ISOLATION OF A BIPHENYL-DEGRADING BACTERIUM FROM A PCB-CONTAMINATED MINE-IMPACTED AREA

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Introduction

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[1-4]. Polychlorinated biphenyls (PCBs), the chlorinated derivatives of biphenyl, have been widely used for a variety of industrial purposes. PCBs cause serious environmental problems because of their high toxicity, low degradability, and persistence. PCBs may have several serious effects on the immune, reproductive, nervous, and endocrine systems[1-4]. Microorganisms present effective tools and may be used in less aggressive bioremediation procedures for cleaning PCB-polluted environments. Many biphenyl-degrading bacteria have been isolated from environmental samples, and these bacteria can co-metabolize PCBs through a biphenyl metabolic pathway[1]. The major catabolic biphenyl biodegradation pathway under aerobic conditions passes through dioxygenation at the 2,3-position[1,2]. The initial step in the aerobic degradation of biphenyl by most microorganisms includes oxidation of biphenyl at the 2,3-position to a *cis*-dihydrodiol, followed by dehydrogenation to a 2,3-dihydroxybiphenyl. The ring is cleaved to form 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), a yellow-colored *meta*-cleavage product, which is then hydrolyzed to benzoic acid and 2-hydroxypenta-2,4-dienoate[1,3]. Coal is composed primarily of the organic elements carbon, hydrogen, oxygen, nitrogen, and sulfur, and it plays an important role in energy generation. Approximately 27% of the world's energy consumption relies on the incineration of coal[5]. Coal mining requires large areas of land to be temporarily disturbed and raises a number of environmental challenges, including organic and inorganic pollution, soil erosion, dust, water pollution, and impacts on the local biodiversity[6]. PCBs are authorized under PCB regulations for use in electrical equipment, primarily as dielectric fluids in electrical equipment. The mining industry has extensively used PCB-containing electrical equipment, and some of this equipment is abandoned underground. This threat is particularly prevalent in the mining industry because mines generally penetrate the water table. When PCBs are spilled or PCB-containing equipment is abandoned underground, PCBs may be released into the ground water, and the contamination source cannot be retrieved. This can result in water pollution for which there may be no solution[6]. In this study, we report the isolation and characterization of a bacterium that is capable of biphenyl degradation in a PCB-contaminated mine-impacted area.

Materials and methods

Bacterial strains and culture conditions. A mixed culture obtained from soil samples collected near an abandoned mine in the eastern part of the Republic of Korea served as the starting material for the isolation of a pure culture. Soil samples were placed in sterilized 50 mL plastic centrifuge tubes. Samples were not refrigerated or frozen during transportation or storage. To identify the newly isolated strain KM-04, the morphological and physiological characteristics of the isolate were examined, and the 16S ribosomal ribonucleic acid (rRNA) gene was amplified by polymerase chain reaction using the primer pair 518F and 800R. The nucleotide sequence was determined by the Macrogen deoxynucleic acid sequencing center.

Growth of the strain KM-04 on biphenyl. The growth of strain KM-04, using biphenyl as the sole source of carbon and energy, was investigated by incubating the culture in 100 mL Erlenmeyer flasks containing 10 mL sterilized MSM (minimal salts medium) and 10 mg/mL biphenyl, as previously described[7-8]. Flasks were inoculated with an optical density at OD_{600} 0.11 and incubated at 28℃ in a rotating shaker set at 160 rpm. Three cultures were removed every 24 h for growth measurements, and the flasks were stored at –70℃ until final samples were collected after 5 days. At this time, the culture media from all harvests were subjected to chemical analysis to determine the extent of biphenyl degradation and metabolic formation. As controls, heat-inactivated (70℃ for 40 min) and poisoned cells (10 mM NaN_3) were employed[7-8].

Analytical methods. Quantitative analysis of the depletion of biphenyl was carried out as follows. Periodically, a set of flasks was removed and 1 mL 85% *ortho*-phosphoric acid was added to each flask to stop the reaction. The flasks were immediately frozen and stored at –70°C. After collecting the last time point sample during incubation (120 h), all flasks were thawed, and liquid-liquid extractions were conducted. The flasks and the corresponding controls were rinsed 8 times with chilled ethyl acetate. After the sixth wash, the remaining water phase was adjusted to pH 3.5 with 85% *ortho*-phosphoric acid. Two final extractions were then conducted. Extracts were dried over anhydrous sodium sulfate, and the ethyl acetate was evaporated under reduced pressure. The biphenyl was identified by comparing the GC-MS retention times and mass spectra with those of authentic standards. The metabolic intermediates derived from biphenyl were identified by LC-MS analysis. Samples were filtered through a porous R2 resin column and analyzed to detect metabolic intermediates using an electrospray ionization/quadruple time-of-flight/mass spectrometer (ESI/Q-TOF/MS) operated in the positive turbo ion spray mode.

Results and discussion:

Identification of the strain Pseudomonas sp. KM-04. From the enriched culture was isolated a bacterial strain, KM-04, that could utilize biphenyl as its sole source of carbon and energy. The strain was Gram-negative, strictly aerobic, and oxidase- and catalase-positive. 16S rRNA analysis revealed that the strain KM-04 exhibited a high sequence similarity to the *Pseudomonas* species (99.5% homology) according to a blast search using the NCBI GenBank.

Growth characteristics of Pseudomonas sp. KM-04 on biphenyl. Under aerobic conditions, *Pseudomonas* sp. KM-04 utilized biphenyl as its sole carbon and energy source. The bacterial growth curve obtained is shown in Fig. 1 and demonstrates that the increase in biomass correlated well with the depletion of biphenyl. Poisoned controls showed that the biphenyl concentration was not reduced abiotically. Within 5 days, the initial quantity of 10 mg/mL biphenyl was almost completely utilized, and the highest cell density was reached with a culture turbidity of 1.25 at OD_{600}. Strain KM-04 could use biphenyl as a sole source of carbon and energy. As such, KM-04 may play an important role in the degradation of biphenyl and chlorinated derivatives in mine-impacted environments.

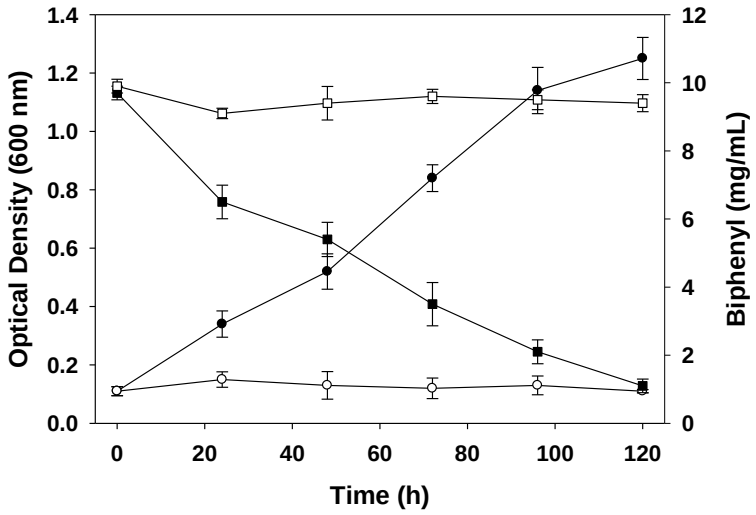
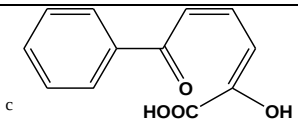
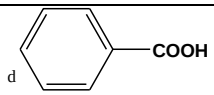


Fig.1. Growth of *Pseudomonas* sp. KM-04 with biphenyl as the sole source of carbon and energy. Poisoned controls showed no growth of the strain KM-04 and no depletion of biphenyl. All values represent the mean of three independent measurements. Symbols: ●, optical density (600 nm) of the live cultures of the strain KM-04; ○, optical density (600 nm) of the poisoned control; ■, biphenyl concentration in the presence of the live strain KM-04 cultures; □, biphenyl concentration in the presence of the poisoned control.

Identification of metabolic intermediates derived from biphenyl. The catabolism of biphenyl by *Pseudomonas* sp. KM-04 gave rise to two metabolic intermediates. The two metabolic intermediates obtained were identified as HOPDA and benzoic acid. The first yellow-colored metabolic intermediate was identified from the diagnostic ESI/Q-TOF/MS peaks observed at *m/z* 219 $[M+H]^+$, which were consistent with the *meta*-cleavage product of biphenyl. The second metabolic intermediate detected was characterized as benzoic acid (ESI/Q-TOF/MS diagnostic peaks at *m/z* 123 $[M+H]^+$), and the mass spectrum of this metabolic intermediate was indistinguishable from that of the authentic standard (Table 1).

Table 1. Metabolic intermediates detected in LC-ESI/Q-TOF/MS after degradation of biphenyl by *Pseudomonas* sp. KM-04.

Metabolic intermediate	RT[a]	Mw[b]	LC-ESI/Q-TOF/MS [M+H]+
 [c]	5.3	218	219
 [d]	16.2	122	123

[a] Retention time (minute).
[b] Molecular weight.
[c] HOPDA (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid).
[d] Benzoic acid.

The expected metabolites, HOPDA and benzoic acid, were identified in the live culture as a result of biphenyl degradation. Further, strain KM-04 grew on benzoic acid and catechol as a sole carbon and energy source. A conventional biphenyl metabolic pathway was proposed for the strain KM-04 (Fig. 2), and this pathway was consistent with previous reports[1,3].

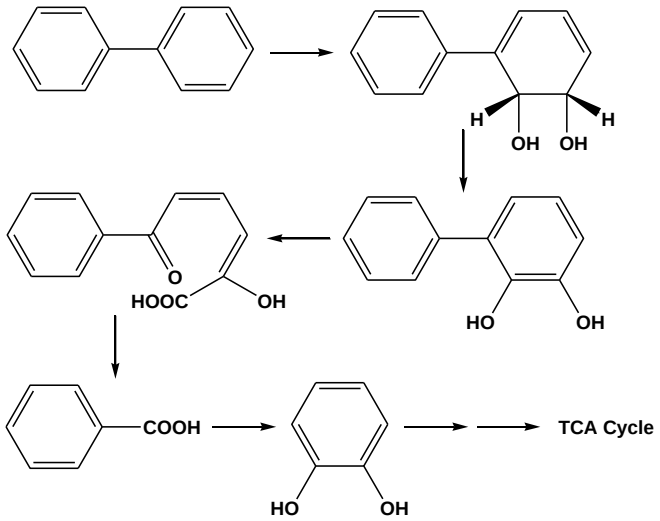


Fig.2. Proposed pathway for the degradation of biphenyl by *Pseudomonas* sp. KM-04.

Most bacteria capable of degrading biphenyl via a pathway through the key intermediates 2,3-dihydroxybiphenyl and its *meta*-cleavage product (HOPDA), the latter of which is then converted to benzoic acid[1-4]. The most important and significant metabolic intermediate is the HOPDA. LC-MS analysis further confirmed the presence of the two key metabolites, HOPDA and benzoic acid. This metabolic pathway differs from the known *bph*A

genes of other aromatic compound degraders (Fig. 2). These results showed that strain KM-04 and the biphenyl-degrading species in other genera share a common biphenyl biodegradation pathway[1-3]. However, strain KM-04 is the first isolate identified that is capable of biphenyl degradation in coal mine-impacted areas. This organism may be an important candidate for remediation of PCB-contaminated abandoned mine areas. It should be emphasized that surface mines and the attendant crushing and milling facilities of both surface and underground mines frequently use PCB-containing electrical equipment.

In conclusion, a bacterial strain capable of degrading biphenyl was isolated from an abandoned coal mine-impacted area. The bacterium was identified as *Pseudomonas* sp. KM-04 according to sequence analysis of the 16S rDNA. Biphenyl, at a concentration of 10 mg/mL, was almost completely removed within 120 h. A biphenyl degradation pathway was tentatively proposed based on identification of the metabolic intermediates. Further studies enzymatic and genetic studies of the biphenyl degradation pathway may improve our understanding of biphenyl degradation in the environment, and may help optimize remediation efforts in PCB-contaminated soil of mine-impacted areas.

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