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Characterization of *gltA::luxCDABE* Fusion in *Escherichia coli* as a Toxicity Biosensor

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Abstract The use of *gltA* gene, as a new biomarker for environmental stress biomonitoring, was investigated because of its key position as the first enzyme of the tricarboxylic acid (TCA) cycle. A recombinant bioluminescent *Escherichia coli* strain, EBJM2, was constructed using a plasmid carrying the citrate synthase (*gltA*) promoter transcribing the *Photorhabdus luminescens luxCDABE* genes (*gltA::luxCDABE*). The responses from this strain were studied with five different classes of toxicants: DNA damage chemicals, phenolics, oxidative-stress chemicals, PAHs, and organic solvents. EBJM2 responded strongly to DNA damage chemicals, such as mitomycin C (MMC) and methyl-nitro-nitrosoguanidine (MNNG) and nalidixic acid with the strongest responses. In contrast, tests with several compounds from the other four classes of toxicants gave no significant response. Therefore, EBJM2 was found to be sensitive to DNA damage chemicals.

Keywords: citrate synthase, DNA damage response, bioluminescence bacteria

INTRODUCTION

Citrate synthase (*gltA*), the ubiquitous enzyme which catalyzes the entry of acetyl-CoA carbons into the Krebs cycle, is potentially a key control point for energy production [1,2]. Because of its key position as the first enzyme of the tricarboxylic acid (TCA) cycle, which performs essential functions both in energy generation and carbon assimilation, citrate synthase is assumed to be an important control point for determining the metabolic rate of the cell [2,3]. Previous research regarding the *gltA* gene focused on more specific issues of its expression, such as the activity of citrate synthase in *Escherichia coli* [4-7], genetic studies on the *Arc* system involved [8,9] and *arcA* gene production-related regulation of the citrate synthase gene and its effects on anaerobiosis and carbon supply [3].

Meanwhile, cell-based biosensors have been developed and used widely, owing primarily to their simplicity of use and high specificity [10,11]. Several reporters, *i.e.*, the green fluorescent protein (GFP), *luxAB*, *luxCDABE*, and *luc* genes have been used extensively for stress identification, functional transcription, and bioimaging studies [12]. Although not as sensitive as using GFP fluorescence, the *luxCDABE* genes have several merits since all the proteins needed for the generation of the bioluminescence are encoded *in vivo* and generate visible light, which can be detected easily and quantified within several minutes. Additionally, biosensors that employ bioluminescence offer a simplicity not seen with other reporters, along with dynamic advantages, especially where multiple samples are needs [13,14].

In this study, a strategy that used a cell-based biosensor to investigate of the effects different environmental stresses have on the expression of the citrate synthase gene (*gltA*) from *E. coli* and its potential use for monitoring are described. To screen the effects on citrate synthase production, DNA damage chemicals, phenolics, oxidative stress chemicals, PAHs, and organic solvents were used. Finally, the possibility of environmental stress detection using the *gltA::luxCDABE* was validated by response to different toxicants.

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MATERIALS AND METHODS

Construction of Strain EBJM2

E. coli strain RFM443 (*strR, glaK2, lacΔ74*) [15] was used as the host strain. To construct the recombinant plasmid, pPDGL, the promoter region for citrate synthase (*gltA*) was amplified by PCR using genomic DNA from *E. coli* strain RFM443. The DNA sequences were obtained from the National Center for Biotechnology Information (NCBI), and the primer pairs used to amplify the promoter region are 5′-TCGGGATCCATTGATGACGA-3′ and 5′-CTAGAATTCCCTGGGGACTA-3′, which included *Bam*HI and *Eco*RI restriction sites. The PCR product was 537 bps in length before being digested with *Bam*HI and *Eco*RI (NEB, USA) and ligated into

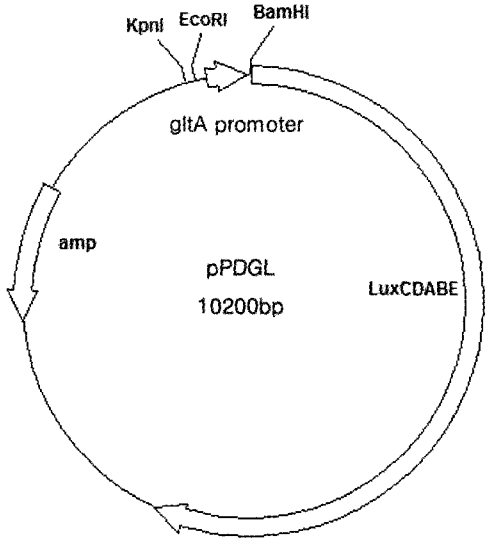

Fig. 1. A schematic diagram of plasmid pPDGL.

pDEW201 [16], a vector that includes a promoter-less *Photorhabdus luminescens luxCDABE* operon. The resultant plasmid, pPDGL (Fig. 1A), was then transformed into *E. coli* strain RFM443 to generate strain EBJM2. To confirm that the construction was correct, both pDEW201 and pPDGL were digested with the same enzymes and the restriction fragments analyzed using gel electrophoresis (data not shown).

Culture Conditions and Chemical Test Protocols

For the experiments, strain EBJM2 was initially grown overnight in 100 mL Luria-Bertani (LB) medium (DIFCO, USA) supplemented with 50 μg/mL ampicillin (Sigma, USA) in 250 mL flasks in a rotary incubator set at 37°C and 250 rpm. Fresh media (100 mL) were then inoculated with 2 mL of this overnight culture and grown until the optical density of the cultures reached 0.08 at 600 nm, measured using a spectrophotometer (Lambda 12, Perkin-Elmer, USA). After reaching this optical density, for flask test 1 mL of chemical solutions were exposed to different concentrations. It was measured optical density and bioluminescence every 10 min for 1 h and every 20 min after that. For 96-well luminometer test, 190 μL of the culture was transferred to the wells of a 96-well plate (Microlite 1, DYNEX Technologies, USA) into which 10 μL of the serially diluted chemical solutions had already been added. The control was exposed to 10 μL of water since this was the solvent used for all of the compounds tested. This plate was then inserted into the 96-well luminometer (Microtitre Plate Reader, MLX, USA) which was set at a temperature of 37°C. The plate shaker was set to high and one second duration before each reading, which was done automatically every 10 min for 5 h. The maximum relative bioluminescence (RBL), which is defined as the maximum ratio between the bioluminescence of the induced cells to that of the control at the same time point, was used to analyze gene expression.

Chemicals

All the chemicals used in this study are listed in Table 1. All, except hydrogen peroxide, were purchased from the Sigma-Aldrich Chemical Company. Hydrogen peroxide was purchased from the Merck Co. (USA). For the DNA damage chemcals, mitomycin C (MMC), methyl-nitro-nitrosoguanidine (MNNG), and nalidixic acid were used. Phenol, 2-chlorophenol, 4-chlorophenol, 2-nitorphenol, and 4-nitorphenol were used for the membrane damage tests. Paraquat, hydrogen peroxide, potassium dichromate, cadmium chloride, and phenazine methosulfate were used for oxidative-stress. Naphthalene and benzo[a] pyrene were used for PAHs test and ethanol, methanol, Isopropanol, acetone, and acetonitrile were tested for organic solvents. The test concentration chose maximum sublethal concentration as middle of the concentration. All chemicals were dissolved in sterile distilled water except PAHs which were used ethanol as a solvent prior to testing.

Data Analysis

All results were taken from three independent samples, from a single culture, conducted simultaneously for error analysis and are shown along with the standard deviation, which is represented by error bars (SigmaPlot (SPSS), Chicago, IL, USA).

RESULTS

Responses of *E. coli* Strain EBJM2 to DNA Damaging Chemicals

To characterize the expression of the *gltA* gene, three well-known mutagens [17] were tested. Fig. 2 shows the time course responses of EBJM2 to mitomycin C (MMC), it clearly shows dose-dependent response as time goes. While Fig. 3A shows the maximum bioluminescent (BL) response seen from each concentration tested. Both clearly show that the BL from EBJM2 increases continuously as greater amounts of mitomycin C (MMC) were added, up to a maximum responsive concentration of 30 ppm. The minimum detectable concentration (MDC) was 1 ppm. With 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), EBJM2 also showed strong responses (Fig. 3B). The maximum relative bioluminescence, about an 80-fold induction, was at a concentration of 5 ppm, with a MDC below 0.5 ppm. Final test chemical nalidixic acid which is bacterial antibiotics, were strongly activated to *gltA* (Fig. 3C).

Responses of *E. coli* Strain EBJM2 to Other Chemicals

In this study, four different phenolic compounds, phenol, 2-chlorophenol, 4-chlorophenol, 2-nitrophenol, and 4-nitrophenol were tested. Choi and Gu [18] found that these four chemicals led to the induction of bioluminescence within another bioluminescent strain DPD2540

Table 1. List of the chemicals tested in this study and their relevant characteristics

Categories	Chemicals	Effect
DNA damage	Mitomycin C	Bioreductive alkylating agent of duplex DNA
	MNNG	DNA alkylation agent
	Nalidixic acid	DNA damaging agent and potent intracellular stress inducer
Phenolics	Phenol	Membrane-damaging microbiocides are transported into the membrane and cause change in the lipid-to-lipid and lipid-to-protein ratios in the membrane.
	2-Chlorophenol	
	4-Chlorophenol	
	2-Nitrophenol	
	4-Nitorphenol	
Oxidative damage	Paraquat	Typical redox-cycling agent
	Hydrogen peroxide	Produced hydroxyl radical
	Potassium dichromate	Transition metal known to generate ROS in a redox-cycling process
	Cadmium chloride	Produces ROS by depleting protein-bound sulfhydryl groups
	Phenazine methosulfate	Produced superoxide radicals
PAHs	Naphthalene	Cause toxicity and inhibit cellular metabolism in *E. coli*
	Benzo[a]pyrene	Cause genotoxic effects in a broad range of prokaryotic and mammalian cell assay
Organic solvent	Methanol	Damage the cell membrane and affect on neurotoxicity to human and animals
	Ethanol	
	Isopropanol	
	Acetone	
	Acetonitrile	

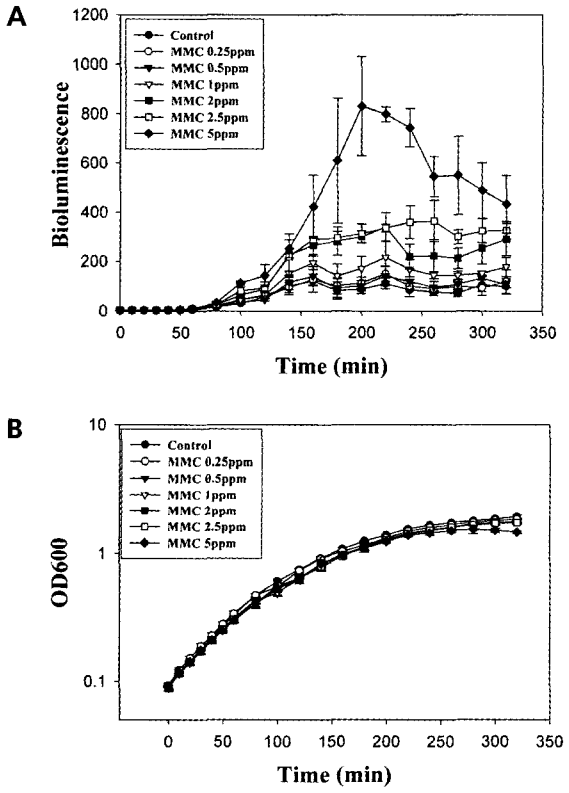

Fig. 2. Time-course profiles showing the growth and specific bioluminescence values from cultures of EBJM2 when exposed to various concentrations of mitomycin C: (A) bioluminescence and (B) growth curve.

which responses to the membrane and protein damaging agents in a dose dependent manner [18]. The results from tests with these phenolics are presented in Fig. 4A. Therefore, the *gltA* gene might not be induced phenolics. Tests with oxidative-stress chemicals were performed to determine if the production of radicals affect *gltA* expression. It is clearly seen that EBJM2 was not responsive to any of these chemicals, indicating that they have no effect on the *gltA* expression (Fig. 4B). Five different organic solvents were tested for study of membrane damage to citrate synthase gene (*gltA*). The result shows in Fig. 4C, effects of organic solvents didn't induced citrate synthase. Finally, test of PAHs, it was expected response on *gltA* causing inhibition of cellular metabolism. However, it also didn't response at all (Fig. 5).

DISCUSSIONS

This study describes the use of the citrate synthase gene (*gltA*) of *E. coli* as a biomarker for environmental whole cell-based biosensor. Because of the importance of citrate synthase within the TCA cycle, we were interested in investigating the effects environmental stresses have on the expression of an energy metabolism related genes. Therefore, in this study, *gltA*, the first enzyme on TCA cycle, was selected to see its response to DNA damaging stresses and its possibility to be used as a whole cell biosensor specific to DNA damage.

Differential adaptive responses in bacteria are caused by the three different mutagens tested in this study, since

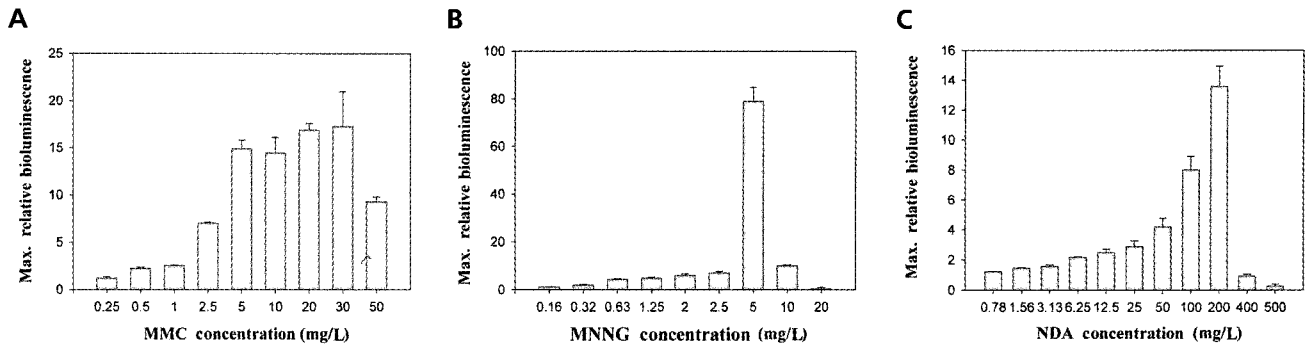

Fig. 3. Maximum relative bioluminescence values seen from cultures of EBJM2 after being exposed to different concentration of mutagens chemicals tested were (A) mitomycin C, (B) 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), and (C) nalidixic acid (NDA).

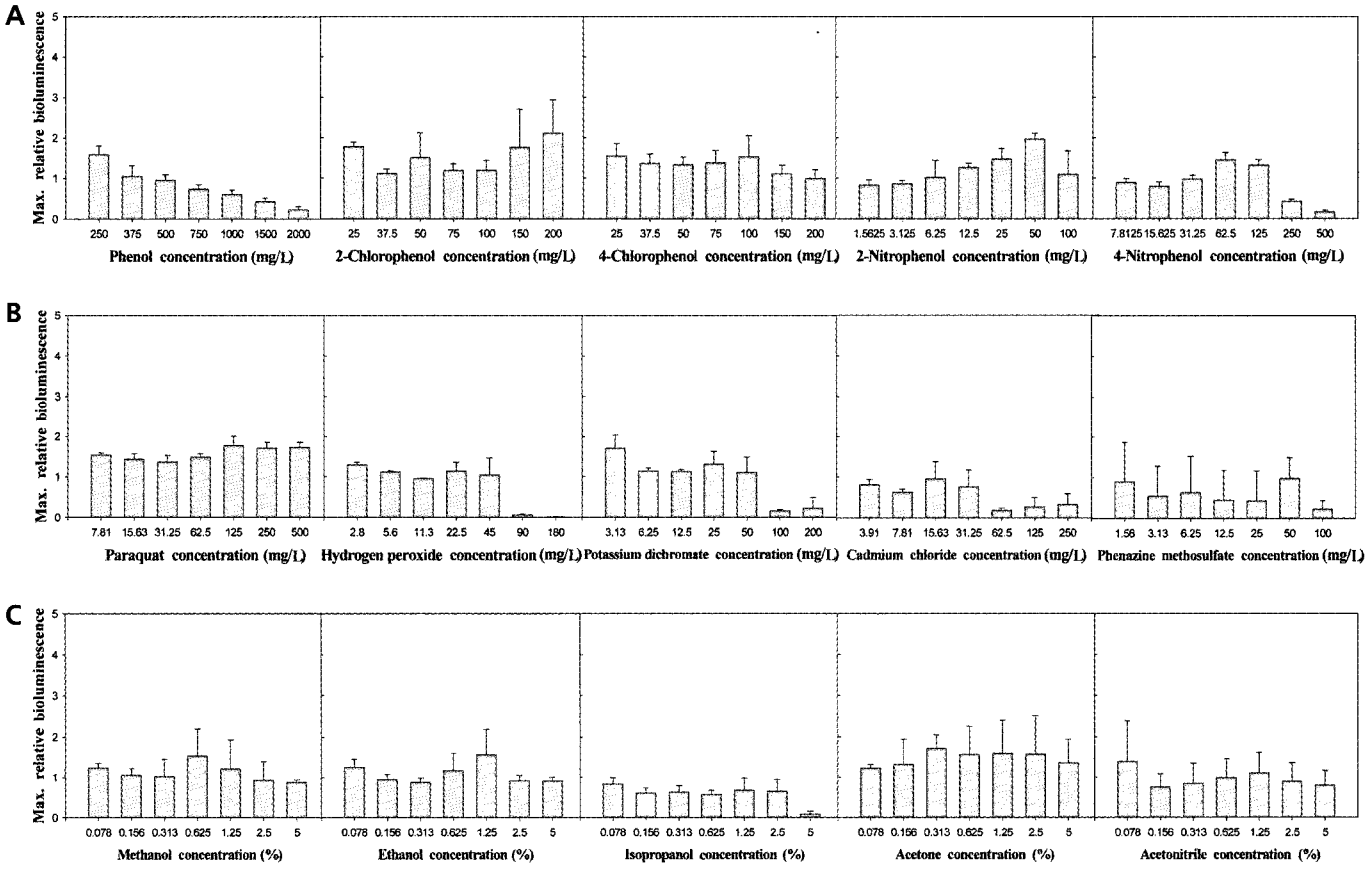

Fig. 4. Maximum relative bioluminescence values seen from cultures of EBJM2 after being exposed to different concentration of phenolics, oxidative stress chemicals, and organic solvents tested were (A) phenolics, (B) oxidative stress chemicals, and (C) organic solvents.

they are by nature an intercalating agent, an alkylation agent, and a hydroxylating agent. Therefore, the repair systems induced are indicative of the type of DNA damage that occurs, *i.e.*, DNA cross-linking and delayed DNA synthesis, alkylation and hydroxylation of DNA [19]. Mitomycin C (MMC) inhibits DNA replication and is known to act as a bioreductive alkylating agent of duplex DNA [20,21] and a DNA cross-link damage agent [22]. Meanwhile, 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) acts through DNA alkylation [23], while nalidixic acid is a DNA-damaging agent and potent intracellular stress inducer.

Although the *gltA* expression increased with some of the mutagens, response mechanism of *gltA* is not clearly explained. If it assumed that it may have chemical specificity, the mutagens that induced *gltA*, MMC, MNNG, and NDA, it is difficult to find similar physical characteristic of these chemicals. On the other hand, it is possible

Table 2. Comparison of EBJM2 (*gltA::*) and DPD2794 (*recA::luxCDABE*) responses to different groups of toxic chemicals

Categories	Chemicals	MDC[a]		MRT[b]	
		EBJM2	DPD2794	EBJM2	DPD2794
DNA damage	Mitomycin C	1.0 ppm	5 ppb	100	230
	MNNG	0.5 ppm	6 ppb	100	230
	Paraquat	ND	100 ppb	–	210
Oxidative damage	Hydrogen peroxide	ND	700 ppb	–	210
	Cadmium chloride	ND	183 ppb	–	210
PAHs	Naphthalene	ND	0.05 ppb	–	210
	Benzo[a]pyrene	ND	0.013 ppb	–	210

[a]MDC: Minimum Detectable Concentration (ND: Not detected).
[b]MRT: Maximum Response Time (min).

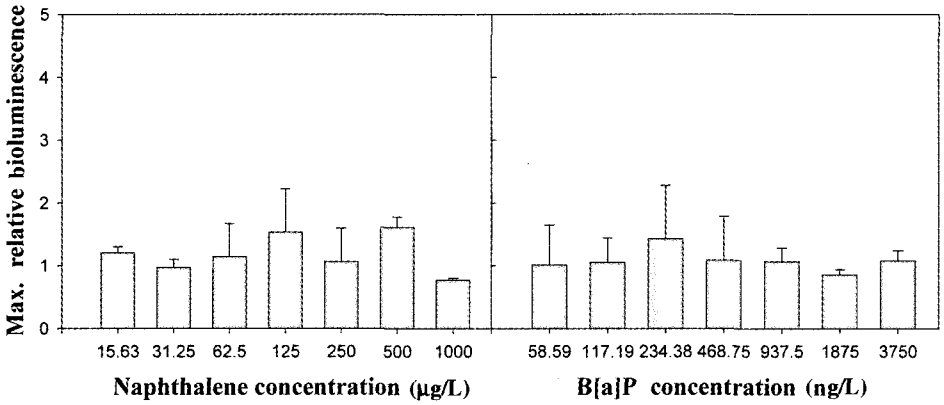

Fig. 5. Maximum relative bioluminescence values seen from cultures of EBJM2 after being exposed to different concentration of naphthalene and benzo[a]pyrene.

to suppose that DNA damage mechanism in bacteria. In bacteria, DNA damage is caused by many kinds of mutagens. Accordingly, bacteria have developed the SOS response to repair its damaged DNA. Repair of DNA damage is essential because DNA must serve as the template for transcription and reproduction. Repair of some types of damage, such as DNA modification, is simply conducted and returns the DNA directly to its original state [24]. For instance, O^6-methylguanine-DNA-methyltransferase (MGMT), under the control of the Ada regulon in *E. coli*, transfers a methyl group from the modified base O^6-methylguanine to itself [25,26]. Therefore, even though MNNG induces an SOS response, the production of O^6-methylguanine by MNNG is not necessarily repaired by the SOS mechanisms. Rather, DNA damage brought on by MNNG is repaired and the bacteria recover promptly because the methyl group is simply removed by MGMT [20,27]. However, it's still unclear *gltA* mechanism of response to DNA damage agents. The last possibility of those mechanism is gltA known genetic study based on literature survey. The expression of *gltA* to DNA damage chemicals were not only related to inhibition of *gltA* according to *arcA* (transcriptional regulator) but also reduced purine nu-cleotides that are used in energy generation via the electron transport-linked phosphorylation reaction [3]. In Gunsalus *et al.* [3] paper, they represented that in the ΔarcA strain, the level of *gltA-lacZ* expression was elevated 2.5- fold compared with that in the wild-type strain during aerobic cell growth. Likewise, the effects of DNA damage chemicals were possibly delayed transcription. And then, the activation of transcriptional regulator was delayed. Therefore, the expression of *gltA* can be increased. *gltA* related to purine nucleotide biosynthesis function also can be induced with mutagens.

The responses from both *gltA* and *recA* biosensors have been compared in terms of the Minimum Detectable C oncentration (MDC), the Maximum Response Time (MRT), and the list of chemicals responsed. The maximum response time (MRT) of *gltA* (in this study) to DNA damaging chemicals was faster than *recA*, but the MDCs of *gltA* for DNA damaging chemicals were slightly higher than that of *recA* (see Table 2). In addition, *gltA* was found to be very specific to only direct DNA damaging chemicals, while *recA* responded to both direct and indirect DNA damaging chemicals significantly.

Finally, the relationship between *gltA* and mutagens still are not clear. However, the DNA damage affects various cellular biosynthesis [19]. Therefore, there are a lot of biomarkers for DNA damage, not only genes which

is related SOS response but also genes like *gltA* which is related energy metabolism.

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