APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1997, p. 1933–1938 Vol. 63, No. 5
0099-2240/97/$04.00+0

Plant Compounds That Induce Polychlorinated Biphenyl Biodegradation by *Arthrobacter* sp. Strain B1B

ERIC S. GILBERT,[1] AND DAVID E. CROWLEY[2]*

Environmental Toxicology Graduate Program,[1] and Department of Soil and Environmental Sciences,[2] University of California, Riverside, California 92521

Received 7 November 1996/Accepted 10 March 1997

Plant compounds that induced *Arthrobacter* sp. strain B1B to cometabolize polychlorinated biphenyls (PCBs) were identified by a screening assay based on the formation of a 4,4′-dichlorobiphenyl ring fission product. A chemical component of spearmint (*Mentha spicata*), *l*-carvone, induced *Arthrobacter* sp. strain B1B to cometabolize Aroclor 1242, resulting in significant degradation of 26 peaks in the mixture, including selected tetra- and pentachlorobiphenyls. Evidence for PCB biodegradation included peak disappearance, formation of a phenylhexadienoate ring fission product, and chlorobenzoate accumulation in the culture supernatant. Carvone was not utilized as a growth substrate and was toxic at concentrations of greater than 500 mg liter^{-1}. Several compounds structurally related to *l*-carvone, including limonene, *p*-cymene, and isoprene, also induced cometabolism of PCBs by *Arthrobacer* sp. strain B1B. A structure-activity analysis showed that chemicals with an unsaturated *p*-menthane structural motif promoted the strongest cometabolism activity. These data suggest that certain plant-derived terpenoids may be useful for promoting enhanced rates of PCB biodegradation by soil bacteria.

Bioaugmentation of soil with bacteria that degrade polychlorinated biphenyls (PCBs) during growth on biphenyl has been studied as a possible strategy for cleanup of PCB-contaminated soils. A large number of biphenyl-utilizing isolates with different degradation capabilities have been identified (1, 2, 13, 14, 24, 27, 29) and shown to promote PCB biodegradation. Unfortunately, use of these microorganisms for bioremediation has been hindered by the inability to use biphenyl as a soil amendment, due to its adverse health effects (19), cost (25), and low water solubility. Several approaches have been investigated to circumvent problems with the use of biphenyl-utilizing bacteria, including the development of field application vectors to support the growth of genetically engineered bacteria that constitutively express the *bph* genes (18). Another approach has been to clone the *bph* genes into a rhizosphere-colonizing pseudomonad that degrades 4-chlorobiphenyl in association with plant roots (4).

Recently, it has been speculated that certain plant compounds or root exudates may serve as natural substrates for induction of the *bph* genes, including flavonoids (10), lignin (12, 16), and terpenes (11). Identification of natural compounds that could be used in lieu of biphenyl would expedite the development of new approaches for bioremediation of contaminated soils. In this research, we used a colorimetric assay to identify plant compounds that promote PCB biodegradation by *Arthrobacter* sp. strain B1B, a known PCB-degrading bacterium (17). After screening several types of plants, we discovered that an extract prepared from spearmint (*Mentha spicata*) was effective in inducing *Arthrobacter* sp. strain B1B to degrade PCB congeners in Aroclor 1242. Further studies were conducted to identify the chemical component of spearmint that promoted PCB biodegradation and to identify the molecular structural properties responsible for this effect.

* Corresponding author. Mailing address: Department of Soil and Environmental Sciences, University of California, Riverside, CA 92521. Phone: (909) 787-3785. Fax: (909) 787-3993. E-mail: Crowley@mail.ucr.edu.

MATERIALS AND METHODS

Culture conditions. *Arthrobacter* sp. strain B1B was maintained on minimal salts agar by using inverted petri plates with biphenyl crystals placed in the lid. The minimal salts medium 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 liter^{-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; $CoSO_4 \cdot 7H_2O$, 10; and H_3BO_3, 5. Liquid cultures were grown in flasks on a rotary shaker at 230 rpm.

Chemicals. *L*-Carvone, *s*-carvone, *s*-limonene, biphenyl, carvacrol, thymol, and cumene were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). PCBs, including 4,4′-dichlorobiphenyl (4,4′-DCBp), Aroclor 1242, and 2,2′,4,5,5′-pentachlorobiphenyl (2,2′,4,5,5′-PCBp), were obtained from Accustandard, Inc. (New Haven, Conn.). Chlorobenzoate standards, (*R*)-limonene, *p*-cymene, and sodium lauryl sulfate (SDS) were purchased from Sigma (St. Louis, Mo.). 2-Chlorobiphenyl (2CBp) and 4,4′-DCBp were purchased from Pfaltz and Bauer (Waterbury, Conn.). Isoprene was purchased from Acros. All solvents were reagent grade or better.

Ring fission product formation assay. Plants, leaf litter, and compost were screened for their abilities to induce PCB cometabolism in *Arthrobacter* sp. strain B1B by measuring the rate of phenylhexadienoate ring fission product formation in response to the addition of 4,4′-DCBp after growth on these substrates in mineral salts medium. Plant materials either were grown in garden plots or were purchased commercially. Leaf litter was collected from an avocado orchard located on the campus of the University of California, Riverside, Calif. Compost was derived from plant material produced in an organic garden located in the city of Riverside, Calif. Fresh roots or shoots of intact plants were liquified with a juice extractor (Hamilton Beach, Washington, N.C.). The extracts were filtered through glass wool and stored frozen in polypropylene bottles. Soluble carbon concentrations of the extracts were determined by the method of Von Wiren et al. (30). Extracts were diluted with minimal salts medium to a final concentration of 500 mg of soluble carbon liter^{-1}. Flasks containing 100 ml of autoclaved plant extract medium were inoculated with *Arthrobacter* sp. strain B1B and cultured as described above. Medium prepared with avocado leaf litter or compost was prepared by twice autoclaving minimal salts medium containing 10% (wt/vol) slurries of these materials.

To assay induction of ring fission activity, cells were grown until mid- to late-log phase, filtered through glass wool to trap any plant residues, washed once, and resuspended in 50 mM phosphate buffer. Resting cell suspensions (optical density at 525 nm of 1.5) were distributed into 20-ml glass vials, and 4,4′-DCBp (5 mg ml^{-1} in hexane) was added to a final concentration of 15 μg ml^{-1}. The accumulation of 4,4′-DCBp ring fission product in the culture supernatant was monitored spectrophotometrically at 434 nm, which is a wavelength characteristic of *para*-substituted chlorobiphenyls (26). Rates of ring fission product formation were converted to units of nanograms of product per milligram of protein per hour by the extinction coefficient for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, the *meta* cleavage ring fission product of biphenyl (6). All

analyses were carried out at least in triplicate, and the experiments were repeated twice.

Resting cell suspensions prepared from cells grown in leaf litter or compost medium were filtered through glass wool and centrifuged at $116 \times g$ for 15 min to remove coarse particulates. The supernatant was decanted and centrifuged at $11,700 \times g$ for 10 min, and the resultant pellet was resuspended in 50 mM phosphate buffer. The resting cell suspensions were distributed in 4-ml aliquots to 20-ml glass vials, and 4,4′-DCBp was added as described above. Cell suspensions without added PCB were used as reference blanks for the spectrophotometric analyses of ring fission product formation.

Individual chemical compounds were also screened for their abilities to induce PCB cometabolism. Flasks containing 100 ml of 1,000 mg of fructose-minimal salts medium liter^{-1} were autoclaved and inoculated with *Arthrobacter* sp. strain B1B. Subsequently, 2.5 to 5 μl of the chemical to be tested was added to the inoculated flasks. *R*-carvone, *s*-carvone cumene, carvacrol, thymol, and *p*-xylene were tested at 300 μM (50 mg liter^{-1}). Isoprene, toluene, and benzene were tested at both 250 and 500 μM. *R*-Limonene, *s*-limonene, and *p*-cymene were added in excess of their solubilities (16, 16, and 35 mg liter^{-1}, respectively [9]). Biphenyl is a solid at room temperature and was added to a concentration of 50 mg liter^{-1}, in excess of its aqueous solubility of 7 mg liter^{-1} (21). *trans*-cinnamic acid was added to a concentration of 50 mg liter^{-1}.

Protein determination. Protein contents were determined by the biuret method (22).

Analysis of spearmint extract. To identify potential PCB degradation-inducing substrates contained in spearmint extract, experiments were conducted with resting cells grown on spearmint extract before and after removal of the polar fraction by using a C_{18} SepPak cartridge (Supelco, Bellefonte, Pa.). After determination that the nonpolar fraction was responsible for induction of PCB biodegradation, the nonpolar fraction was further analyzed by comparing the compositions of the extract before and after growth of *Arthrobacter* sp. strain B1B. A 50-ml volume of autoclaved spearmint-minimal salts medium was sampled aseptically prior to inoculation and again 26 h after inoculation. Ten milliliters of the culture supernatant was withdrawn with a sterile syringe and passed through a C_{18} SepPak to collect the nonpolar components, which were eluted with 750 μl of methanol and analyzed by gas chromatography-flame ionization detection (GC-FID) and Fourier-transform infrared spectrometry (GC-FTIR).

Cometabolism of Aroclor 1242. Cells were harvested in mid- to late-log phase, washed, and resuspended in 50 mM phosphate buffer to a final optical density at 525 nm of 1.6 (1.1 mg of protein ml^{-1}). Four-milliliter aliquots of the cell suspension were placed into 20-ml glass vials, after which 200 μg of Aroclor 1242 in 50 μl of hexane was added to each vial. The cultures were incubated on a rotary shaker for 20 to 24 h. Analyses of Aroclor cometabolism were carried out in triplicate. Vials containing heat-killed cells were used as controls for comparison with live cells.

Immediately prior to extraction, 200 μg of 2CBp or of both 2CBp and 2,2′,4,5,5′-PCBp was added to each culture in 50 μl of hexane to monitor changes in sample concentration or other losses that occurred during the extraction procedure (the recovery of added 2CBp was 91% ± 5%). Subsequently, 4 ml of hexane, 1.5 ml of 20% SDS, and 0.5 ml of distilled deionized water were added to each of the vials, which were then sealed and shaken for 2 h on a platform shaker. The resulting emulsion was separated from the aqueous phase with a Pasteur pipette and transferred to a second vial. Anhydrous sodium sulfate was added to break the emulsion, after which the hexane layer was transferred to autosampler vials for analysis by GC-FID.

The extraction efficiency was determined based on the recovery of peak 41 (2,3,3′,4,4′-PCBp and 2,3′,3,3′,4,6′-hexachlorobiphenyl) (3). These congeners are not significantly degraded by *Arthrobacter* sp. strain B1B (see Table 2). The areas of peak 41 measured in the samples were compared to the corresponding peak areas determined for five independently prepared standards of 50 μg of Aroclor 1242 ml^{-1}. The extraction efficiency for live cells of *Arthrobacter* sp. strain B1B was calculated to be 89% ± 8%. The extraction efficiency for heat-killed cells of *Arthrobacter* sp. strain B1B was found to be 91% ± 7%.

The assignment of Aroclor 1242 congeners to chromatogram peaks was based on their retention indices as described by Bedard et al. (3). The positions of four standards (2CBp, 4,4′-DCBp, 2,4′,5-trichlorobiphenyl, and 2,2′,4,5,5′-PCBp) were determined independently, and GC-MS was used to confirm the identities of selected peaks in the mixture.

Analytical methods. GC analyses were performed with a Hewlett-Packard 5890 GC equipped with an FID (Hewlett-Packard Co., Palo Alto, Calif.). The column was a 25-m HP-5 (5% phenylmethyl-silicone phase; internal diameter, 0.32 mm; film thickness, 0.52 mm). The injector temperature was 250°C, and the detector temperature was 300°C. The carrier gas was helium (30 cm s^{-1}). Detector gases were 30 ml of hydrogen min^{-1} and 300 ml of air min^{-1}. PCBs were analyzed with the following temperature program: initial temperature, 155°C; time, 1 min; ramp at 3°C min^{-1} until 185°C, at 1.5°C min^{-1} until 215°C, and at 3°C until 230°C (holding for 4 min). Spearmint extract was analyzed with the following program: initial temperature, 60°C; time, 1 min; ramp at 7.5°C min^{-1} until 225°C (holding for 10 min).

Chlorobenzoates were analyzed by comparison of retention times with analytical standards with a Hewlett-Packard 1050 series high-pressure liquid chromatograph with UV detection at 230 nm. The column was a C_{18} Hewlett-Packard Spherisorb ODS2 (5 μm, 250 by 4 mm). The samples were eluted isocratically with a mobile phase of 30% acetonitrile and 70% 40 mM acetic acid at a flow rate of 1 ml min^{-1}.

Selected Aroclor 1242 congeners were identified with a Hewlett-Packard 5890 GC equipped with a 5970B series mass selective detector. The column was a 60-m DB-5MS column (J&W Scientific, Folsom, Calif.; inside diameter, 0.25 mm; film thickness, 0.25 mm). The sample was analyzed in the TIC mode and scanned from 100 to 340 m/z U.

An IR spectrum of spearmint extract was obtained with a Hewlett-Packard 5890 series 2 GC equipped with a Hewlett-Packard 5965B infrared detector and an FID in series. The column was a 30-m DB-5MS column (internal diameter, 0.32 mm; film thickness, 0.5 mm). The temperature program was as described above.

Optical densities and ring fission product formation were measured with a Lambda 2 UV-visible light spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.).

TABLE 1. Ring fission product formation by resting cells of *Arthrobacter* sp. strain B1B grown in medium containing plant or plant detritus materials

Compound	Common name	Product formation rate[a]
Biphenyl		1,634 ± 403
TSB		80 ± 5
Mentha spicata	Spearmint	510 ± 59
Mentha pulegium	Pennyroyal	0
Ocimum basilicum	Basil	16 ± 1
Hordeum vulgare	Barley	30 ± 1
Phaseolus vulgaris	Green bean	0
Anethum sp.	Dill	0
Avocado litter		0
Yard compost		0

[a] Values are nanograms of product per milligram of protein per hour.

RESULTS

Screening of plant extracts and soil organic matter for chemical components that induce PCB cometabolism. The ability of *Arthrobacter* sp. strain B1B to cometabolize 4,4′-DCBp was evaluated in a screening assay after growth of cells on selected plant and soil organic matter extracts. Among the materials tested, only spearmint extract induced cometabolism of 4,4′-DCBp at a rate higher than that of control cells grown on tryptic soy broth (TSB), whereas cells grown on the other substrates had negligible activity (Table 1). Crude spearmint plant extract induced degradation activity at a level of approximately 30% of that measured for cells grown on 500 mg of biphenyl liter^{-1}. Cells grown on extracts of barley and basil showed some degradation activity but at a level that was only 20 to 30% of that for cells grown on TSB. Avocado leaf litter and garden compost did not induce any measurable degradation activity.

Since the cells used in the screening assays had been previously maintained on agar medium containing biphenyl, it was relevant to examine whether preculture on biphenyl biased the results for the spearmint extract by carryover of preinduced cells in the inoculum. However, after growth on spearmint extract, cells transferred to fresh spearmint extract continued to cometabolize 4,4′-DCBp at a similar rate (data not shown).

To identify the active inducing component of spearmint extract, spearmint-minimal salts medium was separated into polar and nonpolar fractions with solid-phase extraction on a C_{18} column. Of the total 500 mg of carbon liter^{-1} contained in the extract, only 5 mg liter^{-1} was collected in the nonpolar fraction. However, after removal of this fraction, cells grown on the polar fraction had greatly reduced cometabolism activity. Thus, further efforts aimed at identifying the active component were directed at the nonpolar fraction. Analysis of the nonpolar fraction by GC-FID revealed at least 10 different compo-

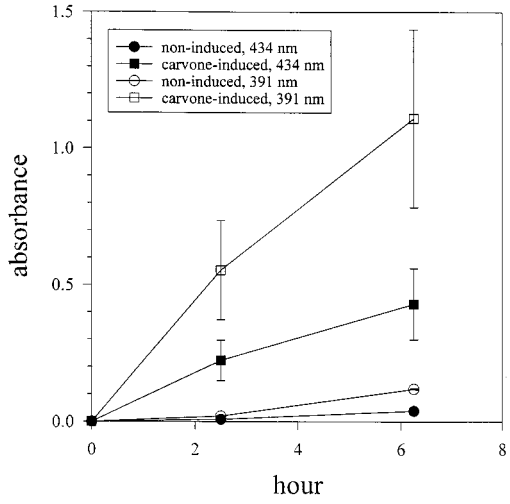

FIG. 1. Phenylhexadienoate ring fission product formation during cometabolism of Aroclor 1242 by resting cells of *Arthrobacter* sp. strain B1B. Cells were grown in medium containing either fructose and carvone (carvone induced) or fructose only (noninduced). Measurements at 391 nm correspond to absorbance by *ortho*-substituted congeners; absorbance at 434 nm is due to *para*-substituted congeners.

nents, of which one accounted for greater than 90% of the total mixture as determined by relative peak areas. After cell growth in spearmint-minimal salts medium, the concentration of the principal nonpolar component decreased in area by 40%, which suggested that this component was responsible for induction of PCB cometabolism.

The identity of the principal nonpolar component was concluded to be carvone, based on two lines of evidence. First, analysis by FTIR revealed that the principal nonpolar component absorbed strongly at 1,696 nm, indicating a C-O stretch characteristic of a carbonyl moiety. A peak at 3,086 nm resulted from the presence of a vinylic C-H bond. Finally, there was excellent correspondence between the fingerprint region of the unknown compound and that of a standard of authentic *l*-carvone. Identification of the principal nonpolar component with the EPA_REVA.L version 3.22 database (HP59963B EPA Library; Hewlett-Packard Co.) determined that it was either *l*- or *d*-carvone, with a match quality of 946 for both stereoisomers. A match quality of 1,000 indicates that two spectra are identical at all points. The second line of evidence supporting the identity of the principal nonpolar component as carvone was the fact of identical retention times for this peak and for that of an authentic standard of *l*-carvone, as determined by GC-FID.

Metabolism of Aroclor 1242 by carvone-induced cells. To confirm the induction of PCB cometabolism by carvone, cells were grown in fructose medium supplemented with a small amount of *l*-carvone. Experiments with these carvone-induced cells were conducted with Aroclor 1242. Three criteria were used to demonstrate cometabolism of Aroclor 1242: (i) PCB congener peak disappearance, (ii) formation of a phenylhexadienoate ring fission product (Fig. 1), and (iii) formation of chlorobenzoates. Compared to heat-killed control cells, live cells grown in fructose-carvone medium cometabolized 62% ± 3% of the total Aroclor over 15 h. Peak area reductions were significantly greater for di-, tri-, and tetrachlorinated biphenyls (TCBps) than for the more highly chlorinated congeners. Ring fission product formation was measured at both 434 and 391 nm, which are characteristic absorption maxima of the *para*- and *ortho*-phenylhexadienoate products, respectively (26). Significant quantities of both PCB ring fission products were observed within 6 h after addition of Aroclor 1242 to cultures of carvone-induced cells. Further intermediate products of aerobic PCB degradation in the supernatants from resting cell assays were shown to include both mono- and dichlorobenzoates. Thus, three lines of evidence confirmed that carvone induced the aerobic degradation of Aroclor 1242 by *Arthrobacter* sp. strain B1B.

Subsequent studies compared carvone-induced cells with cells grown in fructose-minimal salts medium. In samples taken at 8 h after addition of PCBs, 59% ± 2% of the total Aroclor 1242 was degraded by the carvone-induced cells, whereas only 7% ± 6% of the total Aroclor was degraded by the fructose-grown cells (Table 2). Further incubation resulted in only a slight increase in total Aroclor degradation by carvone-induced cells, which reflected slower degradation of the less-abundant, more highly chlorinated congeners. After 15 h, 26 of 32 identified peaks showed a 15% or greater reduction in peak area with carvone-induced cells, compared to 11 peaks after incubation with fructose-grown cells (Table 2).

The tetra- and pentachlorinated congeners were transformed at a rate lower than those of the di- and trichlorobiphenyls. After 8 h of incubation with carvone-induced cells, total peak areas for the TCBps and PCBps showed reductions of 33% ± 6% and 9% ± 3%, respectively (Table 2). After 15 h, there was a further decrease in peak areas, such that 46% ± 3% of the TCBps and 14% ± 6% of the PCBp congeners were degraded (Table 2).

Structure-activity analysis of chemically similar compounds. A structure-activity analysis was conducted to identify the essential molecular features of carvone responsible for inducing PCB cometabolism. The analysis compared the rates of 4,4′-DCBp ring fission product formation by *Arthrobacter* sp. strain B1B after growth in the presence of selected isoprenoid chemicals as a means of evaluating the importance of key molecular features of carvone. A comparison of the rates of ring fission product formation induced by *l*-carvone and *d*-carvone showed that the stereochemistry of the carvone isopropenyl moiety did not influence the rate of cometabolism (Table 3; Fig. 2). Similarly, induction of *Arthrobacter* sp. strain B1B by either of the two stereoisomers of limonene, which differs from carvone by the absence of the ketone moiety, revealed that this functional group did not influence the rate of cometabolism. However, exchanging the ketone moiety of carvone for the hydroxyl functional group (carvacrol) led to a 38% decrease in cometabolism activity. Moving the hydroxyl moiety from the *meta* position of carvacrol to the *ortho* position of thymol led to a 90% reduction in the rate of cometabolism activity (Fig. 2 [structures 8 and 13]).

Induction of cells with *p*-cymene led to a rate of ring fission production that was significantly greater than that with biphenyl and suggested that saturation or unsaturation of the isopropyl moiety was irrelevant for effective cometabolism. Comparison of the rates of ring fission product formation induced by *p*-cymene and cumene showed that the methyl substituent of *p*-cymene was necessary for high induction activity (Fig. 2 [structures 1 and 9]). Lack of significant induction by *p*-xylene revealed that substitution of a methyl group for the isopropyl moiety of *p*-cymene reduced the rate of ring fission product formation by 87%. Growth of cells in the presence of toluene, *p*-xylene, *trans*-cinnamic acid, benzene, and thymol resulted in 4,4′-DCBp cometabolism at rates that were similar to those of noninduced cells grown on fructose.

Nongrowth-linked induction of PCB degradation by carvone. Preliminary experiments showed that providing spear-

TABLE 2. Degradation of Aroclor 1242 by resting cells of *Arthrobacter* sp. strain B1B after 8 and 15 h

Congener(s)	Avg % degradation after 8 h		Avg % degradation after 15 h		Significance[b]
	Carvone-induced cells	Noninduced cells[a]	Carvone-induced cells	Noninduced cells[a]	
2	NI[c]	NI[c]	NI[c]	NI[c]	
2,2′; 2,6	100 ± 0	18 ± 4	100 ± 0	56 ± 47	a
2,4; 2,5	NI[d]	NI[d]	NI[d]	NI[d]	
2,3′	58 ± 15	10 ± 7	100 ± 0	66 ± 30	a
2,3; 2,4′	100 ± 0	34 ± 6	100 ± 0	100 ± 0	a
2,2′,6	6 ± 9	8 ± 7	19 ± 3	−6 ± 41	
2,2′,5; 4,4′; 2,2′,4	78 ± 2	4 ± 7	78 ± 4	40 ± 18	ab
2,3,6; 2,3′,6	36 ± 3	6 ± 7	61 ± 15	29 ± 20	a
2,2′,3; 2,4′,6	67 ± 4	7 ± 3	71 ± 3	19 ± 4	ab
2,3′,5	77 ± 1	6 ± 5	67 ± 16	77 ± 20	a
2,3′,4	66 ± 9	7 ± 11	92 ± 13	77 ± 20	a
2,4′,5; 2,4,4′	80 ± 2	4 ± 6	97 ± 1	25 ± 3	ab
2′,3,4; 2,2′,5,6′	85 ± 2	5 ± 8	89 ± 2	38 ± 6	ab
2,3,4′; 2,2′,4,6′	25 ± 8	2 ± 5	47 ± 5	4 ± 6	ab
2,2′,3,6	1 ± 7	0 ± 4	18 ± 1	−2 ± 9	b
2,2′,3,6′	7 ± 12	0 ± 14	18 ± 9	−6 ± 10	b
2,2′,5,5′	6 ± 8	2 ± 5	17 ± 6	5 ± 4	b
2,2′,4,5′	1 ± 8	3 ± 7	15 ± 9	3 ± 5	
2,2′,4,4′; 2,2′,4,5	14 ± 6	3 ± 6	−8 ± 28	4 ± 5	
2,2′,3,5′	60 ± 6	3 ± 4	77 ± 3	5 ± 8	ab
3,4,4′; 2,2′,3,4′	25 ± 6	2 ± 8	44 ± 5	9 ± 3	ab
2,2′,3,4; 2,3,4′,6; 2,3′,4′,6	1 ± 7	3 ± 4	12 ± 8	1 ± 7	
2,2′,3,3′	18 ± 10	−1 ± 4	42 ± 5	−3 ± 12	ab
2,4,4′,5	40 ± 7	1 ± 10	67 ± 5	2 ± 6	ab
2,3′,4′,5	52 ± 5	2 ± 7	81 ± 5	4 ± 6	ab
2,3′,4,4′; 2,2′,3,5′,6	30 ± 6	2 ± 6	54 ± 7	3 ± 5	ab
2,2′,3,4′,6	NI[d]	NI[d]	NI[d]	NI[d]	
2,3,3′,4′; 2,3,4,4′	12 ± 5	1 ± 6	17 ± 7	3 ± 6	
2,2′,3,3′,6; 2,2′,3,5,5′	13 ± 5	1 ± 6	15 ± 11	3 ± 6	a
2,2′,3,4′,5; 2,2′,4,5,5′	NI[c]	NI[c]	NI[c]	NI[c]	
2,2′,4,4′,5	7 ± 4	9 ± 8	15 ± 6	0 ± 12	
2,2′,3′,4,5; 2,2′,3,5,6,6′	−1 ± 7	10 ± 13	14 ± 8	−3 ± 8	
2,2′,3,4,5′	4 ± 2	−2 ± 6	11 ± 9	4 ± 7	
2,2′,3,4,4′	NI[d]	NI[d]	NI[d]	NI[d]	
2,3,3′,4′,6; 3,3′,4,4′	10 ± 10	2 ± 6	12 ± 7	4 ± 5	
2,2′,3,3′,4	NI[d]	NI[d]	NI[d]	NI[d]	
2,2′,3,4′,5′,6; 2,3′,4,4′,5	9 ± 0	−21 ± 24	18 ± 3	−1 ± 9	b
2,3,3′,4,4′; 2,2′,3,3′,4,6′	NI[d]	NI[d]	8 ± 3	27 ± 35	

[a] Noninduced cells were grown in fructose-minimal salts medium only.
[b] Significant difference in degradation between carvone-induced and noninduced cells ($P < 0.05$). a, 8-h assay; b, 15-h assay. Statistical analysis was by Student's *t* test.
[c] NI, not integrated. Congener was added as a standard during the extraction procedure.
[d] NI, not integrated. There was not sufficient resolution to evaluate degradation for the indicated congener.

mint oil as the sole carbon source for growth of *Arthrobacter* sp. strain B1B resulted in cell lysis. Similar results were observed with cultures provided with carvone as the sole carbon source at 500 mg liter^{-1} or greater. At lower concentrations (100, 200, 300, or 450 mg of carvone liter^{-1}), no growth was detected, although the cells remained viable. Comparison of cell growth in fructose medium with growth in the same medium supplemented with 100 mg of carvone liter^{-1} showed that carvone inhibited the growth of *Arthrobacter* sp. strain B1B (Fig. 3).

DISCUSSION

Identification of natural substrates that induce PCB cometabolism is a first step in devising a practical bioremediation strategy for in situ treatment of PCB-contaminated soils. In this research, a screening assay was devised to identify plant compounds that might induce bacterial cometabolism of PCBs. The results of this assay revealed that several plant-derived chemicals with an unsaturated *para*-menthane structural motif were effective in inducing PCB degradation by *Arthrobacter* sp. strain B1B. The identification of carvone, the principle flavoring agent in spearmint, as a natural cosubstrate for PCB cometabolism was particularly fortuitous, since this compound is nontoxic and is commercially available at low cost.

Structure-activity analyses of related isoprenoid chemicals showed that there was differential induction of PCB biodegradation activity, with the most active inducing agent being *para*-cymene. The rate of degradation induced by carvone was similar to that induced by biphenyl in fructose medium (Table 3), whereas induction by *para*-cymene was similar to that measured after growth of cells on biphenyl in minimal salts medium (Table 1). Direct comparison of the differential induction of PCB cometabolism on the different substrates is complicated by the fact that all of the tested compounds had different solubilities in aqueous media. *para*-Cymene, limonene, and biphenyl all have solubilities of less than 50 mg liter^{-1} and yet were strong inducers of PCB cometabolism. Hence, the relative efficacies of these three substrates in comparison to those of carvone and other substrates with higher solubilities must be considered in relation to their decreased bioavailability.

TABLE 3. Ring fission product formation induced by carvone and structurally similar compounds

Compound[a]	Product formation rate[b]	Significance[c]	Plant source[d]
(1) *p*-Cymene	1,471 ± 343	a	Widely distributed
(2) Isoprene	928 ± 52	b	Widely distributed
(3) (*S*)-(+)-Carvone	860 ± 117	bc	Dill seed and caraway seed
(4) (*R*)-(−)-Carvone	845 ± 187	bc	Spearmint
(5) (*S*)-(−)-Limonene	821 ± 168	bc	Pine needle oil
(6) (*R*)-(+)-Limonene	807 ± 103	bc	Citrus, juniper, and dill seed
(7) Biphenyl	706 ± 72	c	None
(8) Carvacrol	509 ± 19	d	Oregano
(9) Cumene	280 ± 110	e	None
(10) *p*-Xylene	188 ± 80	ef	None
(11) Toluene	138 ± 23	ef	None
(12) *trans*-Cinnamic acid	87 ± 3	f	Storax
(13) Thymol	70 ± 10	f	Thyme
(14) Benzene	61 ± 13	f	None
Fructose only (control)	73 ± 48	f	NA[e]

[a] Numbers correspond to those for structures in Fig. 2.
[b] Values are nanograms of product per milligram of protein per hour.
[c] Rates of compounds marked by same letter are not significantly different ($P < 0.05$; statistical analysis by Fisher's least significant difference test).
[d] Major plant source according to Buckingham (5).
[e] NA, not applicable.

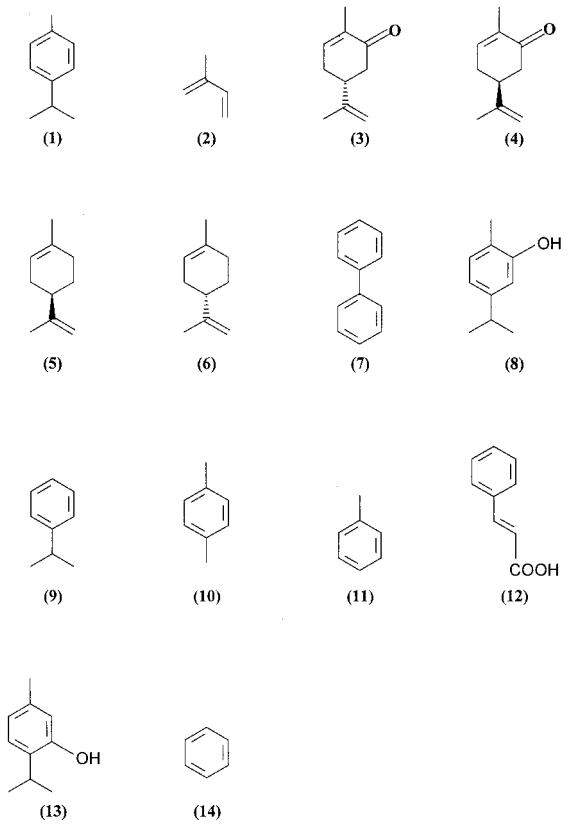

FIG. 2. Structures of compounds used in this study.

Recently, bacteria isolated by enrichment culture on naphthalene (24) and grown on ethylbenzene (27) were shown to cometabolize PCBs, demonstrating that alternative aromatic compounds other than biphenyl can induce the *bph* genes. The observation in the present research that nonaromatic compounds were also effective at inducing PCB oxidation was unexpected. Particularly surprising was the effectiveness of isoprene in inducing PCB cometabolism, since isoprene is not only nonaromatic but also lacks a ring structure. This suggests that a broad specificity receptor molecule, which is responsive to isoprenoid structural features such as those found in the monoterpenes, may be responsible for the upregulation of expression of the *bph* genes. Catabolic pathways for several of the monoterpenes tested here have not been determined; however, limonene, carvone, and *p*-cymene have structural features in common with isopropylbenzene, a compound which has been shown to undergo *meta* cleavage proximal to the isopropyl moiety (7). Further investigation of the metabolism of these compounds will require integrated biochemical and molecular studies to verify induction of the *bph* pathway by carvone in *Arthrobacter* sp. strain B1B.

Several different monoterpenes, including limonene, have been reported to serve as sole carbon sources for certain bacteria (28), but it is not known whether these organisms also cometabolize PCBs. Hernandez et al. (15) have proposed that monoterpenes contained in orange peels and pine needles may serve as natural substrates for biphenyl-utilizing bacteria; however, the utilization of these complex substrates by indigenous PCB degraders has not yet been well investigated. In this research, the monoterpene carvone induced cometabolism of PCBs at a concentration of 50 mg liter^{-1}, which was too low to support growth of *Arthrobacter* sp. strain B1B in the absence of supplemental carbon. Moreover, no growth was observed at higher concentrations of carvone of from 100 to 450 mg liter^{-1}, and concentrations of greater than 500 mg liter^{-1} caused cells of *Arthrobacter* sp. strain B1B to lyse, suggesting that the induced metabolism of carvone by *Arthrobacter* sp. strain B1B was principally a detoxification mechanism. Although speculative, this novel mechanism for induction of PCB cometabolism is consistent with the observation that several monoterpenes with *p*-menthane structural motifs have antimicrobial properties (20, 23). As strategies for PCB bioremediation are devel-

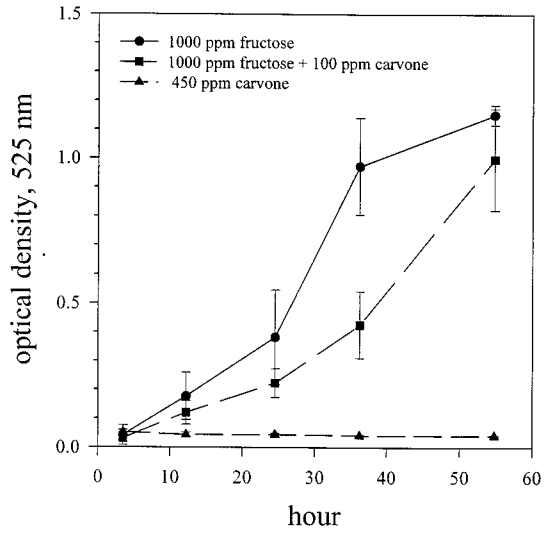

FIG. 3. Growth of *Arthrobacter* sp. strain B1B in selected media. Growth in fructose-minimal salts medium was inhibited by the addition of 100 mg of *l*-carvone liter^{-1}. No growth was detected when *l*-carvone was provided as the sole carbon source at 100, 200, 300, or 450 mg liter^{-1} (shown).

oped, it will be relevant to determine whether isoprenoids that induce PCB cometabolism function in a manner similar to that of biphenyl, by serving as growth substrates, or by induction of a detoxification mechanism, as hypothesized for carvone. For example, selective enrichment procedures that use monoterpenes as growth substrates for isolation of PCB-degrading bacteria may not be useful for identifying bacteria that detoxify monoterpenes while growing on other carbon substrates.

Arthrobacter sp. strain B1B has a limited ability to degrade mixtures of PCB congeners such as Aroclor 1242. When induced by carvone, only 26 of 32 identified peaks were significantly degraded at a level of 15% or more during a 15-h incubation period. Eventually, an effective in situ bioremediation strategy may require the use of bacteria with complementary abilities, such as *Pseudomonas cepacia* LB400 (29) or *Alcaligenes eutrophus* H850 (3), which can degrade a broad spectrum of PCB congeners. These organisms have been shown to grow on selected flavonoid compounds and to retain their ability to metabolize PCBs (8) and could be used as part of a biphenyl-free PCB bioremediation process. The identification of carvone and related monoterpenes as natural substrates for *Arthrobacter* sp. strain B1B provides a starting point for similar investigations of other PCB-degrading microorganisms. The discovery that plant-derived terpenes induce PCB cometabolism also opens up the possibility that plants which secrete monoterpenes into the rhizosphere could eventually be used for in situ bioremediation of PCB-contaminated soils with plant-microbial systems.

ACKNOWLEDGMENTS

We acknowledge Dennis Focht for providing *Arthrobacter* sp. strain B1B, Anthony Hay for advice on the PCB extraction procedures, Alvaro Alvarado for assistance with FTIR, and Jennifer Sasaki for assistance with GC-MS.

This research was supported by a grant from the University of California Toxic Substances Research and Teaching Program.

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