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NOTE

Monitoring Expression of *bphC* Gene from *Ralstonia eutropha* H850 Induced by Plant Terpenes in Soil

Kyung-Ja Jung[1], Byung-Hyuk Kim[1], Eungbin Kim[2], Jae-Seong So[3] and Sung-Cheol Koh[1]*

[1]*Division of Civil and Environmental Systems Engineering, Korea Maritime University, Busan 606-791, Korea*
[2]*Department of Biology, Yonsei University, Seoul 120-749, Korea*
[3]*Department of Biological Engineering and Center for Advanced Bioseparation Technology, Inha University, Inchon 402-751, Korea*

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A PCB degrader, *Ralstonia eutropha* H850 was shown to induce *bphC* gene encoding 2,3-dihydroxybiphenyl-1,2-dioxygenase in a carvone-amended pure culture in our previous study (Park *et al.*, 1999). The present study was carried out to examine how plant terpenes, as natural substrates, would cause an expression of a PCB degradative gene in soil that was amended with terpenes. The population of *Ralstonia eutropha* H850 was maintained at least around 10^8 (CFU/g fresh soil) in the soil amended with carvone or limonene in the presence of succinate as a growth substrate at 50 th day. The gene expression was monitored by RT-PCR using total RNA directly extracted from each soil and *bphC* gene primers. The *bphC* gene expression of the seeded strain H850 was observed in the soil amended with biphenyl (4 days) but not with succinate, carvone and limonene. These results indicate that terpenes widely distributed in nature could be a potential inducing substrate for effective PCB biodegration in the soil but their bioavailability and specific induction behavior should be taken into account before PCB bioremediation implementation.

Key words: PCBs, soil, *bphC* gene, terpenes, *Ralstonia eutropha* H850

Polychlorinated biphenyls (PCBs) have been synthesized and used for a long time as solvent extenders, plasticizers, and flame retardants (Abramowicz, 1990). The compounds, however, have such physical and chemical natures as lipophilicity, heat resistance, and relative inertness (stability). This has led to their persistence and bioaccumulation in biota and has caused health and toxicity problems (Boyle *et al.*, 1992; Johnson *et al.*, 1996). Reductive dechlorination of highly chlorinated PCB congeners occurs in an anaerobic condition (Bedard *et al.*, 1998; Kwon *et al.*, 2001). The dechlorination process is, however, slow and does not lead to the ring cleavage of the congeners, rendering them persistent in the environment. Recent studies have shown that some of the aerobic PCB-degraders are able to cleave the ring structure of PCB congeners effectively in the presence of monoterpenes (*e.g.*, limonene, cymene, carvone, pinene and isoprene) known as an inducer for the PCB-degradation pathway (Gilbert and Crowley, 1997; Park *et al.*, 1999; Jung *et al.*, 2001). The PCB degrader, *Rhodococcus* sp. T104, has been shown to induce the degradation pathway by utilizing limonene, cymene, carvone, and pinene as a sole carbon source which are available in the natural environment (Jung *et al.*, 2001). Limonene is listed as one of the most abundant terpenes found in soil and is produced by more than 300 plants (Burdock, 1995). Carvone as a non-growth substrate could also induce a *bphC* gene expression in *Ralstonia eutropha* H850 (previously *Alcaligenes eutrophus* H850; a Gram-negative bacterium) that was confirmed through RT-PCR (Park *et al.*, 1999). Coinoculation of *Arthrobacter* sp. B1B and *Ralstonia eutropha* H850 enhances Aroclor 1242 degradation up to 59% when induced by carvone that was not used as a carbon source for the degraders (Singer *et al.*, 2000). In this study we attempted to elucidate how *Ralstonia eutropha* H850 could induce the PCB-degradative pathway in some soil conditions amended with carvone and limonene.

Carvone and (*S*)-(−)-limonene were purchased from Aldrich Chemical Co. (Milwaukee, WI.). Biphenyl was purchased from Junsei Chemical Co. (Tokyo, Japan). All solvents and other chemicals were reagent grade or better. The PCB degrader used in this study was *Ralstonia eutropha* H850 because it expressed *bphC* gene in the presence

* To whom correspondence should be addressed.
(Tel) 82-51-410-4418; (Fax) 82-51-410-4415
(E-mail) skoh@hanara.kmaritime.ac.kr

of carvone as an inducer (Park *et al.*, 1999). The degrader was routinely maintained on mineral salts medium (MSM) (McCullar *et al.*, 1994). Soil culture system were set up using Erlenmeyer flasks (250ml) carrying grassland soil from Korea Maritime University (Busan, Korea) amended with biphenyl and terpenes [carvone and (*S*)-(-)-limonene]. *Ralstonia eutropha* H850 was then inoculated (Table 1). The systems were incubated at room temperature in the dark. Five grams of soil were taken periodically for analysis of each degrader population density using the viable count technique. To enumerate the H850 population, 1 g of the soil was serially diluted in phosphate buffer (final concentration of 10 mM K_2HPO_4 and 3 mM $NaHPO_4$), and two appropriate dilutions of each sample were plated out onto MSM plates carrying biphenyl crystals. All of the plates were incubated at 28°C for at least 1 week until identification. Total RNA was extracted by the method of Ogram and Ausubel (Ausubel, 1989; Ogram, 1999). Here soil samples were subjected to the two combined procedures: bead mill homogenization (Ogram, 1999) and hot phenol extraction (Ausubel, 1989) for RNA extraction. Duplicates of each soil system (0.5 g) were taken into 2 ml screw-cap plastic vials containing 0.1 mm diameter zirconium/silica beads (BioSpec Product, Bartlesville, OK). Primers for RT-PCR were designed from the conserved regions of *bphC* gene of *Arthrobacter* sp. M5 (P'eloquin and Greer, 1993) as described in our previous paper (Park *et al.*, 1999). The primers were *bphC2* (forward) 5'-CTGCACTGCAACGAACGCCAC-3' and *bphC3* (reverse) 5'-GACACCATGTGGTGGTGGGT-3'. All primers were custom-synthesized by GenoTec, Inc. (Taejon, Korea). RT-PCR was performed according to the method previously described (Park *et al.*, 1999). One to 3.3 of the total RNA was used as a template.

We showed in a previous report (Park *et al.*, 1999) that a successful amplification (182bp) of mRNA transcribed from *bphC* gene was made in *Ralstonia eutropha* H850 cells, which were grown on biphenyl or induced by carvone. Here in this study soil systems were set up to verify the *bphC* gene expression induced by terpenes (e.g., carvone and limonene) in a sterilized soil system.

The population of strain H850 was maintained at at least 10^8 (CFU/g fresh soil) in all treating conditions even after 50 days (Fig. 1). Since these terpenes were not utilized as the sole carbon source by H850, succinate was provided as a non-inducing growth substrate. After 50 days the amendment of limonene and succinate appeared to give the highest population density (more than 10^9 CFU/g fresh soil). The soil amended with carvone and succinate seemed to show a lower population density (about 10^8 CFU/g fresh soil). Except for the soil amended with biphenyl, the growth of strain H850 population in other soils appeared to be dependent upon the succinate (0.1% w/w) that was readily available in a soluble form. Carvone and limonene were not growth substrates for

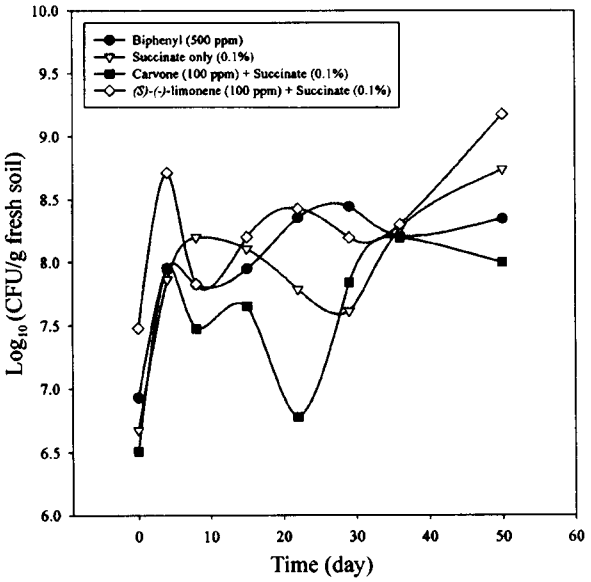

Fig. 1. Population dynamics of the viable *Ralstonia eutropha* H850 in soils amended with biphenyl, succinate, carvone + succinate and limonene + succinate (See Table 1 for detail). Medium used for the viable counting was mineral salts agar plates with biphenyl crystals.

strain H850 but they turned out to be an effective inducer for the degradative pathway of PCB (e.g., 4.4'-DCBp) (Park *et al.*, 1999; unpublished data from the corresponding author). On the other hand, terpenes seemed to be rather toxic to Gram-negative bacteria such as *Ralstonia eutropha* H850. H850 was not able to grow on *p*-cymene, isoprene, terpinene, pinene or abietic acid (each 100 ppm) as a sole carbon source (corresponding authors unpublished data). Gram-positive organisms, however, seemed to be rather tolerant to terpenes, enabling the organisms to utilize them as a carbon source or an inducer for a certain metabolic activity in higher concentrations of terpenes (Hernandez *et al.*, 1997; Van der Werf and Bont, 1999). Moreover, the degree of their tolerances appeared to be strain-specific in some species of Gram-positive organisms such as *Arthrobacter* sp. B1B whose growth was inhibited at the concentration of 100 to 450 mg/l of carvone (Gilbert and Crowley, 1997). In contrast, *Rhodococcus* sp. T104 was able to utilize limonene (300 mg/l) as a sole carbon source (Jung *et al.*, 2001).

The *bphC* gene expression was observed in the soil amended with biphenyl from 4 days after inoculation but was not observed at the 25th day (Fig. 2). This indicates the *bphC* gene could be induced by biphenyl, but the gene expression was not maintained in the soil environment after 25 days. The *bphC* gene expression was not observed in the soil system amended with carvone or limonene even after 4 days. The reason was not clear but it was assumed that the bioavailability (hence induction effect) of carvone and limonene was lower than that of biphenyl due to a

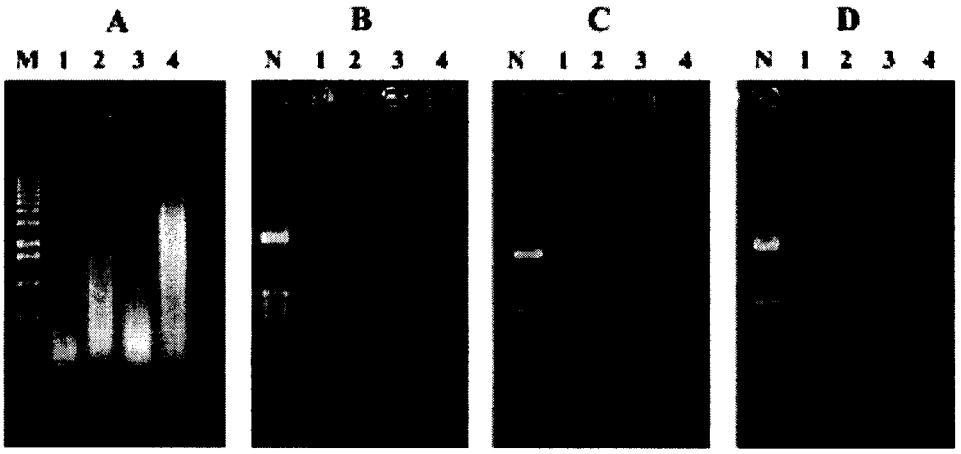

Fig. 2. Pattern of total RNA directly extracted from soils amended with terpenes and inoculated with *Ralstonia eutropha* H850 (A) and the results of RT-PCR in time series (B, 4days: C, 25days; D, 50days). Lanes: 1, biphenyl; 2, succinate; 3, carvone + succinate; 4, limonene + succinate; M, DNA 1kb ladder; N, DNA 50bp ladder.

Table 1. Scheme of soil systems for the test of PCB-degradative gene expression of *Ralstonia eutropha* H850 induced by plant terpenes

Soil system[1]	Soil amount	Growth substrate	Inducer	Inoculum[2]
1	200 g	biphenyl (500 ppm)	-	20 ml
2	200 g	succinate (0.1% w/w)	-	20 ml
3	200 g	succinate (0.1% w/w)	carvone (100 ppm)	20 ml
4	200 g	succinate (0.1% w/w)	limonene (100 ppm)	20 ml

[1]Prepared in duplicate. The final moisture content of each soil was adjusted to 25%
[2]Stationary phase culture of *Ralstonia eutropha* H850 grown on biphenyl (10^8 CFU/ml). The biphenyl in the culture was depleted at the time of inoculation.

potential interaction of these terpenes with the soil matrix (e.g., soil organic matter) or their greater volatility compared with in liquid system. The repeated amendment of a growth substrate and actively induced cells would make a difference in maintaining the PCB degradation activity. Bioavailability of growth substrate, inducer and target PCBs in the soil matrix may be also a critical parameter involved in enhancing PCB biodegradation. Previous reports (Gilbert and Crowley, 1998; Singer *et al.*, 2000) have shown that an effective degradation of Aroclor 1242 by PCB degraders was accomplished by repeated addition of carvone as an inducer, and sorbitan trioleate as both surfactant and growth substrate. Since the strain H850 can not utilize carvone or other terpenes as a growth substrate, it will be worth while to employ *Rhodococcus* sp. strain T104 as a biodegradative agent that is able to utilize (*S*)-(-)-limonene, *p*-cymene, and α-terpinene both as growth substrates and inducers for a PCB-degradative pathway (Jung *et al.*, 2001). Moreover monitoring of the PCB degradative gene expression could be greatly facilitated by construction of a bioluminescent reporter system in the degrader whole cells (Layton *et al.*, 1998; Park *et al.*, 2000). Along with an appropriate degrader selection, employment of an effective surfactant (or biosurfactant-producing organism) will be critical for successful PCB bioremediation in soil system contaminated with PCBs.

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