

Detection of Mega Plasmid from Polycyclic Aromatic Hydrocarbon-Degrading *Sphingomonas* sp. Strain KS14

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Abstract

Polycyclic aromatic hydrocarbon (PAH) degrading *Sphingomonas* sp. strain KS14 was isolated from soil. Strain KS14 was able to utilize phenanthrene and naphthalene as sole source of carbon and energy, and mineralize phenanthrene to carbon dioxide. Strain KS14 cometabolically degraded pyrene using phenanthrene as secondary growth substrate. A large degradative plasmid (>500 kb) was detected from the strain KS14. This study suggests that this mega plasmid could be responsible for the degradation of PAH by strain KS14.

Polycyclic aromatic hydrocarbons (PAHs), a class of fused-ring aromatic compounds, are widespread environmental pollutants. They are commonly found in soil (Jones *et al.*, 1989), estuarine waters and sediment (Guerin, 1989; Shiaris, 1989) as pollutants. PAHs are considered hazardous because of their potential trophic biomagnification and acute toxicity (Darville *et al.*, 1984). Most PAH compounds have mutagenic, teratogenic, and potential carcinogenic effects (Cerniglia, 1989; Chemists, 1983), and it has been generally accepted that these compounds are resistant to the biodegradation processes (Boldrin *et al.*, 1993; Bossert *et al.*, 1984; Herbes, 1981; Walter *et al.*, 1991). Here is described the isolation and characterization of *Sphingomonas* sp. KS14, which is able to degrade phenanthrene as a sole carbon and energy source. This organism contains a stable plasmid of >500 kb. Evidences presented in this paper suggest the possibility that the metabolisms of PAHs by strain KS14 are plasmid mediated.

Soil samples were collected from waste oil disposal site. To isolate PAH degrading bacteria, soil suspensions were prepared by homogenizing 10 g of soil with 100 ml of ice-cold 0.1% sodium pyrophosphate solution (pH 7.0). After sedimenting the coarse soil particles, 1 ml of the supernatant was added to 10 ml of a minimal medium

(Ogunseitan *et al.*, 1991) that contained phenanthrene (100 mg/l) as a sole carbon and energy source. Culture was conducted at 25°C, and after 7 days of incubation, 1 ml of this enrichment culture was transferred to a fresh minimal medium and incubated for additional 7 days. After enrichment cultivation, 0.1 ml of the medium was spread on mineral salt agar (MSA, MSM containing 1.5% Bacto Noble Agar [Difco]) plate, and phenanthrene was uniformly sprayed over the surface of the MSA phenanthrene plate (Kiyohara *et al.*, 1982). The plate was then incubated at 25°C for a week. Colonies that were surrounded with a clear zone were chosen and purified to obtain single strains. Isolates were inoculated to MSM containing phenanthrene (100 mg/l) and incubated for a week in order to confirm the biodegradability. Finally, the strain, which showed notable degradation of fine particles of phenanthrene, was selected as a phenanthrene degrader and designated as strain KS14.

For the biodegradation versatility test, several aromatic compounds were tested. They were naphthalene, anthracene, phenanthrene, pyrene, salicylate and benzoate. Each compound was added to sterile MSM (final conc. 100 mg/l) and then the isolated strain that was grown overnight prior to inoculation was inoculated to the medium. After incubation for 1 week at 25°C in the dark for avoiding photo-destruction of PAH, the ability of the isolated strain to utilize a compound as its sole carbon and energy source was determined. Strain KS14 was able to utilize naphthalene, phenanthrene, benzoate, and salicylate as sole carbon and energy source.

In order to identify strain KS14, fatty acids profile of strain KS14 was analyzed. Fatty acids methyl esters (FAME) were prepared from strain KS14 and analyzed by using MIDI identification system and Sherlock software (MIDI, Inc.). Details were done according to the manufacturer's protocol. Analyses on fatty acids methyl ester profiles showed that strain KS14 belongs to genus *Sphingomonas*. Strain KS14 contained the 2-hydroxymyristate (14:0 2-OH), summed feature 4 (16:1 w7c and 15:0 iso 2-OH), and summed feature 7 (18:1 w7c, 18:1 w9t, and 18:1 w12t) as the dominant fatty acid (78.71%), and did not contain any 3-hydroxylated fatty acids. The presence of 2-hydroxy fatty acids and the absence of 3-hydroxy fatty acids are the main characteristics of genus *Sphingomonas* (Takeuchi *et al.*, 1995; Yabuuchi *et al.*, 1990), and were consistent with our results. Additionally, the analysis of 16S rDNA was performed. Genomic DNA of strain KS14 was extracted, and V3 region of 16S rDNA was amplified and sequenced according to the method of Lee *et al.* (1996). Phylogenetic analysis showed that the strain KS14 belonged to the genus *Sphingomonas* and

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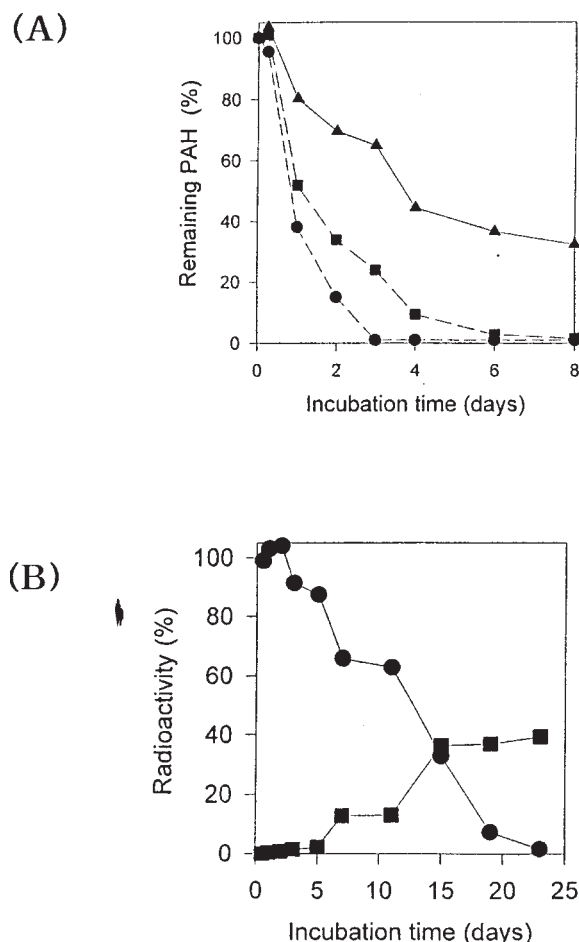


Figure 1. Biodegradation and Mineralization of PAHs by *Spingomonas* sp. Strain KS14. (A) Biodegradation of PAHs, biodegradation of phenanthrene (circle) as sole carbon and energy source; cometabolic degradation of pyrene (triangle) when phenanthrene (square) was used for a growth substrate. (B) Mineralization of [9-¹⁴C] Phenanthrene to ¹⁴CO₂, carbon dioxide, (square); dichloromethane fraction, (circle).

was most closely related to *Spingomonas capsulata* (similarity, 98%). Degradation of aromatic hydrocarbons by *Spingomonas* spp. have been reported by many investigators (Eaton *et al.*, 1996; Fredrickson *et al.*, 1995; Happe *et al.*, 1993), and the catabolic versatility of members of the genus *Spingomonas* is becoming increasingly evident (Armengaud *et al.*, 1988).

To investigate the biodegradability, overnight grown strain KS14 was resuspended in sterile phosphate-buffered saline solution (PBS, pH 7.0), washed twice in PBS and left standing for 1 h starvation period. Aliquots of bacterial suspension were inoculated (final cell conc. approximately 10⁷ cfu/ml) to sterile MSM supplemented with phenanthrene (100 mg/l) as a sole carbon and energy source, and then culture liquid was incubated on a rotary shaker (25°C, 100 rpm) in the dark. The concentration of phenanthrene was determined by gas chromatography after extraction with dichloromethane according to the method of Cho and Kim (2000). Strain KS14 degraded more than 99% (99 mg/l) of phenanthrene added (Figure 1). After 3 days of incubation, the concentration of remaining

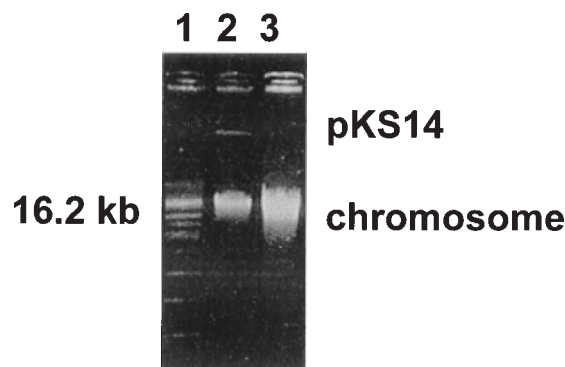


Figure 2. Agarose Gel Electrophoretogram of Plasmid DNA Purified from *Spingomonas* sp. Strain KS14. Lane 1, supercoiled DNA ladder (size marker); lane 2, *Spingomonas* sp. Strain KS14; lane 3, cured strain KS14. DNA molecules were resolved by electrophoresis in 0.7% agarose in E buffer.

phenanthrene was under the detection limit (<1 mg/l). Degradation rate was 4.07 mg/l/h. Final cell concentration was increased about 2 orders over initial cell concentration. Sterile control showed the loss of less than 5% of each PAH examined in all degradation experiments.

All the isolated phenanthrene degrading strains, including strain KS14, could not utilize pyrene as a sole carbon and energy source. In order to select the strain that was able to degrade pyrene cometabolically, PAHs-covered plates that were supplemented with both pyrene and phenanthrene as an opaque thin layer were also used in this experiment. This technique was very useful to isolate the cometabolizers. Strain KS14 could grow on this PAH-double-covered plate and form a clear zone as the result of the degradation of both pyrene and phenanthrene.

To quantify and confirm the cometabolic degradation of pyrene, strain KS14 which was prepared by the procedure same as phenanthrene degradation experiment was inoculated to the minimal medium supplemented with both cosubstrate (pyrene, 100 mg/l) and growth substrate (phenanthrene, 100 mg/l). In the case of the degradation experiment where phenanthrene was used as a growth substrate, 62.5% of pyrene added was degraded by strain KS14 within 8 days of incubation and the concentration of phenanthrene was coincidentally decreased to under the detection limit in this period (Figure 1A). The degradation rate of phenanthrene as a growth substrate (1.12 mg/l/h) was lower than that of phenanthrene as sole carbon and energy source. Strain KS14 was able to degrade pyrene, only when phenanthrene existed in the medium as a primary substrate. It was considered that the enzyme produced by strain KS14 in order to degrade phenanthrene could degrade pyrene, and that pyrene alone could not induce such an enzyme. It was supposed that the lower degradation rate of primary substrate phenanthrene was due to the competition between primary substrate (phenanthrene) and secondary substrate (pyrene) for the same enzyme.

For demonstrating the mineralization by release of $^{14}\text{CO}_2$, strain KS14 was incubated with 500 mg/l phenanthrene supplemented with [9- ^{14}C] phenanthrene in biometric flasks. $^{14}\text{CO}_2$ was trapped in sodium hydroxide solution and remaining phenanthrene was extracted with dichloromethane, and radioactivity was measured with a liquid scintillation analyzer (Tri-Carb 1500, Packard). Figure 1(B) illustrates the time course of phenanthrene mineralization. After 23 days of incubation, 39.6% of the radio-labeled carbon was metabolized to $^{14}\text{CO}_2$, and 1.7% was recovered in the dichloromethane (organic) fraction. Sterile control showed the 98.5% of total radioactivity in the dichloromethane fraction and 1.1% in the $^{14}\text{CO}_2$ absorbing agent. The rest of radioactivity that was not recovered in the mineralization experiments might be due to partially degraded metabolic intermediates and biomass-incorporated ^{14}C .

Catabolic pathways which encode numerous aromatic hydrocarbon degradation pathways are frequently located on plasmids, although the pathways for some xenobiotic compounds such as chlorinated aromatic hydrocarbons can be located on either chromosome or plasmid. In the case of PAHs, it is also supposed that the genes that are responsible for the degradation of PAHs can be located on the plasmid. Therefore, the possibility of the involvement of catabolic plasmid in the degradation of phenanthrene by strain KS14 was investigated.

However, in the initial phase, any plasmids were not detected by using routine alkaline extraction methods. Extremely large plasmid of strain KS14 was detected by the only method using the lysis at 60°C followed by acidic phenol (saturated only with deionized distilled water) treatment (Kado and Liu, 1981). Electrophoresis was carried out at 5 V/cm for 1 h, and plasmid size was calculated from the regression equation for the log of the relative mobility versus the log of the molecular weight for a series of plasmid size standard (supercoiled DNA ladder, Sigma). Extremely large plasmid of strain KS14 was detected (Figure 2). The size of plasmid was approximately 500 kb. It was much larger than NAH plasmids that play a major role in naphthalene biodegradation (Yen *et al.*, 1988). The efficiency of the extraction was not good enough to thoroughly eliminate chromosomal DNA. It was also found that this mega plasmid was easily broken down even during pipetting with micropipette tip and could probably be precipitated with chromosome portion during the extraction step. This large plasmid was designated pKS14. Plasmid pKS14 was maintained well even under the non-selective conditions (repetitive cultivations in nutrient medium without any aromatic hydrocarbons). To elucidate the relationship between pKS14 and PAH-biodegradability of strain KS14, plasmid pKS14 was eliminated from its host strain KS14. This plasmid pKS14 was successfully eliminated in the LB broth containing mitomycin C (5 $\mu\text{g}/\text{ml}$) as a curing agent according to the method of Rheinwald *et al.* (1973). The cured strain (Figure 2) lost both the plasmid and the capability to degrade any PAH tested in this study. However, due to the experimental difficulties caused by the extremely large size of plasmid pKS14, further molecular biological and genetic analyses of the plasmid could not be performed. For studying such mega plasmids, effective

extraction methods are required. This study suggests that the plasmid pKS14 could be involved in phenanthrene and pyrene biodegradation and might play a major role in the biodegradation of PAHs.

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