Biotechnol. Bioprocess Eng. 2000, 5: 395-399

Toxicity Monitoring of Endocrine Disrupting Chemicals (EDCs) Using Freeze-dried Recombinant Bioluminescent Bacteria

Sung Woo Kim, Sue Hyung Choi, Jiho Min, and Man Bock Gu*

Department of Environmental Science and Engineering and Advanced Environmental Monitoring Research Center (ADEMRC), Kwangju Institute of Science and Technology (K-JIST), 1 Oryong-dong, Puk-gu, Kwangju 500-712, Korea

Abstract Five different freeze-dried recombinant bioluminescent bacteria were used for the detection of cellular stresses caused by endocrine disrupting chemicals. These strains were DPD2794 (*recA::luxCDABE*), which is sensitive to DNA damage, DPD2540 (*fabA::luxCDABE*), sensitive to cellular membrane damage, DPD2511 (*katG::luxCDABE*), sensitive to oxidative damage, and TV1061 (*grpE::luxCDABE*), sensitive to protein damage. GC2, which emits bioluminescence constitutively, was also used in this study. The toxicity of several chemicals was determined on the first four freeze-dried bacteria, while nonspecific cellular stresses were measured using GC2. Damage caused by known endocrine disrupting chemicals, such as nonyl phenol, bisphenol A, and styrene, was detected and classified according to toxicity mode, while others, such as phathalate and DDT, were not detected with the bacteria. These results suggest that endocrine disrupting chemicals are toxic in bacteria, and do not act via an estrogenic effect, and that toxicity monitoring and classification of some endocrine disrupting chemicals may be possible in the field using these freeze-dried recombinant bioluminescent bacteria.

Keywords: endocrine disrupting chemicals (EDCs), toxic effect, freeze-dried recombinant bioluminescent bacteria, cellular toxicity

INTRODUCTION

The environmental monitoring of pollutants is becoming increasingly important to the general public. This is particularly true for compounds that pose a potential risk to human health or to the environment. For example, endocrine disrupting chemicals (EDCs) have become a pressing issue, since they may cause adverse effects on human health by interfering with endogenous hormones in the body.

EDCs are known to have an effect related to hormones within animal hosts. Because they are not degraded in the body, and as derivatives of persistent organohalogens and herbicides, they have a high residence period, they may cause serious problems related to bioaccumulation. Therefore, it is necessary to screen and assess the risk due to EDCs in natural systems [1,2]. In this context, most of the studies related to EDCs have proceeded on the basis of their estrogenic effect, using *in vitro* assays for measuring the oestrogen/anti-oestrogen, androgen/anti-androgen and dioxin-like activities of individual compounds or complex mixtures [2]. Some research groups, however, have reported that EDCs also cause cellular toxicity [2,3], while other groups have found that a number of EDCs are cytotoxic and may be detected using bacterial toxicity tests [4-6]. Although bacterial cells were unlikely to experience the estrogenic effects induced in higher organisms, it is certain that bacteria can monitor EDCs toxicity. Therefore, a whole-bacterial cell biosensor has been suggested to monitor EDCs toxicity, even though bacterial cells may not recognize hormogenic mechanisms.

Recently, some ongoing research has constructed genetically engineered bioluminescent bacteria bearing a fusion of stress promoter, which specifically respond to environmental pollutants, and the reporter *lux* genes have used with these strains to monitor toxicants [7-9]. This approach is very useful and simple since light emitted by luciferase is easily monitored and quantified. Various recombinant bacterial strains, DPD2794 (*recA::luxCDABE*), DPD2540 (*fabA::luxCDABE*), TV1061 (*grpE::luxCDABE*), and DPD2511 (*katG::luxCDABE*) [7-9], and GC2 [10], bearing the pLITE2 plasmid (*lac::luxCDABE*) [11], were used for the detection of stress response induction and cellular toxicity, caused by general environmental toxicants, in this study [12]. In addition, a previously developed freeze-drying method was used in this study [13]. Freeze-dried bacteria can easily be used on-site to determine the toxicity of water samples. Another benefit in using freeze-dried cells is that they can be used to measure the toxicity of a sample within a shorter period of time in an flask grown culture. Therefore, freeze-dried genetically engineered bioluminescent bacteria were used for the detection of

*Corresponding author
Tel: +82-62-970-2440 Fax: +82-62-970-2434
e-mail: mbgu@kjist.ac.kr

the toxicity of samples containing EDCs in order to provide information on the mechanism of toxicants and to mimic the effects upon cells during field applications.

MATERIALS AND METHODS

Strains, Growth and Culture Conditions

Various recombinant bacteria, DPD2794 (*recA::luxCDABE*) [7,14], DPD2540 (*fabA::luxCDABE*) [15], TV1061 (*grpE::luxCDABE*) [8,16], and DPD2511 (*katG::luxCDABE*) [9], were employed in this study. Each type has a specific stress promoter fused to the *luxCDABE* operon originating from *Vibrio fischeri*. However, GC2 (*lac::luxCDABE*) has the *luxCDABE* genes of *Xenorhabdus luminescens* under the control of the *lac* promoter, giving constitutive expression [10,11]. Four strains, all but GC2, were grown in Luria-Bertani (LB) medium (Difco Co., USA) supplemented with 25 mg/L kanamycin monosulfate (Sigma Co., USA) to maintain the plasmid, but GC2 was grown using 10 mg/L ampicillin (Sigma Co., USA). The initial pH of the media was adjusted to 7.0 before sterilization. A single colony of each strain, grown on an LB agar plate, was inoculated into 100 mL of sterile LB medium and cultured at 30°C, (37°C for GC2), and 250 rpm in a rotary incubator (New Brunswick Scientific Co., Inc., New Brunswick, NJ, USA). When the optical density at 600 nm (OD_{600}) reached 0.8 (late exponential phase), 2 mL of the preculture were transfered into a new flask containing 100 mL of LB medium and the appropriate antibiotic.

Freeze-drying of the Recombinant Bacteria and Rehydration

The freeze-drying method used has been described previously [15]. Briefly, when the optical density reached 0.8 (late exponential phase) in the second culture, the cells were harvested by centrifugation at 4000 rpm for 40 min at 4°C. After centrifugation, the pellet was resuspended in 10 mL of fresh LB medium with kanamycin (25 mg/L) or ampicillin (10 mg/L) and the resuspension diluted to adjust the O.D. to 0.08, since the viability of the freeze-dried sample was shown to be greatest at this concentration (data not shown). Samples were then mixed with the same volume of 24% sucrose, a cryoprotectant. Aliquots of 1 mL were pipetted into sterile 1.5-mL glass vials and frozen at −70°C for 2 days, dried at a pressure of less than 20 millitorrs for 2 days using a freeze-dryer (Labconco Co., USA), capped and stored at −20°C until used. The freeze-dried cells were rehydrated in 1ml of distilled water, as it was found that distilled water is a more effective rehydrating solution, resulting in a more sensitive biosensing than LB media, saline water, and LB media containing 10% glucose as a rehydrating solution (data not shown). After rehydration, 100-μL samples were removed to luminometer tubes and placed in a water bath at a temperature 30°C or 37°C for 30 min, prior to the chemical additions.

Chemical Induction

All the chemicals used in this study were purchased from the Sigma & Aldrich Chem. Co. Stock solutions were prepared to 1 mg/mL concentrations using sterile distilled water for paraquat and isodrin, ethanol for methyl bromide, DDT, cadmium chloride ($CdCl_2$) and bisphenol A, methanol for methoxychlor and butyl benzyl phathalate (phathalate), and chloroform for preparing a stock solution of ziram. Stock solutions of chemicals were added to the media to a solvent concentration of 0.1%, which did not result in any significant bioluminescent response (Data not shown). Styrene and nonyl-phenol were added directly to the medium. The bioluminescence of cells in the sample tubes was then monitored at set times using a luminometer (Turner Designs, TD-20e) and the relative bioluminescence (bioluminescence of the induced cells/bioluminescence of the control) was determined. This was used as quantitative indicator of toxicity and promoter induction.

RESULTS AND DISCUSSION

Until recently, its majority of studies related to EDCs have focused on their estrogenic effects, but some research groups have reported that EDCs also cause cellular toxicity [2,3]. In this study, it is shown that its detection of specific stresses caused by several EDCs might be possible by using four different inducible bioluminescent bacteria, capable of detecting specific modes of toxicity, and in addition, a constitutive bioluminescent bacterium was used to detect any general cellular toxicity caused by the EDCs.

Stress Responses of Recombinant Bioluminescent Bacteria with Specific Stress Promoters to EDCs

Fig. 1 shows the response of the four different strains to the same dose range of methyl bromide, which is known as an EDC. DPD2540 (sensitive to membrane damage) and TV1061 (sensitive to protein damage) showed a dose-dependent response to methyl bromide, while the other strains did not respond. This indicates that methyl bromide may cause membrane and protein damage in bacteria and, in addition, that it has a harmful effect on bacteria, not an estrogenic effect. Therefore, the detection of EDCs, such as methyl bromide, is plausible using bacteria. In addition, it was found that nonyl-phenol induces bioluminescence in DPD2540 and TV1061 (Fig. 2). This indicates that methyl bromide and nonyl-phenol cause damage to the cellular protein and membrane, but not to the DNA or its oxidative metabolism, after penetrating the cells. The specific responses of the bioluminescent bacteria to the EDCs are summarized in Table 1. Interestingly, as shown in

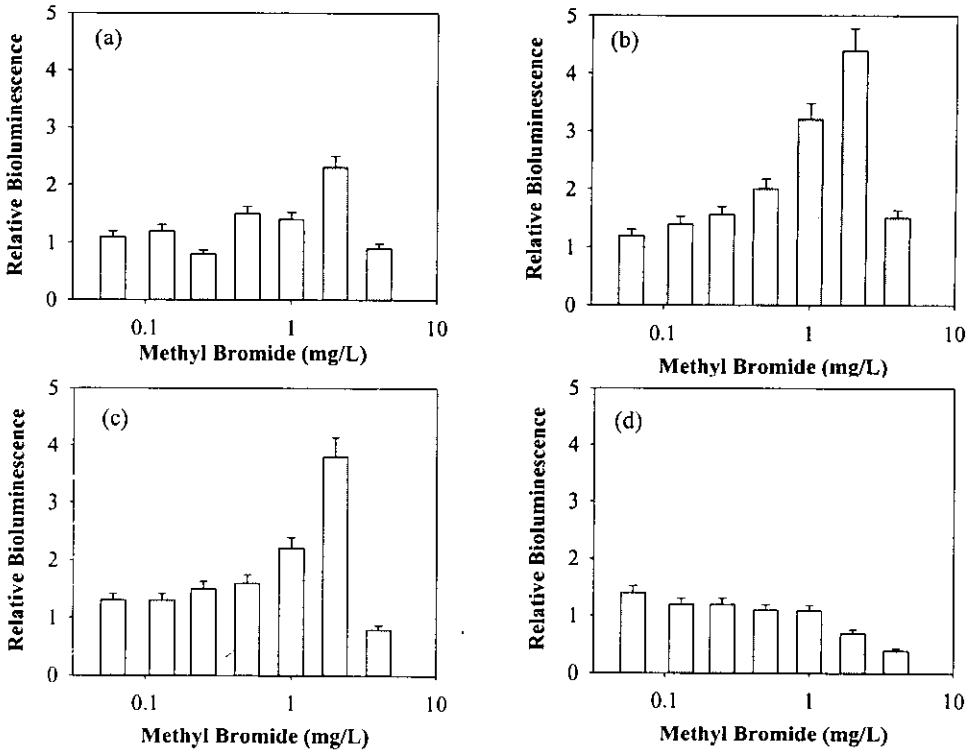

Fig. 1. Dose-dependent response of the four inducible bioluminescent strains to methyl bromide. The RBL is the relative bioluminescence value at 20 min post induction. (a) Response of DPD2794, sensitive to DNA damage, (b) DPD2540, sensitive to membrane damage, (c) TV1061, sensitive to protein damage, and (d) DPD2511, sensitive to oxidative damage.

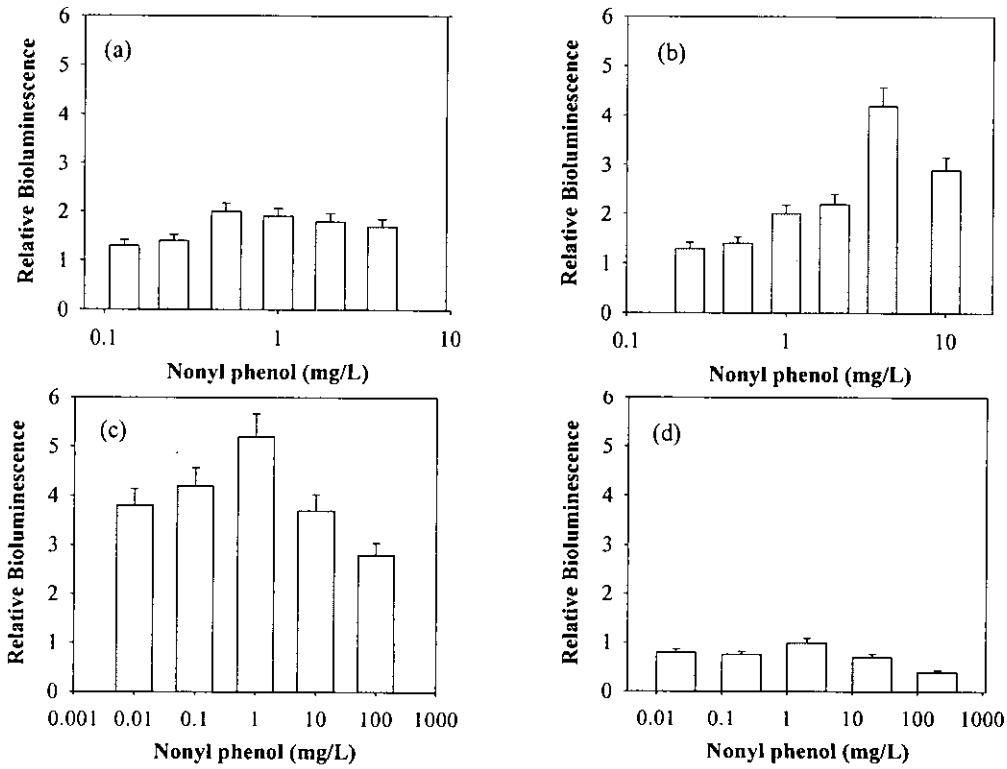

Fig. 2. Dose-dependent response of the four inducible bioluminescent strains to nonly phenol. The RBL is the relative bioluminescence value at 20 min post induction. (a) Response of DPD2794, sensitive to DNA damage, (b) DPD2540, sensitive to membrane damage, (c) TV1061, sensitive to protein damage, and (d) DPD2511, sensitive to oxidative damage.

Table 1. Summary of the maximum relative bioluminescence (RBL) at 20 min post induction for DPD2794, DPD2540, DPD2511 and TV1061, and the effective concentration giving a decrease of 20% bioluminescence (EC_{20}) at 20 min post induction for GC2 by various toxic chemicals having estrogenic effects. The RBL_{max} is the maximum RBL ratio at 20 min post induction. EDC concentrations were from 0.05 ppm to 300 ppm.

Chemicals	DPD2794 (*recA::luxCDABE*)		DPD2540 (*fabA::luxCDABE*)		DPD2511 (*katG::luxCDABE*)		TV1061 (*grpE::luxCDABE*)		GC2 (*lac::luxCDABE*)	
	Concentration giving the RBL_{max} (mg/L)	RBL_{max}	Concentration giving the RBL_{max} (mg/L)	RBL_{max}	Concentration giving the RBL_{max} (mg/L)	RBL_{max}	Concentration giving the RBL_{max} (mg/L)	RBL_{max}	Concentration giving the EC_{20} (mg/L)	RBL
Nonyl phenol	10	1.97	10	2.78	1	1.27	10	4.69	10	0.8
Methyl bromide	2	2.81	2	6.01	0.25	1.38	2	11.4	1	0.8
Styrene	200	0.96	200	1.06	200	1.00	2	2.39	50	0.8
$CdCl_2$	1.83	1.13	1.83	1.02	1.83	1.19	1.83	1.27	1.83	0.8
Bisphenol A	300	1.08	300	1.08	300	1.04	4	2.03	10	0.8
Methoxychlor	1	1.20	1	2.30	N.D.	N.D.	1	1.58	N.D.	N.D.
Ziram	0.01	4.65	0.01	1.08	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Phathalate	N.A.	1.10	N.A.	1.23	N.A.	1.03	N.A.	1.27	N.A.	1.0
DDT	N.A.	1.50	N.A.	1.26	N.A.	1.60	N.A.	1.15	N.A.	1.0

N.D. : Not detected, N.A. : Not available

Table 1, five different strains did not show the response ratio above two fold at the range of dosage from 0.05 ppm to 300 ppm of phathalate and DDT, thus had no effect on any of the strains, which indicates that these chemicals do not cause significant cellular toxicity in the bacterial cells. In other words, these chemicals may be classified as be truly estrogenic in their nature and effect.

Determination of Cellular Toxicity Caused by EDCs

GC2, the constitutive bioluminescent bacterium, was utilized to determine the critical concentration of the various EDCs on cellular metabolism and growth. The RBL (relative bioluminescence) is the ratio of the bioluminescence of the cells exposed to EDCs to that of the control, and the effective concentration is defined as decrease in the bioluminescence by 20% (EC_{20}), 20 min after induction by an EDC. The response of GC2 was similar to the specific response of the inducible bioluminescent bacteria. It was found that GC2 have EC_{20} values for five kinds of EDCs shown in Table 1, indicating that EDCs do cause considerable cellular damage. Also similar to the inducible strains, phathalate and DDT were found to have no effect. Therefore, these chemicals may be classified as truly estrogenic in their nature and effect.

As shown in Table 1, when at least one inducible bioluminescent bacterium showed a specific response to an EDC, it was often accompanied by cellular toxicity in GC2. In all cases, the presence of cellular toxicity was accompanied by a bioluminescent response in at least one strain. On the other hand, if a decrease in bioluminescence was not observed in GC2, no specific stress response was seen in any of the four inducible bioluminescent strains. Therefore, it was found that even though many of the tested EDCs showed toxicity, few have only an estrogenic effect.

Potential for Detection of EDCs Using a Freeze-drying Method

A freeze-drying method was also used in this study, the protocol of which was described previously [12]. Freeze-dried bacteria can easily be used on-site to determine the toxicity of water samples. All data values represent the values 20 min post induction, demonstrating that this method is able to measure the toxicity samples in a shorter time as compared to cultures grown in flasks [4]. Therefore, freeze-dried recombinant bioluminescent bacteria can be used to detect the toxicity of samples containing EDCs, which can be used to classify the mechanism of a toxicant and identify the effects cells experience in the field. This study demonstrates the feasibility of using freeze-dried bacteria as biosensors has been shown and their application of for the monitoring of environmental toxicants in the field.

CONCLUSION

In this study, several endocrine disrupting chemicals were tested with five freeze-dried recombinant bioluminescent bacteria, DPD2794 (*recA::luxCDABE*), DPD-

2540 (*fabA::luxCDABE*), TV1061 (*grpE::luxCDABE*), DPD2511 (*katG::luxCDABE*), and GC (*lac::luxCDABE*) to investigate the natures of its induced cellular stresses and their toxicity. It was found that some known EDCs do cause cellular stresses related to DNA damage, protein damage, oxidative damage, and membrane damage, and that these modes of toxic action are determinable through differences in the response kinetics of the four different inducible bioluminescent bacteria. Detection of EDCs was shown to be very specific and it was demonstrated that the development of a detection method for cellular stresses caused by EDCs using bacteria is possible. In addition, the cellular toxicity caused by EDCs was shown by measuring decreases in the level of bioluminescence in the constitutive bioluminescent bacterium, GC2. Throughout this study, it was found that these five recombinant bioluminescent bacteria could be used for the specific and quantitative analysis of the toxic effect of EDCs, even though they do not have the ability to detect estrogenic effects. We conclude that the detection methods described using recombinant bacteria are suitable for studying the cellular mechanisms of toxicity EDCs. In addition, this study shows that freeze-dried recombinant bacteria are able to monitor the presence of such chemicals within practical dosage ranges. Through this study, the feasibility of using freeze-dried bacteria as biosensors has been shown and their application for the monitoring of environmental toxicants in the field.

Acknowledgements This work was supported by the Minister of the Environment of Korea through the G7 project, and in part by KOSEF through the Advanced Environmental Monitoring Research Center (ADEMRC) at the Kwangju Institute of Science and Technology (K-JIST). Authors are grateful for their support.

REFERENCES

[1] Jimenez, B. (1997) Environmental effects of endocrine disruptors and current Methodologies for assessing wildlife health effects. *Trends Anal. Chem.* 16: 596-606.

[2] Jobling, S. (1998) Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment. *Pure Appl. Chem.* 70: 1805-1827.

[3] Omownumi, A. S. and M. W. Diane (1999) Novel Monitoring Strategies for Endocrine Disrupting Chemicals. *Environ. Sci. Technol.* 33: 368A-374A.

[4] Min, J., E. J. Kim, R. A. LaRossa, and M. B. Gu (1999) Distinct responses of a *recA::luxCDABE Escherichia coli* strain to direct and indirect DNA damaging agents. *Mut. Res.* 442: 61-68.

[5] Burcham, P. C. (1999) Internal hazards: baseline DNA damage by endogenous products of normal metabolism. *Mut. Res.* 443: 11-36.

[6] Schweikl, H., G. Schmalz, and K. Rackebrandt (1998) The mutagenic activity of unpolymerized resin monomers in *Salmonella typhimurium* and V79 cells. *Mut. Res.* 415: 119-130.

[7] Vollmer, A. C., S. Belkin, D. R. Smulski, T. K. Van dyk, and R. A. LaRossa (1997) Detection of DNA damage by use of *Escherichia coli* carrying *recA::lux*, *uvrA::lux*, or *alkA::lux* reporter plasmids. *Appl. Environ. Microbiol.* 63: 2566-2571.

[8] van Dyk, T. K., D. R. Smulski, T. R. Reed, S. Belkin, A. C. Vollmer, and R. A. LaRossa (1995) Responses to toxicants of an *Escherichia coli* strain carrying a *uspA'::lux* genetic fusion and an *E.coli* strain carrying a *grpE'::lux* fusion are similar. *Appl. Environ. Microbiol.* 61: 4124-4127.

[9] Belkin, S., D. R. Smulski, A. C. Vollmer, T. K. Van dyk, and R. A. LaRossa (1996) Oxidative stress detection with *Escherichia coli* harboring a *katG'::lux* fusion. *Appl. Environ. Microbiol.* 62: 2252-2256.

[10] Gil, G. C., R. J. Mitchell, S. T. Chang, and M. B. Gu (2000) A biosensor for the detection of gas toxicity using a recombinant bioluminescent bacterium. *Biosens. Bioelectron.* 15: 23-30.

[11] Marincs, F. and D. W. R. White (1994) Immobilization of *Escherichia coli* expressing the *lux* genes of *Xenorhabdus luminescens*. *Appl. Environ. Microbiol.* 60: 3862-3863.

[12] Cho, C. O. (1991) *Advanced Molecular Biology*. pp. 34-50. The Science of Art Press, Seoul, Korea.

[13] Choi, S. H. and M. B. Gu (2000) Enhancement in the viability and biosensing activity of freeze-dried recombinant bioluminescent bacteria. *Biotechnol. Bioprocess Eng.* 5: 202-206.

[14] Gu, M. B., J. Min, and R. A. LaRossa (1999) Bacterial bioluminescent emission from recombinant *Escherichia coli* harboring a *recA::luxCDABE* fusion. *J. Biochem. Biophys. Methods.* 45: 45-56.

[15] Choi, S. H. and M. B. Gu (1999) A whole cell bioluminescent biosensor for the detection of membrane-damaging toxicity. *Biotechnol. Bioprocess Eng.* 4: 59-62.

[16] van Dyk, T. K., D. R. Smulski, T. R. Reed, S. Belkin, A. C. Vollmer, and R. A. LaRossa (1995) Responses to toxicants of an *Escherichia coli* strain carrying a *uspA'::lux* genetic fusion and an *E.coli* strain carrying a *grpE'::lux* fusion are similar. *Appl. Environ. Microbiol.* 61: 4124-4127.

[Received September 4, 2000; accepted October 30, 2000]