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Inhibitory Effect of Aged Petroleum Hydrocarbons on the Survival of Inoculated Microorganism in a Crude-Oil-Contaminated Site

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We studied the effects of aged total petroleum hydrocarbons (aged TPH) on the survival of allochthonous diesel-degrading *Rhodococcus* sp. strain YS-7 in both laboratory and field investigations. The aged TPH extracted from a crude-oil-contaminated site were fractionized by thin-layer chromatography/flame ionization detection (TLC/FID). The three fractions identified were saturated aliphatic (SA), aromatic hydrocarbon (AH), and asphaltene-resin (AR). The ratio and composition of the separated fractions in the aged TPH were quite different from the crude-oil fractions. In the aged TPH, the SA and AH fractions were reduced and the AR fraction was dramatically increased compared with crude oil. The SA and AH fractions (2 mg/l each) of the aged TPH inhibited the growth of strain YS-7. Unexpectedly, the AR fraction had no effect on the survival of strain YS-7. However, crude oil (1,000 mg/l) did not inhibit the growth of strain YS-7. When strain YS-7 was inoculated into an aged crude-oil-contaminated field and its presence was monitored by fluorescent *in situ* hybridization (FISH), we discovered that it had disappeared on 36 days after the inoculation. For the first time, this study has demonstrated that the SA and AH fractions in aged TPH are more toxic to an allochthonous diesel-degrading strain than the AR fraction.

Keywords: FISH, TLC/FID, saturated hydrocarbon, aromatic hydrocarbon, asphaltene, resin

Crude oils are complex mixtures of a very large number of different hydrocarbons and have a range of chemical properties found in all oils. The hydrocarbons in crude oils can be classified into three major groups: (i) the saturated aliphatic hydrocarbons (SA), the most commonly found molecules, including alkanes (linear or branched) and cycloalkanes, (ii) the aromatic hydrocarbons (AH), and (iii) resin and asphaltene (AR)-like compounds, which are polar, high molecular weight, and more complicated chemicals [11]. The other organic compounds in crude oils contain nitrogen, oxygen, sulfur, and trace amounts of metals [1, 3, 27].

Many researchers have studied the microbial degradation of crude oils and investigated the factors that are involved in successful bioremediation [10, 16, 22]. Biological factors of bioremediation include survival of inoculated microorganisms, indigenous microbial community structure and diversity, and competition with autochthonous microorganisms for either nutrients or electron acceptors [15, 17, 20–22]. A number of reports have been accumulated suggesting that the structure of the active bacterial community changes after heavy oil contamination [4, 8]. Reduction in the diversity of a bacterial community is common after petroleum exposure, but an increase in bacterial diversity has also been reported [14, 29].

It is well known that microbial activities affect the physical characteristics of crude oil [16]. Among the constituents of diesel oil, low molecular weight hydrocarbons such as *n*-alkanes and simple aromatic compounds could be readily removed *via* either microbial biodegradation or volatilization [3], whereas complex compounds including aged hydrocarbons appear to be more persistent in crude-oil-contaminated areas. Specifically, AR fractions are known to be more recalcitrant to microbial biodegradation [28]. Previous studies reported that petroleum hydrocarbons alter microbial membrane structures by changing membrane fatty acids and protein composition [26]. However, much less is known about the toxicity of each fraction and the survival of inoculated microorganisms in a crude-oil-contaminated site. It is still not clear what components in a crude oil are greater environmental risks and cause decreased survival and activity of soil microorganisms. We therefore sought

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to examine the effects and toxicity of each fraction of aged petroleum hydrocarbons collected from a crude-oil-contaminated site using an allochthonous diesel-degrading *Rhodococcus* sp. strain YS-7.

Materials and Methods

Isolation and Identification of Microorganisms

The YS-7 strain was isolated from oil-contaminated soils (Seoul, Korea) using a diesel-oil (LG Caltex, Korea) enrichment culture as a sole carbon source. The 16S rRNA of strain YS-7 was amplified by the polymerase chain reaction (PCR) from DNA extracts with the universal 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGCTACCTTGTTACGACTT-3') primers, and purified. The 16S rRNA partial sequence of strain YS-7 obtained in this study was deposited in GenBank under Accession No. EU821779. The VITEK2 systems were used for physiological characterization, following the manufacturer's instructions. Strain YS-7 was grown on LB agar plates, suspended in 3 ml of autoclaved distilled water, and adjusted to a turbidity equivalent of a 0.6−0.7 McFarland standard using a colorimeter. The prepared solutions were loaded into a VITEK2 (bioMérieux, Marcy-l'Etoile, France) GP (Gram-positive) card, and the data were analyzed using AES parameter version VITEK2 (03.01).

Measurement of Residual Diesel Oil Using Gas Chromatography/Flame Ionization Detector (GC/FID)

Diesel oil (10,000 mg/l) was added to 20 ml of minimal salts basal media (MSB; [23]). Prepared samples were grown at room temperature with shaking at 220 rpm. The residual diesel oil concentration was analyzed using GC/FID. Culture solutions were collected at various time-points (1-day intervals) and mixed with 5 ml of dichloromethane (DCM). The culture−DCM mixture was vortexed and centrifuged at 1,540 ×*g* for 10 min. After the separation, the DCM layers were filtered through nylon 66 filters (0.45 µm; Whatman). Analytes were assayed using an ACME 6100 gas chromatograph (Younglin, Korea). A HP-5MS silica capillary column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, U.S.A.) was used for the GC/FID assay. The assay was performed at the following running conditions: oven temperature, 250°C; detector temperature, 310°C; holding for 3 min at 50°C, 50 to 300°C at 8°C/min, and for 3 min at 300°C.

Thin-Layer Chromatography/Flame Ionization Detection (TLC/FID) and Gas Chromatography/Mass Spectrometry (GC/MS)

An Iatroscan MK6 (Iatroscan Lab., Japan) was used as a TLC/FID system. Silica-coated glass rods (Chromarod-SIII, Japan) were used for the separation of petroleum components extracted from the oil-contaminated soils. One kg of soil was mixed with three volumes of chloroform. The mixture was filtered with 0.45 µm filtration paper (Millipore, U.S.A.), and the filtrate was evaporated using a rotary evaporator (EYELA, Japan). This procedure was repeated. Evaporated samples were resuspended with chloroform and used in further experiments. For TLC, petroleum extracts [1% (w/v)] were prepared with chloroform, and 1 µl of the solution was spotted on the bottom of a glass rod. The spotted rod was developed with *n*-hexane for 30 min. After the solvent was changed to *n*-hexane:toluene [1:4 (v/v)] the glass rod was developed for 10 min. After development, the three spots were placed on the glass rod. The spot with the larger R_f was aliphatic hydrocarbon, and the lower spot was aromatic hydrocarbon. Resin and asphaltene remained on the baseline. The separation conditions were as follows: attenuation=25; chart speed= 10 cm/min; scanning speed=30 s/rod; gas flow=160 ml/min. The relative area of each fraction was measured by the area of the peak on the baseline. The GC/MS analysis of the SA, AH, and AR fractions was conducted using an Agilent 6890N gas chromatograph interfaced to a 5975A mass selective detector (Agilent). A fused-silica capillary column HP-5MS (Agilent) was used for the GC/MS assay. The fractions were dissolved in dichloromethane and injected.

Growth Inhibition by Each Fraction

The SA, AH, and AR fractions survival experiments were carried out in LB medium. Stationary-phase grown cells were inoculated into LB medium at 30°C with agitation (220 rpm), and 2 mg/l of each fraction was added during the exponential growth phase (OD_{600} ~0.3−0.5). Indigenous bacterium *Micrococcus* sp. strain SBS-8 (NCBI Accession No. EU821777) and another allochthonous diesel-oil-degrading *Acinetobacter* sp. strain DR1 (EF520734) were also used for growth inhibition test. At the 1-h interval, the cells were harvested and the OD was measured with a Biophotometer (Eppendorf, Hamburg, Germany). Harvested cells were washed with autoclaved phosphate-buffered saline (PBS, pH 7.5), diluted, and serially plated on LB agar plates. The plates were incubated, lid down, at 30°C for 16 h, and the colonies were counted.

Field Test Design and Fluorescence *In Situ* Hybridization (FISH)

The field survival test of the inoculated mixture of strain YS-7 (50 l solution including 10^6 cells/ml of distilled water) was examined at the crude-oil-contaminated site for more than 10 years (Inchon, Korea). The experimental fields were composed of two areas of 10 m^2, which were surrounded by a ditch. The FISH test for detecting the presence of the targeted bacterium was carried out using a previously described method [2, 12]. Five oligonucleotide probes were used for the FISH test. The eubacterial probes (EUB338, EUB338 II, and EUB338 III), which target most eubacteria, were labeled with Cy3, and two targeted bacteria-specific probes, YS-7FI and SBS-8FI, were labeled with fluorescein isothiocyanate (Table 3). The microscope was equipped with a fluorescence filter cube for detecting signals (Carl Zeiss, Germany). The AxioVision 4.5 software was used to acquire images.

Results

Molecular Characterization of Strain YS-7

Large fractions of crude oil consist of long-chain alkanes. Diesel oils are thought to be good carbon sources for enriching alkane degraders, which might have potential as bioremediation agents in a crude-oil-contaminated site. The diesel-oil degrader, designated as strain YS-7, was isolated from oil-contaminated sites with gasoline. Phylogenic analysis based on 16S rRNA sequences indicated that strain YS-7 showed a high similarity (~99%) with *Rhodococcus erythropolis* (data not shown). Based on the 16S rRNA data, we used the corresponding VITEK2 GP card for the

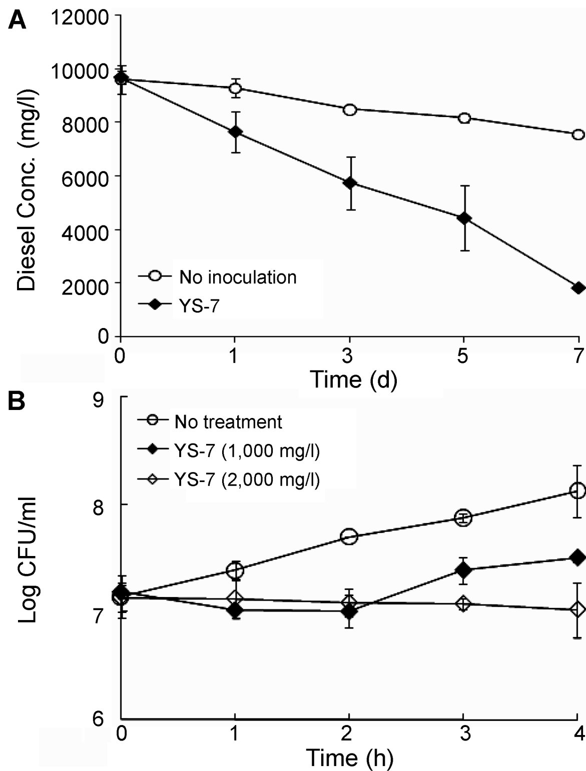

Fig. 1. Diesel oil degradation (**A**) of strain YS-7 in MSB medium using GC/FID and colony counting.

Diesel oil (10,000 mg/l) was added to the MSB medium as a sole carbon source. Crude oil inhibition assay (**B**) of strain YS-7 in LB medium using colony counting. Each sample was incubated at room temperature with shaking (220 rpm). The inhibition assay of strain YS-7 was performed by adding crude oil (1,000 and 2,000 mg/l) to the LB medium. No inoculation: control with a natural decrease of diesel oil. Data are represented as a relative value of initial concentration and the average obtained from individual duplicate samples. No treatment: cells grown without the treatment.

enzymatic analysis. Strain YS-7 positively reacted with arginine dihydrolase 1, leucine arylamidase, L-proline arylamidase, and urease. Thus, the VITEK2 system confirmed that strain YS-7 belongs to the genus *Rhodococcus*.

Table 1. The ratio of the petroleum fractions extracted from soil.

Extracted fractions (TLC/FID)	Crude oil	Aged crude oil
SA fraction (%)	43	33
AH fraction (%)	21	14
AR fraction (%)	36	53

SA, saturated aliphatic hydrocarbons; AH, aromatic hydrocarbons; AR, resin and asphaltene-like.

Diesel Oil Utilization by Strain YS-7

To investigate the utilization of diesel oil as a carbon source, 10,000 mg/l diesel oil was used for the GC/FID analysis. The strain YS-7 could grow on diesel oils (data not shown). Within 7 days, strain YS-7 had almost completely degraded the diesel oil, showing ~1,840 mg/l residual concentration of diesel oil (Fig. 1A). To provide further information of crude oil toxicity to strain YS-7, we performed an inhibition assay [13]. Different concentrations of crude oil were added to exponential-phase-grown strain YS-7 in LB medium, and the colony forming units (CFU) were measured after 4 h. The results showed that the crude oils were toxic, but there was no toxic effect after 2 h at 1,000 mg/l (Fig. 1B). In the presence of ≥2,000 mg/l crude oil, there was a persistent toxicity and cell viability was gradually reduced (data not shown). These results suggested that low concentrations of crude oil have limited toxicity to strain YS-7.

Extraction of the SA, AH, and AR Components in Oil-Contaminated Soils

The ratio of the SA, AH, and AR fractions extracted from crude oil was 43%, 21%, and 36 %, respectively. The crude oil used for our tests was the Forouzan oil with 29.9 API gravity (medium crude oil; Iran). This fresh crude oil we analyzed might be different from original crude oils that had been stored at our study site for more than 10 years. Crude-oil contamination at our study site (a SK petroleum storage site) happened as a result of leaks from pipelines and storage tank-bottom. We compared the composition of

Table 2. Identification of compounds containing SA and AH fractions using GC/MS.

Major compounds in SA fraction	Major compounds in AH fraction
2,6-Dimethyl-undecane	Toluene
2,6-Dimethyl-octane	*p*-Xylene
4-Methyl-1-decene	1,3-Dimethyl-benzene
2-Bromo dodecane	1-Ethyl-2-methyl-benzene
2,6,10-Trimethyl dodecane	1,2,3,5-Tetramethyl-benzene
2,6,10,14-Tetramethyl-heptadecane	1,2,3,4-Tetrahydro-8-methyl-1-naphthalenemethanol
Pentadecane	*N*-[4-(Trimethylsilyl)phenyl]-acetamide
Hexadecane	1-(3-Methylbutyl)-2,3,4-trimethylbenzene
2,6,10,14-Tetramethyl-pentadecane	2,6-Dichloro-3-nitrotoluene
Heptadecane	1,4,6-Trimethyl-naphthalene
2,6,10,14-Tetramethyl-hexadecane	1-(3-Methylbutyl)-2,3,6-trimethylbenzene

SA, saturated aliphatic hydrocarbons; AH, aromatic hydrocarbons.

Table 3. 16S rRNA oligonucleotides used for FISH.

Name	Sequence (5'-3')	Target microorganisms	Reference
EUB386	GCTGCCTCCCGTAGGAGT	Proteobacteria, *Cytophaga-Flavobacterium*, and Gram-positive bacteria	[2]
EUB386 II	GCAGCCACCCGTAGGTGT	Planctomycetes	[6]
EUB386 III	GCTGCCACCCGTAGGTGT	Verrucomicrobia	[6]
YS-7FI	TTGCGCTCCGTCCCTGCT	*Rhodococcus* sp. strain YS-7	This study
SBS-8FI	AGGCGCTCCTATCCGGTA	*Micrococcus* sp. strain SBS-8	This study

fresh crude oil with that of aged crude oil extracted from our study site. Interestingly, the SA and AH compositions were significantly lower and the AR fraction was dramatically higher in the aged petroleum-contaminated soils (Table 1). We therefore conducted GC/MS to identify the compounds in each fraction (Table 2). Many branched alkanes and branched aromatic compounds were found in both the SA and AH fractions (Table 2). However, we could not identify any of the compounds in the AR fraction using our GC/MS approach (data not shown). These results suggested that the components of crude oil could be changed *in situ* through time by increasing AR fraction and more branched alkanes, which might influence the survival of specific microorganisms in the microbial communities of the soil environment.

Growth Inhibition Assay on Each Fraction

We performed a growth inhibition test on all three fractions to investigate their effects on the survival of strain YS-7. The growth of strain YS-7 was greatly reduced by the addition of the SA and AH fractions (Fig. 2A and 2B); however, the AR fraction did not affect growth. It is

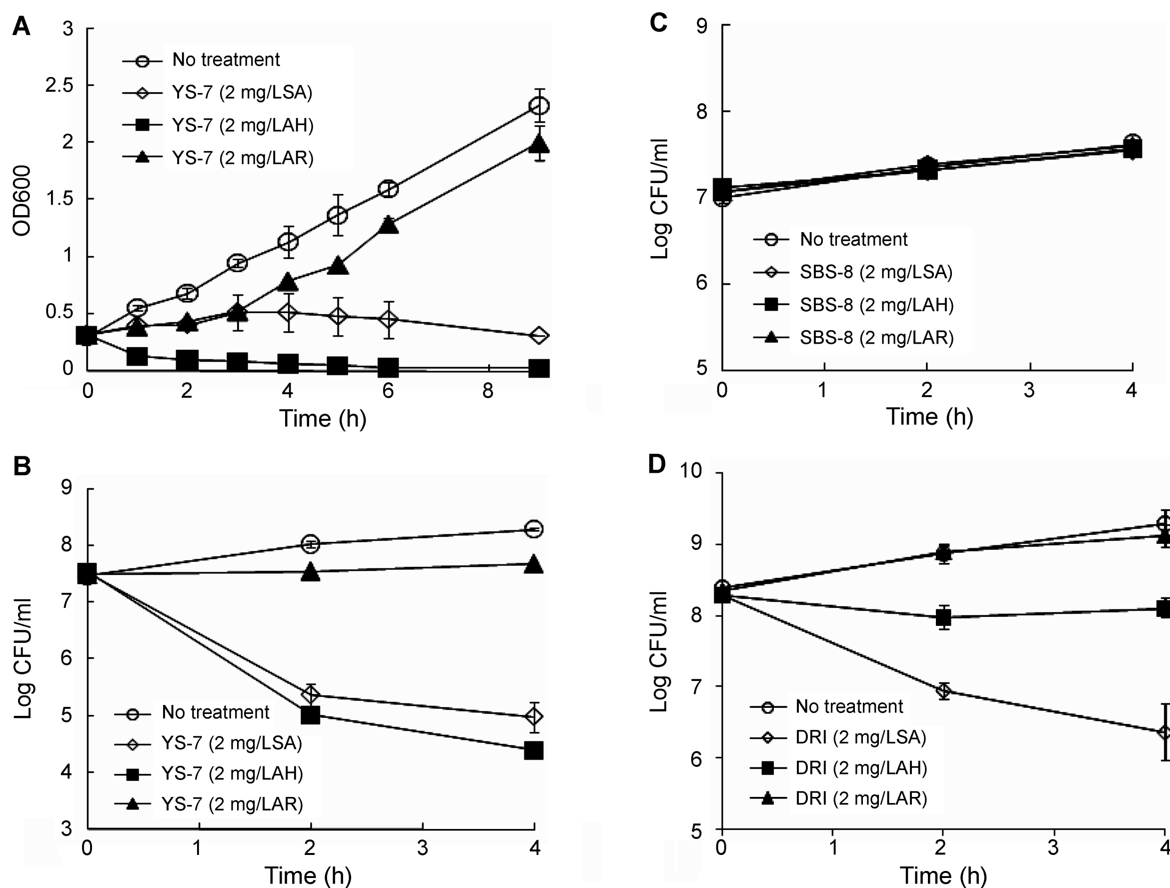

Fig. 2. Inhibition assay of strain YS-7 using the SA, AH, and AR fractions.

The effects of each fraction on the growth of strain YS-7 in LB liquid medium. Each fraction (2 mg/l) was added to exponential-phase-grown cells. Bacterial growth was monitored by measuring the optical density at 600 nm (**A**). The inhibition assays of strain YS-7 (**B**), strain SBS-8 (**C**), and strain DR1 (**D**) were performed by counting viable cells.

interesting that saturated hydrocarbons inhibited the growth of the diesel-oil degrader strain YS-7. From these results above, we speculated that the SA and AH fractions (probably branched hydrocarbons) from the aged petroleum hydrocarbons might be the main compounds inhibiting the survival of strain YS-7 in the oil-contaminated soil. In addition to strain YS-7, we performed the growth inhibition test using strain SBS-8 with another allochthonous diesel-oil-degrading *Acinetobacter* sp. strain DR1 on all three fractions. *Micrococcus* sp. strain SBS-8, isolated from our study site (oil-contaminated soils), was used as a representative of indigenous bacteria. *Acinetobacter* sp. strain DR1 was isolated from the soil of a Korea University paddy field (Deokso, Gyeonggi-Do) by enrichment cultivation, using corn oil as a sole carbon source. The growth inhibition test was accomplished by counting viable cells. Unlike strain YS-7, the growth of strain SBS-8 was not inhibited by all three fractions. However, the only SA fraction inhibited the growth of strain DR1 (Fig. 2C). Saturated aliphatic hydrocarbons were a heavier inhibition factor than AH fractions in the case of strain DR1. However, the growth of strain DR1was not affected by the AR fraction (Fig. 2D).

In Situ Bioremediation and the Monitoring of Introduced Bacteria

To identify the relationship between the survival of introduced microorganisms and the toxicity of aged petroleum hydrocarbons, *Rhodococcus* sp. strain YS-7 was inoculated into oil-contaminated soils that had been exposed to crude oil for over 10 years. The survival of strains YS-7 and SBS-8 was monitored at random time points by FISH (Fig. 3). At time zero, there were no detectable FISH signals for the specific probe in the soil samples tested. The species-specific YS7-FI probe only detected strain YS-7, and that there were no bacteria related to *Rhodococcus* in the oil-contaminated soils that were studied. FISH signals for strain YS-7 were only observed 8 days after inoculation (Fig. 3A), and by 36 days, strain YS-7 was not detected. Strain SBS-8 was detected and distributed in oil-contaminated soils at 8 and 36 days following inoculation (Fig. 3B). These results showed that the survival of strain YS-7 was inhibited by aged petroleum hydrocarbons, especially the SA and AH fractions, in the oil-contaminated soils.

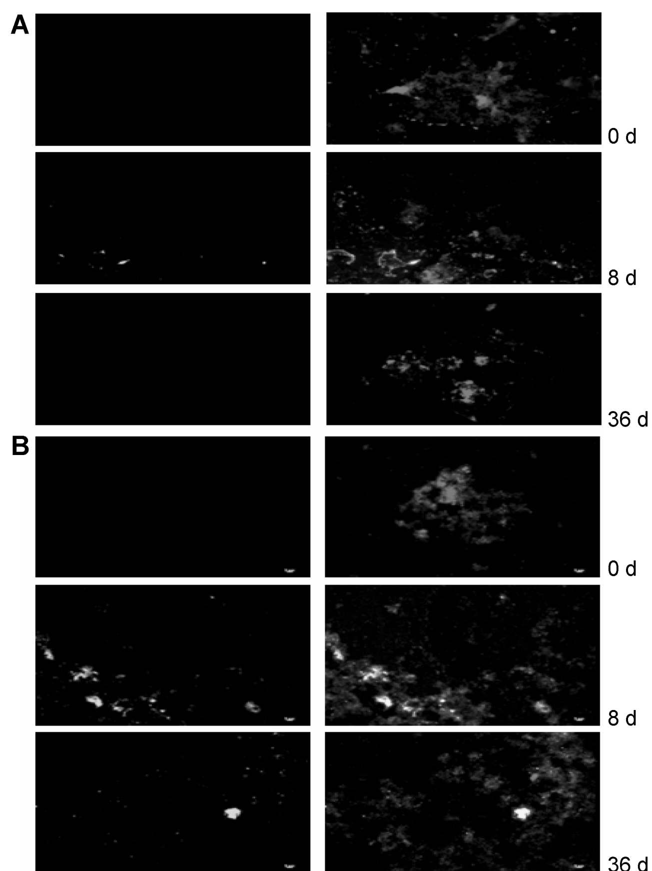

Fig. 3. Fluorescent *in situ* hybridization (FISH) of inoculated microorganisms, strain YS-7 (**A**) and strain SBS-8 (**B**), in oil-contaminated soils during *in situ* bioremediation.
A. A specific probe for strain YS-7 was hybridized with soil samples at random time points. **B**. FISH signal of strain SBS-8 in oil-contaminated soils. Species-specific probes (left column) and the mixture of EUB338 probes (right column) were used to detect the introduced microorganisms. YS-7FI and SBS-8FI were labeled with fluorescein isothiocyanate.

DISCUSSION

To identify the effects of aged petroleum hydrocarbons on the survival of allochthonous diesel-degrading strain YS-7, aged petroleum hydrocarbons were directly extracted from oil-contaminated soils. We showed that the polar fractions (asphaltenes and resins) were relative abundant in our experimental soils (Table 1). This result is a major characteristic of *in situ* bioremediation that is based on unstable mineralization [11, 24]. The growth characterization and inhibition assay on the aged petroleum hydrocarbons showed why strain YS-7 was not detected in oil-contaminated soils 36 days after inoculation (Fig. 2 and 3). The growth inhibition of strain YS-7 by the SA and AH fractions was the most interesting observation in this study. Previous studies showed that some *Rhodococcus* sp. with the ability to degrade crude oil could not mineralize a saturated or aromatic fraction purified from crude oil as a carbon source, and cell growth was also inhibited by the aromatic fraction on medium containing nutrients [13, 25]. A few studies have reported that the AR fraction of aged oil inhibits *in situ* bioremediation [24], and the accumulation of the AR fraction may induce physical and chemical changes in soil characteristics [18]. Until now, there are no reports that the AR fraction in aged crude oil directly affects microbial communities. We tested various concentrations

(0.1. 1.0, 2 mg/l) of each fraction to determine the dose (concentration)-response (survival) relationship data of the SA and AH fractions. The SA and AH fractions with 0.1 mg/l concentration have apparent biostatic activities, but a 1.0 mg/l concentration of SA and AH fractions has a similar level of growth inhibition compared with a 2.0 mg/l concentration (Fig. 2, data not shown). Although a high concentration (>8 mg/l) of AR fraction had biostatic activity (data not shown), less concentration had no growth inhibition of strain YS-7. The co-inhibition assay with two-combined fractions (2 mg/l each) showed that the survival of strain YS-7 is dependent on the presence of SA or AH compounds, but not the AR fraction. Although the actual concentrations of each fraction were not directly measured in soil environments, the dose-dependent relationship of each fraction showed that the AR fraction was not largely associated with microbial survival even if the tested microorganism is limited. These results indicate that the bacteria used in the bioremediation of oil-contaminated environments may need to be highly resistant to petroleum compounds.

The oil-contaminated sites for the field-scale survival test were determined by considering the TPH concentration and the duration of the crude oil contamination. We thought that the duration of exposure to crude oil and the impact of aged petroleum hydrocarbons may be significant factors for microbial activation and survival. A few studies have shown that microbial survival and diversity are highly dependent on the presence of aged petroleum hydrocarbons and the duration of contamination [4, 17]. The inhibition test on each fraction of the aged petroleum hydrocarbons showed that there was a direct interaction between the survival of allochthonous microorganisms and the aged petroleum hydrocarbons in the soil environment, and there was an unexpected decrease in the survival of strain YS-7. Although the FISH assay may have relatively less sensitivity or limited high-background intensity [19], it is useful for directly detecting targeted populations in uncultivated soil environments [5, 9], and it has been used to estimate the survival and distribution of specific bacteria within microbial communities [2, 7]. Our results indicate that the growth of allochthonous strain YS-7 was not adapted to oil-contaminated environments, although it is able to degrade diesel oil. These data suggest that microorganisms with a strong resistance to aged petroleum hydrocarbons may also be responsible for microbial activity in oil-contaminated sites.

Our findings propose that microbial survival might be determined by their resistance to aged petroleum hydrocarbons in oil-contaminated environments where the SA and AH fractions in aged crude oil are clearly major compounds inhibiting microbial survival. Further investigations that test the sensitivity of various kinds of microorganisms to oil contamination will provide clues on the shift in microbial communities in the soil environments after petroleum exposure.

Acknowledgments

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