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Biodegradation of Biphenyl and 2-Chlorobiphenyl by a *Pseudomonas* sp. KM-04 Isolated from PCBs-Contaminated Coal Mine Soil

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Abstract The biphenyl-degrading strain, *Pseudomonas* sp. KM-04, was isolated from polychlorinated biphenyls-contaminated soil sample obtained from the vicinity of a former coal mine. We herein report that strain KM-04 can use biphenyl as a sole carbon source, and resting cells convert biphenyl to its corresponding metabolic intermediates. Incubation of KM-04 with autoclaved mining-contaminated soil for 10 days in a slurry system reduced the levels of biphenyl and 2-chlorobiphenyl by 98.5 % and 82.3 %, respectively. Furthermore, treatment of a mine-soil microcosm with strain KM-04 for 15 days in a composting system under laboratory conditions reduced the levels of biphenyl and 2-chlorobiphenyl by 87.1 % and 68.7 %, respectively. These results suggest that KM-04 is a potential candidate for the biological removal of biphenyl and its chlorinated derivatives from polychlorinated biphenyl-contaminated mining areas.

Keywords Biphenyl · Biodegradation · 2-Chlorobiphenyl · Coal-mine soil · Microcosm study

Biphenyl is a natural component of coal tar, crude oil, and natural gas. It is widely used in organic synthesis, food preservatives, heat transfer fluids, and the synthesis of polychlorinated biphenyls (PCBs). Although the use of biphenyl has decreased in recent years, it persists as a contaminant and is associated with serious environmental problems (Furukawa et al. 2004; Li et al. 2009; Petric et al. 2011; Pieper 2005). PCBs, which are the chlorinated derivatives of biphenyl, are widely used for a variety of industrial purposes. Their properties of low conductivity, high dielectric constants, low volatility, and good chemical stability make them suitable for electrical applications. The use of PCBs in electrical equipment, primarily as dielectric and coolant fluids, is regulated due to their high toxicity, low degradability, persistence, and detrimental effects on the immune, reproductive, nervous, and endocrine systems (Safe 1990; Van Birgelen et al. 1996).

Coal mining disturbs large areas of land and is associated with various environmental challenges, including organic and inorganic pollution, hydrocarbon contamination, soil erosion, dust, water pollution, and reductions in local biodiversity (Bench 2010; Bhuiyan et al. 2010). The mining industry has widely used PCB-containing equipment. When such equipment is abandoned underground, it and can threaten soil and the water table; furthermore, when PCBs (either spilled or leaking from abandoned underground equipment) leach into the soil and groundwater, it can be difficult to identify the contamination source. This can lead to intractable soil and water pollution (Bench 2010).

Many biphenyl-degrading bacteria have been isolated from environmental samples, and have been shown to metabolize PCBs through a biphenyl metabolic pathway (Catelani et al. 1973; Erickson and Mondello 1992; Hayase et al. 1990; Kimbara et al. 1989; Masai et al. 1995; Shuai et al. 2010; Taguchi et al. 2007; Yang et al. 2007). The major biphenyl biodegradation pathway used by most microorganisms under aerobic conditions includes oxidation of biphenyl at the 2,3-position to a *cis*-dihydrodiol, followed by dehydrogenation to 2,3-dihydroxybiphenyl (DHB). The aromatic ring is cleaved to form 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), a yellow-colored

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meta-cleavage product that is subsequently hydrolyzed to benzoic acid and 2-hydroxypenta-2,4-dienoate (Catelani et al. 1973; Erickson and Mondello 1992; Hayase et al. 1990; Kikuchi et al. 1994; Li et al. 2009; Masai et al. 1995; Pieper 2005).

Here, we sought to isolate a bacterium from PCB-contaminated mine soil (referred to the soil affected by mine activity), investigate its ability to degrade biphenyl, and elucidate the relevant metabolites. We found that the isolated bacterium was stimulated and enriched in soil microcosms supplemented with biphenyl or 2-chlorobiphenyl, and subsequently removed the biphenyl and 2-chlorobiphenyl from the soil.

Materials and Methods

Biphenyl (>99.5 % purity), 2,3-dihydroxybiphenyl (>98.0 % purity), catechol, and benzoic acid were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI, USA). Ethyl acetate, *n*-hexane, and acetone (all organic trace analytic grade) were purchased from Merck (Darmstadt, Germany), as were the nutrient broth, *ortho*-phosphoric acid, and agar media. All of the other utilized chemicals and compounds were of the highest commercially available grade.

PCB-contaminated soil was collected from Samchuk; this city, which is located in the eastern part of the Republic of Korea, was greatly impacted by a nearby coal mine. Selective enrichment was used to isolate biphenyl-degrading bacteria. Soil samples were collected in sterilized 50 mL plastic centrifuge tubes, and were not refrigerated or frozen during transportation or storage. Soil samples (0.5 g) were weighed, mixed vigorously with 10 mL of phosphate buffer (pH 7.0), and centrifuged at 1,000×*g* for 10 min. The supernatant was taken as a mixed culture and used as the starting material for the isolation of pure cultures. One hundred microliters of mixed culture was inoculated to 100 mL of 10 mM biphenyl-containing minimal salt medium (MSM) and incubated overnight at 28°C. For selective enrichment, 100 µL of the liquid culture was streaked onto solid MSM agar medium including 10 mM biphenyl. After 3 days of incubation, each colony was inoculated to 1 mL of MSM containing 1 mM biphenyl as the sole carbon source, in a 15 mL sterilized centrifuge tube with a silicon stopper. Single colonies and defined consortia were tested for the ability to utilize biphenyl, as represented by bacterial growth and substrate depletion from the culture medium.

To identify the newly isolated strain, morphological and physiological characteristics were examined. Furthermore, molecular identification was done by PCR mediated 16S rRNA gene sequence analysis using primers set 27F and 1492R (Heuer et al. 1997). The amplified gene was sequenced and deposited in GenBank at the NCBI (National Center for Biotechnology Information).

The growth of strain KM-04, was found to use biphenyl as a sole carbon source, was investigated by culturing the isolate in 100 mL Erlenmeyer flasks containing 10 mL sterilized MSM and 10 µg/mL biphenyl in crystal form, as previously described (Nam et al. 2006). For biphenyl biodegradation experiments, 10 mL of KM-04 cell suspension ($OD_{600} = 0.11$) was added to triplicate 100 mL Erlenmeyer flasks containing 10 µg/mL of biphenyl and incubated at 160 rpm and 28°C for 5 days. The colony-forming units per milliliter (CFU/mL) were measured by conventional plating on 54 solid MSM agar media including biphenyl at 0, 12, 24, 36, 48, 60, 72, 96, and 120 h. At each time point, the six culture media including controls were stored at −70°C until the final flasks were collected at 5 days, and 54 samples were subjected to chemical analysis for measurement of biphenyl degradation and metabolite formation. Cells subjected to heat inactivation (70°C × 40 min) and poisoning (10 mM NaN_3) were used as controls (Nam et al. 2006). Resting cells were subjected to biotransformation experiments, in order to identify metabolic intermediates produced during degradation. To prepare resting cells, KM-04 was pre-grown with biphenyl (100 µg/mL) in 200 mL MSM in a 2-L Erlenmeyer flask to an OD_{600} of 1.0. After 3 days, the culture was harvested by centrifugation (9,000*g* × 20 min, 4°C) and washed three times with sterile 20 mM phosphate buffer (pH 7.0). The OD_{600} of the final resting cell suspension in phosphate buffer was adjusted to OD_{600} 4.0.

To assess the ability of strain KM-04 to metabolize biphenyl and 2-chlorobiphenyl from actual mine soil, slurry experiments were conducted in 250-mL Erlenmeyer flasks using 5 g of autoclaved mine soil and 20 mM phosphate buffer (pH 7.0). The biphenyl and 2-chlorobiphenyl were prepared in acetone (final concentration 10 µg/g soil) and mixed with 25 mL of inoculum ($OD_{600} = 4.0$) in a sterilized flask. Control experiments were performed with heat- or sodium-azide-inactivated KM-04 cells (as described above), and with cell-free mine soil. The flasks were incubated for 10 days at 28°C in a rotating shaker, biphenyl and 2-chlorobiphenyl were extracted from the cultures with ethyl acetate, and their concentrations were determined. Samples were treated with concentrated sulfuric acid prior to extraction in order to recover any adsorbed biphenyl or 2-chlorobiphenyl (EPA 1996; Nam et al. 2005).

For our soil microcosm experiments, we autoclaved the soil from which KM-04 was originally isolated, and evaluated the ability of KM-04 cells to remove biphenyl and 2-chlorobiphenyl from this soil. The biphenyl and 2-chlorobiphenyl concentrations, resting cells inoculation procedures and controls were as described for the slurry

experiments. After 15 days of incubation, biphenyl and 2-chlorobiphenyl were extracted from the soil microcosms, and the concentrations were determined by GC/MS. For the routine determination of recovery rates, 10 μg/g of fluorene was added to each flask prior to extraction (recovery rate, >98.5 %). All data are reported as the means of measurements on three replicate samples.

The concentration of PCBs in the soil used for isolation of KM-04 was determined by batch solvent extraction. The soil (2.5 g) was extracted with 25 mL acetone (solid:liquid extraction ratio, 1:10 g/mL) for 24 h with shaking at 100 rpm, and then separated by centrifugation. Liquid–liquid extraction was performed on the supernatants, and was prepared in *n*-hexane for gas chromatography (GC). The extracts were analyzed for PCBs according to EPA method 8082 using an Agilent Model 6890 GC system (Newark, DE) equipped with an electron capture detector (ECD) (EPA 1996). GC–MS was used to quantitatively analyze the depletion of biphenyl or 2-chlorobiphenyl during bacterial growth and microcosm treatment. The GC–MS analysis was performed on an Agilent GC 6890 N/MSD 5973 equipped with a 60-m DB-5 capillary column. After all samples were collected, the flasks were thawed and liquid–liquid extractions were conducted. The samples and controls were rinsed with chilled ethyl acetate. After the fifth wash, the remaining water phase was adjusted to pH 3.0 with 85 % *ortho*-phosphoric acid. Three final extractions were then conducted. The samples were filtered through a porous R2 resin column, and the metabolic intermediates derived from biphenyl were identified using an HP 1100 mass selective detector liquid chromatography mass spectrometry (MSD LC–MS) system (Agilent) equipped with a Zorbax column (150 mm × 2.1 mm id, 5 μm; Agilent). All experiments were generally performed three times.

Results and Discussion

Soil samples were obtained from near a former coal mine. The total concentration of all PCBs in the soil sample was determined to be 335.1 ng/g dry weight of soil, and total hexachloro biphenyl was found to be the dominant congener rather than the low-chlorinated biphenyl congeners (Fig. 1). The PCBs in this soil could have originated from insulating fluids used for capacitors or transformers that were employed during mining activity several decades ago. *Pseudomonas* sp. KM-04 was selected from among a number of bacterial isolates obtained from this PCB-contaminated soil by selective enrichment culture. It was found to be Gram-negative, strictly aerobic, and oxidase- and catalase-positive. When grown on nutrient agar, KM-04 colonies exhibited a bright, light yellow pigmentation.

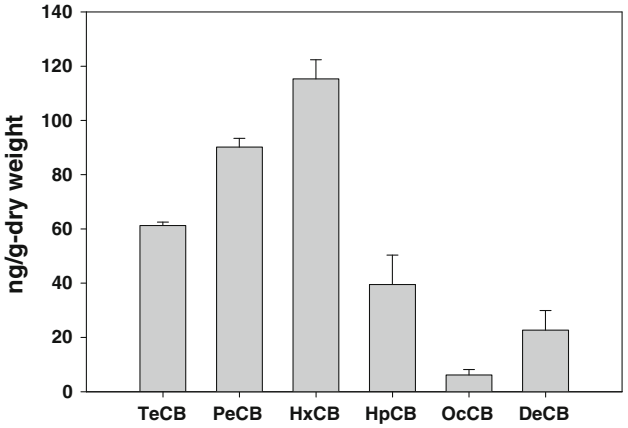


Fig. 1 Concentrations of total PCBs in the coal-mine-contaminated soil from which strain KM-04 was isolated. All values represent the means (*bars*) and standard deviations (*lines*) of three independent replicates. *BP* biphenyl, *TeCB* tetrachloro-biphenyl, *PeCB* pentachloro-BP, *HxCB* hexachloro-BP, *HpCB* heptachloro-BP, *OcCB* octachloro-BP, and *DeCB* decachloro-BP

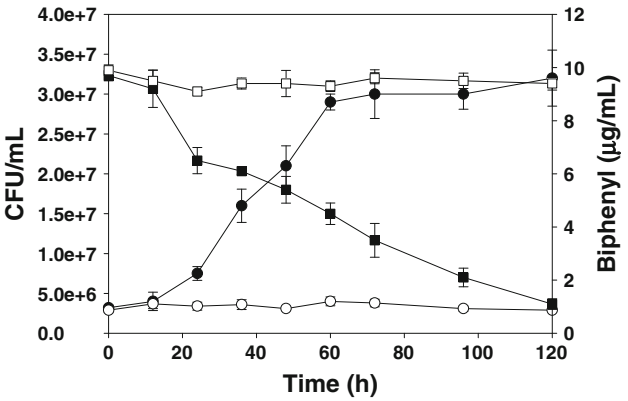
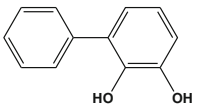
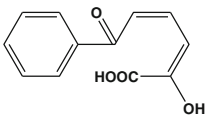
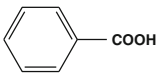


Fig. 2 Growth of *Pseudomonas* sp. KM-04 with biphenyl as the sole carbon source. Poisoned KM-04 cells (control, treated with 10 mM NaN_3) failed to show growth or biphenyl depletion. *Filled circle*, colony-forming units (CFU)/mL of untreated KM-04; *open circle*, colony-forming units/mL of poisoned KM-04; *filled square box*, biphenyl concentration in the presence of untreated KM-04; and *open square box*, biphenyl concentration in the presence of poisoned KM-04

BLAST analysis of the 16S rRNA gene indicated that KM-04 exhibited a high sequence similarity to *Pseudomonas* sp. (99.5 % homology). The obtained sequence was deposited in GenBank (accession No. JN088731). Figure 2 shows the growth curve and biphenyl utilization of *Pseudomonas* sp. KM-04 under aerobic conditions. The biomass (CFU/mL) of strain KM-04 increased with substrate depletion. Within 5 days, the initial biphenyl content (10 μg/mL) was almost completely utilized and the cell density plateaued at ~4 × 107 CFU/mL. The doubling time of bacterial growth during the exponential growth phase was 17.86 h for biphenyl. Control experiments with NaN_3-poisoned

Table 1 Metabolites detected by LC/APCI-MS during the degradation of biphenyl by strain KM-04

Compound structure	Name	Mw	LC/APCI-MS/ $[M + H]^+$
	DHB	186.2	187.2
	HOPDA	218.2	219.2
	Benzoic acid	122.1	123.1

Mw Molecular weight, *DHB* Dihydroxybiphenyl, *HOPDA* 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid

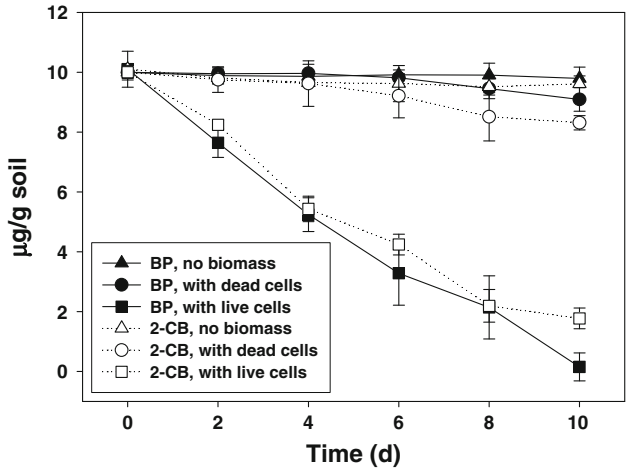


Fig. 3 Time course of the removal of biphenyl and 2-chlorobiphenyl from the mine-soil slurry following addition of living *Pseudomonas* sp. KM-04 cells, dead KM-04 cells, or no KM-04 cells. *BP* biphenyl, and *2-CB* 2-chlorobiphenyl

KM-04 cells did not show evidence of biphenyl degradation, indicating that the observed reduction in biphenyl was not due to abiotic processes. Thus, KM-04 cells appeared to be capable of using biphenyl as a sole carbon source. Furthermore, the cells also appear to use benzoic acid and catechol as sole carbon sources (data not shown). Therefore, KM-04 may play an important role in the natural degradation of biphenyl and its chlorinated derivatives in mining-contaminated soil.

The catabolism of biphenyl by strain KM-04 gave rise to three polar metabolic intermediates: DHB, HOPDA, and benzoic acid. The first metabolic intermediate was DHB. Diagnostic LC–MS displayed a peak at *m/z* 187.2 $[M + H]^+$, which was consistent with the authentic standard. The second metabolic intermediate was yellow-colored and yielded diagnostic LC–MS peaks at *m/z* 219.2 $[M + H]^+$, consistent with the *meta*-cleavage product of biphenyl, HOPDA (Li et al. 2009; Pieper 2005). The third metabolic intermediate was characterized as benzoic acid, showing diagnostic peaks at *m/z* 123.1 $[M + H]^+$ on LC–MS and a mass spectrum indistinguishable from that of the authentic standard (Table 1). These metabolites are consistent with those of a well-known biphenyl metabolic pathway (Li et al. 2009; Pieper 2005) that we propose may be utilized by KM-04. Most biphenyl-degrading bacteria are capable of degrading biphenyl through a pathway involving the key intermediates, 2,3-DHB and HOPDA, with the latter subsequently converted to benzoic acid (Furukawa et al. 2004; Li et al. 2009; Pieper 2005). LC–MS analysis confirmed that the growth of KM-04 on biphenyl generates these key metabolites, suggesting that KM-04 and the biphenyl-degrading species of other genera (Furukawa et al. 2004; Li et al. 2009; Pieper 2005) may share a common biphenyl biodegradation pathway (Table 1).

We further used a slurry system to assess the ability of strain KM-04 to remove biphenyl and 2-chlorobiphenyl from mine soil. After 10 days of incubation, strain KM-04 had removed 98.5 % and 82.3 % of the biphenyl and 2-chlorobiphenyl, respectively, from our slurry system (Fig. 3). These results indicate that the strain maintains its catabolic activity even in mine soil in a slurry system. As previous studies showed that microbial adsorption plays an

Table 2 Removal of biphenyl and 2-chlorobiphenyl by strain KM-04 in a mine-soil microcosm

Substrate	Amount (mean ± SD[a] µg/g dry weight of soil)			Decrease and percentage			
	No biomass	With dead cells	With live cells	By dead cells		By live cells	
				µg/g soil	%[c]	µg/g soil	%[b]
Biphenyl	9.80 ± 0.07	9.25 ± 0.07	1.26 ± 0.55	0.55	5.6	8.54	87.1
2-Chlorobiphenyl	9.75 ± 0.29	8.74 ± 0.76	3.05 ± 0.69	1.01	10.4	6.70	68.7

Biphenyl and 2-chlorobiphenyl were determined after a 15 day incubation

[a] The means and standard deviations were obtained from three independent replicates

[b] %, [(No biomass -With dead cells)/No biomass] × 100

[c] %, [(No biomass -With live cells)/No biomass] × 100

important role in the removal of recalcitrant pollutants, and the adsorption capacity of dead biomass is an important factor in toxin removal rates (Nam et al. 2005; Tsezos and Bell 1989), we tested the extent to which biphenyl and 2-chlorobiphenyl adsorbed to the biomass. To recover biomass-adsorbed biphenyl and 2-chlorobiphenyl, we treated the samples with concentrated sulfuric acid prior to extraction and analysis. We observed a reduction of 16.9 % in the 2-chlorobiphenyl content of the dead-cell culture. This may reflect a strong affinity of 2-chlorobiphenyl for the biomolecules of dead cells, consistent with previous reports that chlorinated compounds exhibit high affinities for lipids and other biomolecules (Dulfer and Govers 1995; D'Angelo and Nunez 2010). We also used a microcosm study to examine the ability of KM-04 to remove biphenyl and 2-chlorobiphenyl from autoclaved mine soil, and found that the removal rates after a 15 day incubation with live KM-04 cells were 87.1 % and 68.7 %, respectively (Table 2). The addition of inactivated strain KM-04 decreased these concentrations by 5.6 % and 10.4 %, respectively, perhaps reflecting adsorption to the dead biomass. This result is consistent with the above-described reports indicating that microbial adsorption plays an important role in the removal of recalcitrant pollutants (Nam et al. 2005; Tsezos and Bell 1989). These findings are important as they indicate that in bioremediation using strain KM-04 both live cells and to a lesser degree, dead ones would remove PCBs.

Our results collectively demonstrate that *Pseudomonas* sp. KM-04 could potentially be used in the bioremediation of biphenyl or low-chlorinated PCBs from contaminated soil. Although the isolated strain may not be capable of dealing with complex congener mixtures, microbial communities can be versatile, with carbon sharing through complex metabolic interactions increasing PCB degradation and allowing the degradation of more highly chlorinated biphenyls. To fully understand the biodegradation of PCBs, we must integrate the results from single-organism studies with efforts to understand the functioning of complex communities. KM-04, which is the first biphenyl-degrading isolate identified from a coal mine-impacted area, may be an important candidate for the remediation of PCB-contaminated mine-impacted areas. This is particularly important because surface mines and the crushing and milling facilities of both surface and underground mines frequently use PCB-containing electrical equipment. Further enzymatic and genetic studies may improve our understanding of biphenyl degradation in the environment and help optimize our efforts to remediate PCB-contaminated soil from mine-impacted areas.

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