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Isolation, identification and alkane hydroxylase genes detection of a marine diesel-degrading bacterial strain (F9)

Dengyong Hou, Zhan Shi, Xianrong Shen*, Ying He, Min Sun, Qun luo and Qingrong Wang

Navy Medical Research Institute, Shanghai, 200433, China.

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A diesel-degrading bacterial strain (F9) was isolated and identified in this study. F9 strain was isolated from diesel polluted seawater. It was a Gram-negative, non-spore-forming bacterium and identified as a strain of *Acinetobacter* sp. according to its physiological and biochemical properties and 16S rDNA sequence analysis. It had efficient diesel-biodegradability and could grow very well with diesel as its sore carbon resource. At the 7th day, F9 could remove more than 90% diesel in culture. Gas chromatography-mass spectrometry (GC-MS) results showed that F9 could degrade all alkanes from C11-C21 in diesel. Using degenerate PCR primers, the alkane hydroxylase genes, including AlkB and CYP153A genes, were detected in F9. Products of PCR were sequenced and analyzed. The results showed that F9 had one CYP153A gene and two AlkB genes. The sequence of CYP153A gene in F9 had 99% identity to sequence of CYP153A gene of *Acinetobacter* sp. EB104. The sequence of one AlkB gene had 99% identity to sequence of AlkM gene of *Acinetobacter venetianus* strain VE-C3. While, the sequence of the other AlkB gene only had 84% identity to sequence of AlkB gene of bacterium alkW42. The presence of multiple alkane hydroxylase genes in F9 may be the reason why it had efficient diesel-degradability and wide substrate range. In conclusion, F9 strain containing multiple alkane hydroxylase genes belonged to genera *Acinetobacter* sp and could degrade diesel efficiently. It has potential to be applied in bioremediation of marine diesel pollution.

Key words: Bacterial strain, diesel-degrading, *Acinetobacter* sp., alkane hydroxylase gene, bioremediation.

INTRODUCTION

Petroleum and its derivatives are important energy resources used by industries and in our daily life. But, due to refuse from coastal oil refineries, offshore oil production, shipping activities, accidental spills and so on, petroleum and its derivatives have became a major pollutant of marine environments (Gentili et al., 2006).

Microbiological decontamination of oil derivatives in polluted environments is an efficient, economic and versatile alternative to physicochemical treatment (Burns et al., 1999). There are two approaches to do bioremediation: biostimulation and bioaumentation (Tyagi et al., 2011). A popular option to favor the clean-up of hydrocarbons polluted marine environments has involved biostimulation, which stimulates the growth of native degrading bacterial. However, for more recalcitrants compounds or if the biodegradable pollutant is introduced to the environment at high concentrations (for example, spills) and a rapid detoxification of the chemical is required, it may not be appropriate to rely on the natural response of members of the native microbial community. For example, a slow biodegradation in an accidental oil spill in coastal seawater may result in the movement of

*Corresponding author. E-mail: xianrong_sh@Yahoo.com. Tel: (+86) 021 81883171. Fax: (+86) 021 81883173.

spilled crude oil to other coastal sites and probably its accumulation in the sediments, so the possibility of undesirable effects on the ecosystem is increased (Bartha, 1986; Alexander, 1999). Bioaugmentation to enhance natural biodegradation is a useful alternative (Vogel, 1996; Jansson et al., 2000; Cunningham et al., 2004). In bioaugmentation, cultured microorganism with degradability is applied to the polluted site to clean up the pollutant.

Isolation and cultivation of indigenous degrading bacteria are essential for bioaugmentation. Degrading bacteria with broad substrate range and high metabolic rates and their formulations are needed to be inoculated into the polluted seawater to enhance the bioremediation rate. Up to now, a lot of degrading bacteria have been isolated (Harayama et al., 2004; AL-Saleh et al., 2009). These bacteria belong to more than 40 genera. Among them, the most frequent bacterial genera include *Pseudomonas, Acinetobacter, Bacillus, Alcanivorax* and so on.

Alkanes are common environmental pollutants, constituting 20 to 50% of crude oil, and alkane-degrading micro-organisms are widely distributed in nature. In spite of their relative inertness, *n*-alkanes, ranging from C12 to C18 and longer, are readily degraded in the presence of oxygen and support abundant growth of many different bacteria (Watkinson and Morgan, 1990). Most of these bacteria are able to oxidize *n*-alkanes by terminal hydroxylases to the corresponding alcohols which are channeled via fatty acids into the *β*-oxidation cycle (Amouric et al., 2010).

In this study, a diesel-degrading bacterium strain was isolated from diesel polluted seawater sample. The strain was named F9 and identified to genera *Acinetobacter* sp. The diesel-degrading ability of F9 was measured in bench and the alkane hydroxylase genes in it were analyzed by polymerase chain reaction (PCR) method.

MATERIALS AND METHODS

Isolation and purification of diesel-degrading bacteria

The diesel oil polluted seawater sample was collected from Dinghai Port, Zhejiang Province of China. The enrichment and isolation of diesel-degrading bacteria were performed in the artificial seawater medium named MMC medium. The MMC medium was prepared from the following composition (g/L): NaCl (24) , KH_2PO_4 (2.0), NH_4NO_3 (1.0), $MgSO_4·7H_2O$ (0.7), KCl (0.7), $Na_2HPO_4·2H_2O$ (3.0). The pH of MMC medium was adjusted to 7.5 with either HCl or NaOH. MMC medium was supplemented with 2% (v/v) trace elements solution of the following composition (g/L): $MgSO_4·7H_2O$ (4.0), $CuSO_4·5H_2O$ (1.0), $MnSO_4·H_2O$ (1.0), $FeSO_4·7H_2O$ (1.0), $CaCl_2$ (1.0). 100 ml of seawater sample was added to 250-ml Erlenmeyer flask containing 2 ml of sterilized diesel oil (as a carbon source), and N, P and Fe sources with concentrations as in MMC medium. The flask was incubated for 10 days at 30°C on a rotary shaker operated at 200 rpm. Then, 5 ml aliquot was transferred to 100 ml fresh MMC medium containing diesel oil and was incubated for 10 days at 30°C on a rotary shaker operated at 200 rpm. The above step was repeated four times. Then, inoculum from the flask was streaked out on LB agar plate and bacterial colonies were selected and purified. LB culture was prepared from the following composition (g/L): NaCl (10), tryptone (10), yeast extract (5.0). The pH of LB media was adjust to 7.2 with either HCl or NaOH.

Assessment of diesel oil-biodegradation ability of the isolates

Oil-biodegradation ability of the isolates was assessed using their growth in MMC media containing 1% (v/v) sterilized diesel oil as sore carbon source. The isolates were firstly inoculated in 5 ml LB liquid medium and cultured at 30°C with shaking (180 rpm) for 12 h. Then, aliquots of approximately 5×10^8 cells were collected, washed twice with sterilized MMC media and inoculated into 50 ml MMC media containing 1% (v/v) diesel oil in 150 ml Erlenmeyer flasks. The flasks were incubated at 30°C with shaking (180 rpm) for 5 days. At the 5^{th}day, the cell density in culture was measured by absorbance at 600 nm.

Identification of bacterial species

The major physiological and biochemical tests including morphology observation, catalase and oxidase, anaerobic growth, growth factor, sugar, salt and temperature were performed as described previously (Olt et al., 1994; Erkeley et al., 1984). The 16S rDNA sequence of diesel-degrading bacterium was analyzed, too. Genomic DNA of bacterial isolate was extracted using the TIANamp Bacteria DNA Kit (TianGen). The 16S rDNA was amplified by PCR using the universal primer set 27F (5′-AGRGTTTGATYV TGGCTCAG-3′) and 1492R (5′-GGHTACCTTGTTACGACTT-3′). The following thermal cycling parameters were used: a 5 min hot start at 95°C; followed by 32 cycles of denaturation for 1 min at 94°C, annealing at 53°C for 1.5 min, and extension at 72°C for 1 min; and a final extension of 20 min at 72°C. Then, the amplified 16S rDNA was sequenced.

Construction of phylogenetic tree

The 16S rDNA sequence was compared with other 16s rDNA in Genbank using Eztaxon server version 2 at http://14 7.47.212.35:8080, and analyzed phylogenetically by Clustalx 1.83 program. Phylogenetic tree was constructed using the Neighbour-Joining method of the Mega 4 program.

Measurement of diesel oil degradation rate

Two sets of samples, with and without bacterial inoculation, were incubated at 30°C, with shaking (180 rpm). After incubation, the diesel oil remaining in each sample was extracted with ligarine and the extracted samples were dried with Na_2SO_4. The diesel in ligarine was measured by UV absorbance at 255nm. The diesel oil degradation rate was determined according to the following equation:

$$R_d = \left(1 - \frac{Xc - X_s}{Xc}\right) \times 100\%$$

R_d = diesel-degradation rate, Xc = diesel remaining in control culture, Xs = diesel remaining in sample culture.

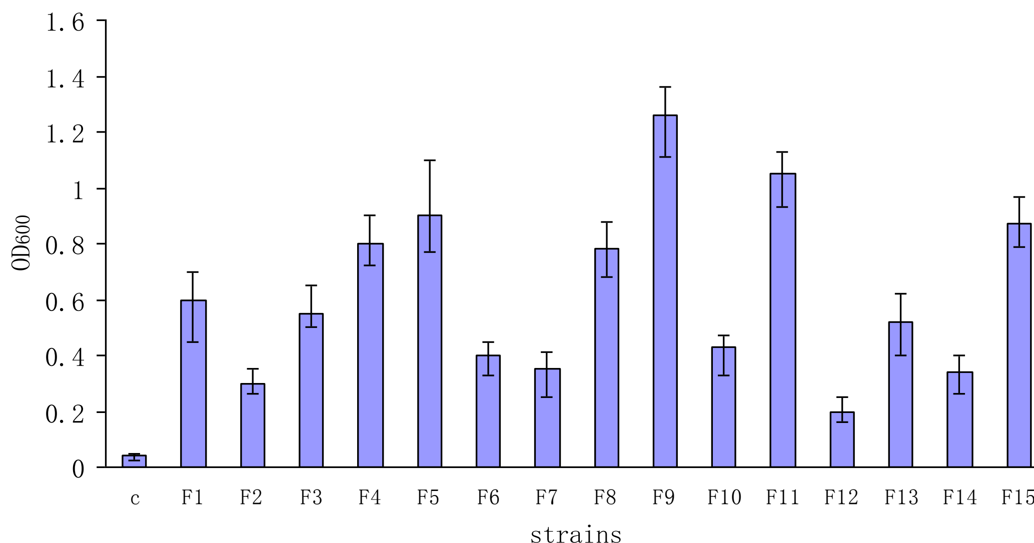


Figure 1. The OD_{600} of 15 isolates culture. c: Blank control; F1 to F15: Bacterial strains isolated from polluted seawater sample.

Gas chromatography-mass spectrometry (GC-MS) analysis of diesel degradation

F9 was inoculated into MMC media which containing 1% (v/v) diesel oil and cultured at 30°C shaking with 180 rpm for 7 days. The control was the same media without inoculation. After 7days, the remaining diesel oil was extracted from the culture with dichloromethane. The extract was analyzed by GC-MS. GC-MS analysis was performed on a Focus DSQ GC-MS instrument (Thermo, USA). An HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used to separate the hydrocarbons. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The injector and detector temperatures were set at 250 and 300°C, respectively. The temperature program was as followings: 2-min hold at 60°C, ramp to 300°C at 20°C/min and 5-min hold at 300°C.

Degenerate PCR primers design

PCR amplification of DNA fragment of CYP153A genes were performed using primers F (5′-TGTCGGTTGAAATGTTCATYGCNMTGGAYCC-3′) and R (5′-TGCAGTTCGGCAAGGCGGTTDCCSRYRCAVCKRTG-3′). This pair of primers was an improvement on the CF and CR primers based on the well-conserved N-terminal (MFIAMDPP) and C-terminal (HTCMGNRL) domains of CYP153A subfamily genes (Kubota et al., 2005).

For AlkB gene detection, degenerate primers AlkBwF (5′-AAYACNGCNCAYGARCTNGGVCAYAA-3′) and AlkBwR (5′-GCRTGRTGRTCHGARTGNCGYTG-3′) were used in PCR amplification. The sites on AlkB gene are the same as the primers designed by Kloos and colleagues (2006) with one base variation in the degeneration degree of each sequence. Their primers were successfully used to detect the genetic diversity of AlkB genes in soil samples (Kloos et al., 2006).

PCR detection of CYP153A and AlkB genes

The two pairs of degenerate primers described above were used for PCR detection of the target genes in F9 strain. The PCR reaction mixture contained 0.04 μM primers, 0.2 mM dNTPs, 1× rTaq buffer (Mg^{2+} plus), 1.25 unit rTaq DNA polymerase (TaKaRa Bio, China) and10 to 50 ng template DNA in a final volume of 50 ml. The PCR amplification was carried out on a MyCycler (Bio-Rad) as follows: initial denaturation for 4 min at 94°C; 32 cycles of 30 s at 94°C, 30 s at 52°C , and 1 min at 72°C; and a final extension at 72°C for 10 min. The PCR products were separated and extracted by electrophoresis on 1.5% agarose gels. Then, the products of PCR were cloned into vector PUC19-T and sequenced. All sequences obtained were compared with reference sequences in the GeneBank databases using the NCBI BLASTN search tool.

RESULTS AND DISCUSSION

Diesel oil-biodegradation ability of isolated strains

Fifteen diesel oil-degrading bacterial strains were isolated from polluted seawater sample. The isolated strains were inoculated in MMC media containing diesel oil as the only carborn resource and cultured for five days. Then, the absorbance value at 600 nm wavelength of the culture was measured. The results showed that F9 strain culture had the highest absorbance value (Figure 1) and it was selected for further study.

Physiological and biochemical characterization of strain F9

The major physiological and biochemical tests were performed and the results showed that colony of F9 were round or nearly round, almost ivory yellow, smooth, moist and with neat edge (Figure 2A). F9 strain was a gram-negative, non-spore-forming bacterium. It was short rod in

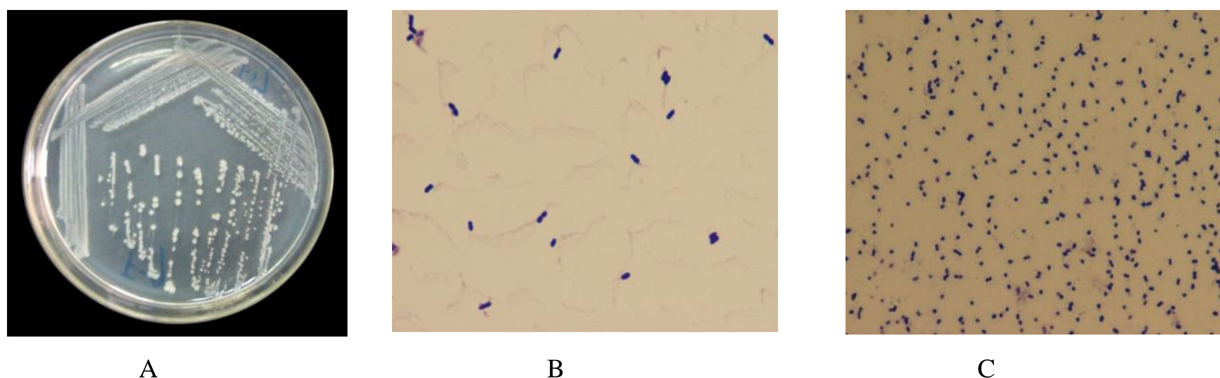


Figure 2. Morphology characteristics of strain F9. A: Colonies of F9 on LB agar plate; B: F9 cells in infant age (10 × 40); C: F9 cells in stationary phase (10 × 40).

Table 1. Physiological and biochemical characteristics of F9.

Characteristic	F9
Catalase	+
Oxidase	—
Anaerobic growth	—
Growth factor required	—
D-glucose	—
D-Ribose	—
D-xylose	—
L-arabinose	—
Sodium citrate	+
37°C	+
44°C	—
Ammonium sulfate	+
Potassium nitrate	+

+, Positive reaction or growth; -,no reaction or growth.

in infant age while cocci in the stationary phase (Figure 2B and C).

F9 was strictly aerobic, oxidase negative, catalase positive and it could not use glucose, D-fructose, L-arabinose and D-ribose. However, it could use sodium citrate, sodium acetate as sole source of carbon, and patassium nitrate and ammonium sulfate as the sole nitrogen source, with no requirement of growth factors (Table 1).

According to The Bergey's manual systematic bacteriology and considering the physiological and biochemical tests performed, F9 was tentatively classified as *Acinetobacter* sp.

The 16S rDNA sequence and phylogenetic tree

The 16S rDNA of F9 was amplified by PCR and sequenced. A phylogenetic tree was constructed based on the 16S rDNA sequence and the sequences retrieved from Genebank (Figure 3). The 16S rDNA sequence of F9 is the closest to that of *Acinetobacter venetianus* RAG-1^T and the similarity is up to 99.9%. They also belong to the same cluster. So, strain F9 was decided as *A. venetianus*.

The genus Acinetobacter appears to be metabolically versatile and has the ability to degrade aliphatic hydrocarbon, thus making it an organism of interest for its possible bioremediational potential (Young et al., 2005). *Acinetobacter* sp. is widespread in nature and can remove or degrade a wide range of organic and inorganic compounds. It has shown the potential in both environmental and biotechnological applications. Previous studies conducted on samples obtained from different sites along Kuwait coast showed the presence of crude oil-degrading bacteria predominantly belonging to the genus Acinetobacter (Radwan et al., 1999, 2002). In this study, the strain F9 was identified as genus *Acinetobacter* according to its physiological and biochemical properties and 16s rDNA sequence. This result indicated that genus Acinetobacter play important role in the degradation of marine oil pollution.

Diesel-degrading ability of F9

The F9 strain was first cultured in LB medium for 8 h and was then inoculated into MMC medium containing diesel as sore carbon resource. The growth rate was assessed indirectly by a turbidity measurement at OD_{600} (Figure 4). F9 could grow very well in MMC medium containing 1% diesel as sore carbon resource. It grew very quickly during the 1 to 4 days and its cell density increased significantly.

Then, the growth rate slowed down during 5 to 7 days. The growth curve of F9 is coincidence with that of other bacteria in their corresponding media. This means that F9 could use diesel as carbon resource perfectly. The removal of the diesel in MMC medium was also assessed (Figure 5). The removal rate of diesel in MMC reached

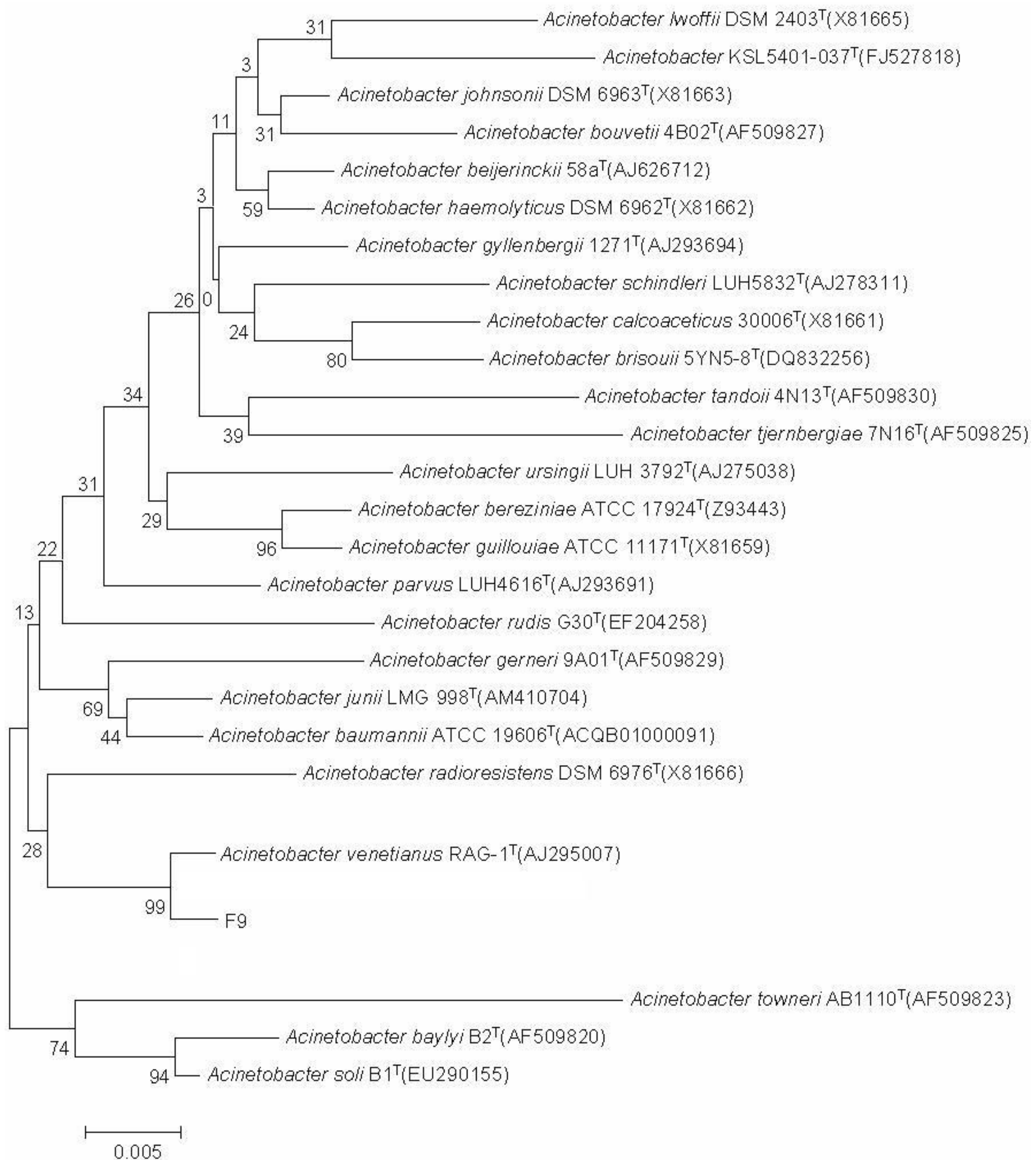


Figure 3. Phylogenetic tree of strain F9 and related species constructed on the basis of 16S rDNA gene sequence using the neighbor-joining method. Bar, 0.005 Knuc unit.

more than 90% at the 7th day which indicated that F9 owned outstanding diesel-degrading ability.

Analysis of component of diesel degraded by F9

After 7days, the remaining diesel oil was extracted from the culture with dichloromethane. The extraction was analyzed by GC-MS. The result showed that diesel oil was mainly composed of C11-C21 alkanes. F9 strain could degrade all of the alkanes in diesel oil. At the 7th day, the alkanes in F9 culture decreased dramatically when compared with that in the control culture (Figure 6).

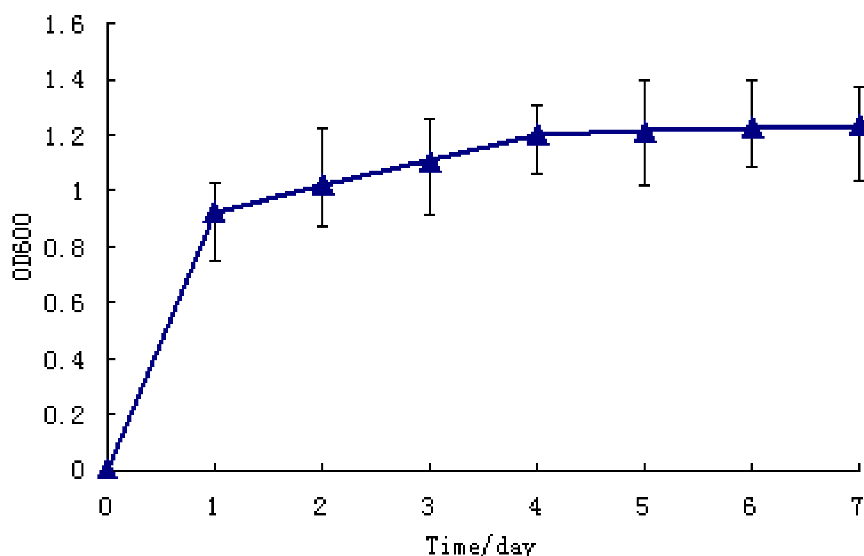


Figure 4. Growth curve of strain F9 in MMC.

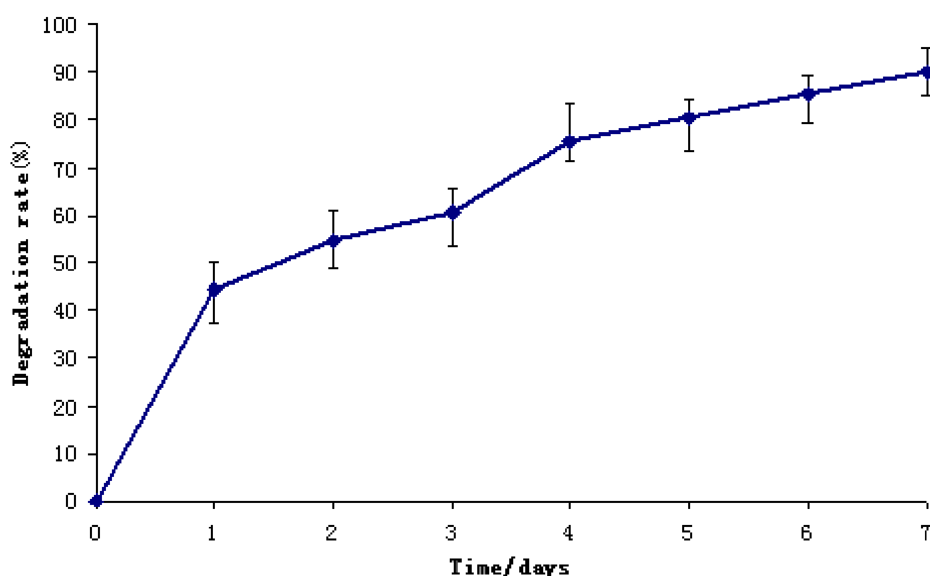
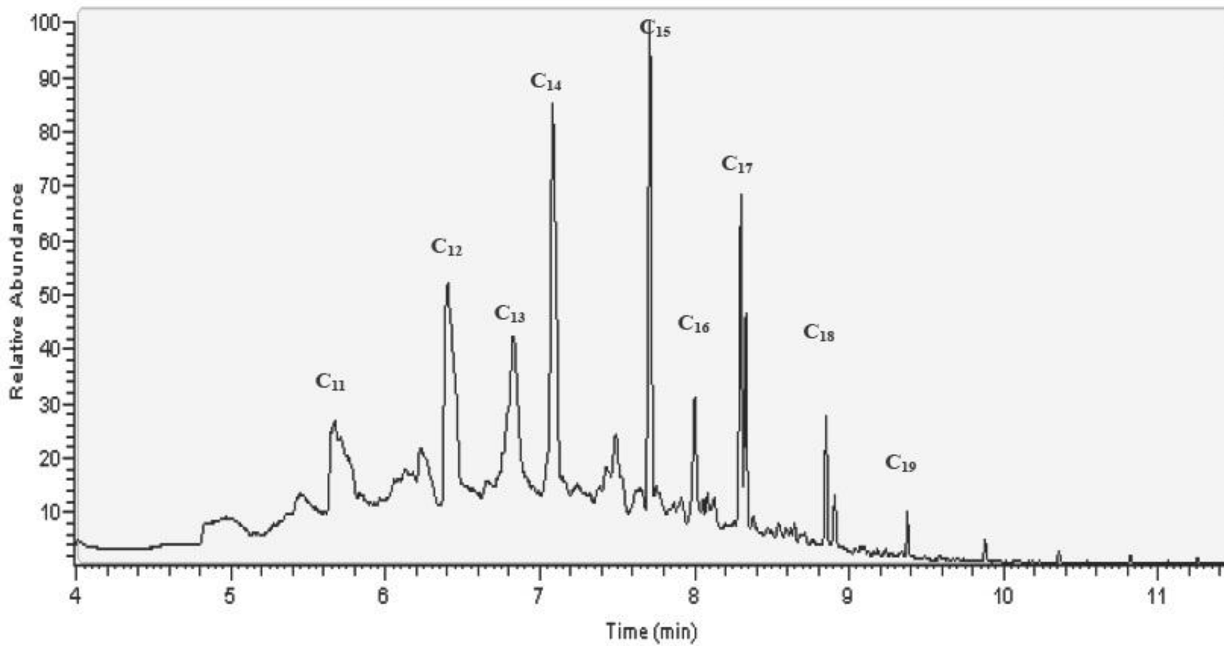


Figure 5. Degradation rate of diesel in culture by F9 strain.

Detection of the alkB and CYP153A genes in F9 by PCR

The genomic DNA and plasmid of F9 was extracted from culture (Figure 7A). The AlkB and CYP153A genes in F9 were amplified by PCR using genomic DNA and plasmid DNA as template, respectively (Figure 7B). Products of PCR were visualized by gel electrophoresis using 1% agarose gel and cloned into vector PUC19 to be sequenced. The results showed that F9 contained more than one plasmid which exhibited 4 bands in gel electrophoresis picture. AlkB and CYP153A genes could be amplified from not only genomic DNA but also plasmid DNA. The length of AlkB fragments amplified by PCR using degenerate primers were about 560 bp, while the length of CYP153A fragments were about 880 bp.

The products of PCR were cloned into vector PUC19-T and sequenced. All sequences obtained were compared with reference sequences in the GeneBank databases using the NCBI BLASTN search tool. Results showed that F9 harbored multiple alkane hydroxylase genes. Sequence of CYP153A gene amplified from genomic DNA



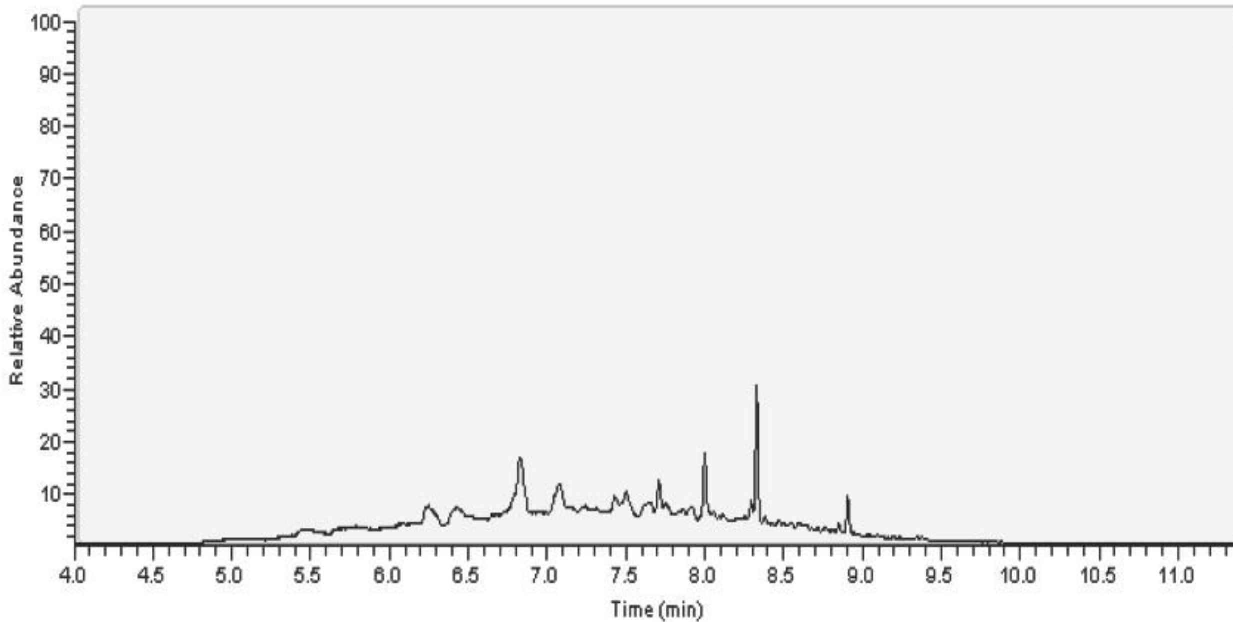


Figure 6. The GC-MS chromatogram of diesel oil extracted from culture. Relative abundance: the highest peak, C15 of the figure with abundance of 1.11 × 108 was assigned value 100, and the height of other peaks was compared with it. The result was relative abundance. A: The GC-MS chromatogram of diesel oil extracted from control culture without F9 inoculation; B: The GC-MS chromatogram of diesel oil extracted from culture with F9 inoculation.

was same as that amplified from plasmid DNA, with 99% identity to sequence of CYP153A gene of *Acinetobacter* sp. EB104 (Genbank No: AJ311718.1). Sequence of AlkB gene amplified from genomic DNA had 99% identity

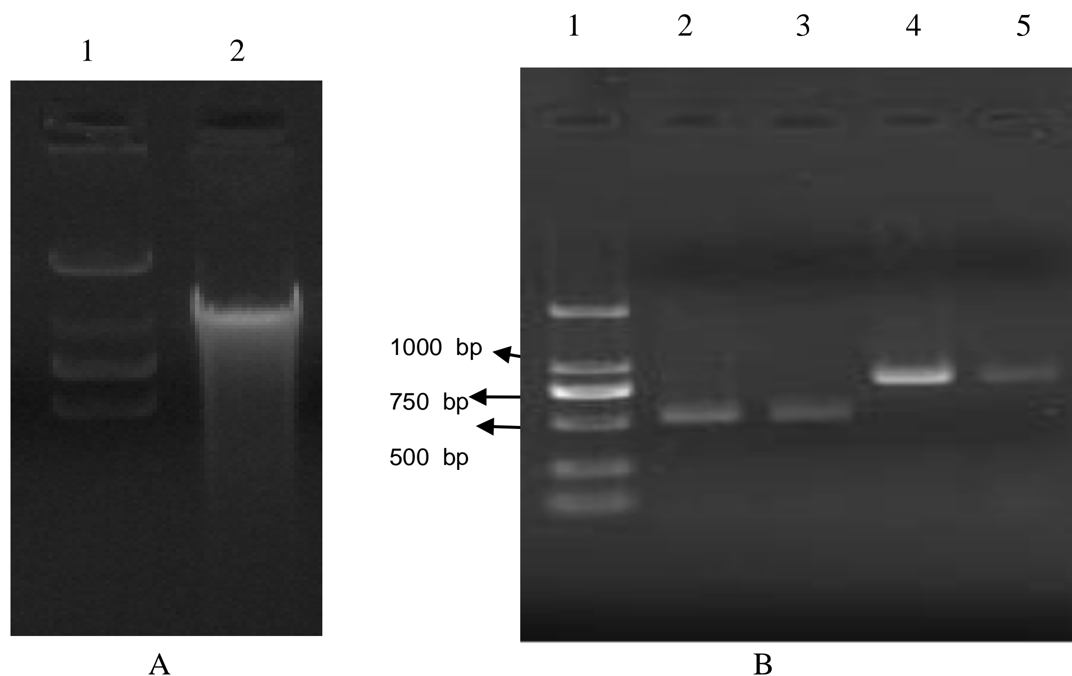


Figure 7. Electrophoresis pictures of DNA. A: Plasmid (Lane 1) and genomic (Lane 2) DNA; B: PCR production, Lane 1 DNA marker, Lane 2 AlkB PCR production with genomic DNA as template, Lane 3 AlkB PCR production with plasmid DNA as template, Lane 4 CYP153A PCR production with genomic DNA as template, Lane 5 CYP153A PCR production with plasmid DNA as template.

to sequence of AlkM gene of *A. venetianus* strain VE-C3 (Genbank No: DQ241788.1). Sequence of AlkB gene amplified from plasmid DNA had 84% identity to sequence of AlkB gene of bacterium alkW42 (Genbank No: DQ287997.1).

Bacterial oxidation of n-alkanes is a very common phenomenon in nature (Buhler and Schindler, 1984). The biodegradability of alkanes by microorganisms is well established. Alkanes are chemically quite inert and have to be activated to allow further metabolic steps to take place. The metalloenzymes that catalyze the oxidation of alkanes in the environment have been reviewed (Austin and Groves, 2011). The first step in the bacterial aerobic degradation of alkanes is that it is catalyzed by oxygenases (Labinger and Bercaw, 2002). Alkane hydroxylase genes have received increasing attention as markers to predict the potential of different environments for oil degradation (van Beilen and Funhoff, 2005). There are two main alkane hydroxylase systems have been found in alkane-degrading bacteria. One is integral membrane non-haem diiron alkane hydroxylases (for example, AlkB and AlkM). The other is the cytochrome P450 CYP153A family (van Beilen et al., 2003; van Beilen and Funhoff, 2007; Rojo, 2009). The former has been extensively studied since it was first described in a hexane-degrading *Pseudomonas oleovorans* (later identified as *Pseudomonas putida* GPo1). The latter was first characterized in *Acinetobacter* sp. EB104 as CYP153A1, which is a soluble haem-containing class I cytochrome P450 (Marin et al., 2001).

To further study the mechanism of high diesel-degradability of F9, alkane hydroxylase genes AlkB and CYP153A harbored in F9 have been detected. One CYP153A gene and two AlkB genes have been amplified from genomic and plasmid DNA. This indicated that F9 had multiple alkane hydroxylase genes.

The presence of multiple alkane hydroxylase genes was also observed in bacteria of other genera. It has been previously reported that *P. aeruginosa* PAO1 has three AlkBs (van Beilen et al., 2003), *Sphingomonas* sp. HXN-200 has five CYP153s (van Beilen et al., 2006) and *Rhodococcus erythropolis* has five AlkBs and two CYP153s (Smits et al., 2002). The coexistence of multiple genes possibly contributes to a wide substrate range and better environmental adaptation of the alkane degraders. The substrate ranges of CYP153 and AlkB have been determined as C5-C16 (van Beilen et al., 2003, 2006; van Beilen and Funhoff, 2007). But, it has been reported that *Parvibaculum* isolates containing only CYP153A utilized n-alkanes from C8 to C15. While, *Salinisphaera* isolates habouring both CYP153A and AlkB were able to utilize alkanes from C5 to C38, as well as pristane (Liping et al., 2010). So, it may be deduced that the coexistence of AlkB and CYP153A has some synergistic effect. The reason

why F9 possesses effective degradability of all n-alkanes from C11 to C21 in diesel may be that it harbors multiple alkane hydroxylase genes.

Conclusion

In this study, a diesel-degrading bacterium named F9 was isolated from diesel-polluted seawater. It was identified as genus *Acinetobacter* according to its physiological and biochemical properties and 16s rDNA sequence. *Acinetobacter* sp. strain F9 could degrade diesel efficiently. At the third day, diesel-degradation rate of F9 reached more than 70% and at the seventh day, more than 90%.

To further study the mechanism of high diesel- degradability of F9, alkane hydroxylase genes AlkB and CYP153A harbored in F9 were detected. One CYP153A gene and two AlkB genes were amplified from genomic and plasmid DNA. This indicated that F9 had multiple alkane hydroxylase genes. The reason why F9 possesses effective degradability of all n-alkanes from C11 to C21 in diesel may be that it harbors multiple alkane hydroxylase genes.

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