significantly higher than the gene expression levels determined earlier.

Fig. 4. Expression of the hsp70 gene in *Folsomia candida* at different exposure times at a concentration of 1 mg Hg/kg soil. Bars are the mean of four relative expressions ± SE. Columns within each time not sharing the same letter are significantly different ($p < 0.05$).

Comparison of toxicity values in the different endpoints

Toxicity parameters based on the three endpoints are shown in Table 3. The EC50 and LOEC values based on reproduction, avoidance, and hsp70 gene expression decreased in that order. The lowest EC50 value was 0.42 mg/kg calculated according to the hsp70 biomarker. In addition, the LOEC based on the reproduction test was 40-fold higher than that based on gene expression. The NOEC values for reproduction and avoidance were 5 and 2 mg/kg, respectively.

DISCUSSION

In present study, tests of chronic toxicity revealed that there were significant effects of soil Hg on the reproduction of *F. candida* at concentrations above 10 mg/kg. The corresponding EC50 value was higher than that measured by Lock and Janssen [3], which could be attributed to the difference between the clones used in the various laboratories [14,38]. It might also be related to the natural variability of *F. candida* populations and the difference in Hg bioavailability depending on soil characteristics. Therefore, it is necessary to use synchronous clones and the same soil when comparing the toxicity of different chemicals. There was no significant variation of mortality among any of the treatments. This lack of sensitivity of mortality was also observed in ecotoxicological tests of cobalt with the same species [39].

The springtail *F. candida* exhibited an increasing tendency to avoid soils with ascending Hg concentrations, which agreed with studies indicating the avoidance of several chemicals by earthworms and isopods [40]. The avoidance test of *F. candida* to soil Hg was more sensitive than the reproduction test. Basically, from this point of view, avoidance in this species is suitable as a screening tool in ecological risk assessment for contaminated land, as suggested in other studies [12,14,41].

The hsp70 gene expression in *F. candida* was measured as an endpoint in this study. Not only was total hsp70 gene relative expression significantly higher at all test concentrations compared with the control but the hsp70 gene could be induced at a very low soil Hg concentration (0.25 mg/kg in this experiment), with which no response was produced in reproduction and avoidance tests. This could be explained by the fact that the up-regulation of the gene should be produced before any physiological variation in the organism could be observed. Therefore, the hsp70 could be a promising biomarker for soil Hg pollution and suitable for early diagnosis of contamination. The relative expression levels of the hsp70 gene seemed to increase with soil Hg concentration, which also fit the dose-response model. On the other hand, approximately four- to sixfold higher gene expression levels were observed after 48-h exposure compared with that in the control or the treatment with 24-h exposure. This finding suggested that the hsp70 protein coding was strongly induced in a short time. However, this trend leveled off with the increase in the exposure time, which could be a result of physiological acclimation that allows the organisms to be tolerant to pollution [42] or because enough hsp70 protein had been produced. Meanwhile, it has been suggested that the up-regulation of some genes was involved in the tolerance to prolonged exposure to stressors [20]. In fact, variations at the individual level following Hg exposure depend, at least in part, on those genes responsible for the corresponding

adaptive mechanisms being regulated or differentially expressed.

The expression of hsp70 gene and the bioassays at individual level were combined to investigate the responses of *F. candida* to soil Hg pollution in this study, which provided more integrated information to evaluate its toxicity and ecological risk from individual to molecular levels. The results of comparison between the different endpoints indicated that expression of the hsp70 gene was the most sensitive in the toxicological bioassays according to their EC50 values; i.e., the hsp70 gene expression had the lowest EC50 value. In addition, the LOEC value of hsp70 gene expression was 0.25 mg/kg, which was much lower than LOEC values according to the tests of reproduction and avoidance. Therefore, the hsp70 gene of *F. candida* should be a more promising biomarker of soil Hg pollution.

The present study provides initial evidence that tests of avoidance and the hsp70 biomarker of the springtail *F. candida* exhibited more sensitive responses to soil Hg stress than reproduction and, moreover, than mortality. The sensitivity of avoidance behavior indicated that the test could be regarded as a fast and sensitive tool in the screening evaluation of soil contamination, which would rapidly yield information for future decisions on the evaluation procedure. Although the results of the reproduction test using *F. candida* were comparable to *Eisenia fetida* and *Enchytraeus albidus* reproduction test results [20], the test was not considered to be a promising method for evaluating polluted soils, because it is time consuming. In particular, the biomarker of hsp70 provided an optional means of quantifying environmental harm when a low concentration of pollutant was present. Thus, environmental monitoring using this hsp70 gene expression in *F. candida* might be helpful in the early diagnosis of potentially polluted sites.

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