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Crude oil depletion by bacterial strains isolated from a petroleum hydrocarbon impacted solid waste management site in California

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ABSTRACT

This research is part of a multidisciplinary research program to develop a bioremediation protocol for a solid waste management (SWM) site in Northern California - a site which is heavily contaminated with petroleum hydrocarbons. In this initial study, 30 bacterial strains were isolated and evaluated for their efficiencies to deplete crude oil. The 3 most efficient bacterial isolates for crude oil depletion were designated as S1BD1, OPKDS2, and OSDS1; they were identified as *Serratia proteamaculans*, *Alcaligenes* sp. and *Rhodococcus erythropolis*, respectively, based on partial 16S rRNA gene sequences. Determination of crude oil depletion efficiency by gas chromatography–mass spectrometry (GC-MS) revealed that *Serratia proteamaculans* S1BD1 was the most efficient (68.0 ± 1.78%), followed by *Alcaligenes* sp. OPKDS2 (63.7 ± 3.28%), and *Rhodococcus erythropolis* OSDS1 (54.9 ± 5.07%). *S. proteamaculans* S1BD1 was able to deplete a wide spectrum of carbon compounds within the individual components of crude oil. *Alcaligenes* sp. OPKDS2 was the most efficient at depleting BTEX (91.2 ± 1.90%), and *R. erythropolis* OSDS1 exhibited a substrate preference of n-alkanes. All three strains exhibited unusually high crude oil depletion efficiencies and tolerated a wide range of salinity and pH levels, which makes them excellent candidates for bioaugmentation of the SWM site.

1. Introduction

Crude oil is an important source of energy and raw materials. However, its accidental release to soils, ground or surface water poses a significant environmental threat to human health (Xia et al., 2014; Zhang et al., 2012). The Deepwater Horizon oil spill in the Gulf of Mexico in 2010 was one of the largest accidental spills, which resulted in the release of 636 million liters of crude oil into sea water (Camilli et al., 2010). This spill impacted 1773 km of coastline (Michel et al., 2013) and resulted in 11 deaths. The oil spill continues to pose a significant threat to the marine environments of the Gulf of Mexico and may take years or even decades to recover (Xia et al., 2014).

Several techniques exist to remove petroleum compounds from the environment, including chemical and physical treatments (Riser-Roberts, 1998). However, these methods are energy-intensive, expensive, and often only partially effective, and some may even create additional uncontrolled hazardous waste (Riser-Roberts, 1998). Bioremediation using bacteria, fungi or yeast is a far less expensive and more eco-friendly approach to removing petroleum hydrocarbons (Harayama et al., 2004). Several species of bacteria have been confirmed that can utilize or degrade hydrocarbons. *Mycobacterium* strains have been shown to degrade 100% of a PAH mix, which included phenanthrene (Phe), fluoranthene (Fla) and pyrene (Pyr), in 14 days (Guo et al., 2010). *Acinetobacter* sp. was reported to be able to utilize n-alkanes of chain length C10–C40 as a sole carbon source (Throne-Holst et al., 2007).

Bioaugmentation of oil-contaminated sites has been carried out using bacterial strains: Mishra et al. (2001) reported that 90% of total petroleum hydrocarbons (TPHs) were removed through bioaugmentation of a field site in one year (compared to only 14% removal in the control). Developing a bioremediation program for a given site depends on the specific characteristics of the site, i.e., the type and variability of contaminants, the soil and climatic

Abbreviation: SWM, Solid waste management.

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environments, as well as the regulatory restrictions placed by local or federal government agencies. In the present study the strategy was to isolate highly efficient bacterial strains for petroleum degradation that could be utilized for bioaugmentation. Certain microorganisms are capable of mineralization and utilization of petroleum hydrocarbons as carbon and energy sources (Montagnolli et al., 2015). However, the rate and extent of depletion of petroleum hydrocarbons depends on the microorganisms' metabolic capabilities. Indigenous microbes associated with the extreme conditions of the site are more adapted to the prevailing conditions and are thus most suitable for use. Such indigenous microbes may be present at low abundances (Sayara et al., 2009) so that isolating the most efficient strains from the contaminated site and then utilizing these for bioaugmentation may be considered as the best strategy for enhancing biodegradation.

In the present study we were fortunate to have access to a solid waste management (SWM) site in Northern California that was contaminated over a period of 30 years (1950–1980) with petroleum hydrocarbons, heavy metals and other contaminants of concern. The overall goal of the present research was to isolate and identify crude oil-degrading bacterial strains present in the soil of this specific SWM site – strains that might eventually prove useful for bioaugmentation. The specific objectives were to: 1) isolate and identify bacterial strains native to the site, 2) characterize their ability to deplete petroleum hydrocarbons, and 3) to determine their tolerance to site levels of salinity and pH.

2. Materials and methods

2.1. Description of the site and sampling

The 29-ha solid waste management site is located in Contra Costa County, California. This site is contaminated with petroleum hydrocarbons (~15,000 mg/kg, especially those with a carbon number under 20 (Laboratories, 2013)), heavy metals, and carbon black (fly ash residue from coal burning industry, particle size <10 μm). Certain areas of the site also contain polycyclic aromatic hydrocarbons (PAHs), especially benzo(ghi)perylene, and pyrene (CH2MHill, 2010). The site is currently covered with a water cap to prevent the release of offensive odors to neighboring public areas. The water and soil are moderately saline and some areas exhibit high or low pH values (Table S1).

Impacted soil samples were collected from different parts of the site, including 1) unplanted soil, 2) saltgrass (*Distichlis spicata*) rhizosphere soil, 3) pickleweed (*Salicornia virginica*) rhizosphere soil and 4) sea-purslane (*Sesuvium verrucosum*) rhizosphere soil (all three plant species are native to the site). Samples were collected using disinfected shovels to dig approximately 6 inches deep, brought back to lab in sterilized containers and stored at 4 °C; the soils collectively weighed ~1200 g.

2.2. Culture media and carbon sources

Soil samples were used as the bacterial source for the enrichment culture in the initial screening step. The enrichment culture used a medium composed of sterilized mineral salts medium (MSM) plus 1% (v/v) of a sole carbon source. The MSM contained g/L 2.42 KH_2PO_4, 5.60 K_2HPO_4, 2.00 $(NH_4)_2SO_4$, 0.30 $MgSO_4 \cdot 7H_2O$, 0.04 $CaCl_2 \cdot 2H_2O$, 4.5×10^{-3} $MnSO_4 \cdot 7H_2O$, 0.1×10^{-3} $CuSO_4 \cdot 5H_2O$ and 0.1×10^{-3} $FeSO_4 \cdot 7H_2O$, the pH was adjusted to 7.0 ± 0.2 before autoclaving (Bury and Miller, 1993). Three kinds of carbon sources (crude oil, a gasoline/diesel mix (v/v = 1) and pyrene) were used as the sole carbon sources for enriching bacterial strains potentially useful for petroleum hydrocarbons depletion. Crude oil was collected from a "Cardinal Field" and stored at room temperature. Gasoline was purchased from Chevron Corporation (USA), diesel was purchased from ConocoPhilips (USA), and pyrene was purchased from Spectrum Chemical (USA).

2.3. Enrichment and isolation of petroleum hydrocarbons degrading bacterial strains

Crude oil, a gasoline/diesel mix (v/v = 1) and pyrene were individually used as the sole carbon source in enrichment culture process. These compounds were selected to represent a wide range of chemical compositions and amount of carbon chains present in petroleum hydrocarbons. The enrichment and isolation methods were modified from previous work (Kumari et al., 2012; Wongsa et al., 2004; Zhang et al., 2012). Five grams of a soil sample were added to a flask containing 200 ml MSM plus 1% (v/v) of a sole carbon source. The step was repeated for each soil sample collected and for each carbon source for a total of 12 enrichment flasks (4 soil samples x 3 carbon sources). The flasks were incubated on a shaker at 130 rpm, room temperature (approximately 25 °C) for the enrichment phase. After approximately 10 days (d) of incubation, 5 ml of inoculum was aseptically transferred from each individual flask to a flask containing fresh MSM and incubated at the same conditions for another 10 d. This was repeated two additional times. After the fourth subculture, the inoculum was serial diluted and spread on Lysogeny broth (LB, formulation per 1 L: 10 g SELECT Peptone 140; 5 g SELECT Yeast Extract; 10 g sodium chloride) agar plates (Thermo Fisher Scientific, USA) for isolation of bacterial strains. Individual colonies on LB agar plates were further isolated using the four quadrant streaking method. Then, individual strains were prepared for storage using a glycerol preservation method and stored in 1 ml cryogenic vials at minus 80 °C until further investigation. In this study, 30 strains were stored in total and were used as the stock source for further experiments.

2.4. Identification and characterization of strains

Genomic DNA was extracted from each bacterial isolate culture broth after 24 h incubation at 30 °C using DNA extraction kit (MO BIO, USA) according to the manufacturer's instructions. The 16S rRNA gene fragment was amplified with a pair of universal primers (27F): 5′ AGA GTT TGA TCM TGG CTC AG 3′ and (1525R): 5′ AAG GAG GTG WTC CAR CC 3′ using the extracted DNA as template. Polymerase chain reaction (PCR) was performed in a thermal cycler using the following conditions: pre-heating for 3 min at 95 °C followed by 30 cycles of 30 s at 95 °C; 45 s at 55 °C; 90 s at 72 °C with a final extension of 10 min at 72 °C. The size of the PCR product was confirmed with electrophoresis using a 1.5% agarose gel. The PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, USA). The amplicons were sequenced at the University of California Berkeley DNA Sequencing Facility. The raw sequences were aligned, edited manually and BLAST searched using a BLAST search option of NCBI GenBenk (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to estimate the degree of similarity to other species.

2.5. Crude oil depletion efficiencies of individual strains

Bacterial inoculum were prepared by growing each bacterial isolate overnight at 30 °C in LB broth. The bacterial cells were harvested by centrifuging at 5000 rpm for 15 min, supernatant was discarded and obtained cells pellets were suspended in sterilized deionized water to adjust OD_{600} of 0.5. Bacterial inoculum (0.3 ml) was transferred to a 60 ml screw-capped vial (to prevent loss of volatile hydrocarbon components) containing 30 ml MSM plus 1% (v/v) crude oil; vials were incubated in a rotary shaker at 30 °C and 130 rpm. MSM plus crude oil vials without cells were used as a