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Sphingobacterium sp. SW-09 Effectively Degrades Phenanthrene, a Polycyclic Aromatic Hydrocarbon, in a Soil Microcosm

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We isolated a potent phenanthrene-degrading bacterium from oil-contaminated soils of Suzhou, China, and assessed the potential use of these bacteria for bioremediation of soils contaminated by polycyclic aromatic hydrocarbons (PAHs) in a microcosm. Based on 16S rDNA sequencing, we identified this bacteria as *Sphigobacterium* sp. SW-09. By PCR amplification, we also identified catechol 2,3-dioxygenase genes (*nahH* genes) mediating PAH degradation. *Staphylococcus* sp. KW-07, which has been identified in our previous study, showed potential for use in bioremediation of oil-contaminated soils. In this experiment, we compared the rate of phenanthrene-degradation between *Staphylococcus* sp. KW-07 and *Sphingobacterium* sp. SW-09 in a microcosm condition. Newly isolated *Sphingobacterium* sp. SW-09 showed a higher phenanthrene-degradation rate than that of *Staphylococcus* sp. KW-07 in soil microcosms. Together, our results suggest that the *Sphingobacterim* sp. SW-09 strain isolated from the Suzhou area may also be useful in bioremediation of PAH-contaminated soils.

Key words : Polycyclic aromatic hydrocarbon (PAH), bioremediation, phenanthrene, *Sphingobacterum* sp. SW-09, *Staphylococcus* sp. KW-07

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are presented in high concentrations in industrial sites including gas stations, petroleum refinery fields, wood preservation plants, and automobile exhaust fumes [1,2,3] (Fig. 1). PAHs are generally formed by two cases, one is natural sources, such as forest fires, volcanic eruptions, and oil seeps, and the other is anthropogenically from fossil fuel burning, oil spills, and accidents in oil transportation [7].

Trace amounts of PAHs can cause cancer, hypoplasia, and hypersensitivity responses in humans [2,6,13]. Accidents such as the Exxon Valdez oil spill (1989), the Sea Prince oil spill (1995), and the West Sea oil spill (2007) have increased public awareness of PAHs as significant environmental pollutants. The US Environmental Protection Agency (EPA) has classified 16 PAHs as "priority pollutants", thereby increasing the attention devoted to controlling the release of such compounds [19]. However, no effective method for removal of existing PAH pollution has yet been established.

One way to eliminate PAHs from contaminated area is to inoculate into the site with microorganism, which degrades PAHs. Currently so many reports described the isolation and characterization of microorganisms that can degrade PAHs [11,17,21]. Most such microorganisms are in the *Pseudomonas* genus, but some others are in the *Alkaligenes, Mycobacterium, Rhodococcus, Staphylococcus* or *Bacillus* genera, and some are fungi [4,9,10,11,15,16,18,22,23].

In an aims to eliminate PAHs from the contaminated soils, we tried to isolate and characterize microorganisms that effectively degrades PAHs from China. Suzhou area of China is one of the industrial sites suffering from significant PAH pollution, so we attempted to isolate the effective microorganisms from Suzhou. As a result, we isolated *Sphingobacterium* sp. SW-09 as a candidate for bioremediation of PAHs-contaminated soils of Suzhou area and this strain shows higher phenanthrene-degradable activity than *Staphylococcus* sp. KW-07 [4], which has been isolated from an army station of South Korea.

Materials and Methods

Materials

Phenanthrene (Lot# 162-00582) was obtained from Wako Chemicals (Osaka, Japan). All reagents for growth media

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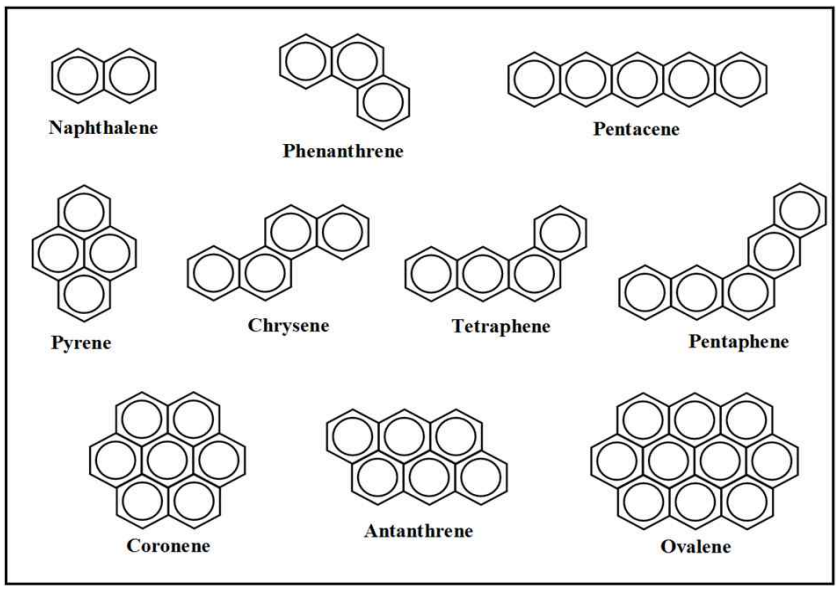

Fig. 1. Chemical structures of various PAHs. PAHs are compounds comprised of two or more benzene rings with no hetero-atoms or substituent groups.

were obtained from Gibco-BRL (California, USA). Organic solvents for HPLC were purchased from Sigma-Aldrich (St. Louis, MO, USA). DNA isolation kits and polymerase chain reaction (PCR) kits were purchased from Core Biosystems (Seoul, Korea) and Takara (Shiga, Japan).

Samplings

Six sites of oil-polluted soil samples were obtained 10 cm below the surface from Suzhou industrial area. Each sample was packed with vinyl pack and stored at 4°C until analysis.

Growth media

Minimal salt medium (MSM), with phenanthrene as the sole carbon source, was employed. First, 5X M9 minimal medium (42.5 g $Na_2HPO_4{\cdot}2H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl, and 2.5 g $MgSO_4{\cdot}7H_2O$ per liter of H_2O) was prepared and autoclaved. Next, mineral elements (73.5 mg $CaCl_2$, 2 mg $CuSO_4$, 20 mg $MnSO_4{\cdot}H_2O$, 20 mg $ZnSO_4{\cdot}7H_2O$, and 10 mg $FeCl_3{\cdot}6H_2O$) were added to this medium [12]. When solid medium, was required, 1.5% (w/v) Bacto-agar was added.

Phenanthrene (0.1%, w/v) was added to liquid medium as a sole carbon source. When we grew the isolates with 0.01, 0.1, 0.5, 1.0, 2.0, or 3.0% (all w/v) phenanthrene in MSM, all had similar growth patterns, suggesting that the concentration of phenanthrene had no effect on bacterial growth. We subsequently cultured all bacteria in MSM with 0.1% (w/v) phenanthrene as sole carbon source.

To prepare phenanthrene-containing solid medium, 1% (w/v) phenanthrene solution dissolved in acetone was sprayed onto solid MSM agar plates, which were next dried for 20 min at room temperature. Luria-Bertani broth (LB) was used as complete medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter of H_2O).

Isolation of phenanthrene-degrading bacteria

One gram of each soil sample was suspended in 10 ml MSM (with 0.1%, w/v, phenanthrene). The suspended solution served as an enrichment culture, and was incubated with shaking (150 rpm) at 25°C for 14 days. Next, 0.2 ml of the supernatant was spread on a solid MSM agar plate and incubated at 25°C. One week later, colonies that formed a clear zone on the spray-coated agar plate were transferred to new MSM agar plates. This process was repeated three times, and pure isolation was successfully done on the phenanthrene layer of the MSM agar plate. From the 20 isolates, a yellow strain showing the largest hollow on a spray-coated phenanthrene agar was selected as a candidate strain. As a negative control, we spread *Escherichia coli* on an MSM agar plate. Bacterial strains propagated from single colonies were stored in 50% (v/v) glycerol solution at -70°C until use.

Identification of phenanthrene-degrading bacteria

Identification of phenanthrene-degrading bacteria was

done by 16S rDNA sequence analysis. We obtained the 16S rDNA sequences of phenanthrene-degrading bacteria. The primer pair 518F (5′-CCA GCA GCC GCG GTA ATA CG-3′) and 800R (5′-TAC CAG GGT ATC TAA TCC-3′) were used for PCR amplification and resulting sequences were analyzed by BLAST (blast.ncbi.nlm.nih.gov).

Optimal temperature and pH of growth of phenanthrene degrading bacteria

The optimal temperature and pH for growth of phenanthrene-degrading bacteria were determined by culturing bacteria in MSM with 0.1% (w/v) phenanthrene as the sole carbon source. Two milliliter aliquots of dense bacterial culture were added to 100 ml fresh MSM with 0.1% (w/v) phenanthrene, and cultured with shaking (150 rpm) at 15°C, 20°C, 25°C, 30°C, 35°C, or 40°C for 5 days. Aliquots (1 ml) were analyzed spectrophotometrically at 600 nm (SmartSpec 3000, Bio-Rad). An analogous method was used to determine the pH optimum, employing pH-adjusted MSM containing 0.1% (w/v) phenanthrene.

Identification of phenanthrene-degrading genes

The phenanthrene-degrading gene, *nahH*, which encodes catechol 2,3-dioxygenase, was amplified from different strains by PCR, using chromosomal DNA as the template. Amplified DNA was resolved on a 1% (w/v) agarose gel and DNA fragments of about 900 bp were collected and re-amplified using same primers as employed in initial PCR. For PCR amplification, the forward primer was 5′-ATG GAA TTC **ATG** AAA AAA GGC GTA ATG CGC-3′ and the reverse primer 5′-ATG CTC GAG **TTA** GGT CAG AAC GGT CAT GAA-3′. The primers contain the initiation and termination regions (the start and stop codons are shown in bold) of catechol 2,3-dioxygenase (*nahH*), which the NCBI database indicates are highly conserved. The underlined regions (GAATTC and CTCGAG) are recognition sites for *Eco*RI and *Xho*I, respectively.

Phenanthrene degradation assay

Bacterial phenanthrene degradation rate was determined by HPLC. Two milliliter amounts of dense bacterial culture were added to 100 ml fresh MSM containing 0.1% (w/v) phenanthrene, and each culture was shaken at 150 rpm for 2 weeks at 25°C. Each day, three aliquots, each of 2 ml, of culture medium were mixed with same volume of acetone by vigorous vortexing. After brief centrifugation, the supernatant (acetone layer) was subjected to HPLC.

Phenanthrene degradation in soil was also determined. In this microcosm assay, cells from 100 ml aliquots of dense cultures of each of *Staphylococcus* sp. KW-07 and *Sphingobacterium* sp. SW-09 were harvested by centrifugation and washed twice with phosphate-buffered saline. Cells were suspended in 5 ml of phosphate-buffered saline to a density of 10^{11} cells/5 ml. Each bacterial suspension (5 ml) was next inoculated into 100 g of autoclaved soil containing 0.1% phenanthrene as a carbon source and incubated at 25°C for 30 days (final density of bacteria was 1×10^{11} cells/ 100 g soil). Each week, 1 g of soil was removed and suspended in 1 ml acetone. After vigorous shaking and centrifugation, the supernatant was analyzed by HPLC to measure phenanthrene concentration. A control experiment was performed by inoculation of *E. coli* into MSM containing 0.1% (w/v) phenanthrene or into soil containing 0.1% (w/v) phenanthrene. Twenty microliter aliquots of organic supernatants were injected into a C-18 column (150 mm × 4.6 mm; Phenomex, California, USA). Solvent (methanol:water = 85:15) flow was 1 ml/min at room temperature. The phenanthrene degradation rate was estimated by examining the HPLC profile of isolated phenanthrene at 254 nm (Waters 2487, Milford, MA, USA).

Results and discussion

Isolation of phenanthrene-degrading bacteria

We isolated 20 strains of phenanthrene-degrading bacteria using MSM with 0.1% (w/v) phenanthrene as the sole carbon source. Colonies that formed on solid MSM differed in morphology and color. Among them, a potent phenanthrene-degrading strain, judged by the size of clear zone on the phenanthrene-coated plates, was selected as a target strain for further experiment. The selected strain yielded as dark-yellow colonies on a plate.

Identification of phenanthrene-degrading bacteria

For identification of the phenanthrene-degrading bacteria, we performed 16S rDNA sequence analysis. Generally, 16S rDNA sequence analysis is the preferred method for identification of bacteria [14]. Our sequence analysis identified the phenanthrene-degrading isolate as *Sphingobacterium* genus (GenBank accession number: JN685591), so we named it as *Sphingobacterium* sp. SW-09. Analysis of the 16S rRNA sequence of our *Sphingobacterium* sp. SW-09 indicated 100% ho-

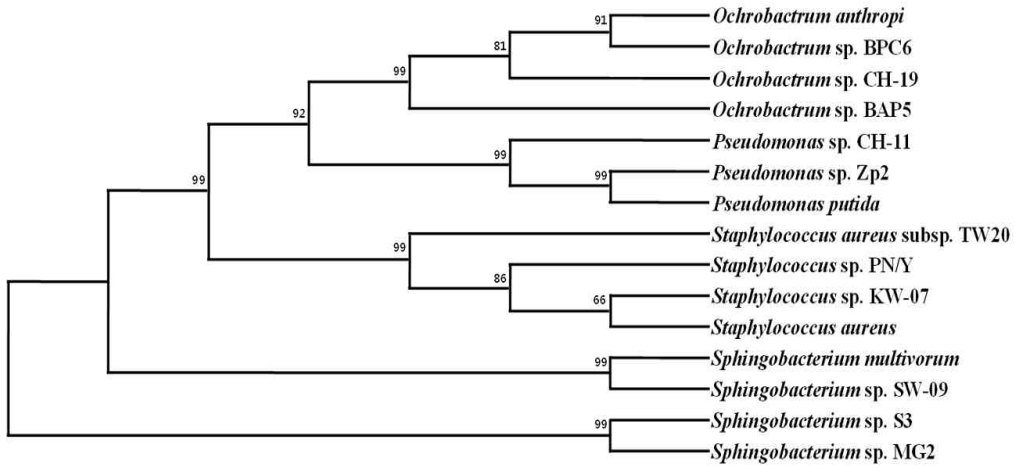

Fig. 2. Phylogenetic tree of *Sphingobacterium* sp. SW-09 and *Staphylococcus* sp. KW-07. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.

mology to several *Sphingobacterium* species including *Sphingobacterium* sp. MG2 (AY556417), and 99% homology to *Sphingobacterium multivorum* (NR040953) (Fig. 2).

Optimal temperature and optimal pH for growth

The optimal growth was done at 25°C and pH 7.0 (data not shown), consistent with the characteristics of many other soil-dwelling bacteria. When we grew the isolates with 0.1, 0.5, 1.0, 2.0, or 3.0% (all w/v) phenanthrene in MSM, all had similar growth patterns (data not shown), suggesting that the concentration of phenanthrene had no effect on bacterial growth. So we decided to culture the bacteria in MSM or soil with 0.1% (w/v) phenanthrene as sole carbon source.

Identification of phenanthrene-degrading gene

To identify phenanthrene-degrading genes, we isolated chromosomal DNA from two strains including *Sphingobacterium* sp. SW-09 and *Staphylococcus* sp. KW-07, and used this DNA as a template for PCR amplification. As shown in Fig. 3, we successfully amplified the 924 bp of *nah*H (encoding catechol 2,3-dioxygenase) from *Sphingobacterium* sp. SW-09 and from *Staphylococcus* sp. KW-07, which has been used as control for *nah*H [4].

Phenanthrene-degrading bacteria exhibit the same degradation pathway under aerobic condition. First, phenanthrene is transformed to dihydrodiol by PAH dioxygenase; dihydrodiol dehydrogenase converts dihydrodiol to catechol; and, finally, catechol is degraded into aldehydes or acids by catechol 2,3-dioxygenase (*nah*H) [4].

To confirm that the amplified DNA fragments contained

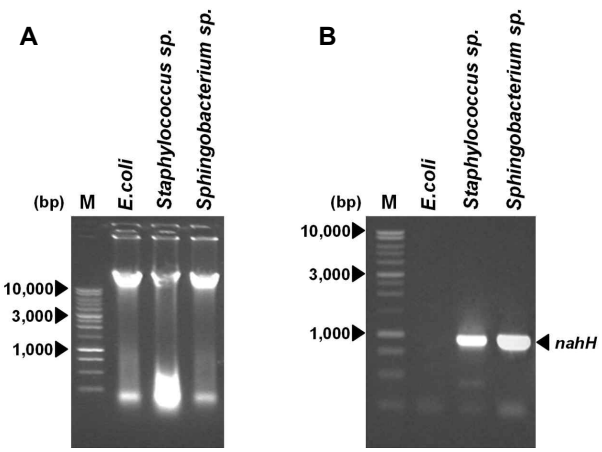

Fig. 3. PCR amplification of the *nah*H gene encoding catechol 2, 3-dioxygenase from *Sphingobacterium* sp. SW-09 and *Staphylococcus* sp. KW-07. (A) Purified chromosomal DNA. (B) PCR amplification of *nah*H genes (924 bp) from each bacterial chromosome.

*nah*H genes, we cloned the amplified fragment into the pGEX vector for DNA sequencing. The *nah*H sequence (924 bp encoding 307 amino acids) of *Sphingobacterium* sp. SW-09 showed 97% homology with that of *Staphylococcus* sp. KW-07 (data not shown).

Phenanthrene degradation rate in MSM

The isolated strain and *Staphylococcus* sp. KW-07 were each cultured in 100 ml MSM with 0.1% (w/v) phenanthrene as the sole carbon source. Each day, we measured optical density at 600 nm to assess bacterial growth, and calculated degradation of phenanthrene by HPLC. We

found that *Sphingobacterium* sp. SW-09 attained the stationary phase at day 10, whereas *Staphylococcus* sp. KW-07 reached at day 14 (Fig. 4A). This result indicates that *Sphingobacterium* sp. SW-09 rapidly grow to effectively degrade phenanthrene.

Our HPLC results indicated that *Sphingobacterium* sp. SW-09 degraded more than 90% of phenanthrene (initial concentration of 0.1%, w/v) in 3 days and degraded almost all of the material in 7 days. However, our previous clone *Staphylococcus* sp. KW-07 showed slightly slower degradation rate compared to the other two species (80% of phenanthrene in 3 days) (Fig. 4B).

Although growth rate of both *Sphingobacterium* sp. SW-09 and *Staphylococcus* sp. KW-07 was still in the latent phase on day 3, the extent of phenanthrene degradation was over 80% at that time. This might because both strains have higher enzyme activities or have different metabolic pathway compared to phenanthrene degrading bacteria has been described elsewhere [5,8,20]

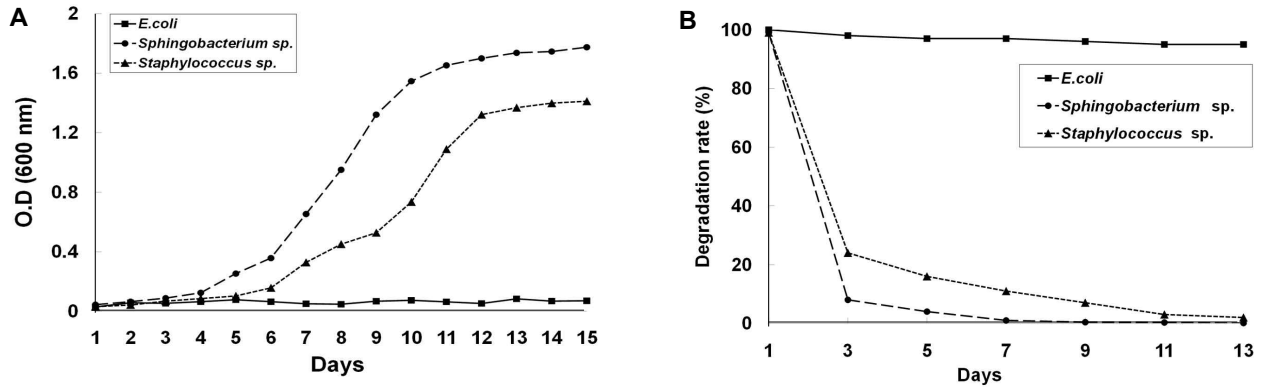

Fig. 4. Growth curves and destruction rate of phenanthrene by phenanthrene-degrading bacteria. (A) Growth curves of phenanthrene-degrading bacteria in minimal medium with 0.1% (w/v) phenanthrene as the sole carbon source. (B) Degradation of phenanthrene by bacteria. Cells were cultured in minimal medium containing 0.1% (w/v) phenanthrene as a sole carbon source, shaking at 25°C, and culture broth aliquots (1 ml) were extracted in triplicate and mixed with equal amounts of acetone. After vigorous vortexing, the organic phase (following centrifugation) was analyzed by HPLC.

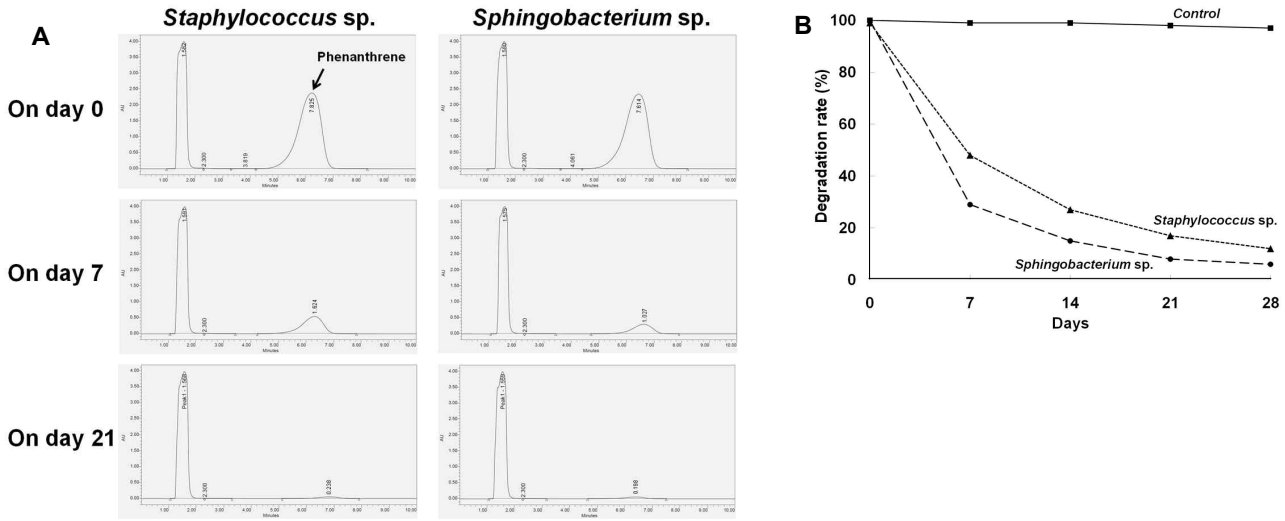

Fig. 5. Phenanthrene degradation in soil soil microcosms. A total of 10^{11} cells of each species were added to 100 g of soil that contained 0.1% (w/v) phenanthrene as sole carbon source, and incubated for 4 weeks at 25°C. At the end of each week, 1 g samples of soil were extracted with 1 ml acetone and the supernatants were analyzed by HPLC. (A) Representative profiles of HPLC analysis. Two peaks within each box represent acetone (left) and phenanthrene (right), respectively. (B) Phenanthrene degradation rates of each bacterial strains obtained by HPLC analysis were indicated at every week.

Phenanthrene degradation in a soil microcosm

We then compared the phenanthrene-degrading ability of both strains in a soil microcosm. We found that after addition of 1×10^{11} cells of *Sphingobacterium* sp. SW-09 to 100 g of soil, the phenanthrene level (0.1%, w/v) was reduced to less than 40% of the initial amount after 1 week (60% degradation for a week). The corresponding figures for our previous clone *Staphylococcus* sp. KW-07 were 50% degradation (Fig. 5). Our results thus strongly suggest that *Sphingobacterium* sp. SW-09 isolated from Suzhou area is more effective than *Staphylococcus* sp. KW-07 for use in bioremediation of PAH-polluted soils. We explored the use of various combinations of bacterial strains, but found no synergistic effect on PAH removal (data not shown).

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초록 : *Sphingobacterium* sp. SW-09에 의한 토양환경에서의 다환 방향족탄화수소인 페난스렌의 분해

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페난스렌은 다환방향족 탄화수소의 일종으로서 미량으로도 인체에 강한 해를 미칠 수 있는 주요 환경오염 물질이다. 미생물을 이용한 페난스렌 제거 목적으로 중국 쑤저우(Suzhou) 지역의 유류 오염토양에서 페난스렌을 강력하게 분해하는 세균을 분리하였다. 16S rDNA 염기서열 결정에 의하여 이 세균은 *Sphingobacterium* sp. SW-09로 동정되었으며 PCR 증폭을 통하여 페난스렌 분해 유전자인 *nah*H를 가지고 있음이 확인되었다. 이전의 연구에서 포천일대의 군부대에서 분리된 강력한 페난스렌 분해세균인 *Staphylococcus* sp. KW-07과 이번에 분리된 *Sphingobacterium* sp. SW-09을 이용하여 이들의 페난스렌 분해능을 비교분석하였다. 그 결과, 쑤저우 지역에서 분리된 *Sphingobacterium* sp. SW-09이 최소배지와 실제토양에서 모두 *Staphylococcus* sp. KW-07보다 강하게 페난스렌을 분해하는 것으로 나타났다. 결과적으로 이번에 분리된 *Sphingobacterium* sp. SW-09을 사용하여 유류 오염토양의 환경정화에 사용할 수 있을 것으로 판단된다.