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Isolation, characterization of *Rhodococcus* sp. P14 capable of degrading high-molecular-weight polycyclic aromatic hydrocarbons and aliphatic hydrocarbons

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ABSTRACT

Rhodococcus sp. P14 was isolated from crude oil-contaminated sediments. This strain was capable of utilizing three to five rings polycyclic aromatic hydrocarbons (PAHs) including phenanthrene (Phe), pyrene (Pyr), and benzo[a]pyrene (BaP) as a sole carbon and energy source. After cultivated with 50 mg/L of each PAH, strain P14 removed 43% Phe, 34% Pyr and 30% BaP in 30 d. Four different hydroxyphenanthrene products derived from Phe by strain P14 (1,2,3,4-hydroxyphenanthrene) were detected using SPME–GC–MS. Strain P14 also was capable of degrading mineral oil with *n*-alkanes of C17 to C21 carbon chain length. Compared with glucose-grown cells, PAHs-grown cells had decreased contents of shorter-chain length fatty acids (⩽C16:0), increased contents of C18:0, Me-C19:0 and disappeared odd-number carbon chain fatty acids. The contents of unsaturated C19:1, Me-C19:0 increased and C18:0 decreased in mineral oil-grown cells. At the same time, the strain P14 tended to float when cultivated in mineral oil-supplemented liquid medium. The degradation capability of P14 to alkane and PAHs and its floating characteristics will be very helpful for future's application in oil-spill bioremediation.

1. Introduction

Petroleum hydrocarbons include *n*-alkanes, cyclo-alkanes and aromatic hydrocarbons are the most widespread contaminants in the environment. Polycyclic aromatic hydrocarbons (PAHs) have long been on the US Environmental Protection Agency (US EPA) list of priority pollutants due to their toxicity, carcinogenicity, teratogenicity and recalcitrance in the environment (Sudip et al., 2002). During the past thirty years, many remediation technologies have been tested in efforts to remove these contaminants. Among them, bioremediation is a safe and cost-effective option (Samanta et al., 2002). A variety of genera of Gram-positive and -negative bacteria, fungi and algae have been isolated and characterized for their ability to utilize alkanes (Wentzel et al., 2007; Sood and Lal, 2008) and PAHs (Chauhan et al., 2008). Bacterial strains with the capability to degrade both PAHs and alkanes were also found (Churchill et al., 1999; Bogan et al., 2003; Dandie et al., 2004).

Two *Pseudomonas* strains that can degrade both naphthalene and alkanes have been isolated by Whyte et al. (1997). *Mycobacterium* sp. strain CH1 (Churchill et al., 1999) capable of degrading larger PAHs (phenanthrene, pyrene) could also metabolize alkanes. *Rhodococcus* sp. strain 1BN degraded medium (C6) and long-chain alkanes (C16–C28), benzene, toluene and naphthalene (Andreoni et al., 2000). However, no *Rhodococcus* has been reported with the capabilities to degrade both high-molecular-weight (HMW) PAHs (e.g. pyrene and benzo[a]pyrene) and *n*-alkanes so far.

Hydrophobic contaminants have been reported to change the cellular fatty acid composition of bacteria (Tsitko et al., 1999; Sokolovska et al., 2003; Wick et al., 2003). Changes include modifying the ratios of saturated to unsaturated fatty acids or inducing cis–trans isomerization and cyclopropanation as a way for bacteria to maintain membrane fluidity and impermeability.

In this study we aimed to isolate and characterize *Rhodococcus* sp. P14, and investigate its capability in degrading phenanthrene, pyrene and benzo[a]pyrene and *n*-alkanes. The changes in cellular fatty acid composition were evaluated as well.

2. Materials and methods

2.1. Chemicals

Phenanthrene (Phe, 98%), Pyrene (Pyr, 98%), Benzo[a]pyrene (BaP, 97%), standard Hexadecane (C16), Octadecane (C18), Tetracosan (C24) and Fatty Acid Methyl Ester (FAME) mixture were purchased from Sigma–Aldrich co. (St. Louis, USA). Methanol, Dichloromethane, *n*-Hexane (all HPLC grade) were purchased from Honeywell Inc. (USA). Mineral oil (light whie) was obtained from Amresco, Inc. (USA). Other chemicals used were all analysis grade.

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2.2. Isolation and identification of bacterial strain

Strain P14 was isolated from the sediments in Xiamen harbor (Xiamen, China) which had been contaminated by crude oil and contained high concentration of PAHs (Tian et al., 2008). Bacteria with PAH-degrading capability was selected by culturing in minimal salts medium (MSM) (Tian et al., 2008) with pyrene as the sole carbon source. A pure culture of strain P14 was obtained by repeated transfer onto 2216E plate (Oppenheimer and Zobell, 1952) incubated at 30 °C.

Morphological studies of strain P14 were done using optical microscope (Olympus, Japan) and scan electron microscope (JSM-6360LA, JOEL Ltd., Japan) after grown on 2216E plate at 30 °C for 24–48 h.

Physiological and biochemical properties of strain P14 were examined using the Biolog identification system (Biolog GP microplate identification system, Biolog lnc., USA) following the manufacturer's protocol.

The 16S rDNA gene sequence was determined using the method described by Dandie et al. (2004). The sequence was analyzed using the Blastn program in NCBI (National Center for Biotechnology Information) databank and was deposited in the NCBI database (Genebank) with the accession number EF634456. A phylogenetic tree was constructed according to the neighbor-joining method with the program MEGA3.1.

2.3. Culture conditions

Strain P14 previously grown on the liquid 2216E medium was centrifuged, washed twice to remove the residual carbon source, resuspended in the MSM medium and used as inoculum for the following biodegradation and floating experiments.

2.4. PAH biodegradation

In the experiment for degradation of single PAH (Phe, Pyr and BaP), the stock PAH dissolved in acetone was added into each of the 100 ml flasks containing 20 ml MSM with a final concentration of 50 mg l^{-1} for each PAH in each culture flask. Before bacterial inoculation, the flasks were kept on a rotary shaker (150 rpm) at 30 °C overnight in order to evenly mix the added PAH with the liquid medium and let the added acetone evaporate. The 200 μl aliquot of the cell suspension as described in Part 2.3 was added to the respective growth medium. A sterilized PAH flask without any bacterial inoculation was used as the abiotic control. Each treatment and control was prepared in triplicate. All cultures were incubated at 30 °C on a rotary shaker (150 rpm) in the dark. The growth of the culture and the residual PAH concentration were measured at days 0, 5, 10, 15, 20, 25, and 30. At each time interval, a 1.0 ml aliquot was taken from the cultures, diluted in MSM solution, plated on 2216E agar medium and incubated for 48 h at 30 °C. Colonies were directly counted and expressed as CFU ml^{-1}. The residual PAH in the cultures were extracted twice with 5 ml CH_2Cl_2. The final extracts were added to 10 ml CH_2Cl_2, and then filtered, transferred into HPLC vials and used for HPLC analysis described below. 50 mg l^{-1} of other PAH in CH_2Cl_2 was added before analysis as an internal standard. For Pyr and BaP, Phe was used as an internal control while Pyr was used when measuring degradation rate of Phe.

Reversed-phase HPLC analysis of the PAH concentrations was performed with a Agilent 1100 series Liquid Chromatograph (Agilent technologies, USA) fitted with a Agilent Hypersil 5 μm particle size, ODS column (4.0 × 250 mm). The mobile phase consisted of methanol and water (90/10 v/v) with a flow rate of 1 ml min^{-1}. Eluted substances were detected at a wavelength of 272 nm. Different PAHs (Phe, Pyr and BaP) were identified by comparing their retention time with those of the authentic chemical.

2.5. Analysis of PAH metabolites by SPME–GC–MS

Strain P14 was grown in 250 ml flasks containing 100 ml MSM with a final concentration of 5 mgl^{-1} single PAH (Phe, Pyr and BaP). An aliquot of 5 ml culture was sampled every 2 days for 2 weeks. The cells were separated from the medium by centrifugation at 8000 rpm at 4 °C. The PAH metabolites were extracted from these cells by an automated on-fiber silylation solid-phase microextraction (SPME) using 85 μm polyacrylate fiber and 100 μl BSTFA derivatization agent. The extract was analyzed by GC–mass spectrometry (GC–MS). The oven temperature program was as follows: 80 °C for 10 min, then linearly increased at a rate of 5 °C min^{-1} up to 260 °C and kept for 20 min.

2.6. Degradation of n-alkanes in the mineral oil by strain P14

Degradation of *n*-alkanes by strain P14 was monitored in 150 ml flasks containing 50 ml MSM with 1% (v/v) of the mineral oil as the sole carbon source. The 500 μl aliquot of the cell suspension was used as the inoculum. Uninoculated controls were maintained to monitor abiotic loss of mineral oil. Each treatment and control was prepared in triplicates. All cultures were incubated on a rotary shaker (150 rpm) at 30 °C. The growth of the culture and the residual mineral oil concentration in the medium were measured at days 0, 7, 15, 21 and 28. Cell growth was directly measured by cell dry weight. The residual undegraded mineral oil was extracted three times with 10 ml CH_2Cl_2, and 250 mg l^{-1} Pyr in CH_2Cl_2 was used as the internal standard. 0.2 μl mineral oil (dissolved in 30 ml CH_2Cl_2) was analyzed by GC–MS QP5050A (Shimadzu, Japan) on a SPB-1701 fused silica column (30 m by 0.25 mm by 0.25 μm). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The oven temperature program was as follows: 80 °C for 10 min, then linearly increased at a rate of 5 °C min^{-1} up to 260 °C and kept for 20 min. *n*-Alkanes were identified by

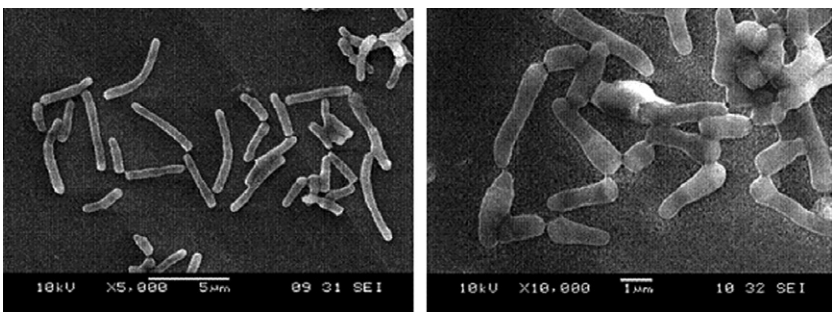

Fig. 1. Scan Electron Microscope (SEM) photo of strain P14.