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Noninvasive monitoring of environmental toxicity through green fluorescent protein expressing *Escherichia coli*

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Abstract−The facile and rapid monitoring of the cellular response to environmental stresses is crucial for understanding the effects of environmental toxicity in living organisms. The overall cell growth can be examined to find a simple monitoring system. Green fluorescent protein (GFP) is advantageous when used as a reporter protein of the cellular stress responses in *Escherichia coli* because it allows the non-invasive monitoring of GFP *in vivo* without affecting the cell metabolism. We compared the environmental toxicities of chemical pollutants using GFP expressing *E. coli* for easy monitoring by incubation in various concentrations of harmful chemicals (ethanol, phenol, para-formaldehyde, paraben, and triclosan). The results showed that all the chemical pollutants act on cell growth and the cell metabolism according to the measured cell density and fluorescent intensity of GFP. In addition, from comparative analysis for quantification, the concentration of unknown ethanol toxicity, which was not determined at that concentration, could be deduced. In conclusion, the degree of toxicity for each chemical pollutant could be estimated or evaluated. This system will be useful for monitoring the toxicity of chemical pollutants in a non-invasive monitoring system.

Keywords: Green Fluorescent Protein (GFP), *Escherichia coli* (*E. coli*), Noninvasive Monitoring, Chemical Pollutants, Stress, Toxicity

INTRODUCTION

Exposure to harmful chemical or physical conditions causes many changes to cells, such as cell growth, alterations in gene expression and protein stability [1]. These changes produce additional upregulated proteins, particularly transcription factor (σ^{32})-induced changes by abnormal increases in temperature, which modulates homeostasis against the stress conditions [2-5]. These evolutionarily conserved heat shock proteins play many important roles in the folding, assembly, degradation, and translocation of proteins [6]. In addition to heat shock, a number of stresses, such as viral infections [7], oxygen limitation, presence of abnormal proteins [8-10], nutrient limitation [11,12], and exposure to various chemical pollutants [1,4,13-15], induce the synthesis of heat shock proteins in many organisms. This upregulation of the heat shock proteins is considered an important mechanism for survival in the presence of environmentally toxic substances. Therefore, the facile and rapid monitoring of the cellular response against environmental stresses is crucial for understanding the effects of environmental toxicity in living organisms [16]. Because different sets of genes and proteins are induced by different stresses [1], it may be difficult to characterize all the proteins. It would be better to examine the overall cell growth to identify an easy monitoring system.

Green fluorescent protein (GFP), which originates from *Jelly fish* [17], has advantages as a reporter protein of the cellular stress responses in *Escherichia coli* because it requires no ATP, co-factors, or staining for fluorescence, and fluorescence is readily visible from outside the cells [18], which makes possible the non-invasive monitoring of GFP *in vivo* without affecting the cell metabolism [19-21].

In the present work, we compared the environmental toxicities of chemical pollutants such as ethanol, phenol, para-formaldehyde, paraben, and triclosan using GFP expressing *E. coli* for easy monitoring. Cell density and fluorescence intensity were analyzed by using bacterial cells incubated with a range of concentrations of harmful chemicals. The level of toxicity was examined and the critical concentration on the cells was determined simply by measuring the GFP fluorescence intensity (Fig. 1).

MATERIALS AND METHODS

1. Bacterial Strains and Plasmids

The plasmid, pTH-GFPuv, which harbors the gfp_{uv} gene under the *trc* promoter originating from the parent vector, pTrcHis (Invitrogen, Carlsbad, CA, USA), was used to express GFP as a model foreign protein. *E. coli* BL21 (Novagen) was used as the host strain. Recombinant *E. coli* cell-transformed pTH-GFPuv were cultured in Luria broth (LB; 0.5% yeast extract, 1% tryptophan, and 1% NaCl) containing 50 μg/mL of ampicillin (Sigma-Aldrich). When the cell density (OD_{600}) reached approximately 1, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich) and the target chemicals were added simultaneously to induce recombinant GFP expression. The cells were cultivated at 250 rpm, 37 °C for 10 h after induction.

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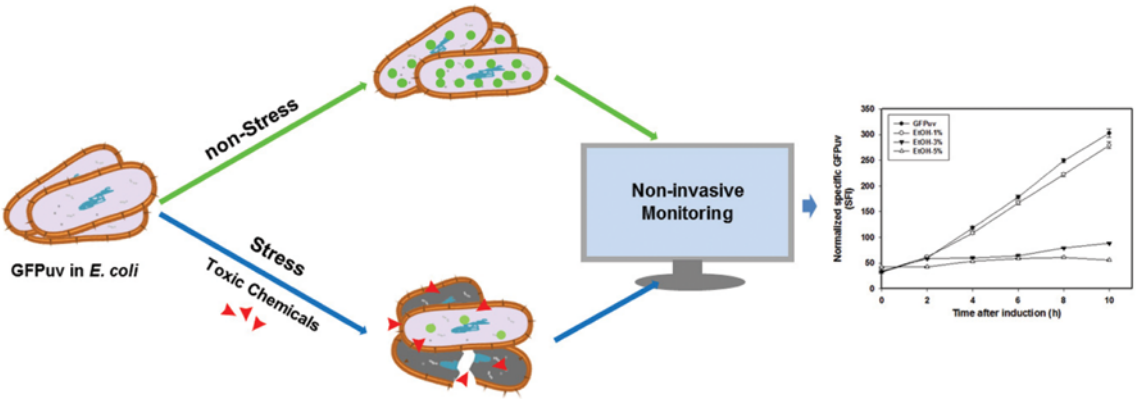

Fig. 1. Schematic representations of non-invasive monitoring of green fluorescent protein expressing *E. coli* against chemical pollutants.

2. Analytical Methods

Cell growth was monitored by the optical density (at 600 nm, OD_{600}) using a UV/VIS spectrophotometer (Evolution 201; Thermo Fisher Scientific, Madison, WI, USA). The GFP assay was performed by measuring the fluorescence intensity with a fluorescence spectrometer (UV-5301PC; Shimadzu, Kyoto, Japan) at an excitation wavelength of 395 nm and an emission wavelength of 509 nm. Each sample collected was centrifuged every 2 h, and the pellet was washed twice with phosphate-buffered saline (1.44 g of Na_2HPO_4 per liter, 0.24 g of KH_2PO_4 per liter, 0.2 g of KCl per liter, and 8 g of NaCl per liter; pH 7.4) before the GFP assay. The data reported below includes the specific fluorescence intensity (SFI) (the raw fluorescence intensity, which was obtained by subtracting the basal fluorescence intensity of the BL21 harboring parent vector (pTrcHisC), divided by the optical density at 600 nm). This allows a comparison of the samples cultured in different optical densities at time zero.

3. Stresses

Chemical pollutants, such as ethanol, methanol, phenol, and heavy metals, are generally considered to induce cellular stress in *E. coli* [14,22]. In the present work, the cellular responses to chemical pollutants were tested by adding 1-5% (vol/vol) ethanol (95%), 0.1-0.3% (vol/vol) paraben (methyl; 100 mg/mL), 0.05-0.15% (vol/vol) triclosan (2 mg/mL), 0.1-0.2% (vol/vol) paraformaldehyde (30 mg/mL), and 0.075-0.2% (vol/vol) phenol (98%). The final concentrations for the chemical pollutants were calculated to be 0.1-0.3 mg/mL for paraben, 0.001-0.003 mg/mL for triclosan and 0.03-0.06 mg/mL for paraformaldehyde.

4. Western Blot Analysis

Each sample was mixed with the sample buffer (10% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, 0.3 M Tris-HCl (pH 6.8), 0.05% bromophenol blue, and 50% glycerol) based on the determined cell density to make the same cell amount, boiled for 5 min, and loaded on a 12.5% (w/v) SDS-polyacrylamide gel for electrophoresis (SDS-PAGE). The proteins were then transferred to Hybond-PVDF membranes (Amersham Pharmacia Biotech, UK) with a transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 20% methanol; pH 9.2) using a Trans-Blot SD Cell (Bio-Rad) at 15 V for 30 min. After blocking for 1 h in TBS buffer (20 mM Tris-HCl and 500 mM NaCl; pH 7.5) containing 5% (w/v) non-fat dry milk, the membrane was incubated for 1 h at room temperature in an antibody solution (1% (w/v) non-fat dry milk in TTBS (TBS with 0.05% Tween-20)) containing a polyclonal anti-GFP antibody (Roche Diagnostics GmbH, Mannheim, Germany) and a secondary anti-mouse antibody conjugated with alkaline phosphatase for GFP detection (Sigma-Aldrich) at a ratio of 1 : 3000 (v/v). After washing with TTBS and TBS, the membranes were developed with a 5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) color development solution (Roche, UK) to detect the recombinant GFP, and the reaction was washed with distilled water to stop development.

RESULTS AND DISCUSSION

1. Cellular Stress Response by Chemical Pollutants

Microorganisms can survive abnormal changes in their environment by evolving an adaptive response [23]. Actually, there may be enormous adaptive responses in bacteria exposed to each chemical pollutant, but the analysis is time-consuming and complicated. Therefore, we examined whether the overall response could be determined simply by measuring the cell growth and fluorescence intensity. Among the abnormal changes, several chemical pollutants (ethanol, paraben, triclosan, paraformaldehyde, and phenol) were employed to examine the effect of their toxicities on the microorganism. Those chemicals are regarded as antiseptics or disinfectants that inhibit microbial contamination. On the other hand, when the levels of the chemical pollutants are sub-inhibitory, the cells can survive by producing transcription factors, chaperons, proteases, etc. [2,4].

The following gives brief descriptions of the chemical pollutants. Ethanol alters the physical characteristics of a cell by causing membrane destruction and the rapid denaturation of proteins, and subsequent interference with the metabolism and cell lysis [24]. Parabens (alkyl esters of p-hydroxybenzoic acid) are a class of antimicrobial agents used extensively as preservatives in cosmetic products, but recent reports have shown that the five most commonly used parabens were detected in human breast cancer tissue [25-28]. Triclosan is widely regarded as a nonspecific biocide that attacks the bacterial membranes and is used in soaps, detergents, etc. [29,30]. Paraformaldehyde is an aqueous type of formal-

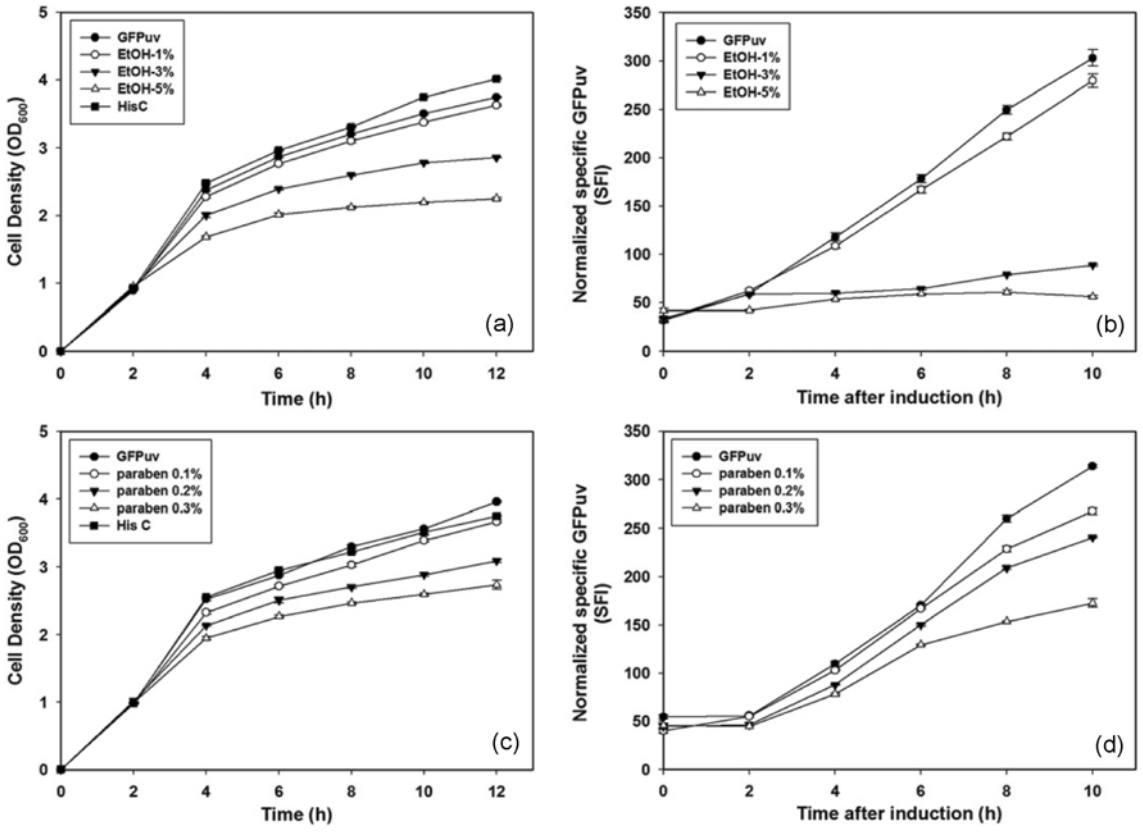

Fig. 2. Cell density (OD_{600} nm) and SFI (normalized fluorescence intensity: fluorescence intensity of GFP - basal fluorescent intensity (HisC)) graph of GFP expressing *E. coli* against ethanol (a) & (b) (1, 3, 5%) and paraben (c) & (d) (0.1, 0.2, 0.3%). GFPuv: no treatment with the chemical pollutant; HisC: parent vector harboring BL21 cell without GFPuv gene.

dehyde with higher polymers and an extremely reactive chemical [31,32]. Phenol induces progressive leakage of the intracellular components, such as the release of K^+, while phenolic-type antimicrobial agents have been used for their antiseptic, disinfectant or preservative properties [24]. Overall, all chemical pollutants employed in this study are believed to be toxic to bacterial growth.

As shown in Fig. 2, cell growth and GFP fluorescence intensities in the presence of three chemical pollutants (ethanol and paraben) were obtained. 1% ethanol appeared to be non-toxic, but the FIs decreased dramatically at more than 3% ethanol (Fig. 2(a)). Interestingly, the FIs were decreased much more than the cell densities, which means that ethanol may affect the cell metabolism rather than cell growth at such concentrations. Such a tendency was also observed in paraben (Fig. 2(c)-(d)), whereas the FIs were not decreased as much as those in ethanol. In all cases, cell growth and FI decreased gradually with increasing concentrations, which suggests that higher concentrations induce higher stress, but the specific responses were not shown in this study. These results indicate that the present non-invasive monitoring system using GFP could represent the environmental toxicity of chemical pollutants. Consequently, a higher concentration resulted in the inhibition of higher cell growth as well as blocking the cell metabolism.

In contrast to previous chemical pollutants, paraformaldehyde, phenol and triclosan showed different patterns in FIs, but not in cell growth (Fig. 3). For paraformaldehyde, the cell densities decreased gradually, whereas the FIs decreased with increasing paraformaldehyde concentration (Fig. 3(a) and 3(b)). These results indicate that because formaldehyde is used generally as a fixation agent, a low concentration of paraformaldehyde inhibits cell division, but is not lethal. Therefore, cell densities decreased with increasing paraformaldehyde concentration. The increase in FIs means that the cell metabolism was unaffected by paraformaldehyde under these conditions. Therefore, the FI increased with increasing concentration. In the case of phenol, some interesting results were obtained. Cell growth decreased with increasing phenol concentration, whereas the FI at 0.1% was higher than that at 0.075% and 0.2%. In addition, an abnormally high fluorescent intensity was observed at 0.4% phenol (data not shown). Pulvertaft and Lumb reported that low phenol concentrations lysed *E. coli* rapidly [33], but lysis did not occur with higher phenol concentrations (0.54%). Srivastava and Thompson proposed that phenol acts more sensitively on young bacterial cells than older cells [34,35]. From previous reports, it is difficult to conclude how phenol influences cell growth depending on the concentration, but higher phenol concentrations might not affect bacterial lysis and the cell metabolism, which is similar to the present results. In the case of triclosan, cell growth reached a stationary phase after the addition of triclosan (Fig. 3(e)), possibly because triclosan blocks cell growth through the inhibition of fatty acid synthesis in the cell [36]. On the other hand, the *E. coli* treated with triclosan showed a higher

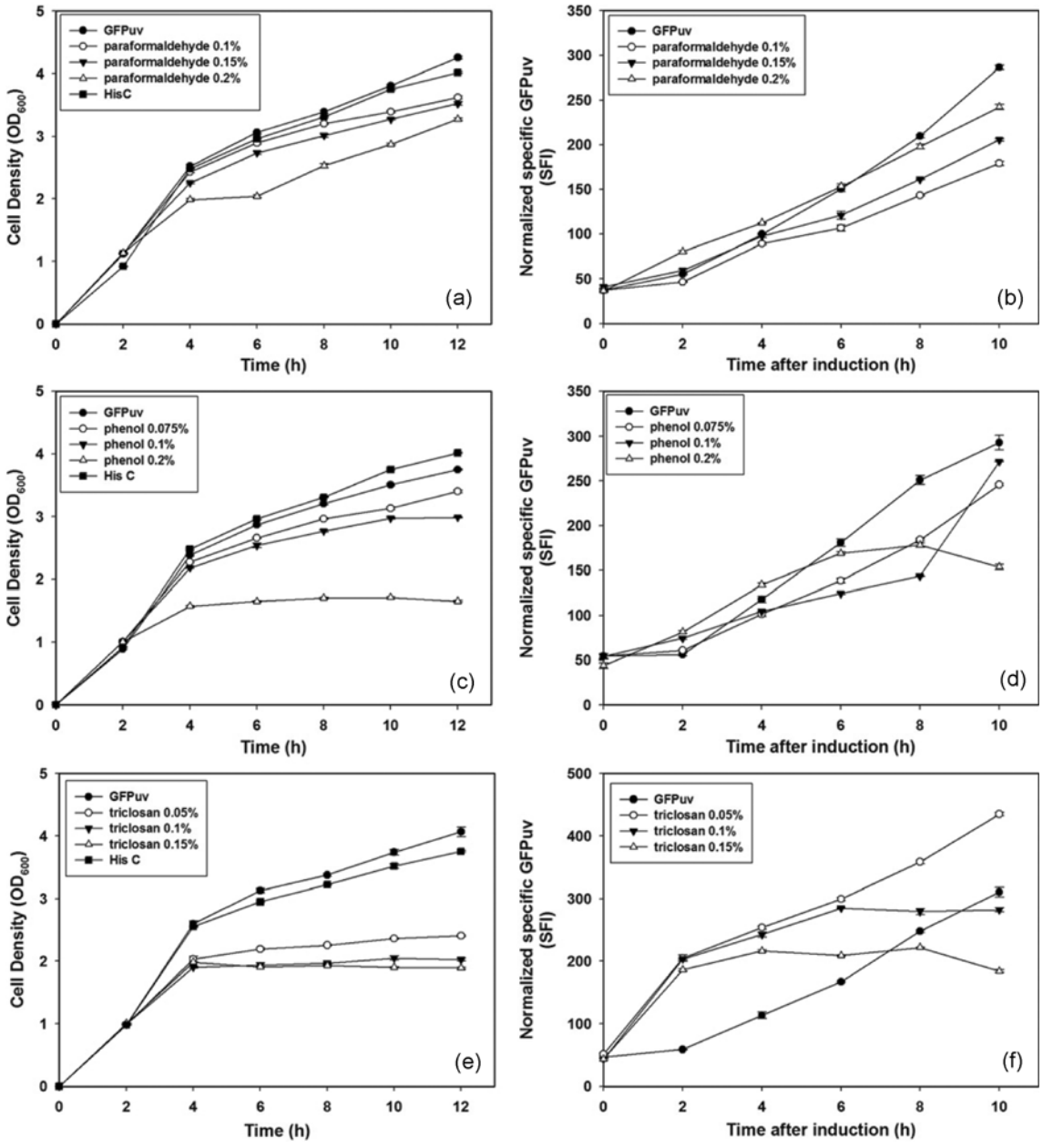

Fig. 3. Cell density (OD_{600} nm) and SFI (normalized fluorescence intensity: fluorescence intensity of GFP - basal fluorescent intensity (HisC)) graph of GFP expressing *E. coli* against paraformaldehyde (a) & (b) (0.1, 0.15, 0.2%), phenol (c) & (d) (0.075, 0.1, 0.2%), and triclosan (e) & (f) (0.05, 0.1, 0.15%). GFPuv: no treatment with the chemical pollutant; HisC: parent vector harboring BL21 cell without GFPuv gene.

FI than that without triclosan (Fig. 3(f)). This suggests that triclosan induces cell damage resulting in leakage of the intracellular contents of the membrane [37,38].

Overall, this study confirmed that all chemical pollutants act on cell growth and cell metabolism according to measurements of the cell density and fluorescent intensity of GFP, even though it is unclear how they act. In addition, some chemical pollutants (e.g., paraformaldehyde and phenol) affected the bacterial cells differently depending on the concentration.

2. Comparative Analysis of the Chemical Pollutants for Quantification

In Figs. 2 and 3, the cell density and FI of GFP expressing recombinant *E. coli* in the presence of chemical pollutants were quantified to examine their effects (toxicity) on bacterial cells. As shown in Fig. 4(a), GFP expression was analyzed under each condition by Western blotting. Although the band intensity was not analyzed quantitatively, the tendencies of fluorescent intensity *vs.* band intensity were similar in principle for all chemical pollutants. When the FIs under highly down-regulated conditions in each chemical pollutant were compared, a similar level of SFIs was observed (approximately 150-200 SFI) except for 5% ethanol (Fig. 4(b)). This analysis suggests that 0.3% paraben, 0.15% triclosan, 0.1% paraformaldehyde, and 0.2% phenol have similar toxicity to bacterial cells, but the repression mechanism is unclear or different for each chemi-

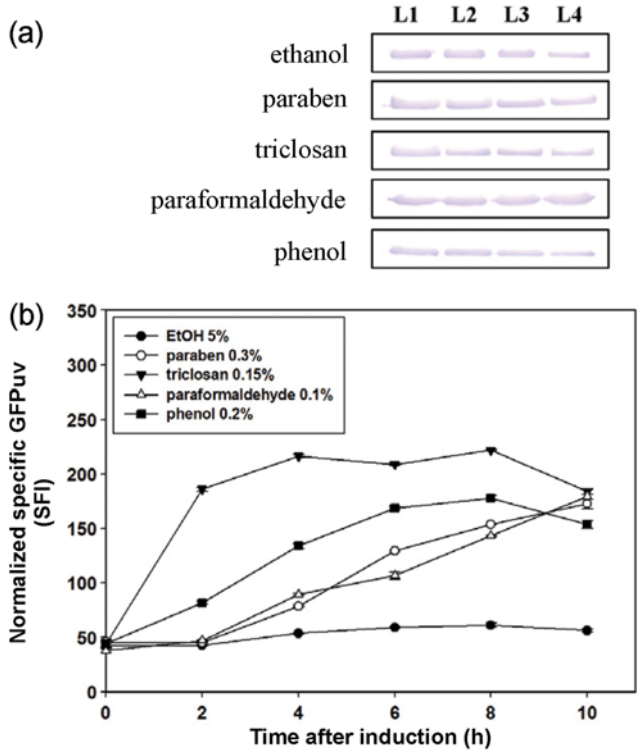

Fig. 4. (a) Western blot analysis for GFP expression against chemical pollutants, L1: 2 h, L2: 4 h, L3: 6 h, L4: 8 h (time after induction). (b) Comparative analysis at the highly down-regulated concentration of chemical pollutants (10 h after induction).

cal. In addition, ethanol had similar toxicity, ~2% ethanol ≈150-200 SFI. From this study, once the data for various chemical pollutants and concentrations are added or provided, it is expected that the degree of toxicity for each chemical pollutant can be estimated or evaluated.

CONCLUSIONS

The environmental toxicity of chemical pollutants such as ethanol, phenol, para-formaldehyde, paraben, and triclosan, was compared using GFP expressing *E. coli* for easy monitoring. GFP was used as a monitoring reporter protein because it requires no ATP, co-factors, or staining for fluorescence, and the fluorescence is readily visible from outside the cells, which allows the non-invasive monitoring of GFP *in vivo* without affecting the cell metabolism. Cell density and fluorescence intensity were analyzed from the cells incubated with diverse concentrations of harmful chemicals, and the level of toxicity was examined and the critical concentration on the cells was characterized simply by measuring the GFP fluorescence intensity. Analysis of the cellular stress response confirmed that all the chemical pollutants act on cell growth and cell metabolism according to measurements of the cell density and fluorescent intensity of GFP. In addition, some chemical pollutants (e.g., paraformaldehyde and phenol) could affect bacterial cells differently depending on the concentration. In addition, from comparative analysis for quantification, the concentration for unknown ethanol toxicity could be determined, even though the experiment was not conducted at that concentration. This non-invasive monitoring system has limitations in that extremely low or high concentrations of chemical pollutants may be difficult to detect because it is basically a cell-based incubation system. In the future, it will be necessary to collect SFI data under a range of concentrations of chemical pollutants and analyze the errors in monitoring and determining the concentration. Overall, the degree of toxicity for each chemical pollutant could be estimated or evaluated, and this system is expected to be useful as a non-invasive monitoring system for monitoring the toxicity of chemical pollutants.

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