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Genotoxicity Assay Using Chromosomally-Integrated Bacterial *recA::Lux*

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Abstract An *Escherichia coli* strain containing the *recA* promoter that fused to the *luxCDABE* operon originating from *Photorhabdus luminescens* was shown to respond sensitively to genotoxic stresses. Two different recombinant bacteria, one (DPD1657) harboring a plasmid with the *recA* promoter that fused to the *luxCDABE* operon, and the other (DPD1710) containing a chromosomally-integrated *recA* promoter that fused with *luxCDABE*, were compared and it was found that the sensitivity of the two strains was significantly different in terms of their bioluminescent level, response time, and the minimum detectable concentration of a chemical causing DNA damaging stress. DPD1710, with a chromosomally-integrated single copy, generally led to lower basal luminescence levels, faster responses, increased response ratios, and an enhanced sensitivity to mutagens, when compared to DPD1657 with a multi-copy plasmid.

Key words: *recA*, chromosomal integration, genotoxicity, sensitivity

Since many industrial wastes and manufacturing sites produce genotoxic agents, monitoring the genotoxicity present in the environment has become very important [7, 12]. Consequently, the need for sensitive detection of genotoxicity to provide a warning against the mutagenic effects of pollutants has led to the development of various bioassays [1, 5, 9, 11, 15].

Assays relevant to human risk assessment often involve mammals, either through direct animal exposure or tissue culture tests. Simpler bacterial assays, justified by the correlation between the genotoxicity of chemicals in bacterial and animal systems, allow faster responses and, thus, have attracted increased usage. Among these, the most widely accepted assay is the Ames test, which quantifies reverse, prototrophic mutation in histidine-requiring strains of *Salmonella typhimurium* [1]. A different approach for the detection of environmental mutagens is based on bacterial SOS activation. Included in this category are the SOS chromotest, rec-lac test, and umu test [16]. Several recent reports have suggested detection of SOS activation through the use of bacterial bioluminescence (*lux*) as a reporter, an approach that monitors bacterial responses in real-time experiments with simple luminometry protocols [2, 3, 8, 15]. Previous studies include the use, within an *Escherichia coli* host, of either *recA* or *uvrA* promoters fused with *Vibrio fischeris luxCDABE* operon [15]. In the Vitotox assay, the same plasmid-borne reporter system is fused to the *recN* promoter of *E. coli* and introduced into *S. typhimurium* [14].

A recombinant *E. coli* strain containing a *recA::luxCDABE* fusion is one of the strains recently constructed, and its responses to known mutagens have been characterized [5, 9, 15, 16]. The recombinant bacterium, DPD1657, contains the *recA* promoter and *lux* fusion on a multi-copy plasmid, thereby enhancing the intensity of the observed response, yet introducing a potential instability into the maintenance of the extrachromosomal genetic element. Another possible disadvantage to using a multi-copy plasmid-based fusion is the loss of responsiveness by the regulatory element, due to the titration effect of hundreds of fusions containing operator-promoters, on a fixed level of the repressor [10, 16]. Accordingly, the current study used the recombinant bacteria, DPD1710, containing a chromosomally-integrated *recA* promoter-*lux* fusion to overcome the disadvantages of a multi-copy plasmid-based fusion.

MATERIALS AND METHODS

Strains and Culture

E. coli strain DPD1657, constructed at DuPont Co. (Wilmington, U.S.A.), harbors a plasmid containing the *recA* promoter fused to a promoterless vector, pDEW201

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with the *luxCDABE* operon from *P. luminescens*, and has DH5α as host. In contrast, *E. coli* strain DPD1710 contains a chromosomally-integrated fusion of the *recA* promoter region from the *Photorhabdus luminescens luxCDABE* operon in the host strain JC7623 [16]. This strain was constructed using the plasmid pDEW14 at the DuPont Co. [16]. Plasmid pDEW14 was constructed by ligating a *Pst*I-*Eco*RI fragment containing a *recA::luxCDABE* fusion from plasmid pDEW201 into plasmid pBRINT [16]. DPD1710 was constructed using strain DPD1657 as the recipient for the transduction of $P1_{vir}$ phage grown on strain JC7623. The *recA::luxCDABE* fusion of strain DPD1710 was integrated into the *lacA* locus of *E. coli* and oriented so that the direction of the transcription was the same as that of the *lacZYA* operon. [16]. The cultures were grown in 100 ml of a Luria-Bertani (LB, initial pH of 7) medium, including 10 mg/l of ampicillin (Sigma Co.) for DPD1657, in 250-ml flasks, which were placed in a rotary incubator (Perkin-Elmer Co., U.S.A.). DPD1710 did not require any antibiotics. The cells were cultivated at 37°C, while mixing and aeration were provided by agitation (250 rpm). After sterilization, each new flask was inoculated with 2 ml of an inoculum derived from a seed culture previously cultivated for 8 h in 100 ml of an LB medium. When the optical density at 600 nm (OD_{600}) reached 0.08 (the early-exponential growth phase), the test chemicals were added to the culture media.

Chemicals

The chemicals used in this study were mitomycin C (MMC, Sigma Co., U.S.A.), methyl-nitro-nitrosoguanidine (MNNG, Aldrich Co., U.S.A.), hydrogen peroxide (H_2O_2, Merck), ethidium bromide (EtBr, Sigma Co., U.S.A.), and phenol (Sigma Co., U.S.A.). The bioluminescence (arbitrary units, AU, U.S.A.) emitted was monitored using a highly sensitive luminometer (Model 20e, Turner Design, CA, U.S.A.), and the cell growth was measured using a UV/Vis spectrophotometer (Perkin Elmer Co., U.S.A.) at 600 nm. The bioluminescence data values were presented as the specific bioluminescence (SBL), i.e. the volumetric light emission (AU) divided by the optical density of the cell suspension (OD_{600}), or response ratio, i.e. the SBL of the induced cells divided by the SBL of the uninduced cells.

Data Analysis

All experiments were performed in triplicate for an error analysis. Three data points were used to calculate

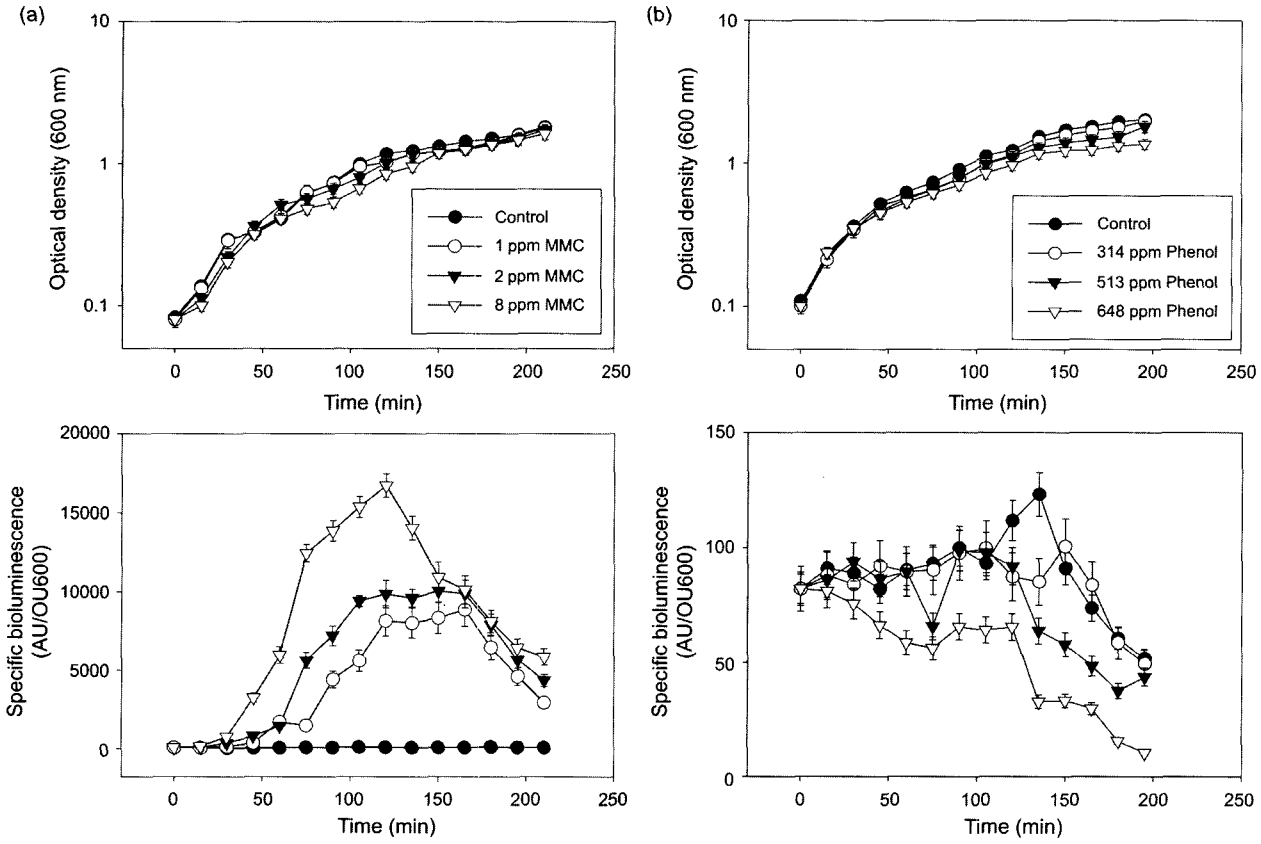

Fig. 1. Representative time course curves for response of DPD1710 to mitomycin C and phenol.
Kinetic response data for cell concentration and SBL in response to addition of mitomycin C (a) and phenol (b).

the standard deviation, which was represented by an error bar.

RESULTS AND DISCUSSION

Strain DPD1710 with a *recA::luxCDABE* fusion was integrated into the chromosome. In addition, it was confirmed that *recA::luxCDABE* was present in DPD1710 as a single copy [16]. As shown in Fig. 1, DPD1710 showed a very sensitive response to DNA-damaging stress caused by MMC, an intercalating agent, however, with no noticeable response to nonmutagenic agents, i.e. phenol. In addition, a higher expression was observed immediately after the exposure to MMC started, indicating a fast response. Conversely, no significant response was observed with any of the concentrations of phenol used in the current study (Fig. 1b). This indicated that the *recA* promoter exhibited a quite specific response to the mutagens. As shown in Fig. 2, in the case of DPD1657, the maximum value for the response ratio appeared 100 min after induction, which was a quite delayed response, whereas DPD1710 gave a faster response after the induction with MMC. These results seemed to indicate that the DNA damage caused by MMC directly affected the *recA* promoter in the chromosome in contrast to the plasmid based *recA* promoter, thereby the bacterium responded to the resulting genotoxicity immediately after the induction. In addition, while the basal bioluminescent value of DPD1657, containing a multi-copy plasmid *recA::luxCDABE* fusion, without induction was around 15,000 (data not shown), DPD1710 exhibited a much lower basal luminescence, i.e. about 10. Therefore, it was found that the single-copy chromosomal integration led to increased response ratios, as compared to the bioluminescent level of the uninduced control (Fig. 2a). This was due to the fact that the background bioluminescence expressed under the *recA* promoter was lower with the single copy in the chromosome. Another aspect related to the decreased background bioluminescence was the responses of the strains to phenol, as shown in Fig. 2b. In a previous study, DPD1657 responded immediately after the introduction of phenol (a nongenotoxic agent) due to the background bioluminescence expressed by the *recA* promoter in the plasmid, even though the maximum bioluminescence level of the strain with phenol was lower than that of the uninduced strain [5]. However, in the current study, DPD1710 did not respond to phenol at all, demonstrating that the background luminescence was significantly reduced in the strain containing the chromosomally-integrated fusion gene. In addition, it was also found that other mutagens exhibited similar responses to MMC, as summarized in Table 1. The minimum detectable concentrations of DPD1710 for several mutagens were almost 30 times lower than those by DPD1657, thus the sensitivity was enhanced due to the chromosomal integration.

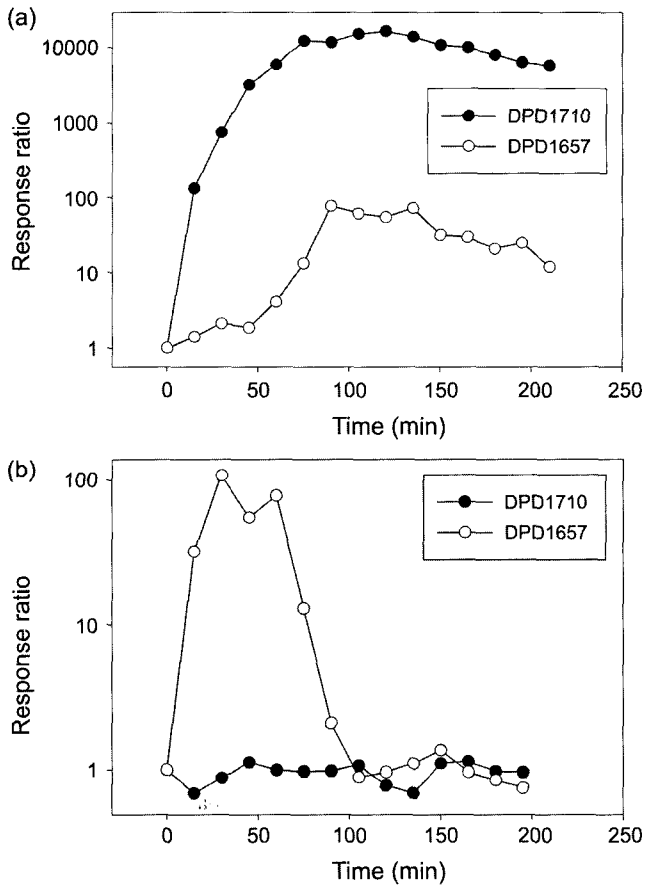

Fig. 2. Comparison of response specificities between DPD1710 and DPD1657.
The chemical concentrations showing the maximum bioluminescence values were used; (a) 2 ppm for MMC and (b) 314 ppm for phenol.

Accordingly, the current study found that a recombinant *E. coli* strain with a chromosomally-integrated fusion of the *recA* promoter with the genes required for bioluminescence exhibited more sensitive response to mutagens than a strain with the fusion located on a plasmid. Since DPD1657 was constructed by using pDEW201, originating from pBR322, its copy number is approximately 55. In contrast, the *recA::luxCDABE* fusion in DPD1710 is a single copy [16]. The use of chromosomal integration generally led to a faster response after exposure to the mutagens tested, as well as to lower basal luminescence levels, increased response ratios, and enhanced sensitivities. Therefore, these results suggested that the addition of mutagens had greater effect on the single-copy *recA* promoter fusion in the chromosome compared to the plasmid-based fusion due to the difference in the copy numbers. In addition, the response was also influenced by the extent of the LexA degradation protein, a repressor in the SOS regulon [11]. The *recA* gene is a rapidly expressed gene by the SOS

Table 1. Comparison between responses of DPD1710 and DPD1657 to several mutagens in terms of minimum detectable concentration and the concentration showing 20% growth inhibition. The minimum detectable concentration was the specific concentration resulting in a 2.5-fold induction of bioluminescence compared to that of the control. In addition, the concentrati on providing 20% growth inhibition was determined using the specific growth rate during the exponential phase of growth.

	Minimum detectable concentration		Concentration showing 20% growth inhibition (EC_{20})	
	DPD1710	DPD1657	DPD1710	DPD1657
Mitomycin C	0.8 ppb	15 ppb	1,000 ppb	2,000 ppb
Methyl-nitro-nitrosoguanidine	0.5 ppb	32 ppb	200 ppb	3,000 ppb
Hydrogen peroxide	0.01 ppb	0.5 ppb	5 ppb	50 ppb
Ethidium bromide	10 ppb	320 ppb	150 ppb	N.T.

*N.T.: Not Tested.

regulon and encodes the RecA protein, which has specific co-protease and general recombinase activities [5, 11]. The rate of the LexA repressor auto-cleavage is substantially elevated by the RecA co-protease action. After LexA is degraded, a swift induction in *recA* expression is seen in response to DNA damage, thereby allowing the *recA* gene fusion to efficiently detect genotoxicity [5, 11]. Consequently, the current results suggest that the existence of a multi-copy plasmid-based *recA* promoter fusion gene requires greater number of mutagen to fully activate all the promoters, while a chromosomally-integrated fusion gene is fully activated with lower mutagen concentration. This also explains the difference in the induction values as a portion of the plasmid-borne fusion genes is repressed, requiring a greater induction to achieve a completely de-repressed state, whereas the chromosomally-integrated fusion gene remains activated longer and complete de-repression of the promoter is easier to achieve. Therefore, modification of the *recA::luxCDABE* to a single-copy system from a multi-copy plasmid system actually enhanced its sensitivity to mutagens and reduced the background bioluminescence emission while providing more stable responses to mutagens and their actions.

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