

Full Research Paper

Construction of a *nrdA::luxCDABE* Fusion and Its Use in *Escherichia coli* as a DNA Damage Biosensor

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Abstract: The promoter of *nrdA* gene which is related with DNA synthesis was used to construct a DNA damage sensitive biosensor. A recombinant bioluminescent *E. coli* strain, BBT*NrdA*, harboring a plasmid with the *nrdA* promoter fused to the *luxCDABE* operon, was successfully constructed. Its response to various chemicals including genotoxic chemicals substantiates it as a DNA damage biosensor. In characterization, three different classes of toxicants were used: DNA damaging chemicals, oxidative stress chemicals, and phenolics. BBT*NrdA* only responded strongly to DNA damaging chemicals, such as nalidixic acid (NDA), mitomycin C (MMC), 1-methyl-1-nitroso-N-methylguanidine (MNNG), and 4-nitroquinoline N-oxide (4-NQO). In contrast, there were no responses from the oxidative stress chemicals and phenolics, except from hydrogen peroxide (H₂O₂) which is known to cause DNA damage indirectly. Therefore, the results of the study demonstrate that BBT*NrdA* can be used as a DNA damage biosensor.

Keywords: ribonucleoside diphosphate reductase, DNA damage response, bioluminescence bacteria, biosensor

1. Introduction

Due to environmental pollution, specific and sensitive detection methods are in need for environmental contaminants. To investigate the impact of toxic agents on organisms, biological test systems have been developed and applied to in various means. These bioassays are thought as good

genotoxicity assessment, as results, rapid and sensitive detection methods for mutagenic condition are in urgent demand for the screening of an overwhelming number of existing substances that are potential DNA-damaging agents. The most widely used indicator, the Ames test is disadvantageous due to the long operation time needed. To overcome that, SOS-dependent bacterial test systems is used for DNA-damaging agents, and their response for those chemicals is known as SOS response. The *umu*-test is the system induced by that SOS response. That employs a fusion between the *umuCD* promoter and *lacZ* gene from *Escherichia coli*. But the *umu*-test also has a weak point in that it has a low sensitivity [1].

In response to these problems, other recombinant bacterial sensors were developed. Several of these biosensors have been characterized and widely used, for instance, in specific stress identification and bio-imaging [2-3]. Such sensors contained a variety of reporter genes, such as *luxCDABE* [4-5], the green fluorescent protein (GFP) [6], *luxAB* [7] and *luc* [8]. Among these, the *luxCDABE* genes can be used to generate bioluminescence *in vivo* without the need for an extraneous addition of substrate. There have been many reports describing the advantages of *luxCDABE*, such as its simplicity of analysis and applicability in detecting multiple samples [9-10]. Furthermore, the reaction time needed to generate the bioluminescent responses is very short. Using this procedure, the *recA*, *sula*, *umuCD* and *recN* promoters have previously been fused with the *luxCDABE* genes and the strains carrying these fusions have been used widely in toxicity assays [11-14]. Furthermore, the use of such fusions can be used to study the functionality of a given promoter. Consequently, the *nrda* gene was selected for further study as a genotoxic biomarker in part due to its functioning in DNA synthesis but also since it is not regulated by the SOS response in *E. coli*.

The *nrda* gene is well known and encodes for the ribonucleoside diphosphate reductase protein, which is involved in DNA synthesis in *Escherichia coli*. The ribonucleoside diphosphate reductase is composed of two subunits, referred to as B1 and B2 [15]. Ribonucleoside diphosphate reductase converts ribonucleotides to deoxyribonucleotides and, in this process, oxidizes the thiol group [16]. As well, the expression of the *nrda* gene is strongly affected by DNA damage, such as after an exposure to UV light, but is not dependent on LexA [17]. To date, many research groups have studied this gene and its protein and have deduced its function, structure and mechanism, but all of these studies only focused on the molecular aspects of this gene and its protein [18-21].

Therefore, in this study we developed BBT*Nrda*, a cell-based genotoxicity sensor which is specific in its responses to genotoxins. This *E. coli* strain harbors a plasmid with the *nrda* promoter fused to the *luxCDABE* operon. Characterization of this strain was performed using exposures to DNA damaging chemicals, oxidative stress-inducing chemicals and phenolics. The results clearly show that BBT*Nrda* strongly responded to only genotoxic compounds.

2. Results and Discussion

2.1. Response of strain BBT*Nrda* to DNA damaging chemicals

Strain BBT*Nrda* was constructed by fusing the *nrda* promoter with the *luxCDABE* operon in plasmid pDEW201 and transforming this plasmid into *E. coli* strain RFM443. This proposed sensor is shown in Figure 1.

To characterize strain *BBTNrdA*, we used four different chemicals that are known DNA mutagens [22], *i.e.*, nalidixic acid (NDA), mitomycin C (MMC), 1-methyl-1-nitroso-N-methylguanidine (MNNG), and 4-nitroquinoline N-oxide (4-NQO). Figure 2 shows the responses from this strain for each chemical. Initially, strain *BBTNrdA* was characterized with NDA and the maximum responses were seen at a concentration of 10 ppm (Figure 2 A), while the minimum detectable concentration (MDC) was 2.5 ppm (Table 1). The strong induction in the RBL (~65-fold) of this strain clearly shows that this strain was responsive to NDA, which is known to inhibit the synthesis of DNA [23]. Since the *nrdA* genes encode for a protein specifically involved in DNA synthesis, the responses of strain *BBTNrdA* to NDA are not surprising.

Likewise, we tested MMC, and found that strain responded very strongly and in a dose-dependent manner (Figure 2 B), with a MRC and MDC of 80 and 0.3125 ppm (Table 1), respectively. In contrast with NDA, MMC crosslinks the two strands of DNA and, as a result, induces apoptosis and arrests the cell cycle in eukaryotic cells [24-26]. From the findings presented here, it is clear that the damage caused by MMC also leads to an induction of the *nrdA* gene.

Lastly, strain *BBTNrdA* was exposed to 1-methyl-1-nitroso-N-methylguanidine (MNNG) and 4-nitroquinoline N-oxide (4-NQO). The results for these responses are shown in Figure 2 C and D, respectively. As with MMC and NDA, the bioluminescence of strain *BBTNrdA* was strongly induced by these compounds and in a dose-dependent manner. MNNG causes alkylation of the cellular proteins and DNA, leading to errors being incorporated in the DNA during replication and repair [27-28]. 4-NQO is also DNA damaging chemical which affects DNA in various ways [29] and its mechanism is similar to the damage caused by exposure to UV [8, 30]. *BBTNrdA* showed an increase in its bioluminescent emission.

Table 1. Summary of the responses from strain *BBTNrdA* with each compound tested

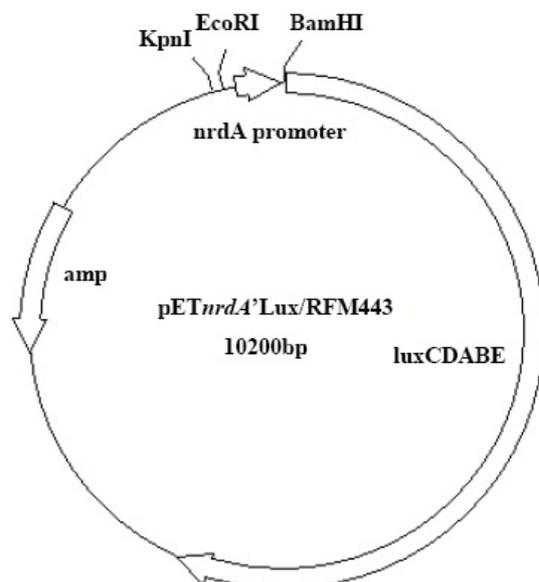
Categories	Chemicals	MDC (ppm)	MRC (ppm)
DNA damage	nalidixic acid	2.5	10
	mitomycin C	0.3125	80
	1-methyl-1-nitroso-N-methylguanidine	0.1563	80
	4-nitroquinoline N-oxide	2.5	20
Oxidative damage	paraquat	ND	ND
	cadmium chloride	ND	ND
	hydrogen peroxide	50	100
Phenol compounds	phenol	ND	ND
	2-chlorophenol	ND	ND
	2,4-dichlorophenol	ND	ND
	2,4,5-trichlorophenol	ND	ND

^aMDC : Minimum Detectable Concentration (ND: Not Detected)

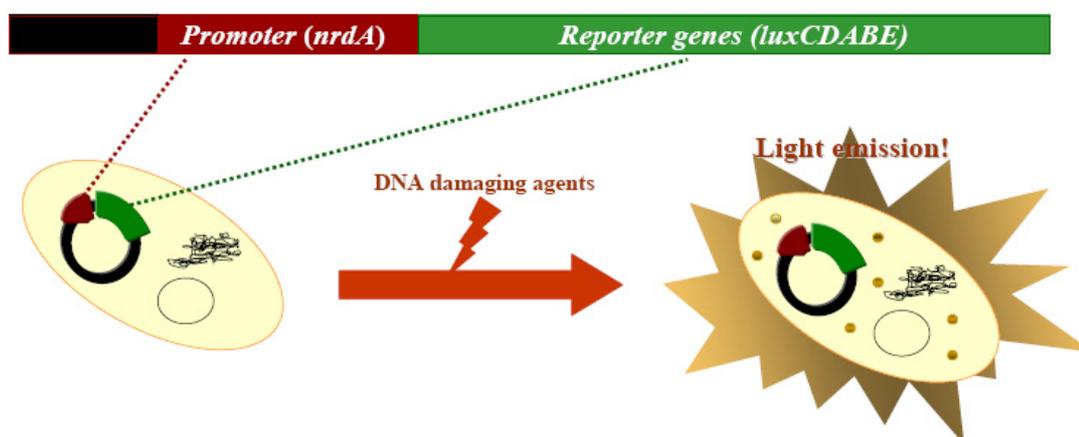
^bMRC : Maximum Response Concentration (ND: Not Detected)

Figure 1. Schematic of the proposed biosensor in this study A) plasmid map of BBT*NrdA* and B) principle of its responses to DNA damaging agents.

A)



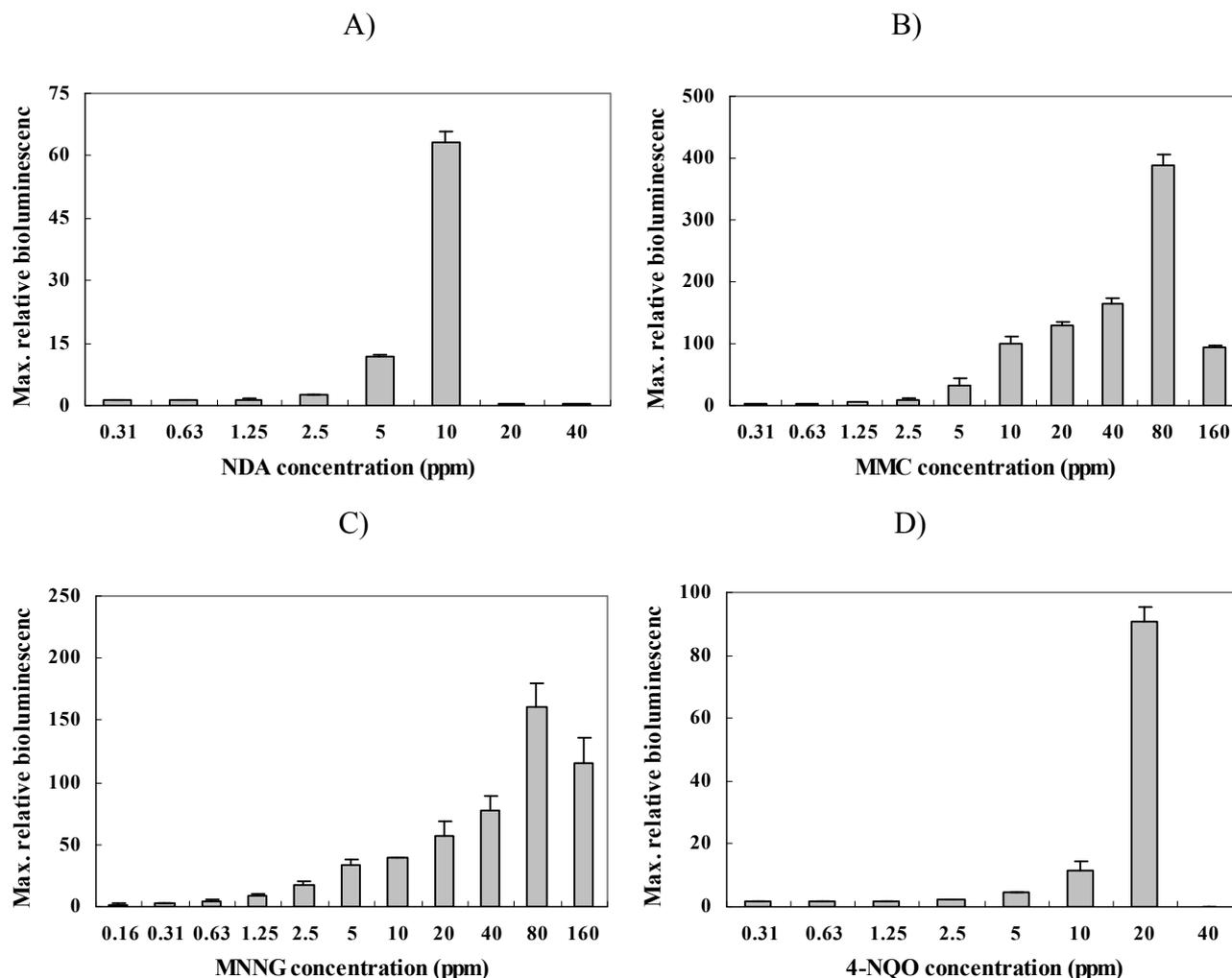
B)



2.2. Response of strain BBT*NrdA* to other chemicals

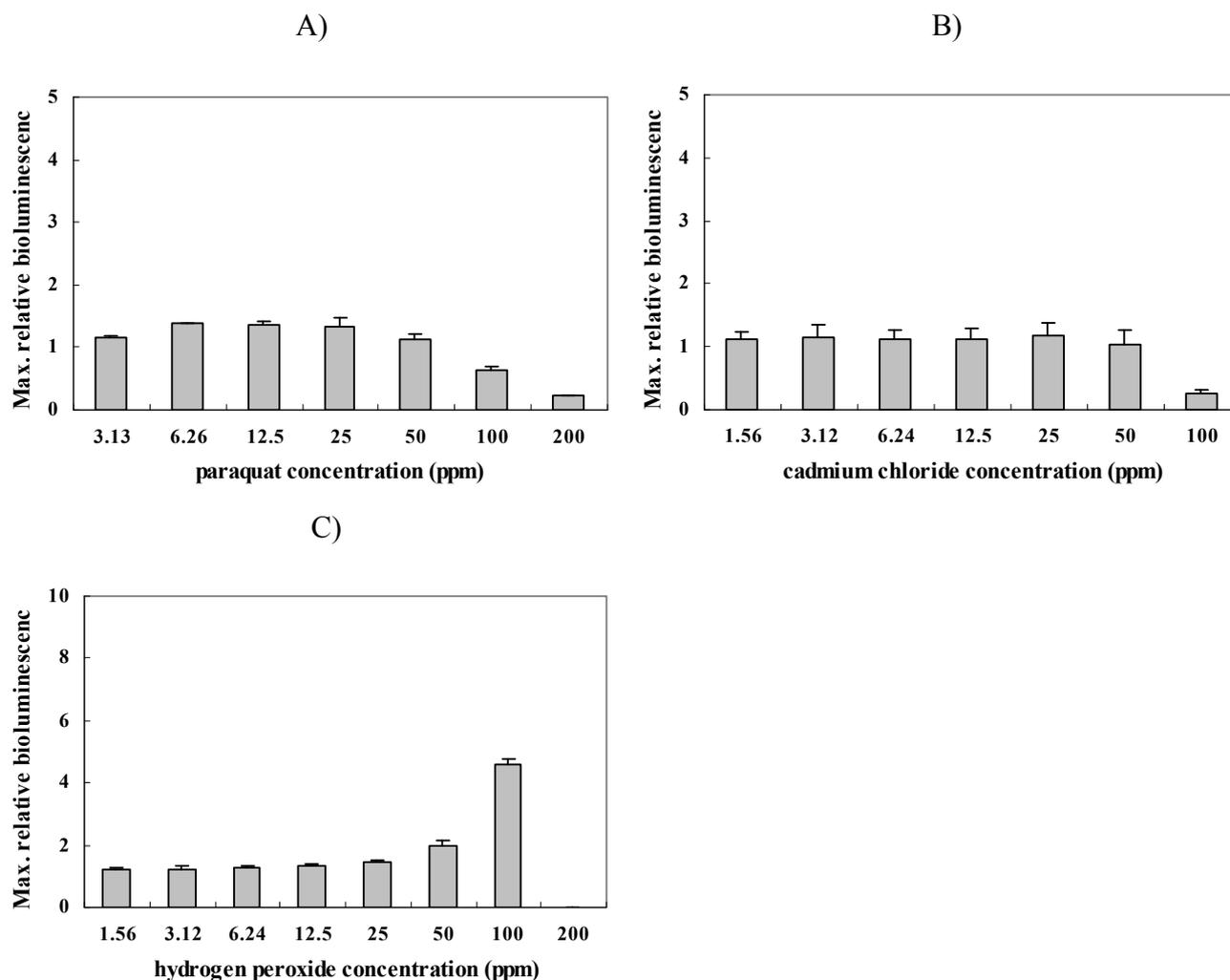
We performed toxicity tests using oxidative stress-inducing chemicals to characterize the *nrdA* gene expression level when *E. coli* is exposed to oxidative radicals [31-32]. For this, paraquat, cadmium chloride and hydrogen peroxide (H₂O₂) were used. Strain BBT*NrdA* gave no response when exposed to paraquat or cadmium chloride (Figure 3 A and B), but a mild response was seen with a hydrogen peroxide (H₂O₂) exposure (Figure 2 C). It is not surprising that the *nrdA::luxCDABE* responded to H₂O₂ since some reports showed that strain DPD2794, another DNA damage-sensitive biosensor, also responded to this compound [25]. Of course, the response mechanisms of each gene for a given chemical are different, but from our results it is clear is that H₂O₂ can lead to DNA damage.

Figure 2. Maximum relative luminescence values seen from strain BBT*NrdA* after being exposed to different concentration of (A) nalidixic acid (NDA), (B) mitomycin C (MMC), (C) 1-methyl-1-nitroso-N-methylguanidine (MNNG), and (D) 4-nitroquinoline N-oxide (4-NQO).



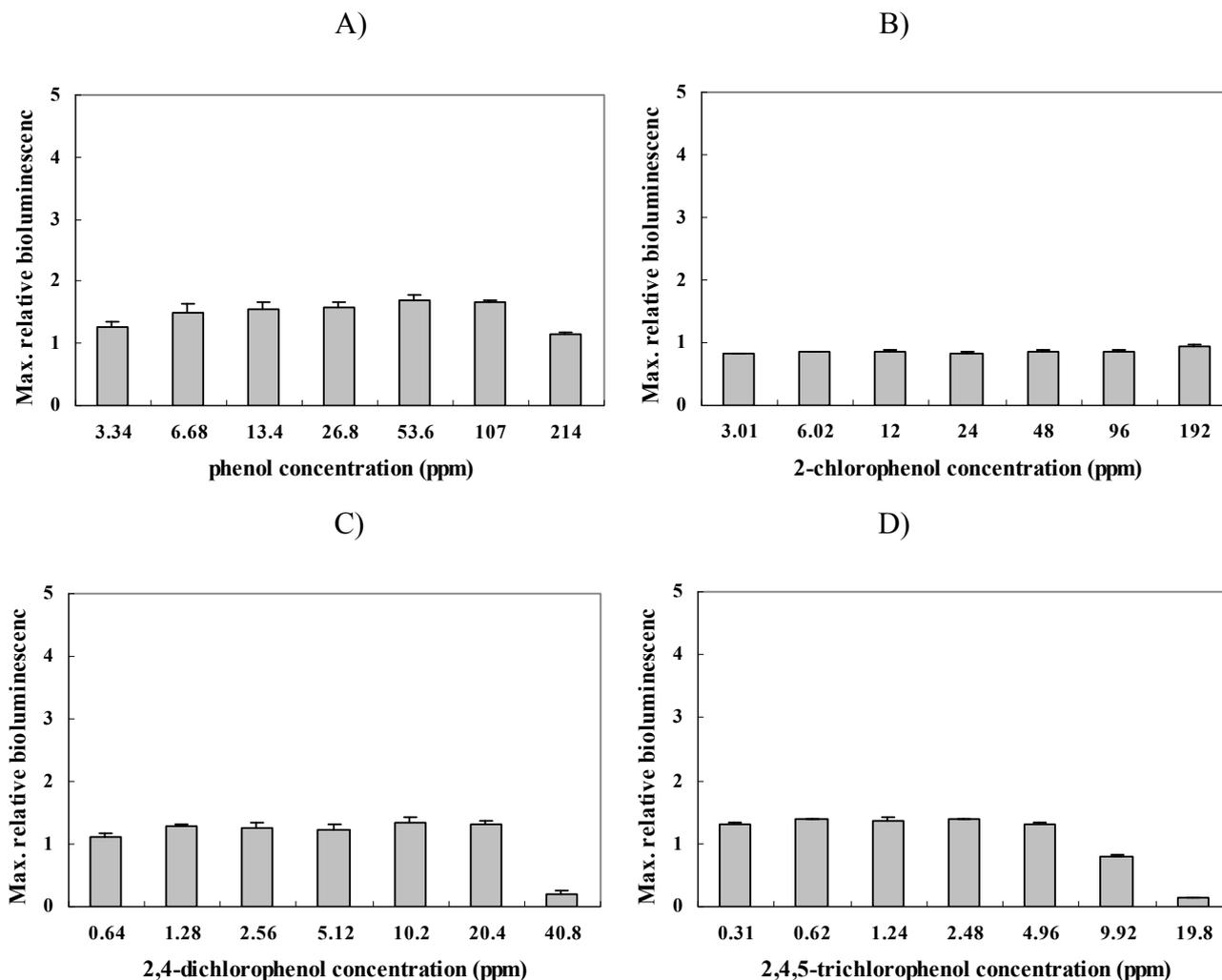
Furthermore, additional experiments were conducted using membrane-damaging chemicals, *i.e.*, phenol, 2-chlorophenol (2-CP), 2,4-dichlorophenol (2,4-DCP), and 2,4,5-trichlorophenol (2,4,5-TCP) [34]. Figure 4 shows that there was no response to these chemicals. This was expected since these compounds should have no effect on the structure or replication of the cellular DNA. Taken together with the results from the oxidative compounds, these results demonstrate that the *nrdA* gene expression level is not induced by membrane damaging or oxidative toxicants, but only by DNA damaging compounds.

Figure 3. Maximum relative luminescence values seen from strain BBT*NrdA* after being exposed to different concentrations of (A) paraquat, (B) cadmium chloride and (C) hydrogen peroxide.



Organisms often encounter abnormal and potentially harmful environments. In response to such conditions, bacterial cells alter their gene expression patterns and, depending on the stress experienced, the production level of specific stress proteins increases to help defend and repair the cells. Consequently, recombinant bioluminescent bacteria are useful tools for examining for toxicants. Depending on the type of promoter region used in the construction of the recombinant bioluminescent bacteria, a variety of sensors can be constructed that respond to a multitude of environmental stimuli. As such, these strains can be utilized to study the mechanisms and classification of specific stress experienced within a given environment. Furthermore, in addition to the analysis of the responses being simple, since it is possible to detect changes in the gene expression levels through the emission of light, these biosensors have other advantages, including their simple usage, quick response time and multiplexing capabilities. As such, they are a powerful tool when applied together in arrays and in the screening of unknown substances.

Figure 4. Maximum relative luminescence values seen from strain BBT*NrdA* after exposure to different concentration of (A) phenol, (B) 2-chlorophenol (2-CP), (C) 2,4-dichlorophenol (2,4-DCP) and (D) 2,4,5-trichlorophenol (2,4,5-TCP).



In this study, we describe a recombinant *nrdA*-dependent bioluminescent strain that is responsive to DNA damaging chemicals. The results found that BBT*NrdA* only responds to genotoxic chemicals and hydrogen peroxide, which is known to cause DNA damage indirectly, and there was no significant induction in the bioluminescence of this strain when exposed to the other oxidative stress- and membrane damage-inducing compounds. These results demonstrate that strain BBT*NrdA* can be used as a sensor for genotoxic conditions.

The *nrdA* gene encodes for the ribonucleoside diphosphate reductase protein. Consequently, BBT*NrdA* responds more sensitively to substances related with inhibition of DNA synthesis. Furthermore, it has the potential to be used as a specific biosensor to detect errors during the conversion of ribonucleotides to deoxyribonucleotides in *Escherichia coli*. Owing to these properties, this strain can be used as a biosensor for detecting genotoxic agents and can be applied in the prescreening of anticancer drugs, which often are used to inhibit DNA synthesis.

3. Experimental Section

3.1. Construction of strain BBTNrda

Escherichia coli strain RFM443 (strR, laK2, ac Δ 74) was used as a host strain [34]. To construct the recombinant plasmid, pET, the sequence of the *nrdA* promoter region obtained from the National Center for Biotechnology Information (NCBI) was amplified by PCR using genomic DNA from strain RFM443. The primers used to amplify the *nrdA* promoter region are 5'-agcagcggaattcaagaaatcgccgaa-3' and 5'-acttaaggatccgcaatagtttcatg-3'. They include *Bam*HI and *Eco*RI (NEB, USA) restriction sites. After the promoter region was amplified, it was ligated into pDEW201 [35], a promoter-less plasmid with the *Photorhabdus luminescens luxCDABE* operon, to yield pET*nrdA*'Lux/RFM443. This plasmid was then transformed into *Escherichia coli* strain RFM443. and measuring their bioluminescent response. A positive clone that showed good responses when exposed to 2 ppm of mitomycin C (MMC) was selected and named BBTNrda.

3.2. Culture Condition and Chemical Test Protocol

For the cell culture, one colony of strain BBTNrda was grown overnight in 3 mL Luria-Bertani (LB) medium (DIFCO, USA) supplemented with 50 μ g/ml ampicillin (Sigma, USA) in a 15mL Falcon tube (Corning, MA, USA) in a shaking incubator at 37°C and 200 rpm. In 100 mL of fresh Luria-Bertani (LB) medium (DIFCO, USA) supplemented with 50 μ g/ml ampicillin (Sigma, USA), 2 mL of the overnight culture was inoculated and grown until the optical density reached 0.8 at 600 nm, measured using a spectrophotometer (Lambda 12, Perin Elmer, USA). Afterwards, the procedure was repeated one more time, but this time the optical density was allowed to reach only 0.08 at 600 nm. Using this BBTNrda culture, a 96-well luminometer test was performed. For the experiment the target chemicals were serially diluted and 10 μ l were added to each well. Then, 190 μ l of the BBTNrda culture solution was transferred to each of wells in the 96-well plate (Microlite 1, DYNEX Technologies, USA) which already contained known concentrations of the target chemicals. For the control, 10 μ l of distilled water was added in place of the target chemicals. This 96-well plate was then placed in the luminometer chamber under controlled conditions (37°C) and constant shaking before measuring the bioluminescence. The measurement interval was at set for every 6 minutes for a total time of 4 hours.

3.3. Chemicals

In this study, all chemicals were purchased from the Sigma-Aldrich Company (USA). They were categorized into three groups depending on the specific stress that they cause. First, the DNA damaging chemicals were nalidixic acid (NDA), mitomycin C (MMC), 1-methyl-1-nitroso-N-methylguanidine (MNNG) and 4-nitroquinoline N-oxide (4-NQO). Likewise, the oxidative stress chemicals included paraquat, cadmium chloride, and hydrogen peroxide (H₂O₂). The last group was membrane damaging chemicals and included several phenolics - phenol, 2-chlorophenol (2-CP), 2,4-dichlorophenol (2,4-DCP) and 2,4,5-trichlorophenol (2,4,5-TCP).

3.4. Data Analysis

All experiments were conducted three times independently and the results are shown as the average with the standard deviations. The relative bioluminescence (RBL) was recorded after exposure to toxicant and is defined as the bioluminescence value of the test sample divided by that of the control for each time point. To further evaluate the responses for each chemical, the minimum detectable concentration (MDC), which represents the lowest concentration showing an RBL value of at least two, was used. As well, the concentration giving the maximum RBL value is referred to as the maximum response concentration (MRC). Using these two parameters, we characterized the responses of strain BBTNrdA with each of the compounds.

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