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Analysis of the Stress Effects of Endocrine Disrupting Chemicals (EDCs) on *Escherichia coli*

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Abstract In this study, three of the representative EDCs, 17β-estradiol, bisphenol A, and styrene, were employed to find their mode of toxic actions in *E. coli*. To accomplish this, four different stress response genes, *recA*, *katG*, *fabA*, and *grpE* genes, were used as a representative for DNA, oxidative, membrane, or protein damage, respectively. The expression levels of these four genes were quantified using a real-time RT-PCR after challenge with three different EDCs individually. Bisphenol A and styrene caused high-level expression of *recA* and *katG* genes, respectively, whereas 17β-estradiol made no significant changes in expression of any of those genes. These results lead to the classification of the mode of toxic actions of EDCs on *E. coli*.

Keywords: Stress effect, endocrine disrupting chemicals (EDCs), real-time RT-PCR, stress-specific responsive gene, gene expression analysis

Many environmental toxic chemicals in the ecosystem could be distributed to humans and accumulated in the human body by various routes. For this reason, public concerns regarding the potential human health effects of environmental toxic chemicals have increased continuously [9]. Endocrine disrupting chemicals (EDCs) may cause adverse effects on human health by interfering with the endocrine system in the body. Although EDCs have drawn some attention because of their estrogenic effects, also to be considered are its cellular toxic actions *via* many different routes such as DNA- or membrane-damages in the cell [1, 5]. Many researchers are performing risk assessments and toxicological studies of EDCs with human cell lines or other organisms by means of physical and chemical measurements, but such physicochemical analyses may not be sufficient to provide detailed information on how they affect the cells on a molecular level [16]. Therefore, a toxicological study looking at the effects they have on the molecular level is required, with gene expression analysis being an appropriate method [22].

Living cells rapidly respond when they are under stressful environmental conditions. This phenomenon is carried out through the changes in the cell's metabolic activities to overcome external stress [10, 12, 20]. In other words, if the cells are exposed to toxic compounds, the metabolic activities in the cell would change based upon the toxicological properties of the chemicals, so as to overcome the adverse effects they cause [3, 6, 8]. Molecular toxicological studies of the stress effect on *E. coli* have been reported using bioluminescent recombinant bacteria, DNA microarray, and real-time RT-PCR, and the comparison among these three methods was also performed [13].

To study the mechanism-based toxicity of EDCs in *E. coli*, therefore, four stress responsive genes, *i.e.*, *recA*, *katG*, *fabA*, and *grpE*, were selected. Each gene responds specifically to DNA damage, oxidative damage caused by hydroxyl radicals, membrane damage, and protein damage, respectively [17, 21, 23, 24]. To perform molecular toxicological studies of different EDCs, transcriptional analyses of these four stress responsive genes, along with the *rrnH* gene as an endogeneous control, were conducted using real-time RT-PCR [4, 7, 11, 14, 18].

Escherichia coli strain RFM443 was used in this study. When the optical density at 600 nm of the cell suspension (100 ml) reached 0.08, 100 ppb of 17β-estradiol in ethanol (0.01% final EtOH conc.), 100 ppb of bisphenol A in ethanol (0.01% final EtOH conc.), or 100 ppb of styrene in ethanol (0.01% final EtOH conc.) were spiked into the flask. The above concentration of each chemical was obtained as a maximum sublethal concentration that does not inhibit the cell growth (data not shown). The cell suspension was harvested at 0, 60, and 90 min after

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Table 1. Primer design for real-time RT-PCR analysis.

Gene	Forward primer (5'->3')	Reverse primer (5'->3')
grpE	ATTGAGGCAGTTGAGCCAG	GCTCAATGCCTTCAACCAT
katG	GGATATGCGTTACGAGTGGA	CCCACTATACGCTGGTAAAGAA
fabA	ACGTTGGCTGAACTGGTTTA	TCCGAAGAACCACAGATCC
recA	GTGAAGAACAAAATCGCTGC	TCTGCTACGCCTTCGCTAT
rrnH	AGTCGAACGGTAACAGGAAGA	GCAATATTCCCCACTGCTG

initiating the exposure by centrifuging samples of the culture at 16,000 ×*g* for 15 min. Total RNA was extracted from the harvested cells using the RNA RNeasy Mini kit (Qiagen, U.S.A.) according to the manufacturer's instructions [13, 15]. The purity of the RNA extracted was checked by using both a gel electrophoresis and UV spectrophotometer. In gel electrophoresis, two bands of 23S and 16S rRNAs were shown clearly (data not shown). In addition, the ratios of UV 260/280 were between 1.8 and 2.1 for all RNA samples. The concentration of total RNA was evaluated by UV/VIS spectrophotometry (Perkin-Elmer Co., U.S.A.). As a negative control, the total RNA from the cells exposed to the solvents alone, *i.e.*, 0.01% of ethanol, was extracted with the same procedure. PCR primers were designed for the four stress responsive genes, *recA*, *katG*, *fabA*, and *grpE*, as well as for the *rrnH* gene as an endogenous control. The primer pairs for each gene are listed in Table 1. The total RNA was reverse-transcribed in a 25 μl reaction mixture containing 100 ng of both the forward and the reverse primers, 40 mM dNTP mix, 2.5 μl 10×HF RT-PCR buffer, and 2.5 U SuperScript reverse transcriptase (Invitrogen). The reaction mixture was placed at 42°C for 30 min and then 95°C for 5 min. Real-time PCR was performed by an ABI Prism 7000 Sequence Detection System (Applied Biosystems, U.S.A.) under the following PCR conditions: an initial cycle at 48°C for 30 min, one cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55°C for one min, and 60°C for 1 min. A total of 10 μl of the cDNAs (target genes and *rrnH*) were amplified in a 25 μl reaction mixture containing 100 ng of gene-specific primers and 12.5 μl 2×SYBR Green PCR Master Mix (Applied Biosystems, U.S.A.) within a single ABI Prism run. Data analysis was performed using ABI Prism 7000 SDS Software (Applied Biosystems, U.S.A.). The induction ratio was calculated using the function $2^{-\Delta\Delta CT}$ according to ABI Prism 7000 SDS Software, where $\Delta\Delta C_T=(C_{T,\text{target gene}}-C_{T,rrnH})_{\text{stressed}}-(C_{T,\text{target gene}}-C_{T,rrnH})_{\text{control}}$ [19].

In this study, the *rrnH* gene, which encodes for the 16S rRNA, was selected and tested as a control to normalize the data obtained from the target genes. The 16S rRNA gene is a useful internal standard because its expression level is less likely to change under the various conditions that may affect the expression of mRNAs [2]. It was confirmed that the *rrnH* gene can be used as an internal standard in this study. Gene expression levels of the *rrnH* gene under the exposure of three different chemicals, 100 ppb of mitomycin C (MMC), 7 ppm of hydrogen peroxide, and 100 ppm of phenol, were not significantly changed compared with the untreated control (Fig. 1). Therefore, all expression values obtained from the target genes could be normalized with the expression level of the *rrnH* gene.

17β-Estradiol, a known representative EDC, did not show any toxic effects in *E. coli* under this experimental condition. As shown in Fig. 2A, the expression levels for all of the four genes by 100 ppb of 17β-estradiol were not increased. This result corresponds to a previous study where the toxicity of EDCs was evaluated with recombinant bioluminescent bacteria [9]. 17β-Estradiol had an only estrogenic potential, which could not be definable using stress-specific genes, *recA*, *katG*, *fabA*, or *grpE*. The interesting result is that the *grpE* gene was slightly suppressed by 17β-estradiol. However, to know the effect of 17β-estradiol on protein damage, further investigation is required. Fig. 2B shows that bisphenol A clearly causes DNA damage in *E. coli*. Only the *recA* gene was highly upregulated and the gene expression level was increased according to the exposure time, whereas *katG*, *fabA* genes did not show any significant response by 100 ppb of bisphenol A. The *grpE* gene was repressed by bisphenol A, which agrees with the result of 17β-estradiol exposure,

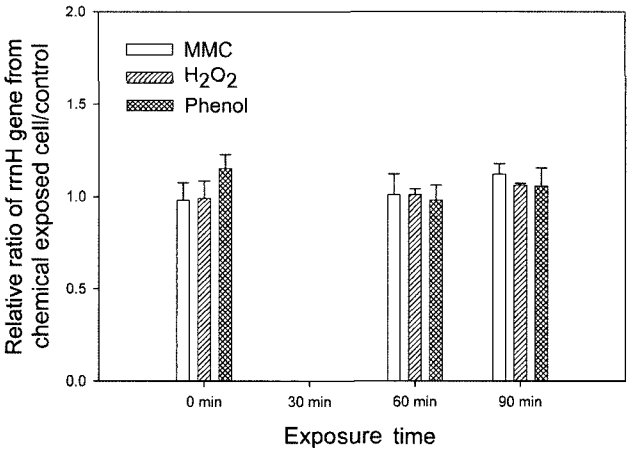

Fig. 1. The *rrnH* gene as an internal standard.
Gene expression levels of the *rrnH* gene under the exposure of three different chemicals were not significantly changed compared with the untreated control.

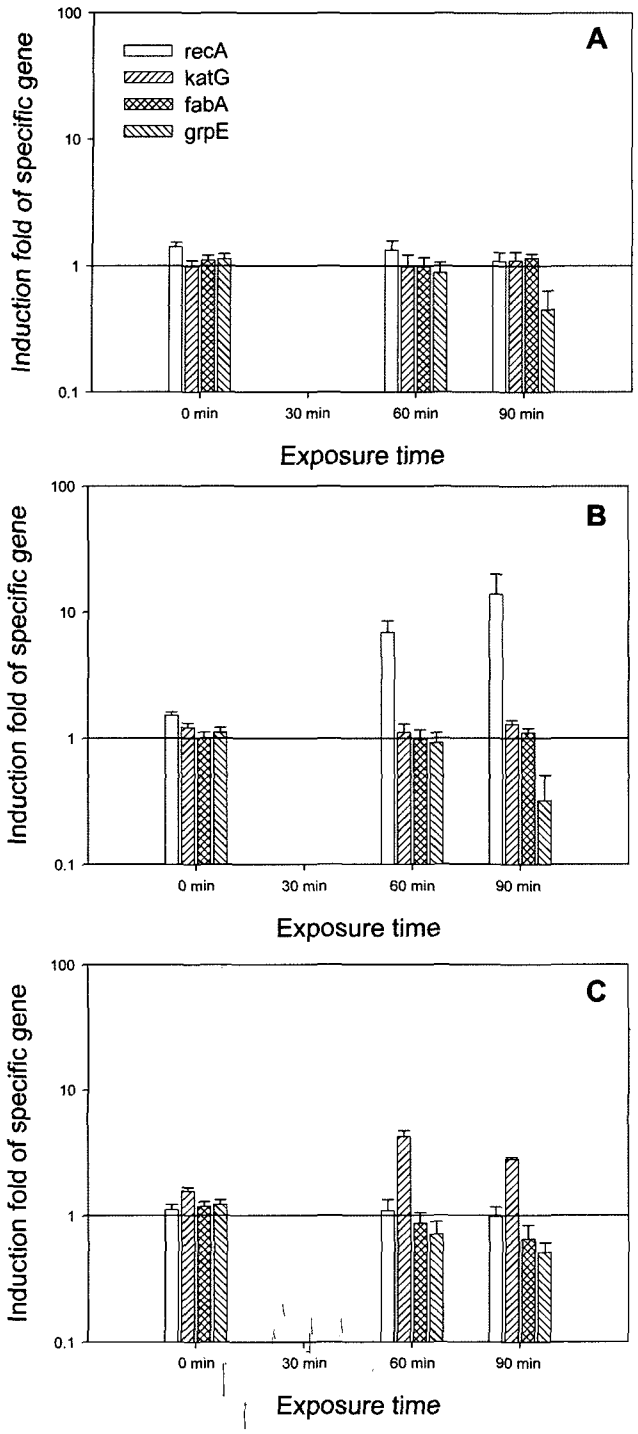

Fig. 2. Relative quantification of *recA*, *katG*, *fabA*, and *grpE* gene expression in *E.coli* after EDCs exposure.
A. 17β-Estradiol (100 ppb). **B.** Bisphenol A (100 ppb). **C.** Styrene (100 ppb).

and the degree of repression was higher than above one. One possibility for this *grpE* gene repression is that EDCs may suppress the genes on heat-shock regulon by an unknown toxic mechanism. In Fig. 2C, only the *katG* gene was highly expressed by styrene, whereas the *recA* gene was not affected. In contrast to the *katG* gene, the expression of the *recA* gene was continuously increased because the cell's response to DNA damage is slow. In particular styrene repressed the *grpE* gene and also the *fabA* gene slightly. Based on the expression level for the four stress-specific responsive genes, the toxic mode of EDCs was evaluated, whereby bisphenol A causes DNA damage and styrene induces oxidative damage by hydroxyl radical.

In this study, from the transcriptional level responses, the modes of toxicity of several EDCs, *i.e.*, 17β-estradiol, bisphenol A, and styrene, in *E. coli* were elucidated. Whereas the four stress-sensitive genes that were selected for analysis provided much information about the stresses experienced when exposed to these EDCs, some of these chemicals need further screening with other stress- or chemical-specific responsive genes to more accurately classify them and their mode of toxicity.

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