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PHYSIOLOGICAL, PHYLOGENETIC AND FUNCTIONAL IDENTIFICATION OF CRUDE OIL-DEGRADING MICROBIAL POPULATIONS

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Abstract

In this study, nine microbial strains with oil-degrading function were isolated from oil-contaminated soil near four oil wells in the 0.6 km^2 area of the Jinnan Oil Field, Huaian, Jiangsu, China (32°57′ N, 119°02′ E). Based on morphological observations, physiological and biochemical responses, and molecular identification, we confirmed that the nine strains belong to five genera: *Pseudomonas*, *Raoultella*, *Lysinibacillus*, *Escherichia*, and *Klebsiella*. The degradation capacity of the nine strains in crude oil medium ranged from 2.43% to 32.63% with an average rate of 21.64%. Further plasmid and chromosome analyses on the oil-degrading genes of the nine microbial strains were conducted. The *GST* gene (degradable polycyclic aromatics) was widely distributed in the chromosomes of eight strains and the plasmids of three strains. The *alkB* gene was detected in the chromosomes of seven strains and the plasmids of two strains. The *LmPH* gene (degradable phenolic substances) was only detected in the chromosome of one strain. Molecular biological analyses on the isolation, identification, and degradation characteristics of oil-degrading microbial strains provide a foundation for microbial remediation in oil-polluted environments.

Key words: biodegradation, functional gene analysis, molecular identification, oil pollution

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1. Introduction

Petroleum, an important energy source and industrial material, consists of various hydrocarbons and corresponding derivatives. With the development of industrial production, oily substances have entered the environment through oil exploration, mining, transportation, and storage. Soil is a major receptor of oil pollution (Afnan, 2014; Banock et al., 2016). Soil bacteria with the ability to degrade various petroleum substances, such as petroleum hydrocarbon have attracted considerable attention. These bacteria include *Pseudomonas*, *Flavobacterium*, *Achromobacter*, and *Ochrobactrum* (Arulazhagan and Vasudevan, 2011; Hirano et al., 2004; Ulea et al., 2017; Zhang et al., 2011). The most common of these bacteria is *Pseudomonas*, which can degrade short-chain and long-chain alkanes and aromatics. Bacterial degradation is also affected by the concentration and molecular structure of petroleum hydrocarbons, biotic factors (e.g., composition of microbial community) and abiotic factors (e.g., temperature, salinity, presence of water etc.) (Das et al., 2011). Laboratory and field studies have shown that some microbial strains have a significant capacity to degrade petroleum contaminants via natural selection (Cojocaru et al., 2016; Head et al., 2006). The distribution of microorganisms depends on the

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geographic locations of sampling points (Liang et al., 2011), regional climate (Maila et al., 2006), and types and characteristics of soil (Hamamura et al., 2006), plants (Joner et al., 2001), and other natural variables. Therefore, targeted research on oil-polluted soil is necessary to isolate oil-degrading strains with local adaptation capacity.

Microbial decomposition of petroleum contaminants is achieved with the catalysis of various degradation enzymes. Identifying the key functional genes of encoded degrading enzymes is critical to understand the degradation capacities of microorganisms and the mechanisms of metabolic processes. Many studies have focused on microbial degradation genes. Yu et al. (2013) found that the *Oxidoreductase* gene is involved in the degradation of alkanes, methylbenzene, naphthalene, and other compounds. Liu et al. (2010) analyzed the *Pseudomonas* genome and found that the *alkB* gene determines the first step in the oxidative decomposition of n-alkanes. In Marinkovic et al. (2013) research, Glutathione s-transferase, which is encoded by *GSTs* gene, is vital in the conjugation and neutralization for oxidation products from the first phase of biotransformation in the PAH (polycyclic aromatic hydrocarbon) metabolism; a phenol-degrading strain with the largest multicomponent phenol hydroxylase (*LmPH*) gene was isolated from oil-contaminated soil in India. This strain was found to be capable of utilizing and tolerating up to 9.5 mM of phenol. Reversed-phase high-performance liquid chromatography showed that the gene could degrade phenol through the catechol ortho fission pathway (Saxena et al., 2013). Ma et al. (2006) conducted a plasmid elimination experiment involving a dominant polycyclic aromatic hydrocarbon PAH-degrading strain from Antarctic soil and verified that PAH-degrading genes are located on the plasmid instead of on the chromosome.

Understanding the functional diversity of microorganisms is important for the biological remediation of petroleum-contaminated soil. In this study, we collected samples of oil-contaminated soil near four oil wells in the 0.6 km^2 area of the Jinnan Oil Field, Huaian, Jiangsu, China (32°57′ N, 119°02′ E). Three functional genes responsible for degrading microorganisms in petroleum-contaminated soil were analyzed. This research aims to isolate and classify oil-degrading bacteria in soil and determine the functional genes of alkanes, phenolic substances, and polycyclic aromatics in the chromosomes and plasmids of oil-degrading strains.

2. Material and methods

2.1. Source of strain and isolation

Soil samples collected from the surroundings of four wells in the Jinnan Oil Field, Huaian, Jiangsu were named JSA, JSB, JSC, and JSD. All were collected from surface soil (0-10 cm deep). After being dried, pulverized, and sieved through 10-mesh nylon mesh, 300 g of each soil was sealed in a sterile bag and then stored in the dark at 4°C.

Enrichment culture was used to screen out oil-degrading bacteria. Approximately 3 g samples of each soil were inoculated into 100 mL of crude oil medium at 1% (v/v) (2 g NH_4NO_3, 0.5 g K_2HPO_4, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.02 g anhydrous $CaCl_2$, 5 g NaCl, 0.02 g $FeCl_3 \cdot 6H_2O$, 1 L distilled water, 10 g crude oil; natural pH range; sterilization at 121°C for 20 min) to create a shaking culture at 30°C and 6 G (RCF) for 48h (Gogoi et al., 2004). The concentration of crude oil that was too high or too low would all lead to a decrease in oil-degrading bacteria (Vyas and Dave, 2007). Up to 10 mL of the enrichment culture sample was inoculated into 100 mL of crude oil medium to create a shaking culture at 30°C and 6 G (RCF) for 48h. These steps were repeated three times, and enrichment culture continued. The final bacterial suspensions were diluted gradually to 10^{-6}. Approximately 0.1 mL of each diluted bacterial suspension with 10^{-4} to 10^{-6} content was inoculated on a solid medium (3 g beef extract, 10 g proteose peptone, 5 g NaCl, 15 g agar, 1 L distilled water; pH 7.0 to 7.2; sterilization at 121 °C for 20 min). Colonies with significantly different physicochemical and morphological characteristics were selected from the solid culture medium, isolated, and then purified. Nine oil-degrading strains were obtained.

2.2. Physiological and biochemical characteristics of bacterial strains and determination of degradation capacity

The nitrogen and citrate usage of the oil-degrading strains was investigated to analyze the physiological and biochemical characteristics of the strains. The Voges-Proskauer (V-P) (Holt et al., 1994), Indole (Miller and Wright, 1982), and methyl red (Barry et al., 1970) tests were used to identify the metabolic characteristics of the strains. The catalase test (Saginur et al., 1982), glucose oxidation fermentation experiments (Bankar et al., 2009), and starch hydrolysis test (Pandey et al., 2000) were performed to analyze the enzyme activity of the strains. Gram staining (Claus, 1992) was used to classify the strains as either Gram-negative or Gram-positive bacteria, providing a foundation for further experimentation.

Bacterial suspensions at the same inoculation amount were added into 150 mL of the crude oil medium to determine the degradation capacity of the nine oil-degrading bacterial strains. Each of the three groups of parallel samples was placed on a shaking table at 30°C to be cultured at 150r/min for 7 d. The gravimetric method was then used to determine the content of the remaining oil.

Chloroform was used to recover the remaining oil with an inoculated liquid medium set as a blank medium (i.e., uninoculated). The liquid was filtered into the weighed beaker for suspension removal and natural drying before weighing. The following

equation was used to calculate the oil degradation capacity (Eq. 1):

$$\eta = \frac{\vartheta_0 - \vartheta_x}{\vartheta_0} x\,100\% \quad (1)$$

where: ϑ_0 = content of the remaining oil in the blank medium; ϑ_x = content of remaining oil in the inoculated medium.

2.3. Identification of bacterial strains and systematic analysis

A Bacterial DNA Extraction Kit from Sangon Biotech Engineering Company (Shanghai) was used to extract the genomic DNA of the nine purely cultured oil-degrading bacterial strains. The 16S rRNA amplification of bacterial genomic DNA with an amplification length of 1500 bp was performed using 27F and 1504R, which are the universal primers of 16S rRNA. Each reaction system comprised 1 μL of DNA template, 0.2 μL of dNTP, 1 μL of 10 × Buffer (Mg^{2+}), 0.25 μL of Primer 27F, 0.25 μL of Primer 1504R, 0.1 μL of Tap DNA polymerase, and 10 μL of dH_2O. The PCR conditions were as follows: 5 min of initial denaturation at 95°C, followed by 30 cycles consisting of 60 s at 94°C, 60 s at 54°C, and 90 s at 72°C, and finally 10 min of extension at 72°C, which was maintained at 4°C.

A SanPrep Column DNA Gel Extraction Kit from Sangon Biotech Engineering Company (Shanghai) was used to purify the PCR products. The purified PCR products were sent to Sangon Biotech Engineering Company (Shanghai) for sequencing. Dideoxy chain termination was the DNA sequencing method used, and an ABI sequencer (3730xl DNA Analyzer) was the sequencing equipment used. The sequencing results of DNA were processed using BLAST software on the NCBI website to obtain log sequence numbers. Bacterial strains that were closely related to this strain were selected from the GenBank database to conduct a comparative analysis using MEGA 5.05 (Tamura et al., 2011) and to construct a phylogenetic tree.

2.4. Plasmid extraction and degradation gene distribution

A SanPrep Column Plasmid DNA Small-scale Extraction Kit from Sangon Biotech Engineering Company (Shanghai) was used to extract the plasmid DNA of the nine purely cultured oil-degrading bacterial strains. Primer Premier 5.0 was used to design four groups of oil-degrading gene amplification primers based on the homologous *alkB*, *LmPH*, and *GST* genes as well as their conserved sequences (Table 1). For the extracted plasmid DNA and the corresponding genomic DNA, the four groups of functional genes were amplified through PCR. Agarose gel (1%) was used for electrophoresis and ultraviolet spectrophotometry.

3. Results and discussion

3.1. Basic characteristics and degradation effects of bacterial strains

The physical and chemical properties of the oil-degrading bacterial strains were preliminary determined through citrate experiments, V-P tests, Indole experiments, glucose oxidation fermentation experiments, Gram staining, and physical and chemical tests. The experiment results are shown in Table 2. The nine oil-degrading bacteria are heterotrophic strains that contain catalase and amylase. Two of these strains are Gram-positive, and seven are Gram-negative. Eight of these strains metabolize glucose in oxidation and produce diacetyl, four strains produce indole, and five strains produce pyruvate.

Temperature and pH were shown to influence degradation. The optimal temperature and pH for the nine strains ranged from 25°C to 30°C and from 7.0 to 7.5; nearly every oil-degrading bacterium began to reach its best growth situation after a 22 h culture (Fig. 1–Fig. 3). Under optimal conditions, significant differences in oil-degradation effects were detected among the nine oil-degrading bacterial strains (Fig. 4). The degradation capacities ranged from 2.43% to 32.63% with an average of 21.64%. The oil-degradation capacity of JSC02 was 32.63% ± 3.11%. Wongsa et al. (2004) screened a highly efficient degrading strain of *Pseudomonas aeruginosa* (No. WATG) from hot spring water contaminated by crude oil medium in Hokkaido, Japan and from water in a coal tank, soil, and slurry. The degradation capacity in their study ranged from 55.0% to 90.8%, which is the optimal degradation of C_8–C_{23} (N-alkanes, cycloalkanes, and aromatics). Rahman et al. (2002) showed that *Pseudomonas* sp. DS10-129, *Bacillus* sp. DS6-86, *Micrococcus* sp. GS2-22, *Corynebacterium* sp. GS5-66, and *Flavobacterium* sp. DS5-73 exhibit high degradation capacities of 66%, 59%, 49%, 43%, and 41%, respectively, under the conditions of pH 7.0, 30°C, and an initial crude oil content of 1%.

Table 1. Functional gene amplification primers of four groups of oil-degrading genes

Primer No.	*Primer sequences*	*Amplified fragment length (bp)*	*Degradation gene*
D6	5′-TGA ATC GGC GGT AAA GA-3′	1007	Alkane hydroxylase gene
	5′-CAG CCT CAG ACT CAG AAG AAG-3′		
D7	5′-CGG ATA ACG CCG TGA ACC A-3′	1881	Alkane hydroxylase gene
	5′-TTG CCA AAG CCG CAC CTC T-3′		
PD39	5′-AGG CAT CAA GAT CAC CGA CTG-3′	684	Phenol hydroxylase gene
	5′-CGC CAG AAC CAT TTA TCG ATC-3′		
GSTs	5′-GAT TTC CTG ACG GTC AAC CC-3′	350	Glutathione S-transferases gene
	5′-GCC CAG CAT CAC GAA CAG ATA G-3′		

Table 2. Physical and chemical characteristics of nine oil-degrading bacterial strains

Name	*Catalase*	*Oxidase*	*Amylase*	*V-P*	*Nitrogen*	*Citrate*	*Indole*	*Methyl red*	*Gram stain*
JSA01	+	+	+	–	+	+–	+	–	–
JSA02	+	+	+	+	+	+	–	+	–
JSA03	+	–	+	+	+	–	–	+–	+
JSB01	+	+	+	+	+	+–	+	+	–
JSB02	+	+	+	+	+	–	+	+	–
JSB03	+	+	+	+	+	–	–	–	+
JSC01	+	+	+	+	+	+	+	–	–
JSC02	+	+	+	+	+	+	–	+–	–
JSD01	+	+	+	+	+	+	–	–	–

"+" represents a positive reaction, "–" represents a negative reaction and "+–" represents a weak positive reaction.

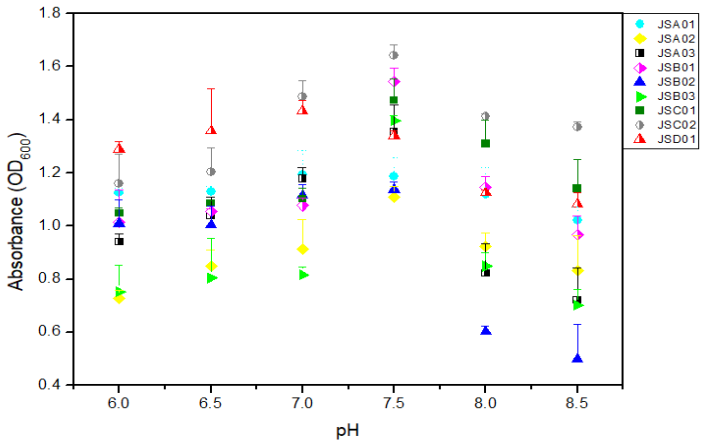

Fig. 1. Effect of pH on the growth of nine oil-degrading bacterial strains

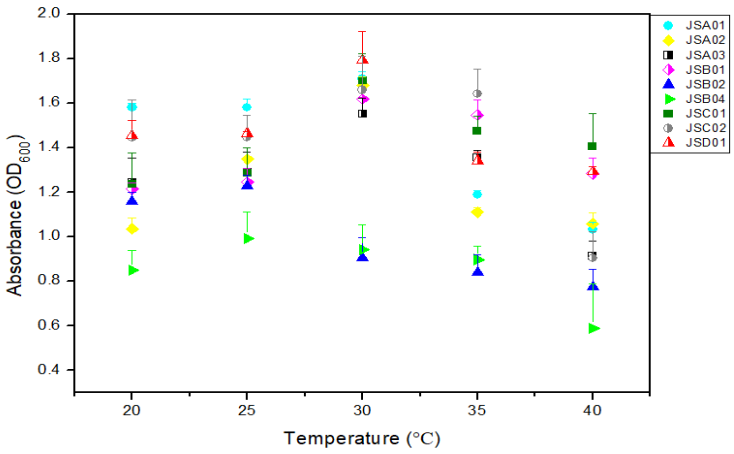

Fig. 2. Effect of temperature on the growth of nine oil-degrading bacterial strains

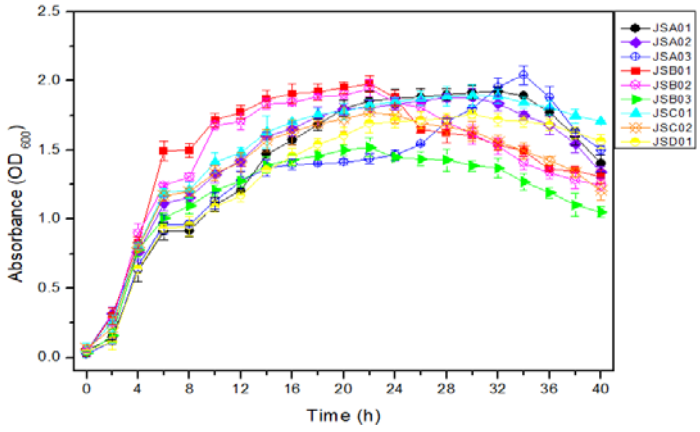

Fig. 3. Effect of culture time on the growth of nine oil-degrading bacterial strains

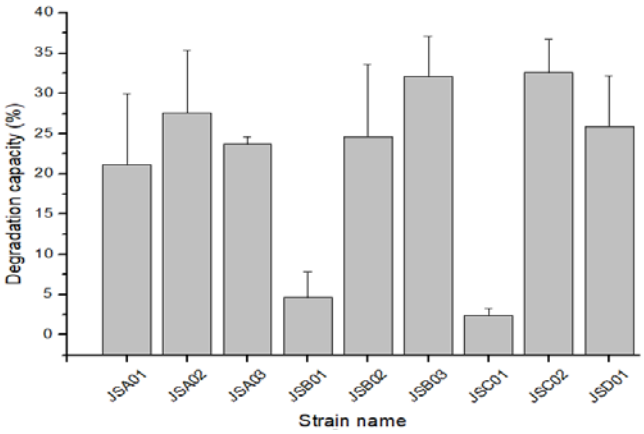

Fig. 4. Degradation capacity of nine oil-degrading bacteria

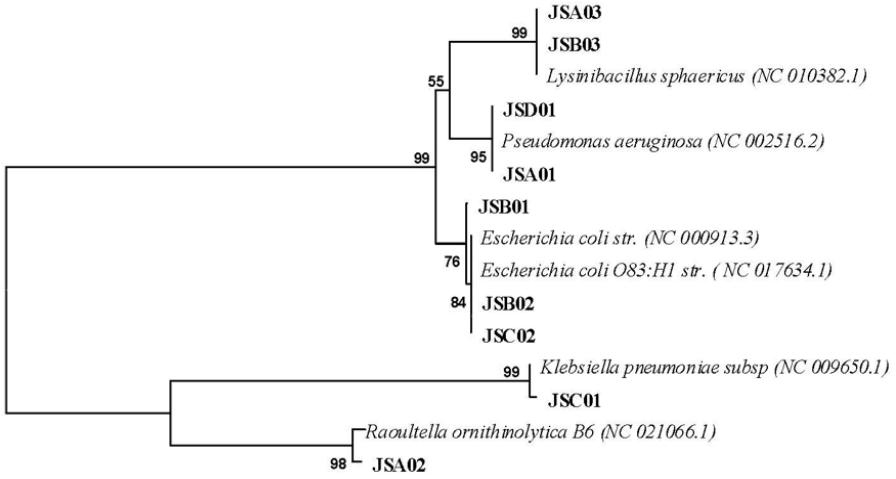

Fig. 5. 16S rRNA phylogenetic trees of nine oil-degrading bacterial strains

The degradation characteristics of different strains are affected by pollutant composition, initial content (Lizardi-Jimenez et al., 2012), environmental conditions (temperature, pH, etc.), and inherent characteristics (Chung and Alexander, 2002; Megharaj et al., 2011; Singh et al., 2003).

3.2. 16S rRNA sequencing result and systems biology analysis

The 16S rRNA of the nine bacterial strains was sequenced based on the results of BLAST software on the NCBI website and the phylogenetic tree from MEGA (Fig. 5).

Analysis of the genotypes, phenotypes (Table 3), and physical and chemical characteristics (Table 2) show that the nine oil-degrading bacterial strains belong to five genera: *Pseudomonas*, *Raoultella*, *Klebsiella*, *Escherichia*, and *Lysinibacill*us.

Existing research results were similar to the data in Tables 2 and 3; for example, JSA03 and JSB03 were both *Lysinibacill*us, have nearly identical morphological properties and are similar to the research of Ahmed et al. (2007), who found that *Lysinibacillus* strains were both Gram-positive, grew as a colony and on the edge of the flat elevation and were opaque.

Table 3. Morphological properties of nine oil-degrading bacterial strains

Name	***Color***	***Surface features***	***Humidity***	***Edge Features***	***Longitudinal profile***
JSA01	Light yellow	Smooth	Wet	Filamentous	Flat
JSA02	Orange	Smooth	Partial desiccation	Foliate	Flat
JSA03	Light yellow	Rough	Partial desiccation	Round	Flat
JSB01	Orange	Rough	Wet	Serrated	Flat
JSB02	White	Rough	Wet	Round	Flat
JSB03	White	Rough	Partial desiccation	Round	Flat
JSC01	Yellow	Rough	Dry	Foliate	Umbilical
JSC02	Light yellow	Smooth	Partial desiccation	Serrated	Flat
JSD01	Yellow	Smooth	Dry	Wavy	Flat

Fig. 6. Agarose gel electrophoresis images of plasmid DNA of nine oil-degrading bacterial strains ("M" represents Marker, and A~I represent JSA01, JSA02, JSA03, JSB01, JSB02, JSB03, JSC01, JSC02, JSD01)

JSA01 and JSD01 strains belong to *Pseudomonas*, which have similar morphological properties, including being Gram-negative and having a yellow color, smooth surface and flat longitudinal profile (Moore et al., 2006). Three *E. coli*. were all found to be similar to the description of Schaechter (2009), who stated that *E. coli*. strains are Gram-negative and have wet and large bacterial colonies.

The petroleum hydrocarbon degradation of *Pseudomonas*, *Klebsiella*, and other genera has also been reported. Wongsa et al. (2004) studied *P. aeruginosa* (No. WATG) screened from the wastewater of a coal tank in Sapporo, Hokkaido in 2004. They found that the degradation effect of this strain on C_8–C_{35} N-alkanes is broadly stable at 62%, and its degradation capacity on C_9–C_{23} N-alkanes is higher than C_{24}-C_{35} and the average degradation capacity. Gai et al. (2012) separated *P. aeruginosa* DQ8 from petroleum-contaminated soil in the Daqing Oil Field of China and determined that this strain can degrade N-alkanes and PAHs. Lors et al. (2012) isolated *Pseudomonas* sp., *Klebsiella planticola*, and 17 other bacterial strains that can degrade polycyclic aromatics from the contaminated soil of a coal tar distillation plant in Northern France. Several studies have reported the crude oil degradation potential of different bacterial strains belonging to the genus *Lysinibacillus* (Das et al., 2015). Sugimori et al. (2013) isolated a *Raoultella* strain 232-2 that is capable of efficiently catabolizing oil. *Escherichia sp*. was isolated from the River Ethiope, Delta State, Nigeria, which can withstand a 2.5% concentration of crude oil (Idise and Owhe-Ureghe, 2015).

3.3. Analysis of degradation genes in genomic and plasmid DNAs

We used Primer Premier 5.0 software to design four groups of primers based on previous research results to further explore the distribution of oil-degrading functional genes in the plasmid and genomic DNAs of the nine bacterial strains. Two groups of *alkB* gene-specific primers include two gene fragments with amplification bands of 1007 and 1881 bp. Vomberg and Klinner (2000) showed that the *alkB* gene in bacteria is strongly correlated with N-alkane degradation and is highly homologous to the known coding amino acid sequence of the *alkB* gene. They obtained important information on the conserved sequence of alkane hydroxylase and on the structure of the active center. The gene sequence of *GST* in GenBank was used to design gene amplification primers. The PCR product for *GST* amplification was approximately 350 bp long. Lloyd-Jones and Lau (1997) verified that bacteria with *GST* could degrade polycyclic aromatics. *LmPH* is involved in phenol degradation in bacteria (Watanabe et al., 1998; Zhang et al., 2004). The amplification band was approximately 684 bp. Seven plasmids were extracted from nine purely cultured oil-degrading bacterial strains (Fig. 6). Four groups of functional genes after PCR amplification for genomic (Fig. 7) and plasmid (Fig. 8) DNAs of the nine oil-degrading bacterial strains were established (Table 4).

JSA02, JSA03, JSB01, and JSB02 were found to have degrading genes. The redundancy rate with degrading genes on genomic DNA was 67%. *GST* (degradable polycyclic aromatics) was widely distributed in the chromosome of eight strains. *GST* was also detected in the plasmids of three strains (e.g., JSA02, JSA03, and JSB01). The *alkB* gene was detected in the chromosomes of seven strains and the plasmids of two strains. The *LmPH* gene (degradable phenol substances) was detected in the chromosome of JSD01. Beilen et al. (1994) studied the metabolism of alkanes from the aspects of genetics and enzymes and found that *alkB* and *alkST* exist in the plasmid of *Psudomonas oleovorans* GPo1.

These two coding areas can form enzymes that are required to degrade alkane and its derivative protein, allowing the strain to decompose and utilize C_6–C_{12} alkanes. Ma et al. (2006) conducted a plasmid removal experiment and found that the PAH-degrading genes of six *Pseudomonas* strains are located in the plasmid instead of the chromosome. Similarly, Wu et al. (2013) proved that *R. ornithinolytica* could decompose polycyclic aromatics. They also found that *GST* exists in two DNA types of *Lysinibacillus sphaericus* JSA03, while no *alkB* gene exists in plasmid DNA.

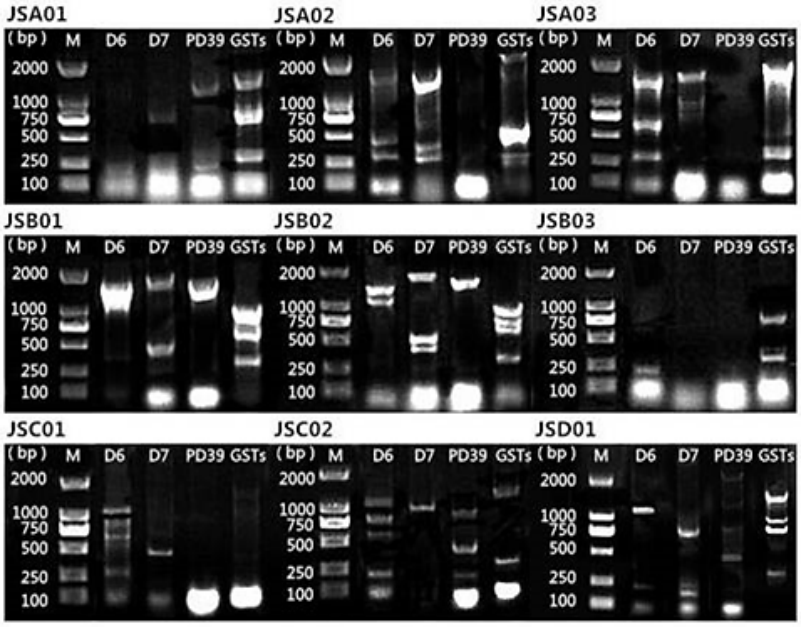

Fig. 7. PCR agarose gel electrophoresis images of genome DNA functional gene primers of nine oil-degrading bacterial strains ("M" represents Marker)

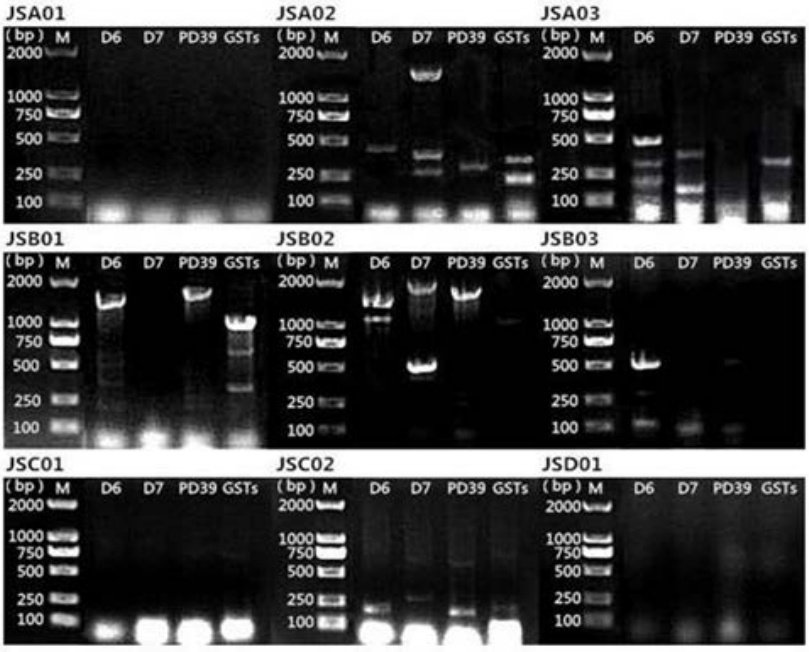

Fig. 8. PCR agarose gel electrophoresis images of plasmid DNA functional gene primers of nine oil-degrading bacterial strains ("M" represents Marker)

Table 4. Distribution of functional genes in the plasmid and genomic DNAs of nine oil-degrading bacterial strains

Name	***D6***	***D7***	***PD39***	***GSTs***
JSA01				√p
JSA02		√p, √g		√p, √g
JSA03		√p		√p, √g
JSB01		√p		√p, √g
JSB02	√p, √g	√p, √g		√p
JSB03				√p
JSC01	√p			
JSC02	√p			√p
JSD01	√p		√p	√p

"√p" represents genomic DNA and "√g" represents plasmid DNA.

However, in contrast to our results, Wu et al. (2009) isolated *L. sphaericus* WH22 from pharmaceutical wastewater and sludge and found that this strain contains a gene that can metabolize alkanes in the plasmid and chromosome. In addition, *alkB* and *GST* were found in the genomic DNAs of the *Escherichia coli* strains JSB01 and JSB02. No *alkB* and *GST* were detected in the plasmid DNAs of JSB01 and JSB02, respectively. Lederberg and Tatum (1946) obtained 24% of the gene information for *E. coli* from horizontal gene transfer by comparison with the gene sequence information of prokaryotes. N-alkanes, phenol, and polycyclic aromatic genes in *E. coli* might be obtained from horizontal gene transfers among bacteria in petroleum-contaminated soil; *P. stutzeri* is known for its natural transformation ability, and naphthalene degradation genes can be horizontally transferred in this species (Mulet et al., 2011). Meintanis et al. (2006) isolated *Lysinibacillus* strains from volcanic soil, seawater and sediment from two bays near the active volcano of Santorini at Nea Kameni island, which were able to grow in liquid cultures with crude oil as the sole carbon source and with horizontal gene transfer of the alkane degradation pathway.

Degrading gene horizontal transfer can strongly promote the degradation of pollutants, thereby enhancing bioremediation (Top and Springael, 2003). This method can be further used in the genetic engineering reconstruction and construction of highly efficient colonies.

4. Conclusions

We collected samples of oil-contaminated soils near four oil wells in the Jinnan Oil Field, Huaian, Jiangsu for microorganism selection and obtained nine oil-degrading bacterial strains with significant characteristics. The results in this study prove the finding that potent hydrocarbon-degrading bacterial consortia exist naturally in the soil near the Jinnan Oil Field. This signifies that hydrocarbon-degrading bacteria exist or evolved to exist with the ever-increasing intensity of soil pollution.

Based on morphologic observations, physiological and biochemical responses, and molecular identification, we confirmed that the nine strains investigated belong to five genera: *Pseudomonas*, *Raoultella*, Lysinibacillus, *Escherichia*, and *Klebsiella*. Except for *Escherichia*, which is rarely referenced with regard to hydrocarbon degrading capacity, the other genera identified have all been reported in similar studies. We also confirmed the distributions of *alkB*, *LmPH*, and *GST* genes in genomic and plasmid DNAs, which was seldom found in other studies.

These methods could be equally applicable for similar bacterial strains, and the technology could be further developed to target any hydrocarbon pollutants that create environmental and health hazards. The results of this study provide reference information for the efficient resource development of oil-degrading bacteria and the biological remediation of petroleum-contaminated sites.

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