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Degradation of Phenanthrene by *Sphingomonas* sp. 1-21 Isolated from Oil-Contaminated Soil

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Abstract A phenanthrene-degrading bacterium, Strain 1-21, was isolated from oil-contaminated soil. This strain was a Gram-negative, aerobic, and rod-shaped bacterium, and exhibited a 99% sequence similarity of 16S rDNA to that of *Sphingomonas subarctica*. The major cellular fatty acid was a summed feature 7 (18:1 w7c, 18:1 w9t, 18:1 w12t), which is a characteristic of the *Sphingomonas* species. When 200 and 1,000 ppm of phenanthrene was added as the sole carbon source, Strain 1-21 degraded 98% and 67% after 10 days of incubation, respectively. Futhermore, this strain was also able to utilize naphthalene and fluorene as sole carbon and energy sources.

Key words: PAH, phenanthrene, *Sphingomonas*

Phenanthrene, a polycyclic aromatic hydrocarbon (PAH) containing three fused benzene rings, is produced by the combustion of fossil fuels, other industrial processes, and natural occurrences in forest fires [12]. PAHs are of particular concern because of their persistence and toxicity. Many of these compounds are known to be mutagenic, and are suspected carcinogens [16]. Because of their relatively poor solubility in water and their hydrophobic nature, PAHs strongly adsorb to particulate material, settle on the seafloor, and accumulate in sediments [10].

Although PAHs may undergo volatilization, chemical oxidation, and photodecomposition, the primary factor affecting the persistence of deposited PAHs is microbial degradation [2, 13]. Microorganisms play an important role in the degradation of aromatic hydrocarbons in terrestrial and aquatic ecosystems.

Phenanthrene has been used as a model compound for studying the biodegradation of PAHs since (i) it is found in high concentrations in PAH-contaminated environmental samples, (ii) many PAHs containing a phenanthrene moiety are carcinogenic, and (iii) the regiospecificity and stereoselectivity of oxygenases can be determined in metabolic studies because phenanthrene is the smallest PAH to have both a "bay-region" and "K-region" [1, 9].

Although most previous research on the microbiology of PAH degradation has been focused on a few *Pseudomonas* and *Mycobacterium* species, other bacterial genera whose members have been reported to degrade PAHs include the genera *Aeromonas*, *Flavobacterium*, *Beijerinckia*, *Alcaligenes*, *Micrococcus*, and *Vibrio* [3, 4, 7, 9, 15].

This paper describes effective degradation of PAH by *Sphingomonas* sp. 1-21 obtained from petroleum-contaminated soil in Korea.

Five-hundred milligram of a soil sample obtained from a petroleum-contaminated area was suspended in 5 ml of a minimal medium containing 1,000 ppm of phenanthrene as the sole carbon and energy source, and incubated for 1 week at 28°C with shaking at 130 rpm. The minimal medium contained 30 mg of $(NH_4)_2SO_4$, 2 g of NaCl, 0.5 g of K_2HPO_4, 50 mg of $MgSO_4 \cdot 7H_2O$, 20 mg of $CaCl_2 \cdot H_2O$, 10 mg of KNO_3, and 1 ml of a trace metal solution in 1 L of deioinized water. One-hundred microliter of the culture was transferred to a fresh medium and incubated for 1 week. This procedure was repeated 3 times. The enriched culture was then plated on a minimal agar plate, 0.5% phenanthrene in acetone was sprayed uniformly onto the plate, and the plate was incubated at 28°C for 1 week with frequent observation [14]. Colonies forming clear zones were transferred to a tryptic soy agar plate (Difco, Detroit, U.S.A.) to obtain isolated colonies. Each isolated colony was grown in a minimal medium supplemented with 500 ppm of phenanthrene for 10 days, and then its phenanthrene-degrading capability was analyzed by HPLC.

A DNA sequencing analysis of the 16S rDNA was performed according to the method of Giovannoni *et al.* [8] with a slight modification. The primers used for the amplification of the 16S rRNA gene were 27F and 1492R, which were known to bind specifically to the 16S rDNA

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domain of eubacteria. The PCR products were isolated in an agarose gel and sequenced. The DNA sequence was compared with the GeneBank and EMBL databases, and used to generate a phylogenetic tree by the neighbor-joining method [18]. Neighbor-joining trees were constructed with the program TreeconW, version 1.3b [21], based on evolutionary distances estimated by using the Juckes-Cantor correction. The tree reliability was then assessed by the bootstrap method [5] with 100 pseudoreplicates. A value of 95% was considered to be statistically significant, however, values above 50% are also reported, since a bootstrap is a rather conservative estimate for the reliability of a clade.

To estimate the growth rates, the liquid culture was sampled and mixed with 4 volumes of a solvent mixture (acetone-methanol-hydrochloric acid, 10:10:1 (v/v)) to dissolve the solid phenanthrene, then its O.D. at 660 nm was measured to estimate the number of bacterial cells [11].

The isolated strain was grown in a tryptic soy broth (TSB) for 24 h, washed 3 times with minimal media and resuspended in the minimal medium to be used as an inoculum. A minimal medium containing phenanthrene was inoculated to have an O.D. value of 0.3 at 440 nm and incubated at 28°C at 130 rpm with shaking.

The culture was extracted with 3 volumes of ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated in a vacuum dryer. The extracted phenanthrene was dissolved in 2 ml of acetonitrile and analyzed isocratically by an HPLC equipped with an LC-PAH column (Supelco, U.S.A.) combined with a UV detector. Twenty microliters of the sample were injected, the flow rate was 1 ml of acetonitrile per min, and the peak was observed at 254 nm.

More than 250 colonies were isolated that formed clear zones on the phenanthrene sprayed plates. These colonies were transferred onto plates containing 1,000 ppm of phenanthrene, incubated for 10 days, and a medium color change which is indicative of the syntheses of metabolic intermediates was observed. Twenty-six strains were selected and their phenanthrene-degrading abilities were measured. Of the 26 strains, Strain 1-21 exhibited the best phenanthrene degradation and was used in all the subsequent studies.

Strain 1-21 was a Gram-negative rod 1~1.5 μm long, and formed smooth light yellowish and semi-translucent colonies. Strain 1-21 was positive on both oxidase and catalase. When its fatty acid composition was analyzed, both the summed feature 4 (16:1 w7c/15 iso 2OH) and the summed feature 7 (18:1 w7c/w9t/w12t) were obtained, and these are common characteristics of the *Sphingomonas* species [20]. Interestingly, 2-hydroxylated fatty acids (14:0 2OH, 15:0 2OH, 16:0 2OH) were also detected, which are very rarely found in other bacteria. However, the presence of 2-hydroxylated fatty acids makes this strain unique among other known species of *Sphingomonas*.

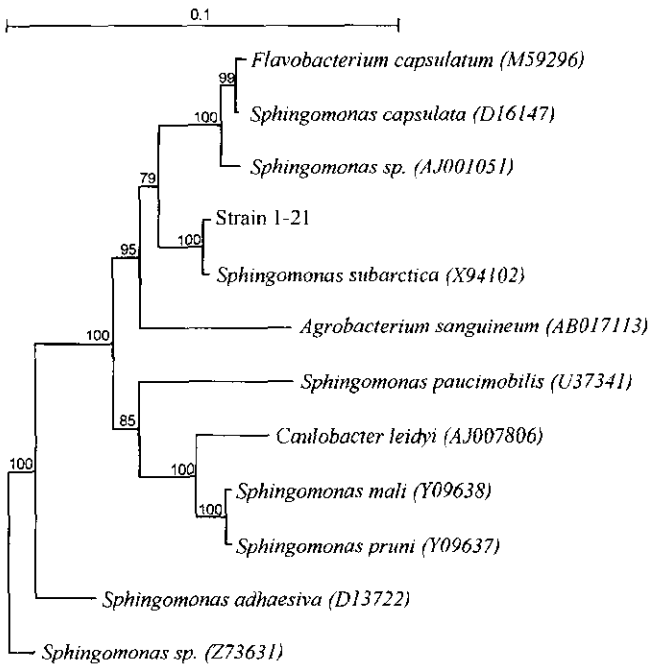

Fig. 1. Phylogenetic tree showing the relationship of Strain 1-21 within the radiation of members of the genus *Sphingomonas* and related taxa.

The 16S rDNA sequence data of the Strain 1-21 showed a 99% similarity to *Sphingomonas subarctica* and a 98% similarity to *Sphingomonas capsulata*. *Sphingomonas subarctica*, first known as a polychlorophenol-degrading bacterium, was originally classified as a member of *Pseudomonas* before being newly classified by Nohynek *et al.* [18]. The present result confirms that the Strain 1-21 is a *Sphingomonas* (Fig. 1).

Sphingomonas is widely distributed in water, soil, and sediment, and has various catabolic capabilities that can be used in bioremediation [6]. Many species of *Sphingomonas* are known to be capable of degrading PAHs, such as toluene, naphthalene, xylene, dibenzofuran, hexachlorohexan, and chlorinated biphenyls. Since many species of *Pseudomonas* and *Flavobacterium* have been reclassified as members of *Sphingomonas*, *Sphingomonas* are expected to degrade other contaminating compounds such as PAHs.

The phenanthrene degrading ability of the Strain 1-21 was measured with various concentrations of phenanthrene in the media. With 100 ppm or 200 ppm of phenanthrene, the strain degraded more than 80% after 5 days and more than 95% after 10 days. This is better than *Mycobacterium sp.* BG1, which degraded more than 90% of 200 ppm of phenanthrene after 2 weeks [17]. Strain 1-21 also degraded 55% of 500 ppm of phenanthrene after 5 days and 93% after 10 days (Fig. 2). With 1,000 ppm, it degraded about 60% after 10 days, which is similar to *Pseudomonas* sp. AJ1 [11]. These results showed that the Strain 1-21 is excellent in phenanthrene degradation.

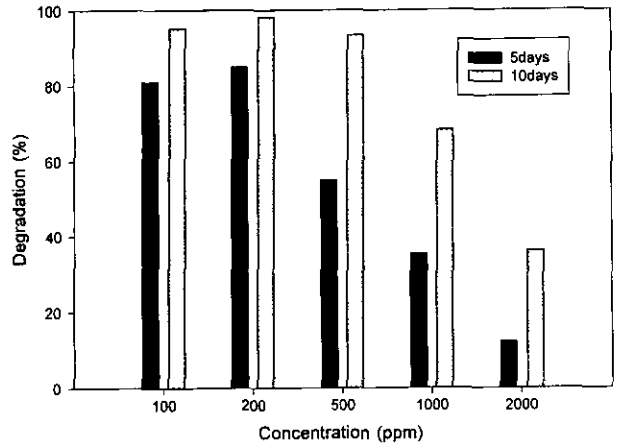

Fig. 2. Degradation of variable concentrations of phenanthrene by Strain 1-21.

Normally, PAHs exist as particles in liquid media and PAH-degrading bacteria grow by adsorbing to these particles, which causes problems in measuring the cell numbers by a plate count or spectrophotometer. However, a new method proposed by Iwabuchi *et al.* [11] suggests that the addition of a solvent mixture (acetone:methanol: hydrochloric acid=10:10:1) to dissolve the remaining phenanthrene particles in the liquid medium makes it possible to assess the cell numbers.

In a 200 ppm phenanthrene-containing minimal medium, the cells grew exponentially after a 2-day induction period and entered the stationary period after 4 days (Fig. 3). The phenanthrene degradation was almost proportional to the cell growth. Essentially, there was no phenanthrene degradation during the first 2 days of incubation, however, the phenanthrene thereafter was degraded slowly for 2 days, then rapidly for 3 days, and finally slowly again until day-9. This result indicates that the Strain 1-21 can grow

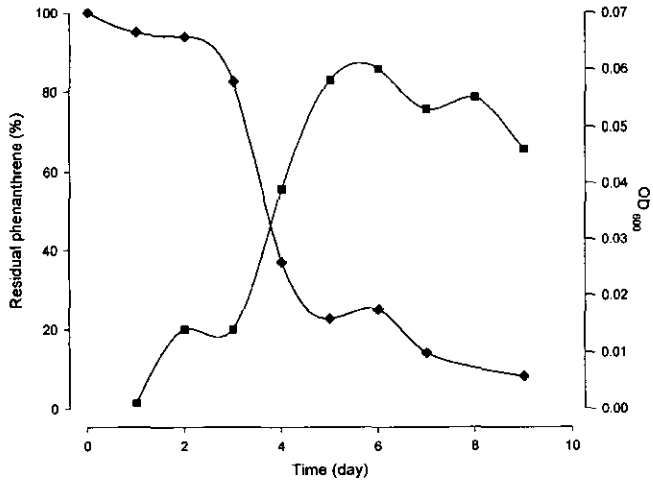

Fig. 3. Relationship between phenanthrene degradation (◆) and growth (■) of Strain 1-21.

Table 1. Degradation of polycyclic aromatic hydrocarbon compounds by Strain 1-21.

Substrate	Degradation
Naphthalene	+
Anthrathene	-
Phenanthrene	+
Fluorene	+
Fluoranthene	-
Pyrene	-

using phenanthrene as the sole carbon and energy source. A previous report [11] stated that bacteria grow very slowly when they use PAHs as the sole carbon and energy source, because they secrete large amounts of metabolic intermediates into the media when they degrade PAHs [11]. When phenanthrene is degraded, 1-hydroxy-2-naphtoic acid is detected in the media (data not shown). When *Pseudomonas* sp. AJ1 is grown in 1,000 ppm of phenenthrene, the maximum O.D. value of 0.1 is obtained at 660 nm [11]. Strain 1-21 exhibited an even lower value of 0.06 in 200 ppm of phenanthrene, thereby indicating a very low growth rate (Fig. 3). Despite these low growth rates, the reason that PAH-degrading bacteria continue to survive in nature is that they do not have to compete with other bacteria for PAHs to survive, since PAHs are not easily degraded or used by other bacteria [11].

To examine whether the Strain 1-21 could degrade other PAHs, the strain was inoculated on minimal medium plates and then uniformly sprayed with 0.5% naphthalene, anthracene, fluorene, fluoranthene, or pyrene in acetone. Strain 1-21 formed clear zones in the naphthalene and fluorene-sprayed plates after 4-5 days and changed the color of the media to yellow. However, it did not degrade anthracene, pyrene, and fluoranthene (Table 1). In contrast, *Sphingomonas paucimobilis* [6], formerly known as *Pseudomonas paucimobilis* [17], can degrade naphthalene, phenanthrene, fluoranthene, and anthracene, but not fluorene and pyrene. This strain is excellent in phenanthrene degradation when compared with other known phenanthrene-degrading bacteria. It also degrades other PAHs such as naphthalene and fluorene. Therefore, the Strain 1-21 could be used in combination with other PAH-degrading bacteria in the bioremediation of petroleum-contaminated areas.

Acknowledgments

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