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Response of Bioluminescent Bacteria to Sixteen Azo Dyes

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Abstract Recombinant bioluminescent bacteria were used to monitor and classify the to xicity of azo dyes. Two constitutive bioluminescent bacteria, *Photobacterium phosphoreum* and *Escherichia coli, E. coli* GC2 (*lac::luxCDABE*), were used to detect the cellular toxicity of the azo dyes. In addition, four stress-inducible bioluminescent *E. coli*, DPD2794 (*recA::luxCDABE*), a DNA damage sensitive strain; DPD2540 (*fabA::luxCDABE*), a membrane damage sensitive strain; DPD2511 (*katG::luxCDABE*), an oxidative damage sensitive strain; and TV1061 (*grpE::luxCDABE*), a protein damage sensitive strain, were used to provide information about the type of toxicity caused by crystal violet, the most toxic dye of the 16 azo dyes tested. These results suggest that azo dyes result in serious cellular toxicity in bacteria, and that toxicity monitoring and classific a-tion of some azo dyes, in the field, may be possible using these recombinant bi oluminescent bacteria.

Keywords: Azo dyes, cellular toxic effect, stress responses, recombinant bioluminescent bacteria

INTRODUCTION

Azo dyes, are widely used in textiles, printed goods, cosmetics, drugs, food coloring and other consumer goods. They are also used extensively in laboratories for either biological staining or as pH indicators. The extent of their use within a society is related to the degree of industrialization [1,2], and it is possible they may cause cancer. Azo dyes have essentially been regarded as safe [2]. In fact, many azo dyes have been shown to be genotoxic in short-term genotoxicity tests and carcinogenic in tests on laboratories animals and microorganisms [1,2].

Crystal violet is known to be toxic to microorganisms, and has been demonstrated to bind to DNA [3,4]. In mammalian cell cultures, gentian violet is a strong mutagen, while prolonged exposure in the drinking water of mice led to toxic effects in their bone-marrow cells, which was evident from a decrease in the mitotic activity [3,4].

However, very little data has been published on the genetic and cellular toxicities of most of these dyes. Many rapid and sensitive toxicity assay methods have been developed by necessity. Researchers in Germany, France and the Netherlands, use the higher organisms, such as fish or daphnia, to detect the toxic substances discharged into their environments [5], but these methods were reported to be less sensitive to hazard materials.

Bioluminescent bacteria have been highlighted as toxicity biosensors because of their rapid response, low cost and improved reproducibility [6-8]. Typically, two types of bioluminescent bacteria have been utilized. The first types are strains of constitutive bioluminescent bacteria, such as *Photobacterium phosphoreum* and *Vibrio fischeri*. With these strains, the toxicity of chemicals can be evaluated through their reduced bioluminescent intensity in response when the cells experience a toxic or lethal condition. The other types of bioluminescent strain are the stress-inducible bioluminescent bacteria, containing a stress promoter, fused to the *luxCDABE* gene operon, which acts as a transcription reporter from these promoters [6-8]. A very wide range of stress promoters are known to exist in prokaryotic cells and fusion of these promoters with the *luxCDABE* results in cellular biosensing strains that emit light when stressed.

In this study, several recombinant bioluminescent bacteria were used to monitor and classify the toxicity of azo dyes, as they have been shown to be very reliable and stable biosensing tools [9]. Of these, two constitutive bioluminescent bacteria, *P. phosphoreum* and *Escherichia coli* GC2 (*lac::luxCDABE*) [10], were used to detect the cellular toxicity of the dyes, while four stress-inducible bioluminescent bacteria were used to provide information about the type of toxicity caused, depending on their toxic action. These bacteria were *E. coli* DPD2794 (*recA::luxCDABE*) [11], a DNA-damage sensitive strain, DPD2540 (*fabA::luxCDABE*) [6], a membrane-damage sensitive strain, DPD2511 (*katG::luxCDABE*) [12], an oxidative-damage sensitive strain and TV1061 (*grpE::lux CDABE*) [13], a protein-damage sensitive strain.

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MATERIALS AND METHODS

Strains, Media, and Freeze-drying Methods

The recombinant inducible bioluminescent strains, DPD2794, DPD2540, DPD2511 and TV1061, all were constructed with *E. coli* strain RFM443 (*strR, galK2, lacΔ74*) as a host, but harboring different plasmids [6, 11-13]. These recombinant plasmids contain a transcriptional fusion of the *E. coli* stress promoter to the *Vibrio fischeri luxCDABE* operon. Therefore, the cells harboring these plasmids will produce bioluminescence when the stress promoters are stimulated by toxic chemicals. All of these plasmids confer resistance to ampicillin and kanamycin.

Another recombinant bacterium, *E. coli* GC2, which also has the host, *E. coli* strain RFM443 was also used, but was transformed with pLITE201 that has the *lac* promoter fused to the genes from *P. luminescens,* which confers the resistance to ampicillin [10]. This strain produces bioluminescence constitutively.

Luria-Bertani (LB) medium, supplemented with 25 mg/mL kanamycin monosulfate, was used for all strains, with the exception of *E. coli* GC2, which was supplemented with 100 μg /mL ampicillin. The media pH was adjusted to 7 prior to autoclaving. Seed cultures (100 mL) were grown in 250-mL culture flasks in a shaking incubator, set at 250 rpm., and a temperature of 30°C, for the inducible strains, and 37°C for the *E. coli* GC2, until they reached an O.D. of 0.8, measured using UV/Vis spectrophotometer at 600 nm. For sample preparation, a new 100-mL flask, containing LB, with kanamycin or ampicillin, was inoculated with 2 mL of the seed culture. For the *P. phosphoreum*, the growth conditions of Ribo and Kaiser [14] were used. The sub-cultured *E. coli* GC2 and *P. phosphoreum* cells were grown to the mid-exponential phase, *i.e.*, an optical density of 0.8 at 600 nm, centrifuged at 2,600 g for 30 min (Ultra 21 K, Hanil Co., Korea), and the pellets resuspended in 10 mL of the LB medium and mixed with 10 mL of 24 %(w/v) sucrose. Aliquots (1 mL) were added to sterile 1.5 mL glass vials, frozen at -70°C for 2 days, followed by freeze-drying at -50°C at less than 20 milli-torrs for a further 2 days [9,15].

For the flask experiments, the stress-inducible bioluminescent strains were grown in Luria-Bertani (LB) medium (Difco) with 25 mg/L kanamycin monosulfate (Sigma Co.), at 30°C and an initial pH of 7.0, with aeration provided by agitation on a rotary shaker at 250 rpm. A flask containing 100 mL of sterile medium was inoculated with 2 mL of inoculum from an overnight culture. When the optical density, at 600 nm (OD_{600}), reached 0.08, azo dayes were added to the culture media, respectively.

Chemical Treatment

All the azo dyes, *i.e.*, crystal violet, basic violet 1 and 2, direct blue 71, congo red, thiazol yellow G, orange II, acid green 25, reactive black 5 and 15, disperse yellow 3 and disperse orange 11, used in this study were purchased from the Sigma & Aldrich Chem. Co. The domestic production dyes, *i.e.*, navy blue, yellow, brown, blue and black, were obtained from Company A. Stock solutions of all 16 dyes were prepared in ethanol, and diluted into the media to a final ethanol concentration of below 0.1%, a level that resulted in no significant stress response from the bioluminescent bacteria. The bioluminescence of the cells in the sample tubes was monitored at set times using a luminometer (Turner Designs, TD-20e). The maximum BL ratio was defined as the ratio of the maximum BL induced by the cells, to the maximum BL of the control cells exposed to ethanol alone. However in the cases of the *E. coli* GC2 and *P. phosphoreum*, the constitutive strains, the relative bioluminescence (RBL) was defined as the ratio the BL induced by the cells to the BL of the control cells 15 min post treatment. Freeze-dried *E. coli* GC2 and *P. phosphoreum* showed a significant reduction in their bioluminescence for the first 15 min and then gradually decreased. Therefore, a time of 15 min was selected for measuring the EC20 value.

RESULTS AND DISCUSSION

Toxicity Response of the Bioluminescent Bacteria to Sixteen Different Azo Dyes

Two constitutively bioluminescent bacteria were used to detect the cellular toxicity caused by 16 different azo dyes. These bacteria, the recombinant strain *E. coli* GC2, harboring a recombinant plasmid-borne fusion of the *lac* promoter and *luxCDABE* operon from *Photorhabdus luminescence*, and *Photobacterium phosphoreum,* emit high levels of bioluminescence under non-toxic conditions, but these decrease in intensity under toxic conditions. The toxicity was measured using the freeze-dried *P. phosphoreum* and *E. coli* GC2's bioluminescence changes, and correlating these with the bioluminescence of the control. The concentration resulting in a 20% reduction after 15 min (EC_{20}) was utilized to evaluate the toxicities of the dyes tested in these experiments, and were similar to those reported elsewhere that showed that some dyes had a limited toxicity toward microorganisms, whereas others were highly toxic [1,2].

As shown in Fig. 1, crystal violet (basic violet 3) causes significant toxicity toward the bacterial cells. The recombinant strain, *E. coli* GC2, showed a dose dependent decrease in its bioluminescence response, clearly demonstrating that crystal violet is toxic to bacteria (Fig. 1a). The effective concentration (EC_{20}), which is defined as the concentration of a chemical that produces a 20% decrease in the bioluminescence 15 min after treatment, was 0.1 mg/L for crystal violet. In addition, tests with *P. phosphoreum* gave similar results. However, the EC_{20} for crystal violet obtained with *P. phosphoreum* was higher than that of the *E. coli* GC2, indicating that the recombinant bioluminescent bacterium is more sensitive than the naturally bioluminescent *P. phosphoreum*.

As shown in Table 1, all 16 azo dyes caused serious

Table 1. The effective concentration of the 16 different dyes using freeze-dried bioluminescent bacteria

Dye		EC20 (mg/L)	
		E. coli GC2 (*lac::luxCDABE*)	*Photobacterium phosphoreum*
Basic dyes	Crystal violet	0.1	0.30
	Basic violet 1	0.1	0.25
	Basic violet 2	1	1
Direct dyes	Direct Blue 71	25	30
	Congo red	10	10
	Thiazol yellow G	10	30
Acid dyes	Orange II	50	50
	Acid green 25	100	100
Reactive dyes	Reactive Black 5	300	450
	Reactive Blue 15	400	500
Disperse dyes	Disperse yellow 3	30	50
	Disperse orange 11	30	50
Domestic dyes	Navy blue	10	30
	Yellow brown	30	50
	Blue	30	50
	Black	30	50

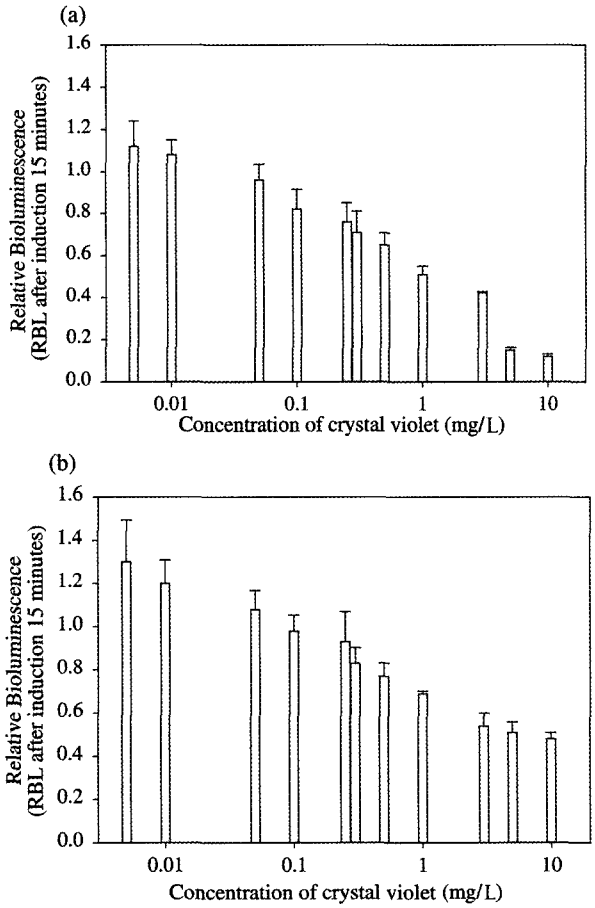

Fig. 1. Dose-dependent response of the two constitutive bioluminescent strains to crystal violet. The RBL is the relative bioluminescence value at 15 min post treatment. Response of (a) *E. coli* GC2 and (b) *Photobacterium phosphoreum*.

cellular toxicity within the two bioluminescent bacteria. These dyes are classified into 6 different groups, according to the staining methods used in their industrial applications [1,2]. Within the basic dyes group, crystal violet was the most toxic, as well as the most toxic of all 16 azo dyes, based on its EC_{20} values obtained with *E. coli* GC2 and *P. phosphoreum*. It was found that the level of cellular toxicity, due to the addition of the azo dye groups, decreased in the following order: basic dyes, direct dyes, disperse dyes, domestic dyes, acid dyes and reactive dyes. The toxicity caused by each group was found to be dependent on their water solubility [2]. All the groups, with the exception of the basic dyes, are also classified as water-soluble, while only the basic dyes are classed as lipid soluble [2]. Moller and Wallin reported that the water insolubility of these dyes is the most important factor in the detection of their toxicological properties [2]. This suggests that the solubility of azo dyes is an important factor in their uptake and bioavailability. In addition, the basic dyes were more toxic than the other groups due to their higher adsorption into cellular membranes due to their hydrophobicity.

Determination of Specific Stresses Caused by Crystal Violet

Crystal violet, the most toxic among the 16 azo dyes tested, has long been known to be mutagenic to organisms. Therefore, in this study, four stress-inducible bioluminescent bacteria; DPD2794 (*recA::luxCDABE*), a DNA-damage sensitive strain; DPD2540 (*fabA::luxCDABE*), a membrane-damage sensitive strain; DPD2511 (*katG::luxCDABE*), an oxidative-damage sensitive strain and TV1061 (*grpE::luxCDABE*), a protein damage-sensitive strain, were used to provide information about the type of toxicity caused by crystal violet.

The DPD2794, DPD2540 and TV1061 all showed

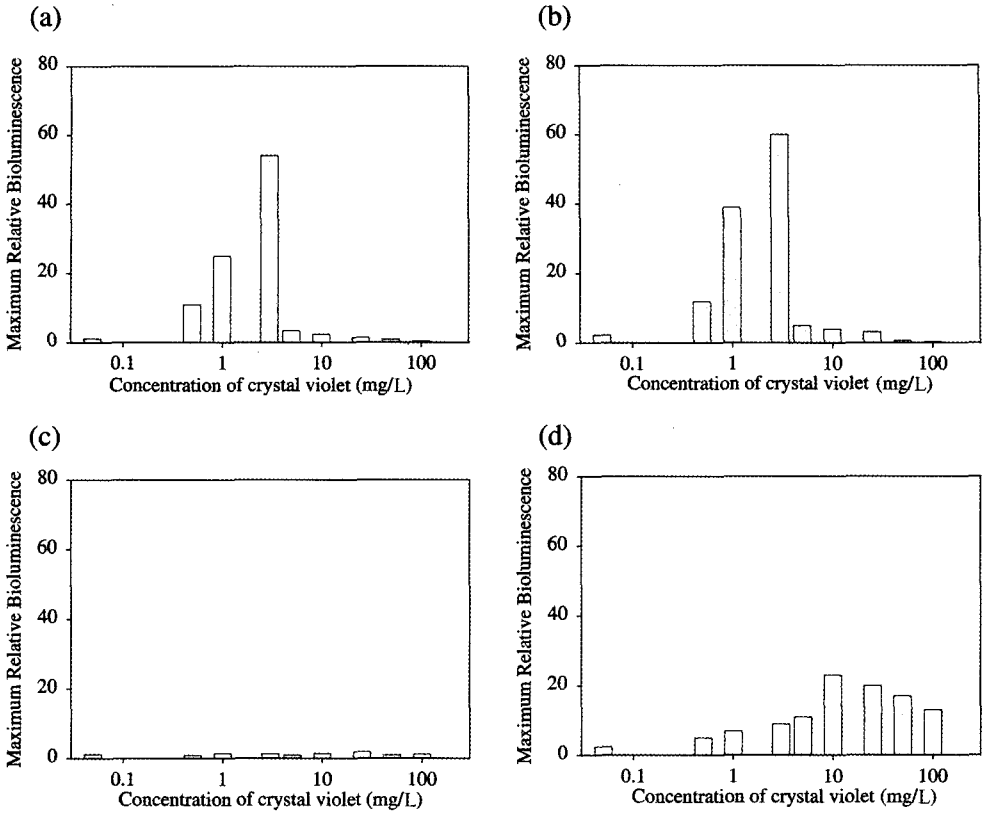

Fig. 2. Dose-dependent response of the four inducible bioluminescent strains to crystal violet. The RBL is the maximum relative bioluminescence value during the experiments. Response of (a) DPD2794, (b) DPD2540, (c) DPD2511, and (d) TV1061.

significant bioluminescent responses to several concentrations of crystal violet (Fig. 2a, b and d). In other words, a dose-dependent bioluminescent response was obtained with DPD2794, DPD2540 and TV1061 with the maximum bioluminescent response seen at a concentration of 2.5 mg/L for DPD2794 and DPD2540, and 10 mg/L for TV1061, are defined as the critical concentration for each strain. Above this concentration, depending upon the strain, the induction of bioluminescence was significantly hindered due to inhibition of cellular metabolism due to crystal violet's toxicity. Conversely, DPD2511, which is very responsive to hydroxyl radicals, showed no response to its addition (Fig. 2c). Comparing the response ratios of the four inducible strains, the major damage caused by the crystal violet was to the membrane, indicating that its adsorption into the cellular membrane cause serious damage to the bacterial membrane.

CONCLUSION

Various azo dyes were tested using *E. coli* GC2 (*lac::luxCDABE*) and a wild type bioluminescent bacterium, *Photobacterium phosphoreum* to investigate the cellular stresses and the toxicities induced. It was found that the cellular toxicity caused by the azo dyes could be detected via a decrease in the levels of bioluminescence induced in the constitutive bioluminescent bacteria, *E. coli* GC2 and *P. phosphoreum,* and the levels toxicity experienced due to their addition, based on their grouping, decreased in the following order: basic dyes, direct dyes, disperse dyes, domestic dyes, acid dyes and reactive dyes. It was additionally found that crystal violet caused cellular damage, which resulted in damage to the DNA, membranes and proteins, and that these modes of toxicity are determinable through differences in the response kinetics of the four different inducible bioluminescent bacteria. The results of this study clearly demonstrate the usefulness of these bioluminescent bacteria for specific and quantitative analysis, and the monitoring of toxic effects and cellular toxicities of azo dyes.

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