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Degradation of polycyclic aromatic hydrocarbons by microbial consortia enriched from three soils using two different culture media

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ABSTRACT

A consortium composed of many different bacterial species is required to efficiently degrade polycyclic aromatic hydrocarbons (PAH) in oil-contaminated soil. We obtained six PAH-degrading microbial consortia from three oil-contaminated soils using two different isolation culture media. Denaturing gradient gel electrophoresis (DGGE) and sequence analyses of amplified 16s rRNA genes confirmed the bacterial community was greatly affected by both the culture medium and the soil from which the consortia were enriched. Three bacterial consortia enriched using malt yeast extract (MYE) medium showed higher degradation rates of PAHs than consortia enriched using Luria broth (LB) medium. Consortia obtained from a soil and then added back to that same soil was more effective in degrading PAHs than adding, to the same soil, consortia isolated from other, unrelated soils. This suggests that inoculum used for bioremediation should be from the same, or very similar nearby soils, as the soil that is actually being bioremediated.

1. Introduction

Contamination of soil with crude oil or refined petroleum compounds is of concern worldwide (Keith and Telliard, 1979; Snape et al., 2001). Petroleum is a complex mixture of aliphatic and aromatic hydrocarbons (Leahy and Colwell, 1990). The polycyclic aromatic hydrocarbons (PAHs) are of intense public concern due to their persistence in the environment and potential deleterious effects on human health. Many PAHs are known carcinogens and/or mutagens making remediation of soils polluted with petroleum compounds a priority (Miller and Miller, 1981; Kimber and White, 1986; Palhmann and Pelkonen, 1987).

The PAHs composed of four or more benzene rings are strongly adsorbed to soil colloids, relatively insoluble in water, and rarely leach (Edwards, 1983). Volatilization and plant uptake are also minimal due to low vapor pressures and rapid adsorption (Edwards, 1983; Reilley et al., 1996). Consequently, their environmental fate is governed primarily by colloidal adsorption and microbiological degradation (Cerniglia, 1992; Reilley et al., 1996).

Degradation of PAHs in contaminated soils by indigenous microorganisms is often a slow process. It is therefore of interest to isolate and identify PAH-degrading microorganisms, and many isolates have been obtained by using different culture media. These isolated bacteria have been assigned to a number of genera including *Pseudomonas*, *Alcanivorax*, *Microbulbifer*, *Sphingomonas*, *Micrococcus*, *Cellulomonas*, *Dietzia*, *Gordonia* and *Marinobacter* (Canet et al., 2001; Brito et al., 2006) and are capable of degrading alkanes and aromatic hydrocarbons under aerobic or anaerobic condition. Molds belonging to the genera *Penicillium*, *Fusarium*, *Amorphoteca*, *Neosartorya*, *Paecilomyces*, *Talaromyces*, *Graphium* and the yeasts *Candida*, *Yarrowia* and *Pichia* have also been implicated in hydrocarbon degradation (Chaillan et al., 2004). Some of them have been used for bioremediation of petroleum-contaminated environments (Hanson, 1997; Kasai, 2002; Olivier et al., 2004; Silva et al., 2009; Teng et al., 2010). However, no single microbial species has the ability to metabolize more than two or three classes of compounds typically found in crude oil. A consortium composed of many different bacterial species is thus required to efficiently degrade crude oil.

The use of a bacterial consortium as inoculum provides certain advantages over biostimulation of indigenous microorganisms in cases where there is pollutant toxicity or a lack of appropriate

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microorganisms (both quantity and quality) (Westlake, 1982). Consequently, research has focused on the characteristics of bacterial community structures in petroleum-contaminated sites, as well as changes in these community structures associated with petroleum contamination (Popp, 2006; Labbé et al., 2007). Reports are available about the bacterial community structure associated with bioremediation of different kinds of environmental pollutants (Popp, 2006; Labbé et al., 2007). However, less is known about the selectivity of culture media that often creates this diversity when conducting studies related to remediation using culture methods. A combined culture and molecular approach can provide both information about diversity of petroleum-degrading microorganisms as well as then having a culture available for further microbiological and biochemical studies.

In an effort to more precisely determine the effect of different laboratory culture media on the selectivity and growth of the PAH-degrading consortia, malt yeast extract (MYE) and Luria broth (LB) media were chosen to culture PAH-degrading microorganisms. Microbial diversity within each of these commonly used laboratory media was analyzed using polymerase chain reaction and denaturing gradient gel electrophoresis (PCR–DGGE) as the profiling tool. Bacteria in the consortia were isolated and identified by sequencing 16S rRNA genes. PAH degradation, in modified Barth's solution (MBS) buffer and soil, due to inoculation by these consortia was also evaluated.

2. Materials and methods

2.1. Soils and chemicals

Three soil samples were collected using a small shovel to a depth of about 10–15 cm from different petroleum-contaminated locations in Wooster, OH, USA. Sample C was from an abandoned gasoline filling station and Sample G was from an actively operating gasoline filling station. Sample J was soil that was collected from the immediate area surrounding a leaking petroleum storage tank that received crude oil from an adjacent oil well. All the sites have a history of at least some petroleum contamination over periods of years and samples were collected from areas where there was visual evidence of previous contamination.

Selected chemical properties of these three soils are listed in Table 1. Soil pH, Bray-1 P, exchangeable bases (i.e. K, Ca, Mg), cation exchange capacity, and total N were determined as described in Sparks (1996). Concentrations of all other elements shown in Table 1 were determined via inductively coupled plasma atomic emission (ICP-AES) spectrometry after perchloric acid digestion (Hossner, 1996; Jackson, 1958; Sommers and Nelson, 1972). Concentrations of total petroleum hydrocarbon were determined as described in Das and Mukherjee (2007).

2.2. Enrichment and isolation of PAH-degrading consortia from petroleum-contaminated soils

Two culture media (MYE and LB) were used for enriching PAH-degrading microbial communities from the three petroleum-polluted soils. Initially, 8 g of petroleum-contaminated soil were suspended in 50 mL MYE (20 g malt extract, 2 g yeast extract, 1 L distilled water) or LB (10 g tryptone, 5 g yeast extract, 10 g NaCl, 1 L distilled water) media. These media were buffered at pH 7 and contained 0.2% each of anthracene, phenanthrene and pyrene. Incubation was conducted at 25 °C by shaking on a reciprocal shaker (150 strokes per minute) for 7 days. Enrichment continued by subculturing in the same medium using a 1% inoculum from the previous culture. The initial enrichment step was repeated for a total of three cycles, including the first cycle. Two consortia from each soil for each medium were harvested at the end of the enrichment cycles by low-speed centrifugation (5000 rpm) for 15 min.

The six microbial consortia were then transferred into 50 mL of MBS (1 g $(NH_4)_2SO_4$, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, and 5 mg $FeSO_4 \cdot 7H_2O$ in 1 L distilled water, pH 7) medium in which the sole carbon and energy sources were a mixture of anthracene (200 mg L^{-1}), phenanthrene (80 mg L^{-1}), and pyrene (40 mg L^{-1}). The samples were incubated as described above for 10 days and then 2% of the culture was inoculated into fresh MBS medium containing the same concentrations of PAH compounds. After three cycles that included the first cycle in MBS medium, these six microbial consortia were stored in a refrigerator until further tests were conducted. They are hereafter referred to as C-1, G-1, and J-1 for those originally isolated from soil samples C, G, and J using MYE and C-2, G-2 and J-2 for those originally isolated from corresponding soil samples using LB.

Table 1
Selected chemical properties of three petroleum-contaminated soils.

Chemical properties	Soil C	Soil G	Soil J
Total petroleum hydrocarbons (mg kg^{-1})	10,400	12,200	12,700
pH	8.5	7.6	5.7
Bray-1 P (mg kg^{-1})	16.9	20.0	26.6
Exchangeable K (mg kg^{-1})	86	16	98
Exchangeable Ca (mg kg^{-1})	1590	696	756
Exchangeable Mg (mg kg^{-1})	27	25	86
Cation exchange capacity (meq 100 g^{-1})	8.4	3.7	7.5
Total concentrations of elements (mg kg^{-1})			
N	2160	1680	2010
P	664	454	732
K	4560	2050	5030
Ca	38,600	37,700	2550
Mg	17,900	10,300	2960
S	3260	3620	593
Al	25,100	8420	24,700
B	72.1	67.4	75.4
Cu	90.3	25.7	9.45
Fe	38,300	116,000	22,800
Mn	614	971	327
Mo	4.46	4.35	1.12
Na	3050	191	279
Zn	1198	714	77.8
As	15.1	4.91	9.82
Ba	139	79.3	145
Cd	1.17	2.39	0.37
Co	10.5	8.47	7.99
Cr	86.6	106	27.1
Ni	37.3	33.9	16.3
Pb	61.2	43.9	7.86
Si	180	31.3	108
Sr	68.3	79.9	53.0

2.3. PCR and DGGE

A polymerase chain reaction was conducted using a set of universal bacterial primers, PRBA 338 and PRUN518R, that amplifies the 338 to 518 bp region of the 16s rRNA gene. For the PCR reactions, the 100 μL of final mixture volume contained 1 μM of each primer, 50 μL of GoTaq Green Master Mix (Promega, Madison, WI) and 1 μL DNA (10–20 ng) template. The PCR reactions were performed using an automated thermal cycler (PTC-100, MJ Research, Waltham, MA). The temperature program for the PCR reaction started with a 94 °C denaturation step for 9 min. Then 30 cycles were conducted in which each cycle included a denaturing step of 94 °C for 30 s, an annealing step of 55 °C for 30 s and an extension step of 72 °C for 30 s. The last step in the PCR program was a final extension at 72 °C for 7 min.

Denaturing gradient gel electrophoresis analysis was used for separation of PCR products obtained as described above. Denaturing gradients in 8% (w/v) polyacrylamide gels (16 cm by 16 cm) ranged from 35 to 65%, and urea and formamide were used as denaturants. DGGE was performed using the Dcode Universal Mutation Detection System (BioRad Laboratories). The gel was loaded and run in 1× TAE (20 mM tris–Cl, 10 mM acetate, 0.5 mM Na_2EDTA) buffer at 60 °C for a total of 780 V h (constant voltage of 130 V for six hours). Gels were then stained with ethidium bromide and visualized on a UV transilluminator and photographed (Gel Logic Unit, Kodak, California, USA).

Images of DGGE gels were digitized, and DGGE bands were processed using the Quantity One image analysis software (version 4.1) and manually corrected. A covariance principal-component analysis of band types was carried out for C-1, C-2, G-1, G-2, J-1 and J-2 by a similarity cluster analysis. The DGGE data were used to generate a binary matrix, showing the presence (1) or absence (0) of bands. The unweighted pair-group method with arithmetic means (UPGMA) method was used to perform the clustering analysis of the DGGE banding patterns, using the Euclidean distance coefficient by SPSS 13.0 software.

2.4. PAHs degradation in buffer after inoculating with PAH-degrading consortia

Twenty-one 100-mL baffled Erlenmeyer flasks, containing 50 mL of MBS medium amended with 100 mg L^{-1} PAHs (40 mg L^{-1} anthracene, 40 mg L^{-1} phenanthrene, and 20 mg L^{-1} pyrene) were inoculated with C-1, C-2, G-1, G-2, J-1, and J-2 with three replicates. Three flasks were not inoculated with any consortium and served as negative controls. Incubation was conducted at 25 °C by shaking on a reciprocal shaker (150 strokes per minute). On day 10, samples were extracted using liquid–liquid extraction. The culture was extracted three times with 10 mL of dichloromethane. The combined organic phase was dried over anhydrous sodium