J. Microbiol. Biotechnol. (2002), **12**(3), 417–422

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Detection of Aromatic Pollutants by Bacterial Biosensors Bearing Gene Fusions Constructed with the *dnaK* Promoter of *Pseudomonas* sp. DJ-12

PARK, SANG-HO, DONG-HUN LEE, KYE-HEON OH[1], KYOUNG LEE[2], AND CHI-KYUNG KIM*

Department of Microbiology and Biotechnology, and Biotechnology Research Institute, Chungbuk National University, Cheongju 361-763, Korea
[1]Department of Life Science, Soonchunhyang University, Asan 336-600, Korea
[2]Department of Microbiology, Changwon National University, Changwon 641-773, Korea

Received: November 23, 2001
Accepted: March 25, 2002

Abstract Gene fusions were constructed by the transcriptional fusion of the *dnaK* promoter of *Pseudomonas* sp. DJ-12 or *E. coli* to the *lux* or *luc* marker gene. The *dnaKp-DJ::luxCDABE* bioluminescent fusion in the biosensor using the *Pseudomonas* sp. DJ-12 *dnaK* promoter exhibited about 5-fold more extensive response to ethanol than that of *dnaKp-EC::luxCDABE*. The bioluminescent response of the *dnaK-DJ::luc* fusion to ethanol was much weaker than those of the other fusions. The biosensor harboring the *dnaKp-DJ::luxCDABE* fusion was examined for its bioluminescence production based on exposure to aromatic compounds, such as biphenyl, 4-chlorobiphenyl (4CB), 4-hydroxybenzoate (4HBA), and catechol. In particular, the bioluminescence produced by the *dnaKp-DJ::luxCDABE* fusion was most sensitive to 1 mM biphenyl and 4CB when exposed for 80 min, and the responses were also very strong to other aromatics. Therefore, the biosensor bearing the *dnaKp-DJ::luxCDABE* fusion would appear to be the most useful for the detection of aromatics and other pollutants.

Key words: Biosensor, *Pseudomonas* sp. DJ-12, *dnaK* promoter, *dnaKp-DJ::luxCDABE* fusion, aromatic pollutants

Aromatic compounds, particularly chlorinated aromatics, are serious environmental pollutants due to their lipophilic, persistant, and toxic properties. Although many countries strictly control the use of such aromatic pollutants, their distribution in nature is still one of the major problems contaminating our environment [17, 22].

To detect the presence of such chemical pollutants in complex environments, biosensors have been widely applied. Some of these biosensors are able to detect and quantify various compounds, including BTEX compounds [1, 25], naphthalene [10], monochlorinated biphenyl [12], and mercury [6]. These biosensors are made by gene fusions constructed with the promoters of *nahG* coding for salicylate hydroxylase [7, 10], *bphA* for biphenyl dioxygenase [12], and *todR* for toluene degradation [1].

Van Dyk *et al.* [23, 24] and Belkin *et al.* [2] constructed biosensors by fusing *luxCDABE* bioluminescent genes to the promoters of stress shock genes, including *dnaK*, *grpE*, *uspA*, and *katG* from *E. coli* strains. These biosensors have been examined by monitoring various kinds of toxicants, such as ethanol, phenol, and heavy metals. Among them, the gene fusion with the *dnaK* promoter has been reported to be more sensitive to toxicants than the fusions with other promoters. However, a biosensor bearing the fusion of the *dnaK* promoter and *luxCDABE* responds better to ethanol than aromatic pollutants, including pentachlorophenol, 2-nitrophenol, and phenol [23]. Furthermore, the bioluminescence by fusion was found to be inhibited by phenol at concentrations higher than 4,170 ppm. Therefore, it is still necessary to construct biosensors that can detect diverse aromatic pollutants more sensitively.

The *dnaK* gene in *Pseudomonas* sp. DJ-12 has been reported to be induced by various aromatic pollutants, such as biphenyl, 4-chlorobiphenyl (4CB), 4-hydroxybenzoate (4HBA), benzoate, and catechol [11, 14, 15]. Accordingly, in the current study, the *dnaK* gene of *Pseudomonas* sp. DJ-12 was used to construct bacterial biosensors for the detection of aromatic pollutants in water by fusing the *dnaK* promoter to the *luxCDABE* or *luc* gene. The resulting biosensors produced bioluminescence much more extensively than biosensors bearing the *E. coli dnaK* promoter or *luc* gene as a marker.

*Corresponding author
Phone: 82-43-261-2300; Fax: 82-43-264-9600;
E-mail: environ@trut.chungbuk.ac.kr

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in the current study were *E. coli* W3101 [8] and *Pseudomonas* sp. DJ-12, which is an isolate capable of utilizing biphenyl and 4-chlorobiphenyl as carbon and energy sources [9]. The expression of the DnaK protein in *Pseudomonas* sp. DJ-12 was previously studied by treatment with several aromatic hydrocarbons [15, 16]. *E. coli* XL1-Blue was used as the host strain for the gene fusions to construct the bacterial biosensors. The biosensors bearing the gene fusions to measure the bioluminescence were grown to the log phase at 25°C in a Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). The initial pH of the medium was adjusted to 7.0 before sterilization.

Enzymes, Reagents, and Aromatic Compounds

The restriction enzymes, DNA modifying enzymes, and T4 DNA ligase were purchased from Posco Co. (Seoul, Korea). All the aromatic compounds used in this study were purchased from Sigma Co. (St. Louis, MO, U.S.A.). Stock solutions (100 mM) of 4-hydrobenzoate (4HBA) or catechol were prepared in sterile distilled water, while stock solutions (100 mM) of biphenyl or 4-chlorobiphenyl (4CB) were prepared in ethanol. The appropriate amount of the stock solutions was added directly into the culture of the biosensors for the production of bioluminescence, as described by Van Dyk *et al.* [23].

Construction of Bioluminescent Reporter Strain

The *dnaK* promoter (*dnaKp*) was obtained by PCR amplification from the genomic DNA of *Pseudomonas* sp. DJ-12 or *E. coli* W3101 using the primers 5'-GTTAGCGGATCCAAAAGCA CAAAAAAT-3' and 5'-AGCAGTGAATTCCATCTAAACGTCTCCA-3', as shown in Fig. 1. The primers were designed based on the promoter region of the *dnaK* operon [5]. Following digestion with *Bam*HI and *Eco*RI, the PCR products obtained from the *Pseudomonas* DJ-12 genomic DNA or *E. coli* W3101 *dnaK* gene were

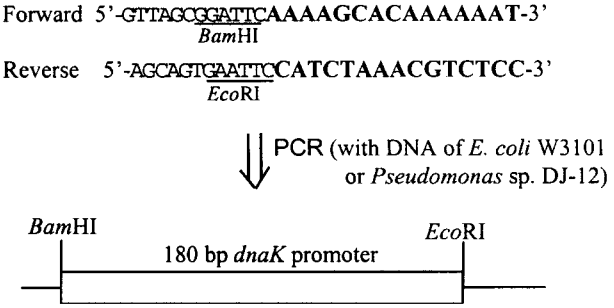


Fig. 1. Construction of *dnaK* promoter by PCR with designed primers and DNA from *E. coli* W3101 and *Pseudomonas* sp. DJ-12.

directionally ligated to *Bam*HI- and *Eco*RI-digested pUCD615 [18], a plasmid containing the promoterless *luxCDABE* operon of *V. fischeri*, to create the bioluminescent gene fusions, *dnaKp-DJ::luxCDABE* (Fig. 2A) and *dnaKp-EC::luxCDABE* (Fig. 2B). In the case of *Pseudomonas* sp. DJ-12, the PCR product was inserted into the pBluescript SK(+/−) vector (Stratagene, La Jolla, CA, U.S.A.) according to the method described by Sambrook *et al.* [19]. The *dnaK* promoter regions digested with *Sac*I and *Xho*I were subsequently inserted into the pGL3-basic vector (Promega Co., Madison, WI, U.S.A.) carrying the *luc* gene to construct *dnaKp-DJ::luc* (Fig. 2C). All the gene fusions were transformed into *E. coli* XL1-Blue to measure the bioluminescence.

Measurement of Bioluminescence

The bioluminescence produced by the biosensors was measured according to the *in vivo* method described by Tauriainen *et al.* [21]. One-hundred-eighty μl of the bacterial biosensor culture (10^6 CFU/ml cells) was transferred into 96-well microtiter plates, and 0.1 to 5 mM aromatic compounds was added directly into the reaction wells. The luminescence was measured using a Berthold luminometer (Autolumat LB935, EG & G Berthold Analytical Instruments, Oak Ridge, TN, U.S.A.) by incubating at 25°C. Triplicate experiments for each concentration of chemicals were conducted, and the relative bioluminescence unit was

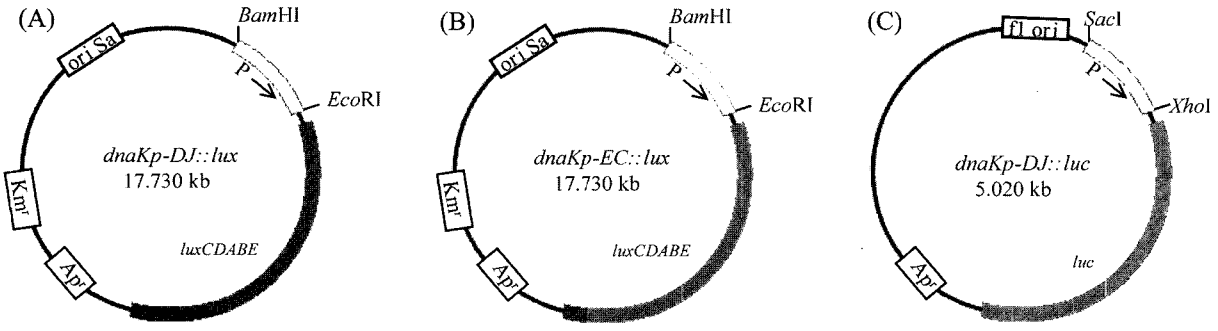


Fig. 2. Bioluminescent gene fusions of *dnaK* promoter of *Pseudomonas* sp. DJ-12 (A) and *E. coli* W3101 (B) with *luxCDABE* reporter gene. The *dnaKp-DJ::luc* (C) was constructed by fusing the *dnaK* promoter of *Pseudomonas* sp. DJ-12 with the *luc* reporter gene of fireflies.

determined by the difference of the bioluminescence between that produced by the biosensors when exposed to the aromatics and the bioluminescence of the untreated biosensors. In the case of biphenyl and 4CB dissolved in ethanol, the bioluminescence of the biosensors induced by ethanol was subtracted from the bioluminescence produced by the biosensor with each aromatic.

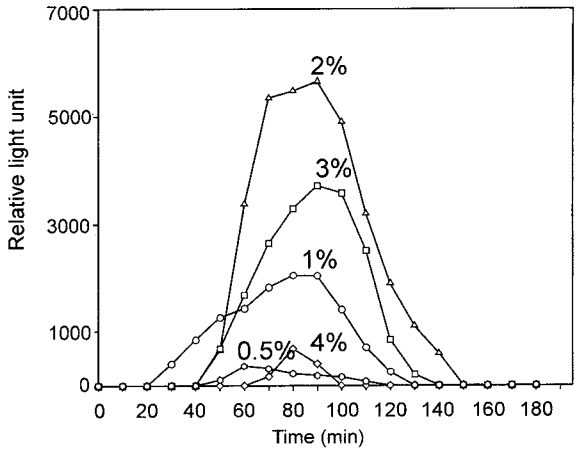
RESULTS AND DISCUSSION

Bioluminescent Responses of Bacterial Biosensors to Ethanol

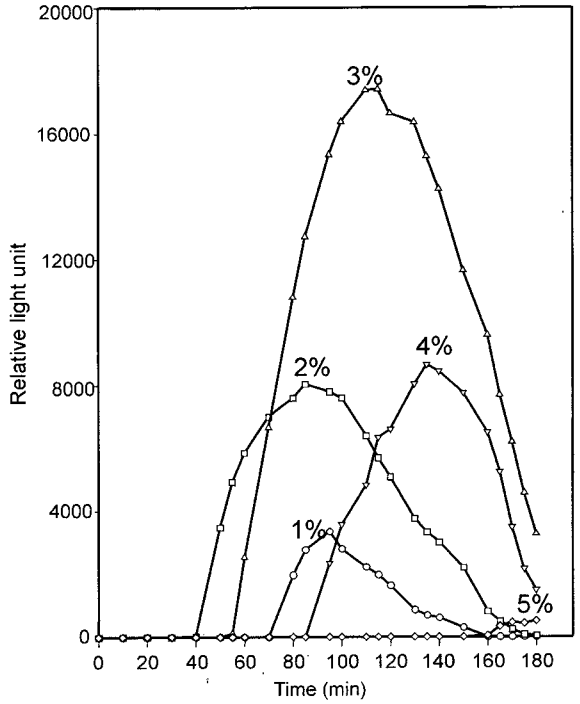
The bacterial biosensor bearing *dnaKp-DJ::luxCDABE,* which was constructed using the *Pseudomonas* sp. DJ-12 *dnaK* promoter, was examined for its bioluminescence production by exposure to various concentrations of ethanol. This was done to compare the bioluminescence with that of *dnaKp-EC::luxCDABE,* previously constructed by Van Dyk *et al.* [23] using the *E. coli dnaK* promoter under the same condition as in the current study. The results are shown in Fig. 3. The light production by *dnaKp-DJ::luxCDABE* fusion was much stronger than that of the *dnaKp-EC::luxCDABE* fusion. Specifically, the bioluminescence produced by the *dnaKp-DJ::luxCDABE* fusion was about 5-fold brighter than that of *dnaKp-EC::luxCDABE* when exposed to 3% ethanol for 100 min. The bioluminescent response of the *dnaK-DJ::luc* fusion to ethanol was much weaker than those of the other fusions. The extent of the bioluminescent responses of the biosensors to ethanol was in the order of *dnaKp-DJ::luxCDABE>dnaKp-EC::luxCDABE>dnaKp-DJ::luc.*

Ethanol has been reported as an efficient inducer for the production of DnaK proteins [13]. Accordingly, the light production induced by a biosensor using the *dnaK* promoter has been recommended to evaluate the response sensitivity to environmental stresses. Cha *et al.* [4] reported that the light production by a *dnaK::gfp*$_{uv}$ fusion using the *E. coli dnaK* promoter measured within a range of 2 to 4% ethanol concentrations, which also increased after 4 h exposure. The *dnaKp-DJ::luxCDABE* fusion constructed in the current study exhibited a similar pattern in response to ethanol. However, the luminescence produced by the *dnaKp-DJ::luxCDABE* fusion was much stronger than that by the *dnaK::gfp*$_{uv}$ fusion.

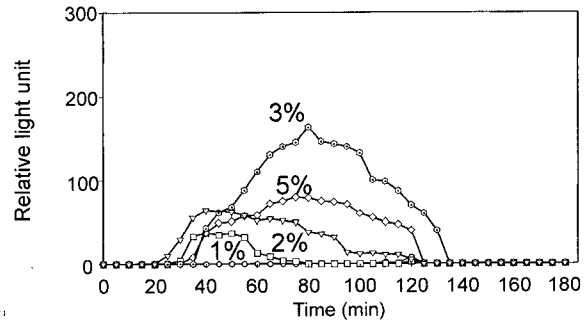
Van Dyk *et al.* [23] reported that a *dnaKp::luxCDABE* fusion constructed with the *E. coli dnaK* promoter responded to 4% ethanol when exposed for 60 min. However, the fusion produced a weaker bioluminescence when exposed to pentachlorophenol rather than ethanol. A lag time prior to the maximum induction was also observed, as anticipated from the requirements for transcription and translation, and the rate of response was transient. Such a transient response was also observed in the response of *dnaKp-*



(A) *dnaKp-EC::lux*



(B) *dnaKp-DJ::lux*



(C) *dnaKp-DJ::luc*

Fig. 3. Light production by *dnaKp-EC::luxCDABE* (A), *dnaKp-DJ::luxCDABE* (B), and *dnaKp-DJ::luc* (C) biosensors when exposed to different concentrations of ethanol for 180 min.

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Pseudomonas 5'- AAAAGCACAAAAAATTTTAGCATCTCCCCCTTGATGACGT
E. coli         AAAAGCACAAAAAATTTTTGCATCTCCCCCTTGATGACGT
                                  *

Pseudomonas     GGTTTACGACCCCATTTAGTAGTCAACCGCAGTGAGTGAG
E. coli         GGTTTACGACCCCATTTAGTAGTCAACCGCAGTGAGTGAG

Pseudomonas     TCTGCAAAAAAGTGAAATTGGGCAGTTGAAACCAGACGTT
E. coli         TCTGCAAAAAAATGAAATTGGGCAGTTGAAACCAGACGTT
                          *

Pseudomonas     TCGCCCGTATTACAGACTCACAACCACATGATGACCGAAT
E. coli         TCGCCCCTATTACAGACTCACAACCACATGATGACCGAAT
                 *

Pseudomonas     ATATTAGTGGAGACGTGGTAGATG - 3
E. coli         ATAT-AGTGGAGACGTT-TAGATG
                 *             **
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Fig. 4. Alignment of nucleotide sequences of *dnaK* promoters from *Pseudomonas* sp. DJ-12 and *E. coli* (GenBank accession number, M10420).
Asterisks indicate the different nucleotides between the two strains.

DJ::lux to ethanol in the current study (Fig. 3). That is, less bioluminescence was produced by the biosensor after exposure for a certain period of time, as indicated by Van Dyk *et al.* [23]. The bioluminescence production by *dnaKp-DJ::luxCDABE*, which was constructed with the *dnaK* promoter of *Pseudomonas* sp. DJ-12, was much stronger than that of the *dnaKp-EC::luxCDABE* fusion constructed with the *E. coli dnaK* promoter. This difference in response might have been due to the different nucleotide sequences of the promoters. The nucleotide sequences of the *dnaK* promoters from *Pseudomonas* sp. DJ-12 and *E. coli* (GenBank accession number, M10420) had six different base pairs, as shown in Fig. 4. As such, this might explain the significant difference in the bioluminescent response to ethanol between the *dnaKp-DJ::luxCDABE* and *dnaKp-EC::luxCDABE* fusions.

Bioluminescence Responses by *dnaKp-DJ::luxCDABE* to Aromatics

The biosensor bearing the *dnaKp-DJ::luxCDABE* fusion was examined for its luminescent response to several aromatics, and the results are shown in Fig. 5. The bioluminescence began to be produced by the *dnaKp-DJ::luxCDABE* fusion when it was exposed to biphenyl, 4CB, and 4HBA at 1 mM concentration. The bioluminescent response was the highest when exposed to 1 mM 4CB or 0.2 mM catechol for 90 min. The bioluminescent responses of the *dnaKp-DJ::luxCDABE* fusion to biphenyl, 4CB, and 4HBA were the strongest at 1 mM concentration of each

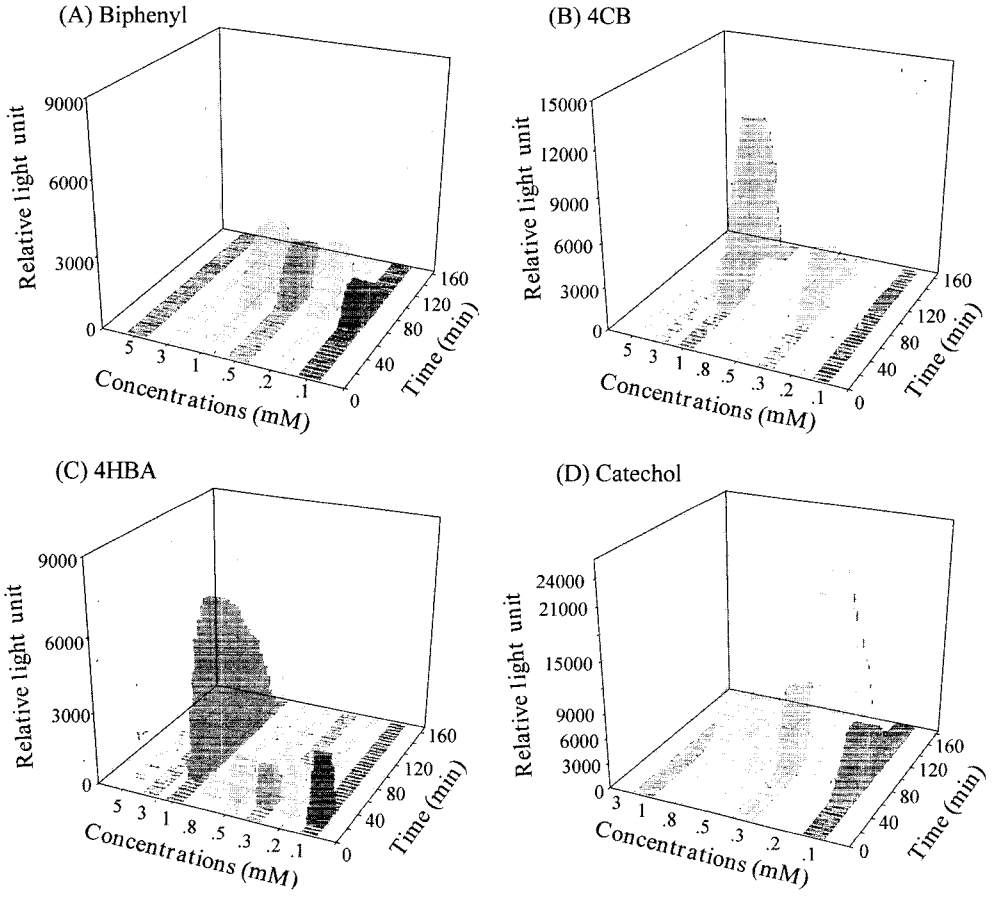

Fig. 5. Light production by *dnaKp-DJ::luxCDABE* fusion when exposed to biphenyl (A), 4CB (B), 4HBA (C), and catechol (D).

aromatic. However, no bioluminescence was produced at higher concentrations of the aromatic pollutants.

Layton *et al.* [12] detected several aromatic hydrocarbons with a *bphAp::luxCDABE* fusion constructed with the *bphA* gene promoter of *Ralstonia eutropha* ENV307. They observed that the bioluminescence was only three- to four-fold brighter than the background when exposed to aromatic pollutants, such as biphenyl, nonochlorinated biphenyls, and Aroclor 1242. Various aromatic pollutants have been reported to act as good inducers for the production of the DnaK stress-shock protein in *Pseudomonas* sp. DJ-12 [14, 15] and *E. coli* cells [3]. Van Dyk *et al.* [23, 24] showed that a gene fusion using the promoters of the *dnaK* or *grpE* stress-shock genes and *luxCDABE* reporter genes, respectively, could be used to detect chemical toxicants, such as phenol, 2-nitrophenol, copper sulfate, and ethanol. However, the *dnaKp-DJ::luxCDABE* fusion constructed in the current study was found to respond much more extensively to aromatics, compared to the fusions using the promoter of the catabolic gene (ex, *bphA* gene) or stress-shock gene from *E. coli* strains.

Bioluminescent Responses by *dnaKp-DJ::luc* to Aromatics

The bioluminescent response of the *dnaKp-DJ::luc* fusion to aromatic pollutants was also examined, and the results are shown in Fig. 6. The bioluminescence produced by the *dnaKp-DJ::luc* fusion was generally well induced by biphenyl, 4CB, and 4HBA at concentrations of 0.8 mM to 3 mM when incubated for 80 to 120 min. However, the extent of light produced by the biosensor bearing the *dnaKp-DJ::luc* fusion was much lower than that by the biosensor bearing the *dnaKp-DJ::luxCDABE* fusion, even though the same promoter was used for the gene fusions.

Tauriainen *et al.* [20, 21] constructed biosensors using *luc* as the marker gene for measuring toxic metals, such as arsenite, antimonite, and cadmium. The *luc* gene has also been used for the construction of a biosensor by fusion with the Pu promoter of *xylR* from the TOL plasmid of *Pseudomonas putida* mt-2 [25]. This biosensor was specifically examined for its luminescent response to BTEX and toluene-like molecules, however the light production was not very strong. Accordingly, the current results indicate that the gene fusion constructed with the *dnaK*

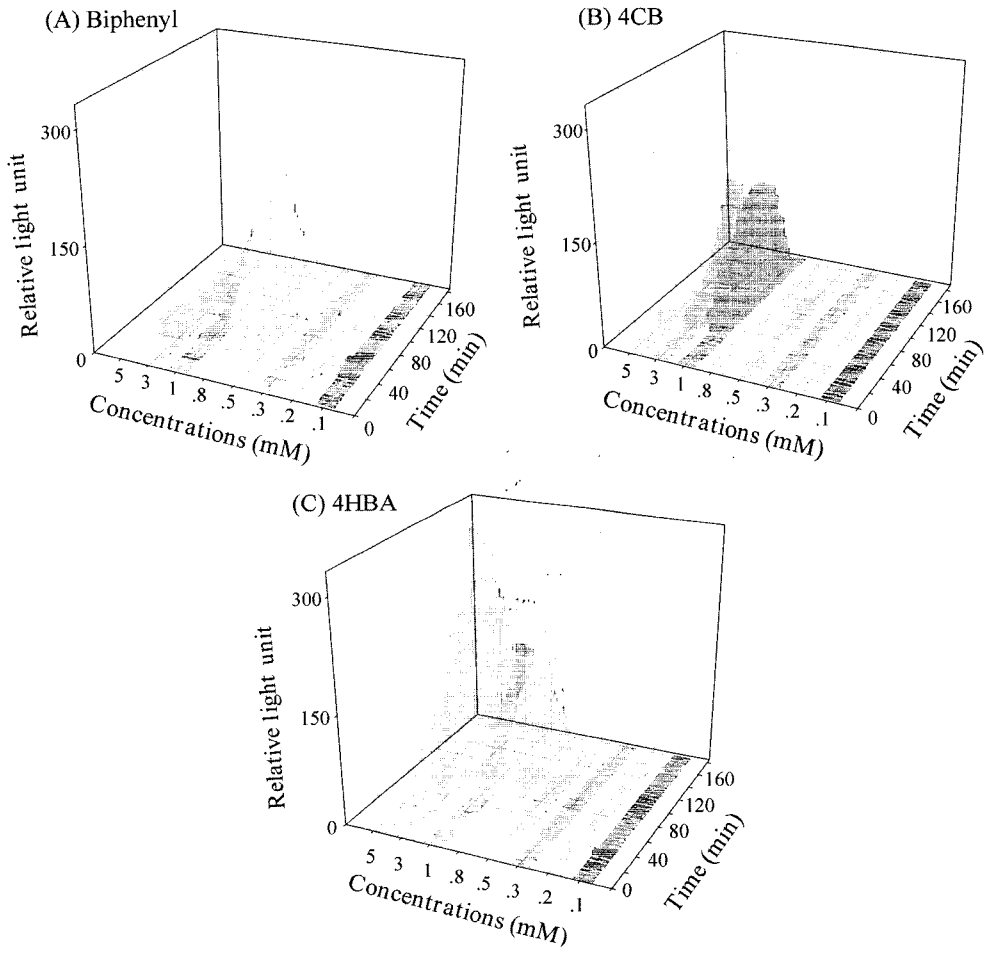

Fig. 6. Light production by the *dnaKp-DJ::luc* fusion when exposed to biphenyl (A), 4CB (B), and 4HBA (C).

promoter from *Pseudomonas* sp. DJ-12 and the *luxCDABE* reporter gene has potential for use in the detection of aromatics, such as 4CB, biphenyl, 4HBA, and other pollutants contaminating the environment.

Acknowledgment

This work was supported by a grant (KRF-2000-015-DP0338) from the Korea Research Foundation. We gratefully acknowledge Marco Bazzicalupo for helpful discussion which was provided by a visiting program (01H020000500) of International Cooperative Research Project between Korea and Italy.

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