Isolation and identification of biodegradation ability of alkane-degrading bacteria: Molecular detection and analysis of alkane hydroxylase genes

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This study was conducted to characterize and to detect the alkane degradation capacity of the bacterial strain (strain Y9) isolated from the sea mud of the crude oil-polluted Dinghai area in China. A Gram-negative, strictly aerobic, oxidase negative, and catalase positive bacterium, strain Y9 was isolated and identified as Acinetobacter sp. based on its physiological characteristics and its 16S rRNA gene sequence analysis. The 16S rDNA of strain Y9 sequence is 99.8% identical with Acinetobacter venetianus RAG-1T. The Y9 strain had an obligate requirement for NaCl but could not tolerate high salt concentrations. Strain Y9 was able to degrade C9~C22 n-alkanes from diesel oil as the sole carbon source, and the degradation rate was up to 53.28% in 7 days at 30°C, pH 8.0. The concentrations of the initial diesel oil and initial bacteria were 4 and 2% (v/v), respectively. The length of alkane hydroxylase gene (alkB) and CYP153A obtained by PCR are 544 and 864 bp, and displays 84 and 98% with Acinetobacter sp. M-1 and Acinetobacter sp. OC4, respectively.

Key words: Oil spills, biodegradation, alkB, CYP153A, alkane degradation, petroleum degradation.

INTRODUCTION

Natural disasters, shipping activities, accidental spillage of fuels and other petroleum products cause serious ecological damage to coastal fauna and flora. Hydrocarbons present in the fuel are major pollutants of marine environment (Yamamoto et al., 2003). Once the oil leaks into the natural environment, it transforms due to evaporation and photo-oxidation (Albert et al., 2003). The conventional methods used to handle petroleum pollution are restricted to physical and chemical suppression. However, these are expensive and helpful measures only to clean-up high concentrations of oil releasing secondary pollutants into the environment (Pritchard et al., 1992). Generally, ultimate and complete degradation is accomplished mainly by bio-degradation due to the activities of marine micro flora.

Microbial degradation is considered to be the best method for the breakdown of hydrocarbons (Harayama et al., 1999; Nakamura et al., 2007). It has been claimed to be an efficient, economic, and versatile alternative to physicochemical treatment. The main components of diesel are medium and long-chain alkanes, and most of them degraded naturally by the water circulation. However, the residual part of the long-term alkanes stays in the environment for a long time and causes toxicity hazards to the marine life and environment. Some marine bacteria, capable of degrading petroleum hydrocarbons have been isolated, which include the genera Pseudomonas, Cycloclasticus (Dyksterhouse et al., 1995), Alcanivorax (Yakimov et al., 1998), Neptunomonas (Hedlund et al., 1999), Oleiphilus (Golyshin et al., 2002), Oleispira (Yakimov et al., 2003) etc. Unfortunately, natural microbial degradation occurs relatively slowly in marine environments due to limited availability of nitrogen and phosphorous salts, and due to

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low temperatures. It was reported that the assimilation/degradation process that occurs in bacteria is mediated by enzymes and the degradation by genes (Tomohisa et al., 2010).

Previous studies showed that indigenous bacteria were useful for oil degradation (Swannell et al., 1996), and Alcanivorax sp. is found in higher abundances in oil-polluted waters and coastal lines than in unpolluted waters (Kasai et al., 2002; Syutsubo et al., 2001). Moreover, Alcanivorax sp. has been dominant in the field and microcosm experiments when excess of nitrogen and phosphorus fertilizers were added to stimulate microbial degradation of oil (Roling et al., 2002). The study of Shogo Nakamura (Nakamura et al., 2007) indicates that Alcanivorax sp. has played an important role in the process of biodegradation of oil contaminated environments when incorporated with other bacteria. In recent times, different approaches have been researched; petroleum-metabolizing of bacteria, petroleum-degradation mechanisms, factors affecting microbial degradation (Ilori et al., 2005), the way of assimilation into bacteria activities of the degradation related enzyme and discovering the degradation-related gene etc. (Tomohisa et al., 2010). A great variety of organisms have been cultivated from the isolates of oil and water samples (Harayama et al., 2004). And a wide range of metabolically diverse bacteria have been successfully isolated from petroleum samples such as soil, seawater. Pseudomonas species were often isolated from oil-contaminated wastewater (Hasanuzzaman et al., 2007) which plays an important role in the petroleum biodegradation (Ko and Lebeault, 1999; Oluwafemi et al., 2009). Members of this genus have broad affinity for hydrocarbon and can degrade alkanes, salicylic, thiophenes, aromatics, etc. (Abalos et al., 2004; Norman et al., 2002; Mohammad et al., 2007).

In recent years, bioremediation technology has been popular for low investment with no secondary pollution and for good results of the basic advantages controlling marine oil pollution. There must be efficient degrading micro flora as executives of biological repair (Shigeaki et al., 2004; Syutsubo et al., 2001). The purpose of this study was to isolate diesel oil degrading bacteria, screening and identification. Also to obtain efficient diesel-degrading bacteria with degrading enzyme genes like alkane hydroxylase (alkB), P450 etc. The aim of this study was to describe the phenotypic characteristics of strain Y9 isolated from the sea water and to study its phylogenetic placement, based upon the analysis of its 16S rRNA sequences. In order to exploit further applications of the effective bioremediation technology, the distribution of degrading gene was also analysed.

MATERIALS AND METHODS

Bacterial strain Y9 was isolated from sea water of the Dianghai Port using enrichment culture techniques with diesel oil as sole carbon source. Recipient strain DH 5a (E. coli), was collected from Second Military Medical University.

Media

Enriched medium was prepared by dissolving 5 g yeast extract, 10 g typtone and 15 g NaCl in 1 L ddH2O, and adjusted the pH value of the medium to 7.2~7.4, then sterilizing the solution, autoclave at 121°C for 30 min. Solid plate was prepared by adding 1.5% (m/v) agar. Luria-Bertani (LB) medium Synthetic sea water (SM) medium: SM contained (1L distilled water, the pH value of medium was adjusted to 7.5 by the addition of 10% solution of NaOH): 24 g NaCl, 7.0 g MgSO4·7H2O, 1.0 g NH4NO3, 0.7 g KCl, 2.0 g KH2PO4, 3.0 g Na2HPO4 and added 200 μl trace elements in solution, sterilized by 0.22 μm membrane filter. The medium was supplemented with appropriate sterilized diesel oil as the sole carbon source. The trace element solution included 2 mg CaCl2, 50 mg FeCl3·6H2O, 0.5 mg CuSO4, 0.5 mg MnSO4·H2O, 10 mg ZnSO4·7H2O in per liter.

Phenotypic analysis

For all phenotypic tests, cultures were grown in LB medium: routine tests were applied according to Molecular Cloning: A Laboratory Manual.

Chemicals

All chemicals used were of analytical grade. All organic solvents used were high-performance liquid chromatographic (HPLC) grade and were purchased from Guoyao Company in Shanghai of China. Commercial marine diesel (density 832 g/L) Petroleum ether (Medicines, boiling range is 60~90°C, light transmittance is greater than 90%). Anhydrous sodium sulfate (300°C drying 1 h, cooling equipment spare bottle); Sulphuric acid; Dichloromethane (HPLC grade).

Kits

In following kits were used in this study: Bacterial micro biochemical reaction tube; AxyPrep a small amount of bacterial genomic DNA kit; AxyPrep DNA gel extraction kit; San Prep column a small amount of plasmid DNA extraction kit; Ins TAclone TM PCR Cloning Kit (Fermentas).

Description of sampling site

Strain Y9 was isolated from diesel oil-polluted sea water sampled from Dinghai Port, Zhejiang Province of China. The sampling site had a mean pH of 8.12, a DO of 5.60 mg/L and an oil content of 1.031 mg/L. This site has been polluted with diesel oil over years and the oil concentration was as twice as the fourth class quality according to the sea water quality standard of the People’s Republic of China.

Morphological and physiological characteristics tests

For colony morphology and microscopic examination, cultures were grown on agar plates and examined by microscopy. All the tests were conducted using standard procedures (Gerhardt et al., 1994;
Molecular and phylogenetic analysis

For PCR of 16S rDNA genes, DNA was extracted using the AxyPrep™ Bacterial Genomic DNA Miniprep Kit (AXYGEN BIOSCIENCES), and used as template for PCR amplification of 16S rDNA, alkB and CYP153A genes. The 16S rDNA gene was amplified using the set of primers (Lane, 1991) 27F and 1492R. Amplification was done as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 90s, and elongation at 72°C for 90s, and final elongation at 72°C for 10 min. AxyPrep™ PCR Cleanup Kit (AXYGEN BIOSCIENCES) was used for PCR product purifications. Sequencing was performed on both strands by the commercial services of Shanghai Sangon Bio Technology Co. (Shanghai, China). 16S rDNA sequences were compared using EzTaxon Server version 2.1, and phylogenetically analyzed by ClustalX 1.83 program (Thompson et al., 1997). Phylogenetic trees were constructed using the Neighbour-Joining method of the MEGA4 program 10 (Tamura et al., 2007). Sequences were deposited at the GenBank database under accession numbers EU647556e64 (16S rRNA genes).

Gas chromatography-mass spectrometry (GC-MS) analysis

The effect of diesel oil-degradation was analysed by gas chromatography-mass spectrometry (GC-MS, Thermo Focus DSQ GC-MS, USA). Under optimal conditions, the Y9 strain was incubated for 7 days and the residual diesel oil was extracted from the culture with dichloromethane. The organic phase was dehydrated with anhydrous sodium sulfate. 1 μl of the organic phase was analysed by gas chromatography equipped with an Agilent Technologies HP-5 MS column (30m×0.25 mm×0.25μm). Helium was used as carrier gas (ca. 1ml min-1) and was adjusted to a linear velocity of 1.0 ml/min and the oven temperature program was 60~280°C (held 5 min) at 10 min-1, the initially oven temperature was 60°C for 2 min. The injector (250°C) was operated in the split mode (10:1). MS conditions: The temperature of the ion source and the interface were set at 250°C and 1.50 kV of electron impact and 1.000 amu.

Diesel degrading enzyme gene analysis of indigenous bacteria

To amplify the partial fragment of the putative alkane hydroxylase gene (alkB) and CYP153A gene, highly degenerate primers, AlkBF (5'-AAYACNGCNAYGARCT NGGVCAYAA-3') and AlkBR (5'-GCRTGRTGRTCHGARTGNCGYTG-3'); CypF (5'-TGTGGCCTTTAATGTCATGNCMTGGAYGC-3') and CypR (5'-TGCAGTTCGCCAGGCGTDCSRYRCAVCRTG-3'), were generated based on the conserved regions of several alkane hydroxylase gene sequences available in the Gen-Bank database. The genomic DNA of train Y9 was obtained by the method of kit. The alkB and CYP153A genes were obtained and purified, then ligated to pUCm-T vector and then introduced into component cells of E. coli DH5α by transduction.

Screening for the positive clone

E. coli transformants carrying this library were cultured on LB plate supplemented with Amp (100 μg/ml). Then the positive clones were further confirmed.

Plasmid isolation

For isolation of recombined plasmids bearing DNA fragments, gene-T of the positive clone, Axygen kit was used.

DNA sequencing, sequence assembly and analysis

The recombinant plasmid gene-T containing a DNA fragment about 550 bp from stain Y9 was sequenced by Sangon. Sequence comparisons were made against the sequences in the GenBank using the BLASTx program (Altschul et al., 1990).

Construction of phylogenetic tree

Nucleotide sequences of various enzymes or subunits were extracted from NCBI (Altschul et al., 1990). Phylogenetic trees were generated using the neighbour joining method of Saitou and Nei (1986) and multiple sequence alignment was done using ClustalX (Thompson et al., 1997). The length of each branch represents the evolutionary distance between the sequences. The thermal cycles were taken in a Peltier thermal cycler (Bio- rad). PCR products were purified and recovered by using an AxyPrep™ PCR Cleanup Kit (Axygen). Sequence data were manually aligned with nucleotide sequences obtained from GenBank by using DNASTAR. Alignments and phylogenetic analysis of alkB and CYP153A sequences were also carried out by using the DNASTAR program.

RESULTS AND DISCUSSION

Isolation of diesel oil-degrading bacteria

One of the isolates (Y9) that grew in SM with crude oil was selected for further characterization for its high rate of diesel oil degradation.

Morphological and physiological characteristics tests

Bacterial strain Y9 had an obligate requirement for NaCl but could not tolerate high salt concentrations. Through, the morphological and biochemical identification of colonies of strain Y9 which had grown on nutrient agar plates for three days (Figure 1) were round or nearly round, 1~2 mm in diameter, characteristically small, ivory yellow color, smooth, moist, translucent, non-pigmented and slightly raised in the centre, with regular, transparent and halo-like peripheries. The cells of Strain Y9 were 1.2~1.9 μm long and 0.9~1.1 μm wide, motile, lophotrichous, non-spor-forming, Gram-negative rods, and cells were short rods in young period while cocci cells in the stationary phase. Spore formation was not observed in its life. Y9 was strictly aerobic, oxidase negative, catalase positive and it could not utilise glucose, D-fructose, L-arabinose, and D-ribose. However, it has utilized sodium citrate, sodium acetate as sole source of carbon, and potassium nitrate and ammonium sulfate as the sole nitrogen source, with no requirement of growth factors. Table 1 showed the characteristics used to distinguish strain Y9 from other members of the genus Acinetobacter.
Table 1. Characteristics that differentiate Y9 from other members of the genus Acinetobacter.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Y9</th>
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<tbody>
<tr>
<td>Motility, flagella arrangement</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>-</td>
</tr>
<tr>
<td>Growth factors required</td>
<td>-</td>
</tr>
<tr>
<td>NO₃⁻ reduction</td>
<td>+</td>
</tr>
<tr>
<td>Ionic requirements</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of organic acids</td>
<td></td>
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<tr>
<td>Glucose</td>
<td>-</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>-</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>-</td>
</tr>
<tr>
<td>D-Irbose</td>
<td>-</td>
</tr>
<tr>
<td>Na-Citrate</td>
<td>+</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>+</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>+</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>+</td>
</tr>
<tr>
<td>Optimal temperature (°C)</td>
<td>30°C</td>
</tr>
<tr>
<td>Growth at 4°C</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 47°C</td>
<td>-</td>
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</tbody>
</table>

+, positive reaction or growth. -, no reaction or growth.

The biodegradation rate of diesel oil was high when the initial oil concentration was 3% (v/v) (Figure 2). This was probably because when the bacteria contacted with diesel oil, diesel oil was emulsified strongly, the oil-degrading bacteria concentrated in the oil-water interface, diesel oil provided a better carbon source for the growth of bacteria, and thus with the increase of the concentration of diesel oil; the fast growth and reproduction of bacteria promoted the biodegradation of diesel oil. When the initial concentration of diesel oil was too high, the oil would cover the surface of water and prevent oxygen to dissolve into liquid culture which hinders the usage of oxygen and nutrients to some extent. Oxygen transfer and the toxicity of volatile hydrocarbons would inhibit biodegradation of oil (Rambeloarisoa et al., 1984).

Molecular and phylogenetic analysis

Nearly full-length 16S rRNA gene sequences (1504 nt) of strain Y9 was determined. In the phylogenetic tree, Y9 was included in the Acinetobacter cluster. And the closest relative was Acinetobacter venetianus RAG-1T (99.8%).

Gas chromatography-mass spectrometry (GC-MS) analysis

Petroleum is a complex mixture of hydrophobic components like n-alkanes, aromatics, resins and asphaltethenes (Leahy and Colwell, 1990). Microorganisms are known to attack and degrade a specific component as compared with other components of oil. It has been observed that the same compounds in different crude oil samples were degraded to different extents by the same organisms (Obayori et al., 2009).

The chromatogram of diesel oil-degrading effect in the optimal conditions after 7 days incubation was shown in Figure 3. Strain Y9 has degraded 53.28% diesel under the optimum conditions, and the ratio of degradation of C9–C14 and C15–C22 were 57.19 and 49.69%, respectively. The components of diesel oil were mixed medium-chain n-alkanes (C9–C22), and the chromatogram shows nearly all components were degraded by Acinetobacter sp. strain Y9. The Acinetobacter are one of the representative species capable of growing on n-alkanes (Tani et al., 2002). Therefore, these bacteria are frequently detected in waste water treatment procedures, seawater, and sewage sludge (Phrommanich et al., 2009; Gottsching and Schmidt, 2007). Acinetobacter sp. strain Y9 has substantial use in bioremediation.
Y9 has the unusual characteristics of being able to grow on a broad range of n-alkanes. Previous studies showed the crucial role of *Acinetobacter* sp., that is, it played an important role in the process of biodegradation of oil in the marine environment (Liu et al., 2010). These results showed that strain Y9 could potentially be useful for the remediation of hydrocarbon pollution in the marine environment. It could be seen from the photos of the degradation that the strain Y9 was apparently grew in the SM culture in 3 and 5 days compared with the abiotic control. It indicated that the strain Y9 could grow in the SM using the diesel as sole carbon, nitrogen and energy sources.

**Diesel degrading enzyme gene analysis of indigenous bacteria**

For *alkB* and CYP153A sequence comparison, PCR amplifications yielded one-band product in both DNA chroms and plasmid. The length of alkane hydroxylase gene (*alkB*) and CYP153A obtained by PCR are 544 and 864 bp, and displays 84 and 98% with Acinetobacter sp. M-1 and Acinetobacter sp. OC4, respectively (Figure 4). This indicates the presence of two n-alkane biodegradation systems in strain. The *alkB* and CYP153A sequences of Y9 formed a deep cluster and a separate group from other taxa investigated so far. According to van Beilen et al. (2003), there was no clear linkage between the diversity of the *alkB* genes and phylogenetic lines. Nevertheless, when a particular genus, such as *Mycobacterium* or *Burkholderia*, was analysed independently, the phylogenetic tree of its partial *alkB* was highly matches with that of its 16S rRNA gene sequence, as was the case for *Alcanivorax*.

According to the present results, strain Y9 showed similarities in phenotypic and genotypic characteristics with *Acinetobacter venetianus* RAG-1T, with 99.8% 16S rRNA gene sequence similarity.

**Description of *Acinetobacter* strain Y9**

Cells are 1.2~1.9 μm long and 0.9~1.1 μm wide, motile, lophotrichous, non-spore-forming, Gram-negative rods. Colonies on LB agar are characteristically small, translucent, non-pigmented and slightly raised in the centre, with regular, transparent and halo-like peripheries. The optimum growth temperature is 37°C. NaCl is required for growth. Actively degrades Tween 80; catalase- and oxidase-positive, but negative for agarase, arginine dihydrolase, amylase and gelatinase. Nitrate reduced to nitrite. Among 5 carbon sources, positive for citric acid but negative for Glucose, Arabinose, and Ribose. Cells are able to degrade n-alkanes with chain length C5 to C23. It was capable of degrading n-alkanes in diesel oil from C11~C22. Comparative 16S rDNA sequence analysis placed the isolate with members of the genus *Acinetobacter*. That finding was corroborated by various morphological and physiological characteristics data. And the 16S rDNA of strain Y9 is 99.8% sequence identity with the type strain of *Acinetobacter venetianus* RAG-1T.

Phylogenetic analysis based on 16S rRNA gene sequence comparison (Figure 5) showed that strain Y9 represented one of the species of the γ-Proteobacteria, branching within the clade of *Acinetobacter* and forming a distinct branch with *Acinetobacter venetianus* RAG-1T. We initially considered that Y9 should be classified in a genus based on its close relationship in 16S rRNA, *alkB* and CYP153A gene sequence data.

**ACKNOWLEDGEMENTS**

We extend our sincere thanks to Dr. Peter Pothula, BioQuest for his valuable editorial guidance in the
Figure 3. The chromatogram of diesel oil. (a) abiotic control (b) *Acinetobacter* sp. strain Y9 culture.
Figure 4. The gel electrophoresis of the PCR product of alkB and CYP153A from strain Y9. (M. Marker; 1. alkB gene of genomic DNA; 2. alkB gene of plasmid; 3. CYP153A of genomic DNA; 4. CYP153A of plasmid).

Figure 5. Phylogenetic tree of *Acinetobacter* sp. strain Y9 and related species constructed on the basis of 16S rDNA sequence using the neighbor-joining method. Bar, 0.005 Knuc unit.
REFERENCES


