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Technical Note

Degradation of hexadecane by *Enterobacter cloacae* strain TU that secretes an exopolysaccharide as a bioemulsifier

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ABSTRACT

A Gram-negative rod-shaped bacterium, previously shown to utilize alkanes and polycyclic aromatic hydrocarbons (PAHs), was identified as *Enterobacter cloacae* (GenBank accession number, GQ426323) by 16S rRNA sequence analysis and was designated as strain TU. During growing on *n*-hexadecane as the sole carbon source, the strain TU extracellularly released an exopolysaccharide (EPS) exhibiting bioemulsifying activity into the surrounding medium. The EPS was found to be composed of glucose and galactose with molecular weight of 12.4 ± 0.4 kDa. The structure of the EPS was postulated according to by 1D/2D NMR, as follows: -D-Glc*p*-(1 → 3)-α-D-Glc*p*Ac-(1 → 3)-α-D-Gal*p*-(1 → 4)-α-D-Gal*p*-(1 →. While an enhanced emulsification and aqueous partitioning of *n*-hexadecane was displayed as functions of the EPS concentration, the EPS neutralized the ζ potential of *E. cloacae* TU cell and elevated the surface hydrophobicity of the cells, as determined by the microorganisms adhering to hydrocarbon assay (MATH). This was found to favor the bioavailability of *n*-hexadecane when it served as the sole carbon source for *E. cloacae* TU and thereby contributed to the accelerated degradation of this hydrocarbon.

1. Introduction

Petroleum hydrocarbons are an important source of soil contamination due to their extensive applications at unprecedented rates and scales (García-Borboroglu et al., 2006). These compounds are immobilized or encapsulated by soil particles and have poor solubility in aqueous solutions; consequently, the availability of hydrophobic hydrocarbons for degradation by microbes is poor. Therefore, enhancement of the bioavailability of petroleum hydrocarbons is crucial to their efficient and rapid degradation. The use of chemical surfactants has been extensively studied (Mulder et al., 1998; Volkering et al., 1998; Willumsen and Arvin, 1999); however, the toxicity and biodegradability of these substances are major concerns that restrict their application (Volkering et al., 1998). Separation of a petroleum hydrocarbon from the cell surface by the addition of a chemical surfactant has been described earlier (Churchill and Churchill, 1999); however, it should be noted that the addition of the chemical surfactant may also affect the uptake of contaminants by microbial cells, leading to unsatisfactory degradation. In recent years, increasing efforts have been made to develop and increase the applications of microbial surfactants to bioremediation. In comparison to synthetic surfactants, microbial surfactants have the advantages of lower toxicity, higher biodegradability, and greater environmental compatibility (Cameotra and Makkar, 2004).

Microbial surfactants obtained from a wide range of oil-degrading microorganisms can be grouped into two major categories depending on their molecular weight. Low-molecular-weight surfactants include biosurfactant such as glycolipids and lipopeptides, while high-molecular-weight compounds include emulsan, alasan, biodispersan, and extracellular or cell membrane-bound bioemulsifiers (such as exopolysaccharide (EPS)) (Calvo et al., 2008; Lee et al., 2008). Enhancement of the biodegradation of a petroleum hydrocarbon by an EPS was first reported by Hino and coworkers (1997) using *Pseudomonas* sp. SLI and SLK as the working strains. Subsequently, production of an EPS and its application to the biodegradation of hydrocarbons was reported in the case of certain *Halomonas* species such as *Halomonas maura*, *Halomonas eurihalina*, and *Halomonas ventosae* (Calvo et al., 1998; Martínez-Checa et al., 2007). In 2006, Iyer and coworkers (2006) found that the EPS excreted by a marine *Enterobacter cloacae* could emulsify various hydrocarbons, mineral oils, and vegetable oils. In addition to enhancing the solubility of hydrocarbons, the protective functions of EPSs have also been noted—this compound shields bacterial cells from direct exposure to toxic substances (Costerton, 1985; Wang et al., 2008; Kang and Park, 2009; Gutiérrez et al., 2009). Based on these results, EPSs are promising agents for the bioremediation of oil contaminants.

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In an earlier study on on-site remediation of petroleum-contaminated soil in Henan providence, Liu and coworkers isolated a Gram-negative rod-shaped bacterium that utilizes alkanes and polycyclic aromatic hydrocarbons (PAHs) as carbon sources (Han et al., 2008a,b; Zhang et al., 2008). Of great interest was the fact that this strain could produce bioemulsifiers that enhanced the solubility of hydrocarbons. In the present study, we first characterized the above-mentioned strain by employing 16S rRNA sequence analysis. The composition and structure of the EPS were determined by acid hydrolysis and 1D/2D NMR, respectively. The effects of the EPS on the utilization and degradation of *n*-hexadecane were examined in the case where the latter served as the sole source of carbon and energy. Finally, the effects of the EPS on the solubility of *n*-hexadecane and the surface properties of the cells in terms of hydrophobicity and ζ potential were examined in order to elucidate the mechanism underlying the enhanced degradation.

2. Experimental

2.1. Microorganism, cultivation conditions and the degradation of n-hexadecane

The oil-degrading microorganism was isolated from a sample of petroleum-contaminated soil obtained from the Zhongyuan oilfield, Henan province, China; this microbial sample was stored in our laboratory (Han et al., 2008a,b; Zhang et al., 2008). For inoculums preparation, stored stocks (−70 °C) were grown in Luria–Bertani (LB) medium prior to use.

2.2. Identification of the oil-degrading microorganism

The cell morphology was examined under a JEM 1400 transmission electron microscope (JEOL, Japan). The copper grids were immersed in the cell suspension for 10 min and then blotted dry with filter paper. The grids were examined after negative staining with 1% (w/v) uranyl acetate for 10 min and then blotted with filter paper.

Phylogenetic analysis based on the 16S rDNA sequences was performed with the aid of the MEGA software package using the neighbor-joining method (Saitou and Nei, 1987; Quillaguamán et al., 2004). Partial rDNA sequences were analyzed using a basic local alignment search tool (BLAST) to estimate the degree of similarity to other rDNA sequences obtained from NCBI/GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

The API 20E system was used to analyze biochemical properties such as the enzymatic activities of tryptophan deaminase, gelatinase, urease, β-galactosidase, ornithine decarboxylase, lysine decarboxylase, and arginine dihydrolase. It was also used to test for H_2S, indole, nitrate reduction, and Voges–Proskauer.

2.3. Production and purification of the EPS by the strain TU

The cultivation of the strain TU at 30 °C using *n*-hexadecane as the sole source of carbon and energy was conducted by inoculating the strain in sterile 300-mL Erlenmeyer flasks containing 100 mL of the mineral salt medium (Bouchez-Naïtali et al., 1999) and 0.5 mL *n*-hexadecane. After 72 h cultivation, the culture medium was centrifuged for 20 min at 8000*g* and 4 °C. Trichloroacetic acid of 80% (w/v) was then added to the supernatant to a final concentration of 4% (w/v) and incubated overnight, followed by 20 min centrifugation at 8000*g* and 4 °C. Three volumes of cold 95% EtOH were added to the supernatant, and precipitation was carried out at −20 °C for 2 h. The precipitate was dissolved in hot water, centrifuged for 20 min at 8000*g* and 4 °C, dialyzed for 3 d against pure water, and lyophilized for 24 h (Prasertsan et al., 2008), yielding crude EPS.

Purification of the crude EPS started from anion-exchange chromatography using 1 mL DEAE FF HiTrap column (GE Healthcare, USA), in which the 1 mL of EPS solution at concentration of 10 g L^{-1} which dissolved in 10 mM Tris–HCl buffer (pH 8.5) was applied to the column, followed by elution with pH 8.5, 10 mM Tris–HCl buffer containing 1 M NaCl at a flow rate of 0.5 mL min^{-1}. Each 3-mL fraction was analyzed for its carbohydrate content by the phenol–sulfuric acid method (Dubois et al., 1956). After dialysis against pure water for 3 d and freeze-drying, the purified EPS was applied to Superdex 75 column and eluted with pH 7.0, 50 mM PBS buffer at a flow rate of 0.5 mL min^{-1}.

2.4. Determination of the molecular weight of the EPS

M_n and M_w represent the number and weight average molecular weights, respectively, and these values together with the polydispersity index (M_n/M_w) were determined using the size exclusion chromatography (SEC) and a multi-angle laser light scattering system (MALLS) (DAWN EOS; Wyatt Technology, Santa Barbara, CA, USA) coupled to a refractive index (RI) detector (Water 410) (Hwang et al., 2003; Ayala-Hernández et al., 2008). The EPS was dissolved in deionized water, filtered through 0.22-μm filter membranes (Millex HV type; Millipore Corp., Bedford, MA, USA), and injected into the SEC/MALLS system. The injection volume and concentration were 100 μL and 1 g L^{-1}, respectively. The compound was eluted with deionized water at a flow rate of 0.5 mL min^{-1}.

2.5. Determination of the monosaccharide components of the EPSs

The monosaccharide composition of EPS was determined by the HPLC method described by Freitas et al. (2009). During a run, 25 mg of the EPS sample was dissolved in 16.5 mL of 4% (v/v) H_2SO_4 and hydrolyzed for 1 h at 121 °C. The solution was adjusted to pH 6.0 with calcium carbonate and filtered through a 0.22-μm filter paper prior to injection into a Biorad Aminex HPX-87P column coupled to an RI detector. Elution with deionized water was performed at a flow rate of 0.6 mL min^{-1} and at a temperature of 85 °C.

2.6. NMR spectroscopy

The EPS sample was deuterium exchanged three times by freeze-drying from D_2O and then tested in a 99.99% D_2O solution (25 g L^{-1}). The 600 MHz ^1H NMR spectrum and 150.9 MHz ^{13}C NMR spectrum of the EPS solution were recorded at room temperature on a JNM-ECA600 spectrometer (JEOL Ltd., Japan). The chemical shifts were expressed in ppm using signal (4.6672 ppm for ^1H) and internal DSS (54.41, 19.14, 15.03, and −2.64 ppm for ^{13}C). The signals were assigned from the COSY, TOCSY, HMQC, and HMBC experiments. The TOCSY data were acquired using a data matrix of 512 × 2048 points and 16 scans per increment; the isotropic mixing time was set at 80 ms. HMQC and HMBC data were acquired using data matrices of 512 × 1024 and 256 × 2048 points, respectively, and 16 and 64 scans per increment, respectively.

2.7. Determination of the emulsification capability and its effect on the aqueous solubility of n-hexadecane

While incubated with or without exogenously added 500 mg L^{-1} EPS, the effect of EPS on the biodegradation of *n*-hexadecane by the EPS-producing strain was determined by measuring at intervals of 24 h the residual amount of *n*-hexadecane using GC analysis after extracted from the test and control flasks with an