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# Induction by Carvone of the Polychlorinated Biphenyl (PCB)-Degradative Pathway in *Alcaligenes eutrophus* H850 and Its Molecular Monitoring

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**Abstract** There is a possibility that carvone, a monoterpene from spearmint (*Mentha spicata*), could induce the *bph* degradative pathway and genes in *Alcaligenes eutrophus* H850, which is a known Gram-negative PCB degrader with a broad substrate specificity that was thoroughly investigated with *Arthrobacter* sp. B1B, a Gram-positive PCB degrader. The strains B1B and H850 were unable to utilize and grow on the plant terpene [(*R*)-(-)-carvone] (50 ppm) to be recognized as a sole carbon source. Nevertheless, the carvone did induce 2,3-dihydroxybiphenyl 1,2-dioxygenase (encoded by *bphC*) in the strain B1B, as observed by a resting cell assay that monitors accumulation of a yellow *meta* ring fission product from 4,4'-dichlorobiphenyl (DCBp). The monoterpene, however, did not appear to induce the *meta* cleavage pathway in the strain H850. Instead, an assumption was made that the strain might be using an alternative pathway, probably the *ortho*-cleavage pathway. A reverse transcription (RT)-PCR system, utilizing primers designed from a conserved region of the *bphC* gene of *Arthrobacter* sp. M5, was employed to verify the occurrence of the alternative pathway. A successful amplification (182 bp) of mRNA transcribed from the N-terminal region of the *bphC* gene was accomplished in H850 cells induced by carvone (50 ppm) as well as in biphenyl-growth cells. It is, therefore, likely that H850 possesses a specific PCB degradation pathway and hence a different substrate specificity compared with B1B. This study will contribute to an elucidation of the dynamic aspects of PCB bioremediation in terms of roles played by PCB degraders and plant terpenes as natural inducer substrates that are ubiquitous and environmentally compatible.

**Key words:** Carvone, PCB, *bph* genes, RT-PCR, bioremediation

Polychlorinated biphenyls (PCBs) have been widely used for more than half a century for various industrial purposes such as dielectric fluids solvent extenders and flame retardants. Unfortunately, they are strictly regulated because of its potential toxicity (e.g., mutagenicity, endocrine disruption, etc.). They are generally recalcitrant, but decontamination may be possible through physicochemical treatment technology (e.g. combustion and photolysis); however, this can possibly generate other wastes and is very costly. On the other hand, treatment of PCBs may also be possible at a relatively lower cost through a bioremediation process utilizing PCB-degraders [4].

Reductive dechlorination of PCBs in anaerobic condition can reduce their toxicity while making the compounds more vulnerable to aerobic biodegradation in which a ring cleavage reaction occurs, and subsequently generates chlorobenzoate and chlorinated aliphatics that are also biodegradable under an aerobic condition [6, 26]. Biphenyl has usually been used as a growth substrate (C-source) to isolate and grow bacteria that degrades PCB congeners [3], and to enhance their biodegradation in soil [11] and sediment. [14]. Biphenyl, however, is not a normal component of soil from which biphenyl/PCB-degrading bacteria are routinely isolated. It is, therefore, assumed that the biphenyl/PCB metabolic pathway is involved in attacking natural substrates (e.g. plant terpenes such as carvone) having structural similarity to biphenyl [15] (Fig. 1). This assumption has been confirmed in some reports showing that certain plant origin compounds or root extracts could serve as substrates for induction of the biphenyl/PCB-degradative pathway [8, 9, 15]. Carvone, a chemical component of spearmint but not a carbon source, could successfully induce the PCB degradative pathway in the Gram-positive bacteria *Arthrobacter* sp. B1B [12]. Limonene is also the most abundant terpene found on earth and it is produced by more than 300 plants [7]. Carvone has been suggested as a monooxgenation product of the limonene [27], indicating the possibility of utilizing limonene as a potential co-substrate in PCB bioremediation. To the best of our knowledge, there has been no report regarding an induction by pure terpenes of PCB-degradative pathway in Gram-negative bacteria.

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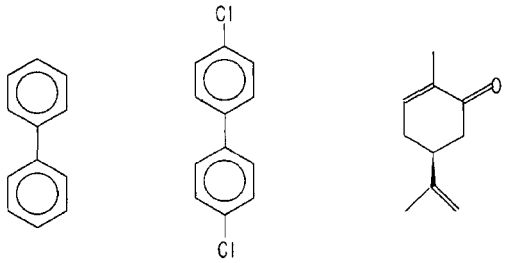


**Fig. 1.** Structural similarity of a plant terpene (carvone) to biphenyl and 4,4'-dichlorobiphenyl.

Rapid and specific monitoring of *in situ* gene expression has been made possible by mRNA extraction and quantification during the microbiological processes such as naphthalene metabolism in soil and groundwater [28] and mercury volatilization in freshwater [16].

This study was undertaken to determine whether or not carvone could induce the *bph* degradative pathway and genes in a well-characterized PCB degrader, *Alcaligenes eutrophus* H850 [3], which is a Gram-negative bacterium. The results showed that carvone had a possibility of inducing the 4,4'-DCBp-degradative pathway in the strain H850, and the pathway induction was successfully monitored by reverse-transcription PCR (RT-PCR) utilizing *bphC* primers originated from a Gram-positive bacteria *Arthrobacter* sp. M5.

## MATERIALS AND METHODS

### Bacterial Strains, Culture Maintenance, and Growth Conditions

Bacteria used in the study were *A. eutrophus* H850 [3], *Arthrobacter* sp. B1B [12], *Corynebacterium* sp. T104 [15], and *Pseudomonas* sp. LB400 [5]. All PCB-degraders were maintained on mineral salts agar by using inverted Petri plates with biphenyl crystals placed on the lid. The mineral salts medium (MSM) [18] consisted of 10 mM $K_2HPO_4$, 5 mM $(NH_4)_2SO_4$, 3 mM $NaH_2PO_4$, 1 mM $MgSO_4$, and 10 ml of chloride-free trace element stock solution, which contained the following (in milligrams $l^{-1}$): $CaSO_4$, 200; $FeSO_4 \cdot 7H_2O$, 200; $MnSO_4 \cdot H_2O$, 20; $NaMoO_4 \cdot 2H_2O$, 10; $CuSO_4$, 20; $CoSO_4 7H_2O$, 10; and $H_3BO_3$, 5. Then, the liquid cultures were grown in flasks on a rotary shaker at 200 rpm and 26°C.

### Terpene (Carvone) Utilization

The cultures tested were *Arthrobacter* sp. B1B, *Pseudomonas* sp. LB400, *Corynebacterium* sp. T104, and *A. eutrophus* H850. The experiment was based upon a protocol modified from the method by Gilbert and Crowley [12]. Each culture was inoculated into an Erlenmeyer flask containing 100 ml of mineral salts medium with *R-(-)*-carvone (Sigma-Aldrich; 50, 150 ppm; Fig. 1) as a sole carbon source, and then incubated at 26°C and 200 rpm. Cultural growth was monitored by measuring absorbance level at 525 nm using a spectrophotometer (Jasco, model V-550, Tokyo, Japan).

### Carvone Induction of PCB-Degradative Pathway

Unless otherwise stated, carvone was utilized as a sole carbon source, and the induction of the PCB-degradative pathway was performed by adding carvone (50 ppm) into MSM containing fructose or succinate (0.1% w/v) as a carbon source. Here, two representative PCB degraders were tested; *Arthrobacter* sp. B1B and *A. eutrophus* H850. The carbon sources such as biphenyl (150 ppm) and fructose (or succinate) were used as positive and negative controls, respectively.

### Resting Cell Assay of PCB Co-Metabolism

To assay induction of the ring fission activity, cells were grown until the mid- to late-log phase, filtered through the glass wool to trap substrate residues, and then washed twice and resuspended in a 50 mM phosphate buffer. The resting cell suspensions (30 ml; adjusted to 1.5 at 525 nm) were aliquoted into 100-ml Erlenmeyer flasks, and then the stock solution of 4,4'-dichlorobiphenyl (DCBp) (Allied Signal, Inc., Seelze, Germanay) prepared in hexane was added up to 5-100 μM as final concentrations. The accumulation of *meta* ring fission product of 4,4'-DCBp was monitored spectrophotometrically at 434 nm, which is a characteristic wavelength of *para*-substituted chlorobiphenyls [22]. 4,4'-DCBp is one of the *para*-substituted congeners which are the most vulnerable in being attacked by *Rhodococcus* sp., while *A. eutrophus* H850 and *Pseudomonas* sp. LB400 prefer 2,5-substitution patterns [26].

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for *bphC* Gene

The total RNA purification for RT-PCR was performed according to the method discribed by Ausubel *et al.* [2] with a slight modification. Cells grown by following the induction scheme were washed once with an ice-cold AE buffer (20 mM sodium acetate, pH 5.5 and 1 mM disodium EDTA) and resuspended in 250 μl of AE. Then, 30 μl of 10% SDS, 200 μl of AE buffer, and 1 ml phenol were added and then mixed. The reaction mixture was incubated at 60°C for 15 min with a brief vortexing every 5 min. The emulsion was chilled on ice at 0-4°C and centrifuged at 4°C for 15 min. Aqueous phases were taken and 3 M of the sodium acetate (pH 5.2) was added up to 0.25 M. The solution was then extracted with phenol-chloroform (25:24) 3 times and was ethanol-precipitated. The dried

pellet was resuspended in TM buffer (40 mM Tris-HCl, pH 7.5; 6 mM $MgCl_2$) according to DNase (Promega) and this was completed by the manufacturer's protocol. Concentration of the RNA was measured by the spectrophotometric method as described in Ausubel *et al.* [2].

The primers used in the PCR were designed from a conserved region of the chromosomally located *bphC* gene which encoded 2,3-dihydroxybiphenyl dioxygenase of *Arthrobacter* sp. M5, a Gram-positive bacterium. This region turned out to be homologous to the equivalent gene of *Pseudomonas pseudoalcaligenes* KF707 [25]. Forward and reverse primers were designed from the gene fragment (182 bp) of the N-terminal sequence, and their sequences were 5'-CTGCACTGCAACGAACGCCAC-3' (primer 1) and 5'-GACACCATGTGGTGGTTGGT-3' (primer 2), which were custom-synthesized by GenoTec, Inc. (Taejon, Korea). One to 2 μg of the total RNA was used as a template. For a positive control, the *bphC* gene, carried on pKTF18 which was cloned from *P. pseudoalcaligenes* KF707 [25], was also employed. Each reaction mixture (50 μl) contained the following reagents: 5 μl of *Taq* DNA polymerase. 10× buffer. DTT (10 mM), $MgCl_2$ (1.5 mM), dNTPs (250 μM, each), RNasin (10 U) (Promega), Primer 1 (310 nM), Primer 2 (310 nM), AMV-RT (15 U) (Promega), and *Taq* polymerase (1.3 U) (Promega). RT-PCR was performed in a DNA thermocycler (Perkin Elmer model; GeneAmp PCR System 2400). Reverse transcription (RT) and the subsequent DNA amplification were conducted in the same tube. The RT thermal condition was 65°C, 10 min for RNA denaturation and 50°C, and 8 min for reverse transcription. The PCR conditions were denaturation (94°C, 5 min), 25 cycles of the standard PCR (94°C, 1 min; 60°C, 30 sec, 72°C, 30 sec), and a final chase reaction of (72, 7 min).

## RESULTS AND DISCUSSION

### Growth of PCB Degraders on Biphenyl and Carvone

The growth responses of PCB degraders on biphenyl and plant terpene (*(R)-(-)*-carvone) were tested before initiating the experiment for their PCB degradative pathway induced by carvone (Fig. 2). The growth was monitored daily at 525 nm as described by McCullar *et al.* [18]. First, we tested the growth on biphenyl (150 ppm) of the representative PCB degraders such as *A. eutrophus* H850, *Pseudomonas* sp. LB400, *Arthrobacter* sp. B1B, and *Corynebacterium* sp. T104 (Fig. 2A). The strains of H850 and T104 showed relatively reasonable growth and reached more than 1.0 O.D. within 5 days, while strains B1B and LB400 demonstrated somewhat slower growth. In this particular experiment, LB400 had a rather long lag phase. Biomasses of all strains, enough for the resting cell assay, were routinely obtained within one week. There was no growth observed for these biphenyl/PCB degraders when *(R)-(-)*-carvone (50 ppm) replaced biphenyl as a sole carbon source (Fig. 2B). This indicated that carvone could not be utilized as a carbon source by the degraders; but, it is important to mention that it could be an inducer. The strain B1B did not grow on higher concentration of carvone (100–450 ppm) either [12].

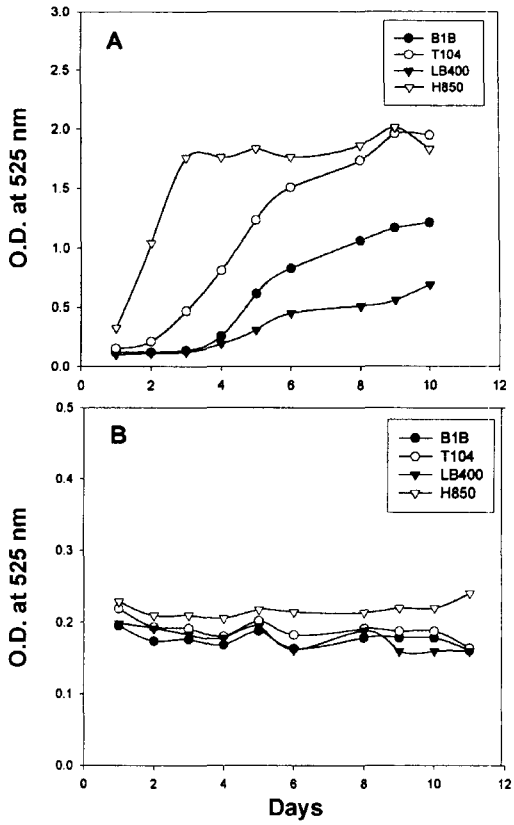


**Fig. 2.** Growth of the representative Gram-positive and Gram-negative PCB degraders, *Arthrobacter* sp. B1B, *Corynebacterium* sp. T104, *Pseudomonas* sp. LB400, and *Alcaligenes eutrophus* H850 on biphenyl (150 ppm; panel A) and carvone (50 ppm; panel B).

Microbial utilization of terpenes, however, did occur, and appeared to be strain-specific and more prevalent in a Gram-positive bacteria such as *Rhodococcus* sp. [15, 27]. Cymene and limonene were shown to be both substrate and inducer for the PCB-degradative pathway in several Gram-positive environmental isolates (*Rhodococcus* sp., *Corynebacterium*, and *Cellulomonas* sp.) [15]. This indicated that the degraders should be provided with a supplementary carbon source for their growth, therefore, fructose and succinate were chosen in this study as growth substrates for B1B and H850.

### Cometabolism of 4,4'-DCBp by the Resting Cells of B1B and H850

Since carvone was not utilized as a carbon source by the strains B1B and H850, cells used for the resting cell assay

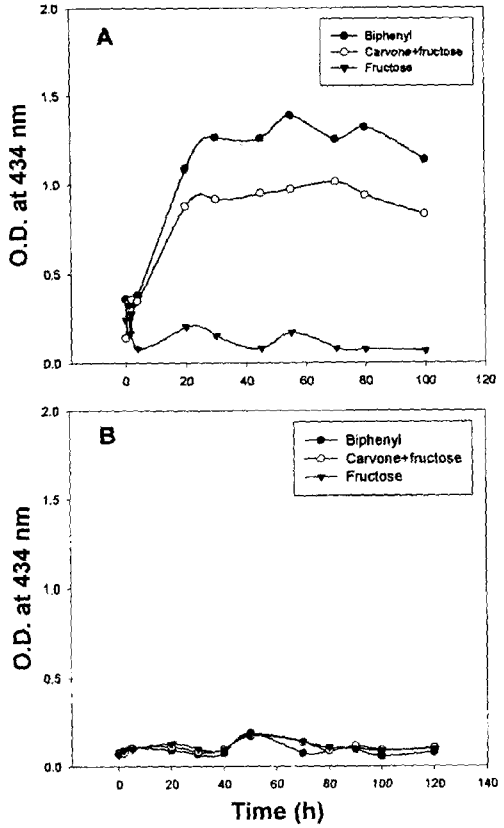


**Fig. 3.** Time course of accumulation of *meta* ring fission product from 4,4'-DCBp by the resting cells of *Arthrobacter* sp. B1B (panel A) and *Alcaligenes eutrophus* H850 (panel B) grown on biphenyl (150 ppm), carvone (50 ppm) + fructose (0.1%, w/v), and fructose only (0.1%, w/v).

were prepared by initiating growth on fructose (B1B), and fructose or succinate (H850) in the presence of carvone as an inducer. B1B could not utilize succinate as a sole carbon source. The results of co-metabolism of 4,4'-DCBp by resting cells of B1B and H850 are shown in Fig. 3. 4,4'-DCBp (100 μM as a final concentration) was added to the B1B cells that was washed twice with phosphate buffer and adjusted to 1.5 O.D (at 525 nm) in density. The accumulation of 4,4'-DCBp *meta* ring cleavage product in the cell supernatant was monitored spectrophotometrically at 434 nm, which was predicted to be a characteristic of *para*-substituted chlorobiphenyls [22]. The cleavage product was 2-hydroxy-4-chloro-6-oxo-6-(4'-chlorophenyl)hexa-2,4-dienoic acid based upon the report by Ahmad *et al.* [1]. The formation of the yellow ring fission product was clear in B1B cells grown in the presence of carvone as well as in biphenyl as a positive control (Fig. 3A). The degree of maximum accumulation of the product in biphenyl growth condition was as much as 27% higher than in carvone induction. When B1B cells were grown only on fructose, they produced little amount of the yellow ring cleavage product, with a light pink color observed at the relatively

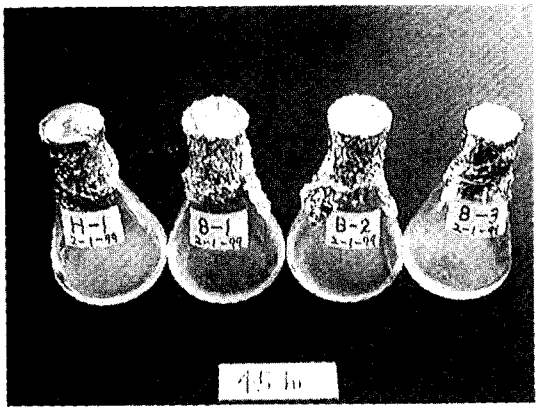


**Fig. 4.** Visualization of cometabolic products by the resting cell assays (at 45 h) described in Fig. 3. H850 grown on biphenyl (150 ppm) (H-1); B1B on biphenyl (150 ppm) (B-1); B1B on carvone (50 ppm) + fructose (0.1%, w/v) (B-2); B1B on fructose only (0.1%, w/v) (B-3). Note the yellow color in B-1, B-2, and a light pink color in H-1 and B-3.

later time during the incubation (B-3, Fig. 4). This suggested an induction of the PCB-degradative pathway (at least the first three steps encoded by *bphA, bphB, bphC* genes, respectively) by carvone. The metabolic products produced by the resting cell assay (after 45 h) were also visualized (Fig. 4). Here, biphenyl-grown cells of B1B (B-1, Fig. 4) and its carvone-induced cells (B-2, Fig. 4) readily co-metabolized 4,4'-DCBp and its yellow ring fission product was seen in just 1 h. This is in good agreement with the previous report that carvone and limonene were inducers in B1B, equivalent to biphenyl [12]. The resting cell assay for H850 was also completed in a similar fashion with an exception that the concentration of 4,4'-DCBp was 5 μM. The substrate concentration was determined based on a previous report [3] that the degradation of 4,4'-DCBp by H850 occurred up to 60% at 5 μM while the rate dropped to 0% at 25 μM or higher. As observed in Fig. 3B, H850 did not appear to produce the *meta* cleavage product in all three induction conditions. Instead, a light pink colored compound was observed in those induction conditions, and this was visible after 35 h incubation. This may suggest the presence of the *cis-, cis*-muconate radical, typically resulting from the *ortho* cleavage pathway in a mono aromatic compound degradation [24].

### Visual Identification of Degradation Products of Methylcatechols from *Alcaligenes eutrophus* H850

Since H850 could not accumulate significant amounts of *meta* cleavage product from 4,4'-DCBp (5 μM), we tried to demonstrate with higher concentration (5 mM) of methylcatechols. To accomplish this, H850 was streaked and grown on MSM plates with biphenyl on the lid until distinctive colonies were shown. Then, monoaromatic substrates, 4-methylcatechol and 3-methylcatechol (0.5- 1 ml; 20 mM dissolved in acetone), were applied to the

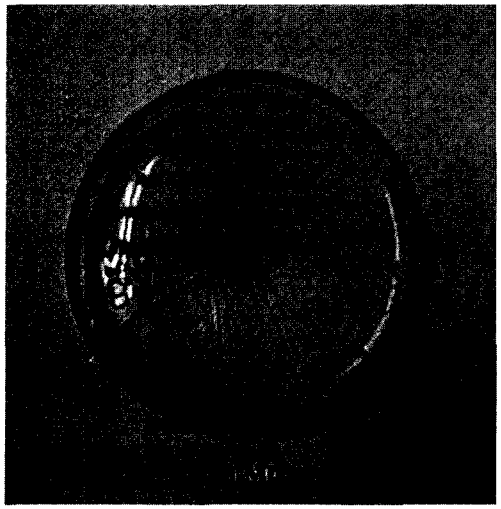

**Fig. 5.** Transformation of 3-methyl catechol by *Alcaligenes eutrophus* H850 to generate yellow color ("1") and pink color ("2") compounds, putative *meta*- and *ortho*-cleavage products of the catechol, respectively. H850 was grown on a MSM plate under biphenyl vapour for several days and flooded with 3-methyl catechol (20 mM). Formation of yellow color was seen in minutes and preceded that of the pink one.

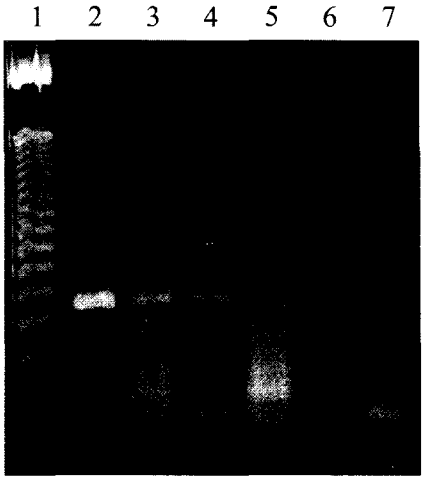


**Fig. 6.** Monitoring *bphC* gene expression through RT-PCR in *Alcaligenes eutrophus* H850.
Lane 1, 50 bp DNA ladder; lane 2, PCR product from the template pKTF18 carrying *bphC* gene of *Pseudomonas pseudoalcaligenes* KF707 [25]. The subsequent lanes show RT-PCR products (182 bp) from the total RNA template prepared from cells grown on biphenyl (150 ppm) (lane 3), carvone (50 ppm) + fructose (0.1%, w/v) (lane 4), fructose (0.1%, w/v) only (lane 5), and succinate (0.1%) only (lane 6). A negative control (lane 7): RNA template of lane 4 was treated with RNase and amplified at the same conditions.

colonies of H850. After an hour of incubation at room temperature, the colors of the halo around colonies were observed. A yellow halo zone appeared in a few minutes while a pink halo zone appeared later and became stronger in color density, compared to the yellow halo in a longer incubation period (Fig. 5). Therefore, it was assumed that yellow and pink halo zones might have resulted from induction of the *meta* and *ortho* cleavage pathways, respectively, with the *ortho* pathway being much preferred. Presence of both pathways are common in *Pseudomonas* strains capable of degrading catechols [20].

### Monitoring *bphC* Gene Expression through RT-PCR

It was clear from the above experiments that *A. eutrophus* H850 could potentially induce the *ortho* cleavage pathway in degradation of 4,4'-DCBp at a lower concentration level (5 μM). In order to demonstrate the occurrence of the degradation pathway, a reverse transcription PCR system was employed utilizing primers designed from a conserved region of *bphC* genes of *Arthrobacter* sp. M5 [21] and *P. pseudoalcaligenes* KF707 [25]. H850 cells grown under these four different induction conditions were subjected to both total RNA extraction and RT-PCR. A successful amplification (182 bp) of mRNA transcribed *bphC* gene was made in H850 cells, which were grown on biphenyl or induced by carvone. The degree of induction by biphenyl was higher than by carvone (Fig. 6). Fructose also induced the transcript, but this was not surprising because *Arthrobacter* sp. B1B cells grown on fructose only were observed to degrade 4,4'-DCBP up to 40% [12]. The *bphC* gene amplification was authentic because the corresponding primer regions of the *bphC* gene in *A. eutrophus* A5 [19] had a high DNA sequence homology with those of H850 and KF707 (e.g., 95.2% and 85% for forward and reverse primers, respectively, based on a BLAST search for H850).

Monitoring techniques that target markers such as short-lived metabolites and mRNA of *in situ* metabolism may offer a possibility of measuring a real-time metabolic activity. The metabolic examples would be stress proteins [17] and semialdehyde [23]. In this study, the formation of metabolites such as *meta* and *ortho* ring cleavage products of 4,4'-DCBp was compared with mRNA transcription of the relevant gene, *bphC* in *A. eutrophus* H850. Here, a putative *ortho* ring cleavage product was monitored spectrophotometrically (data not shown) and the concomittant expression of the *bphC* was successfully tracked by RT-PCR in carvone-induced cells of H850. Since mRNA of prokaryotic cells is short-lived (e.g., a few minutes), its presence may be linked to an on-going metabolic activity in the cells. Recently, naphthalene dioxygenase mRNA transcripts from groundwater samples contaminated with coal tar wastes were successfully monitored by RT-PCR technology and characterized on a real-time basis [28]. Quantity of naphthalene dioxygenase mRNA transcripts was well correlated with mineralization rate and concentration in soils polluted with PAH including naphthalene [10].

It is likely that the *bphA* gene can also be induced by carvone because the *bph* genes (*bphA, bphB,* and *bphC*)

are linked as an operon as shown in *P. pseudoalcaligenes* KF707 [25]. Furthermore, this assumption may support a hypothesis that carvone can also be an inducer for the *bphA* gene encoding for the dioxygenase involved in the first dioxygenation of biphenyl and PCB. Gilbert and Crowley [12] reported that there was no statistical difference between biphenyl and carvone in the induction of the *meta* cleavage pathway of *Arthrobacter* sp. B1B. This indicated that an induction of the PCB-degradative pathway by carvones could strongly depend upon the species (or strains) involved. Catechol 2,3-dioxygenase was also reported to be strain-specific [13]. In particular, Gram-positive organisms appear to be resilient to various terpenes and this allows the organisms to utilize them as a sole carbon source [15, 27]. However, there has yet been no report on induction of a PCB-degradative pathway by carvone in Gram-negative organisms which seemed to be readily affected by the toxicity of terpenes (personal communication from Dr. Crowley, University of California, Reverside).

The discovery that carvone can induce PCB co-metabolism in *A. eutrophus* H850 as a representative Gram-negative degrader indicates the importance of Gram-negative bacteria to utilize plant terpenes as natural substrates *in situ* PCB bioremediation, replacing biphenyl which is a synthetic carcinogenic chemical banned for environmental uses. We believe that this study will contribute to elucidation of dynamic aspects of PCB biodegradation and bioremediation to utilize plant terpenes as natural inducer substrates that are ubiquitous and environmentally compatible.

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