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NOTE

Construction of a Bioluminescent Reporter Using the *luc* Gene and *meta*-Cleavage Dioxygenase Promoter for Detection of Catecholic Compounds

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Several types of bioluminescent reporter strains have been developed for the detection and monitoring of pollutant aromatics contaminating the environment. In this study, a bioluminescent reporter strain, *E. coli* SHP3, was constructed by fusing the *luc* gene of firefly luciferase with the promoter of *pcbC* responsible for the *meta*-cleavage of aromatic hydrocarbons. The bioluminescence expressed by the *luc* gene in the reporter was well triggered by the promoter when it was exposed to 2,3-dihydroxybiphenyl (2,3-DHBP) at 0.5 to 1 mM concentrations. The bioluminescent response was more extensive when the reporter strain was exposed to 5 mM catechol and 2 mM 4-chlorocatechol. These different types of bioluminescent responses by *E. coli* SHP3 appeared to be characterized by the nature of the aromatics to stress. Since *E. coli* SHP3 responded to 2,3-DHBP quite sensitively, this reporter strain could be applied for detecting some catecholic pollutants.

Key words: Bioluminescent reporter, *luc* gene, *pcbC* promoter, 2,3-dihydroxybiphenyl, catechol, 4-chlorocatechol

The increasing level of environmental pollution demands specific and sensitive detection methods for monitoring environmentally toxic pollutants. There have been two approaches that are currently practised for the detection of such environmental contaminants: A direct analytical method that allows the determination of specific contaminants and a bioassay method for measuring the harmful effects on organisms. However, such methods do not involve continuous measures for specific chemicals affecting living organisms (14).

Microbial reporter assays have gained importance because of rapid responses, low cost, and improved reproducibility (14). These reporter strains are characterized to be constructed as a promoter-reporter gene concept by the coupling of promoters of particular gene and reporter gene. One of them are the bioluminescent reporter strains made with *luxCDABE* genes to detect various toxic inorganics and xenobiotic compounds. The bioluminescent reporters have been developed in order to detect a variety of environmental pollutants, such as pentachlorophenol (14), 4-nitrophenol (2), ethanol (14, 15), benzen, toluene, ethylbenzene, and xylene (BTEX) (1), and the presence of mercury (10). These bioluminescent sensors were made with the promoters of *nahG* coding for salicylate hydroxylase (4, 7), *bphA* for biphenyl dioxygenase (8), and *todR* for toluene degradation (1).

Recently, the firefly luciferase gene (*lucFF*) has been recommended as a reporter gene to construct recombinant bioluminescent bacteria, because the *luc* gene has advantages over bacterial *luxAB* luciferase genes in terms of the broad host range and high quantum yield (3). Tauriainen *et al.* (12, 13) constructed luminescent bacterial strains using the firefly *luc* gene as a reporter and *ars* promoter for measuring toxic metals of arsenite, antimonite, and cadmium. The *luc* gene was also used for the construction of a reporter plasmid by fusion with the Pu promoter of *xylR* from the TOL plasmid of *Pseudomonas putida* mt-2 (16). The *E. coli* biosensor cells were reported to respond specifically to BTEX and toluene-like molecules. However, the need for a bacterial reporter that can detect other kinds of aromatic pollutants still remains.

Catecholic compounds are generated as by-products through the biodegradation of herbicides and other aromatic hydrocarbons, such as polychlorinated biphenyls,

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benzoates, DDT, and bidicin. The cleavage of benzene ring structures is one of the most crucial steps in the biodegradation of catecholic compounds. The ring-cleavage conducted by *meta*-cleavage dioxygenase is more common in the bacterial catabolism of aromatics. In this study, therefore, the promoter of a *pcbC* gene encoding *meta*-cleavage in the dioxygenase for degradation of 2,3-dihydroxybiphenyl (2,3-DHBP) and catechol was utilized to construct a bioluminescent reporter by fusing with the firefly *luc* gene to detect various catecholic and chlorocatecholic compounds dissolved in water.

Construction of bioluminescent reporters

Pseudomonas sp. DJ-12 is an isolate capable of utilizing 4-chlorobiphenyl (4CB), biphenyl, 4-chlorobenzoate (4CBA), and 4-hydroxybenzoate (4HBA) (6). Its metabolic pathway from 4CB to 4CBA was elucidated by cloning the corresponding genes, *pcbABCD* (5, 6), and the nucleotide sequences of *pcbCD* genes and the corresponding promoters were analyzed (6). In this study, the promoter of *pcbC* for *meta*-cleavage dioxygenase was fused with the *luc* genes which were purchased from Promega Co. (Madison, WI, USA). *E. coli* XL1-Blue was used as the host strain for transformation and subcloning studies. The transformed *E. coli* strains were cultivated at 30°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with ampicillin (50 μg/ml) as a selective marker. The cultivated cells were used immediately for the bioluminescence induction test. The survival of the *E. coli* SHP3 was examined at regular intervals by plating the culture aliquot on LB agar during exposure to aromatic chemicals.

The reporter plasmids were constructed using the promoter of the *pcbC* gene in pCU1031 (6) as shown in Fig. 1. A 2.2-kb *Pst*I fragment of pCU1031 carrying the *pcbCD* gene was inserted into the pBluescript KS(+/–) vector according to the method described by Sambrook *et al.* (9). This recombinant plasmid, pSHP1, was digested with *Cla*I and allowed to self-ligate with T4 DNA ligase. Accordingly, the resulting pSHP2 plasmid constituted the vector and the *pcbC* promoter through the deletion of the *pcbCD* structural gene. 180-bp of pSHP2 digested with *Sac*I and *Xho*I were subsequently inserted into the pGL3-basic vector carrying the *luc* gene which was digested with the same enzymes. Thereafter, the bioluminescent reporter plasmid, pSHP3, was constructed by inserting the *pcbC* promoter upstream of the *luc* gene in the pGL3 vector. The reporter plasmid DNAs were digested with appropriate restriction enzymes, separated by electrophoresis in a 0.7 or 1.5% agarose gel, and then purified with UltraClean™15 (Mol Bio Laboratories, Inc., Solana Beach, CA, USA). This pSHP3 reporter plasmid was transformed into *E. coli* XL1-Blue to make a reporter strain designated as *E. coli* SHP3.

The promoter of *pcbC* was inserted upstream of the *luc* gene to create bioluminescent reporter plasmids. The plasmids of pSHP2 and pSHP3 were examined by digestion with restriction enzymes followed by gel electrophoresis as shown in Fig. 2. The 180-bp *pcbC* promoter region from pSHP2 was confirmed by DNA bands digested with *Pst*I and *Cla*I restriction enzymes (Fig. 2A, lane 4). Thereafter, the promoter inserted in the pGL3-basic vector carrying the *luc* gene was identified as the DNA fragment digested with *Sac*I and *Xho*I, as seen in lane 5 of Fig. 2B.

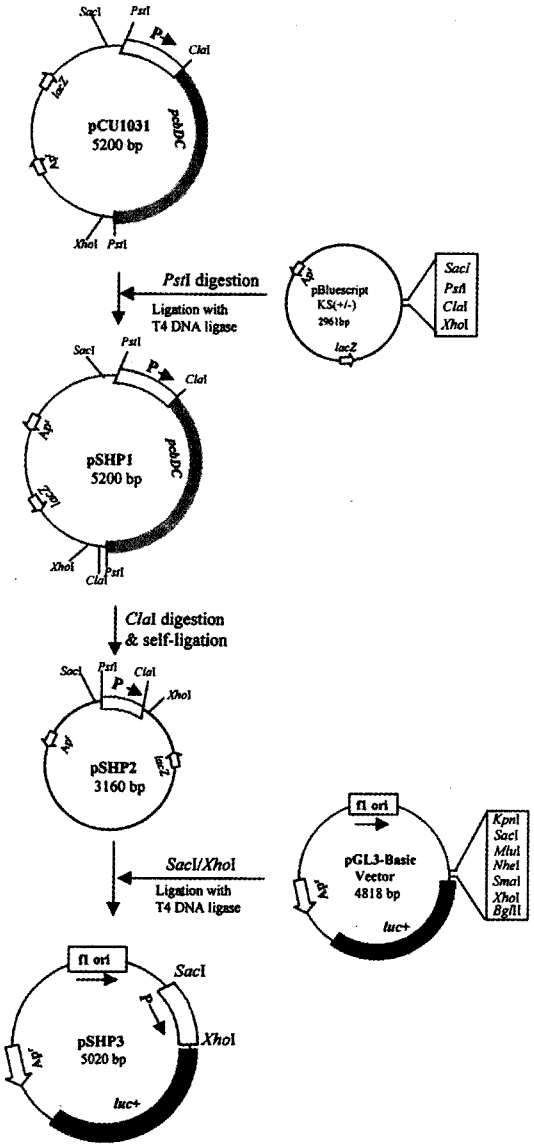

Fig. 1. Scheme for construction of a bioluminescent reporter plasmid, pSHP3.

Production of bioluminescence in E. coli SHP3 by 2,3-DHBP

The bioluminescence produced by the reporter strain was measured according to the *in vivo* method described by Tauriainen *et al.* (13). 180 μl of the bacterial culture was

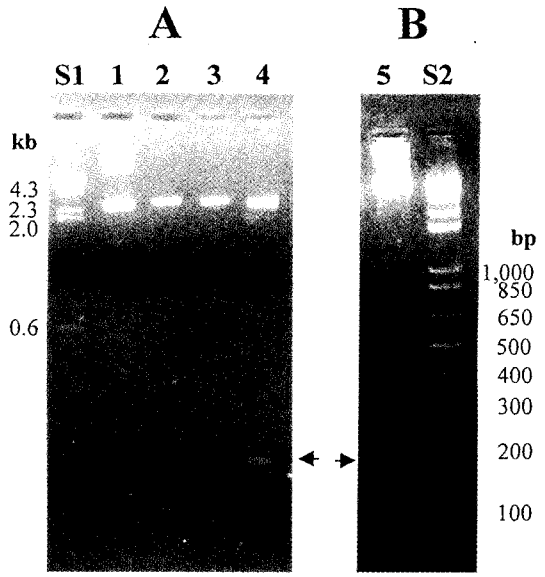

Fig. 2. Restriction analysis of pSHP2 (A) and pSHP3 (B). Lanes: S1, λ-*Hin*dIII DNA size marker; 1, plasmid DNA of SHP2; 2, pSHP2 digested with *Pst*I; 3, pSHP2 digested with *Sal*I, 4, pSHP2 digested with *Pst*I and *Cla*I; 5, pSHP3 digested with *Sac*I and *Xho*I; S2, 1-kb size ladder. The arrows indicate 180-bp DNA inserts carrying the *pcbC* promoter and *luc* genes.

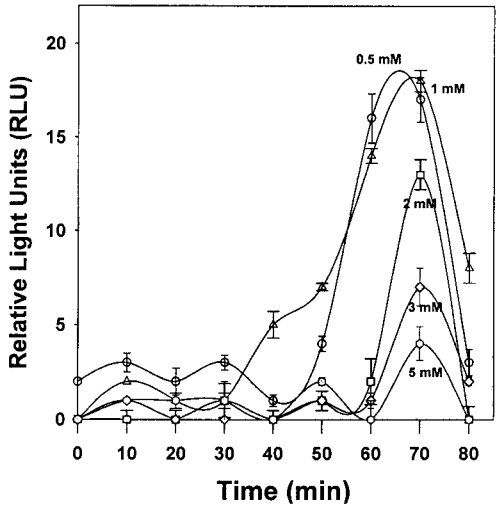

Fig. 3. Production of bioluminescence in *E. coli* SHP3 by exposure to 2,3-dihydroxybiphenyl at various concentrations.

transferred into 96-well microtiter plates and 1 mM luciferin was added as the substrate. Appropriate amount of aromatic compounds were added directly into the reaction wells. The luminescence was measured with a Berthold luminometer (Autolumat LB935, EG & G Berthold Analytical Instruments, Oak Ridge, TN, USA) by incubating at 25°C. The unit of bioluminescence was expressed as relative light units (RLU).

The bioluminescence produced by the reporter strain, *E. coli* SHP3, was measured by exposing them to 2,3-dihydroxybiphenyl (2,3-DHBP) at various concentrations. The results are shown in Fig. 3. The maximum bioluminescence was produced at 70 min when exposed to 0.5 mM and 1 mM 2,3-DHBP. Light production was lowered at concentrations higher than 1 mM 2,3-DHBP. This decrease in bioluminescence may be due to a decrease in the survival rate caused by the toxicity of the compound, because the survival rates of the reporter cells gradually decreased in LB broth containing 2 mM or higher concentrations of 2,3-DHBP, as seen in Fig. 4. However, 2,3-DHBP concentrations of 0.5 and 1 mM did not have much of an affect on the survival rate of the organism during 80 min of exposure.

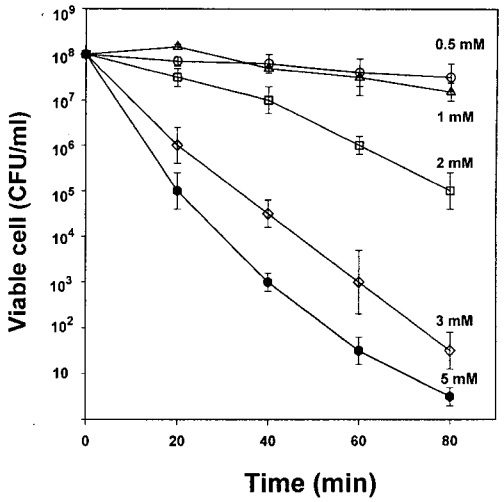

Fig. 4. Survival of *E. coli* SHP3 in LB broth containing 2,3-dihydroxybiphenyl at different concentrations.

Selifonova and Eaton (11) reported that the production of bioluminescence in a reporter strain containing *ipbRo/pA-luxCDABE* was related to the concentrations of the inducing chemicals, such as isopropylbenzene, monoalkylbenzenes, benzene, and toluene. That is, the production of bioluminescence by isopropylbenzene was increased from 1 to 100 μM. However, the level of bioluminescence decreased within higher concentrations of the chemical because of its toxicity towards the reporter strains. The reporter strains with *grpE'::luxCDABE* constructed by Van Dyk *et al.* (14) showed reduced bioluminescence with high concentrations of pentachlorophenol. Therefore, it can be concluded that the *E. coli* SHP3 reporter strain constructed in this study can be used for detection of biphenyl compounds such as 2,3-DHBP at concentrations up to 1 mM.

Bioluminescent responses to catecholic compounds

The induction of bioluminescence in *E. coli* SHP3 was examined with catechol and 4-chlorocatechol. The bioluminescence and survival rates of the reporter cells during exposure to those aromatics are summarized in Table 1.

Table 1. Production of bioluminescence and survival rates of *E. coli* SHP3 during exposures to catechol and 4-chlorocatechol

Aromatic pollutants	Reaction time (min)	Concn. (mM)	Relative light units (RLU)	Initial CFU/ml ($\times 10^7$)	CFU/ml after reaction ($\times 10^7$)
Catechol	50	0.5	4	-[a]	-
		1	17	-	-
		2	110	9.2	8.0
		3	164	7.2	4.0
		5	207	8.2	2.7
4-Chlorocatechol	50	0.5	3	-	-
		1	8	9.9	8.9
		2	48	10.2	7.0
		3	3	10.0	5.0
		5	6	-	-

[a]Not tested

The bioluminescence was sensitively induced by the aromatics. The highest response of bioluminescence was observed when *E. coli* SHP3 was exposed to 5 mM catechol and 2 mM 4-chlorocatechol for 50 min. Unlike the case with 2,3-DHBP, the survival rates of the organism in each compound were not proportional to the intensity of the bioluminescent response. Van Dyk *et al.* (14) reported that sublethal concentrations of many aromatic compounds could induce bioluminescence. Therefore, the bioluminescent responses of *E. coli* SHP3 to pollutant aromatics were specified and characterized to be different depending on the nature of the aromatics. The bioluminescent reporter strain, *E. coli* SHP3, constructed in this study exhibited quite sensitive responses to aromatic compounds such as 2,3-DHBP and catecholic compounds.

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