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Degradation potential and diversity of oil-degrading bacteria isolated from the sediments of the Jiaozhou Bay, China

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Abstract

A great deal of oil contaminated the shoreline by the Qingdao oil pipeline explosion in 2013. The four oil-degrading consortia were enriched from sediment samples with crude oil as sole carbon and energy sources. The biodiversity and community analysis showed that the *Luteibacter*, *Parvibaculum* and a genus belonging to *Alcanivoracaceae* were found predominant bacteria in the four consortia, which belonged to *Proteobacteria*. Nine strains exhibiting distinct 16S rRNA gene sequences were isolated from the consortia. These strains were identified to eight genera based on 16S rRNA gene sequences. Five of the nine strains degraded more than 30% of the crude oil in two weeks by gravimetric method. From the analysis of GC-MS, most of the isolated strains tended to degrade n-alkanes rather than PAHs. Five strains showed high degrading ability of the total n-alkanes. Only Strain D2 showed great PAHs degrading ability and the degrading rates ranged from 34.9% to 77.5%. The sequencing analysis of the oil-degrading consortia confirmed that the genus of *Alcanivorax* was one of the dominant bacteria in Consortia A and E and Strain E4 might be one of the dominant bacteria. The strains obtained in this study demonstrated the potential for oil bioremediation in oil-contaminated beach ecosystems.

Key words: oil spill, biodegradation, microbial consortium, dominant bacteria

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1 Introduction

Millions of liters of petroleum hydrocarbons from both natural and anthropogenic sources contaminated the marine environment annually (Hassanshahian et al., 2012). Bioremediation technologies to degrade these pollutants had long been investigated in laboratory and in field studies (Mortazavi et al., 2013; Cai et al., 2013). However, new knowledge on the microbial physiological activities was required to improve the degradation efficiency in practice (Samanta et al., 2002). The key step to enhance the efficiency was to eliminate the adverse effects of the biotic and abiotic factors on the functional communities. It was generally acknowledged that the abundant bacteria were the best adapting to the environment. So screening the predominant microorganisms with high degrading rate should be a sound approach. To screen the dominant bacteria, the abundance of each bacterium should be exhibited firstly and high-throughput sequencing analysis provided an efficient way to get the abundance information. Combining the sequencing analysis and isolation of bacteria would give a sight to understand the abundance of each isolate. The dominant bacteria would be more efficiently screened based on the abundance information.

Changes in composition and abundance of oil lead the rapid succession on the associated bacterial community (Mason et al., 2014). The bacteria would be totally different between the fresh and weathered oil. Most of the studies used oil samples collected after more than 10 d after oil-spill, and in this circumstance the oil composition varies significantly due to weathering effect (Fingas, 2012; Kleindienst et al., 2015). The consortia in these samples would change to adapt to the altered conditions. The dominant strains were more suitable for the biodegradation of

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weathered oil, but not for the quick response of oil-spill. This may be one reason for the low efficiency of bioremediation in large scaled application. The Qingdao blowout on November 22, 2013 (known as “11.22”) resulted in an intermittent spill of an estimated 2 000 metric tons of crude oil on the seashore. Our team collected the sediment samples polluted with fresh oil. We wanted to study whether there were differences in oil-degrading bacteria isolation between these samples and the weathered oil polluted samples. And then we expected to isolate the dominant strains with the high oil-degrading ability under the direction of high through sequencing analysis. All the strains we screened would be useful for directing the cleanup in oil-contaminated beach ecosystems.

2 Materials and methods

2.1 *Sites and sampling protocol*

After the “11.22” explosion in Qingdao on November 22, 2013, the neighboring seashore was contaminated by heavy crude oil. Within the following week, we collected sediments from four sites (Sites A, B, C, D and E) along the oil-polluted seashore (Fig. A1). The consortium from Site A (Site C, Site D and Site E) which showed high degrading performance was defined as Consortium A (C, D and E), specifically shown in Table A1. Samples were collected using sterilized, site-specific sampling shovels. Excavated samples were wrapped in aluminum foil, immediately transported to the lab on ice and kept at 4°C before further processing. Each site consisted of sandy sediment with an oil content of 0.92–1.40 g per 100 g of sediment (Table A1).

2.2 *Isolation of the oil-degrading bacteria*

A total of 2 g contaminated sediment was added to 100 mL ONR7a medium with 1% (w/v) crude oil as the sole carbon and energy source (Dyksterhouse et al., 1995). The medium was autoclaved in 121°C for 15 min. After incubation for two weeks under aerobic conditions at 25°C, cultures that showed visible degrading changes to the oil were transferred as a 2% inoculum to fresh medium as above, and cultivated under the same conditions. This consortium enrichment was repeated three times to obtain a stable bacterial consortium. A culture without added sediment was used as a negative control. The consortia that caused most visible degradation of the oil from Sites A, C, D and E were defined as Consortia A, C, D and E respectively.

Then the culture (100 μL) was serially diluted and plated on M8 agar plates (Cui et al., 2008) and incubated at 25°C for 5 d. Bacterial strains displaying different morphologies were streaked on fresh M8 plates for purification. The consortia and all isolates were stored in 20% of glycerol in cryogenic vial at –80°C.

2.3 *DNA extraction and Illumina MiSeq sequencing*

DNA was extracted from cell biomass pelleted from 10 mL of the final cultures through centrifugation at 9 660 *g* for 10 min. Total genomic DNA was extracted following the method described by Rochelle (Rochelle, 2001). The V3–V4 region of the 16S ribosomal RNA gene was amplified by PCR (95°C for 2 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension stage at 72°C for 10 min) using primers 338F and 806R. All PCR reactions were performed in triplicate with a reaction volume of 20 μL (4 μL of 5× FastPfu buffer, 2 μL of 2.5 mmol/L dNTPs, 0.8 μL of each primer (5 μmol/L), 0.4 μL of FastPfu Polymerase, and 10 ng of template) on an ABI GeneAmp® 9700 thermal cycler. Products were run on 2% agarose gels, from which amplicons were extracted, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, US) according to the manufacturer’s instructions, and quantified using QuantiFluor™-ST (Promega, US). Purified amplicons were pooled in equimolar mixtures and paired-end sequenced (2×250 bp) on an Illumina MiSeq platform according to standard protocols.

2.4 *Bacterial community analysis*

Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.17) with the following criteria: (1) The 300 bp reads were truncated at any site with an average quality score <20 over a 50 bp sliding window, discarding any reads with less than 50 bp remaining; (2) exact barcode matching, two nucleotide mismatch in primer matching, reads containing ambiguous characters were removed; (3) only sequences that overlapped by more than 10 bp were assembled according to their overlap sequence, and reads that could not be assembled were discarded.

Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (Version 7.1 http://drive5.com/uparse/), chimeric sequences were identified and removed using UCHIME. Each 16S rRNA gene sequence was taxonomically classified against the SILVA (SSU115) 16S rRNA database using the RDP Classifier (http://rdp.cme.msu.edu/) with a confidence threshold of 70% (Amato et al., 2013). All the sequences data were submitted to the Genebank and the Bioproject accession numbers were PRJNA399603. The alpha-diversity indices (ACE, Chao1, Shannon and Simpson) were analyzed with Mothurv (version 1.30.1) at a 97% similarity level (Schloss et al., 2011).

2.5 *Identification and phylogenetic analysis*

Purified strains were cultured in M8 medium at 25°C for 3 d (cell concentration at OD_{600}: 1.0–1.2). Genomic DNA was extracted and 16S rRNA amplified as above from pellets obtained by centrifugation at 9 660 *g* for 5 min, except for the primer set replaced by 16SR and 16SF (Cui et al., 2008). The amplified 16S rRNA gene fragments were sequenced using the ABI 3730xl sequencing system by TsingKe Biological Technology (Qingdao). Sequences were aligned using BLAST and used to construct the phylogenetic tree according to the methods described by Shao et al. (2010). The dominant Strain E4 isolated was deposited in China General Microbiological Culture Collection Center with the number CGMCC 1.16160.

2.6 *The characterization of Thioclava isolates*

The six strains belonged to genus *Thioclava* were discriminated by the house keep gene and multilocus sequence analysis. PCR amplification of *gyrB*, *rpoD*, *recA*, *trpB*, and *dnaK* gene were performed using the primers as described previously (Lai et al., 2014; Liu et al., 2017). The PCR products were sequenced with the ABI 3730xl sequencing system by TsingKe Biological Technology (Qingdao). All sequences were submitted to GenBank database. The accession numbers were assigned and listed in Table A2. The type strain *Rhodovulum sulfidophilum* DSM 1374^T was chosen as an outgroup for the phylogenetic analysis. The multilocus sequence analysis (MLSA) was followed the study of Liu et al. (2017). Phylogenetic tree was constructed by neighbor-joining method based on MLSA sequences with MEGA software (Version 7.0.26). Bootstrapping analysis was carried out with 1 000 replicated. And the similarities of different genes were calculated by the DNAMAN software (Version 6.0.3).

2.7 *Degradation rate and analysis of characteristics*

Next, the rates at which the bacterial consortia and isolated strains degraded crude oil were quantified. All four consortia and

9 of the 15 isolated strains with distinct 16S rRNA gene sequence were tested. Of each enriched consortium or isolate, 1 mL was transferred to 100 mL of sterilized ONR7a medium containing 1% (w/v) crude oil (1% inoculum volume) in three replicates, and incubated at 25°C on a rotator shaker for two weeks. After this period of biodegradation, the total residual hydrocarbons and their derivatives were extracted from the control (medium without any inoculum) and the degraded medium using 50 mL of dichloromethane.

The rate at which oil was degraded was calculated using the residual weight of oil, determined by gravimetric analysis as described by Zheng et al. (2012). Degrading rates (A) were obtained using the formula:

$$A = [(m_0 - m_1) \div m_0] \times 100\%,$$

where m_0 is the residual weight of the control and m_1 is the residual weight of the sample after degradation.

More specifically, the total utilization of alkanes and polycyclic aromatic hydrocarbons (PAHs) were quantified using the gas chromatography–mass spectrometer (GC-MS) method. One milliliter of the residual organic phase was dehydrated in a column with 2 g of anhydrous Na_2SO_4 and filtered with a 0.22 μm nylon membrane (JINTENG, Tianjin, China). After evaporation under a stream of nitrogen, the residue was re-dissolved in chromatography-grade n-hexane. N-Tetracosane-D50 and P-Terphenyl-D14 were set as internal standards (10 μg/mL each). The samples were then analyzed using a 6890A gas chromatograph (Agilent Technologies) in an HP-5 MS capillary column (30 m long×250 μm internal diameter, 0.22 μm thickness) and a 5 973 mass spectrometer equipped with a quadrupole axis detector. Hydrocarbons were quantified following the protocols described by Zheng et al. (2012) and degradation rates calculated as a percentage relative to each hydrocarbon's concentration in the control.

The utilization of alkanes and PAHs as a sole carbon and energy source was determined. One of the following compounds was contained in the culture. The compounds were Hexadecane, Dotriacontane, Napthalene, Dibenzothiopene, Fluorene, Phenanthrene, and Pyrene. The bacteria were centrifugated, washed and resuspended in ONR7a medium. The culture, containing 0.3% (w/v) alkane and PAH respectively, was inoculated by 2% (v/v) bacterial suspension and incubated on a rotary shaker at 25°C for 7 d. The medium without innoculation was served as control. All the experiments were performed in triplicate. The growth was obtained by measurement of the optical density at 600 nm. The utilization of carbon sources was measured by the increase of the bacteria (Balachandran et al., 2012).

3 Results

3.1 *Structure of oil-degrading microbial consortia*

Bacterial consortia that visibly degraded oil yielded a total of 55 548 effective reads with a length of 427–440 bp (Table 1). Sequence coverage was 99.9%, indicating that the sequencing depth captured the composition of the prokaryotic consortia well. The RDP classifier gave a total of 262 OTUs, with Consortium A containing 86 OTUs and Consortia C, D, and E less than 70 OTUs each (Table 1). Moreover, community richness was considerably higher in Consortium A than in the other consortia, while biodiversity was highest in Consortium D (Table 1). Consortia A, C and E showed similar levels of biodiversity (Simpson index 0.23–0.28, Table 1).

Table 1. OTU richness and diversity indexes of culturable oil-degrading consortia with similarity level of 97%

Sample ID	Reads	OTU	Ace	Chao	Coverage	Shannon	Simpson
A	17 359	86	92	98	0.999	2.12	0.26
			(88, 106)	(89, 140)		(2.09, 2.14)	(0.26, 0.27)
C	9 269	69	76	73	0.999	2.06	0.28
			(71, 93)	(70, 88)		(2.02, 2.09)	(0.27, 0.29)
D	13 215	53	62	76	0.999	2.28	0.16
			(56, 83)	(58, 150)		(2.26, 2.3)	(0.16, 0.16)
E	15 705	54	61	60	0.999	1.86	0.23
			(56, 76)	(55, 81)		(1.85, 1.88)	(0.23, 0.24)

The bacterial community composition was analyzed at different taxonomic levels (Figs 1 and 2). Across all the consortia, eight phyla were identified, with *Proteobacteria* the most abundant phylum in all the samples (98% in Consortium A, 90% in Consortium C, 98% in Consortium D and 95% in Consortium E; Fig. 1). Consortia C and E showed a broadly different structure to Consortia A and D. *Bacteroidetes* was the second most abundant phylum, at a similar abundance in both Consortia C (4.3%) and E (4.7%), while *Actinobacteria* represented 5.4% of Consortium C but only 0.6% in Consortium E (Fig. 1).

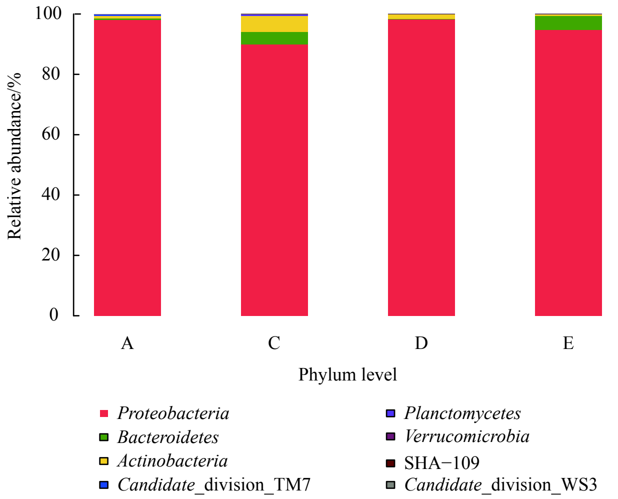

Fig. 1. The community structure of the bacterial consortia at the phylum level.

At a lower taxonomic level, 80 genera were identified (Fig. 2). The different sites again showed substantial differences in community structure. A genus belonging to *Alcanivoraceae* (*Alcanivoraceae*-norank in Fig. 2) was the main genus in Consortia A and E, reaching up to 45.1% and 40.7%. The other dominant taxa (>10%) were totally different in the four consortia (Fig. 2). In Consortium A, *Thalassospira* accounted for 23.3% of the reads, while in Consortium E, *Defluviimonas* (17.2%), other genus belonging to *Rhodobacteraceae* (14.2%), and *Parvibaculum* (11.5%)

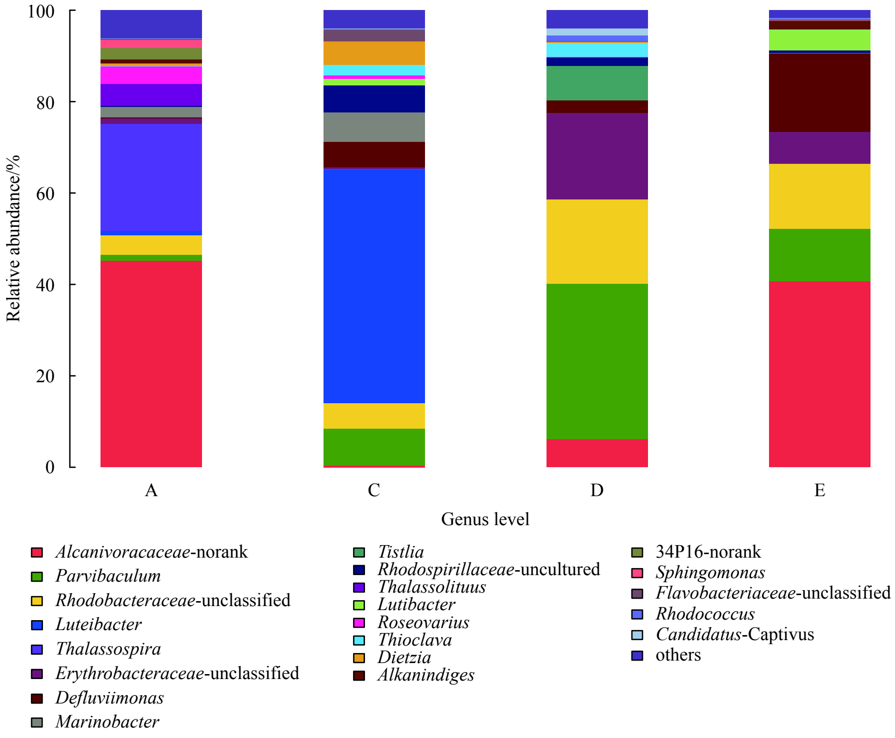

Fig. 2. Biodiversity and community structure of the bacterial consortia at the genus level.

were the most abundant genera after *Alcanivoracaceae*-norank. In contrast, in Consortium C the dominant genus was *Luteibacter* (51%), which contributed <1.1% to other consortia. Consortium D showed a richer structure, with 71.5% of reads consisting of three main genera (*Parvibaculum* 34%, genus belonging to *Erythrobacteraceae* 18.9%, genus belonging to *Rhodobacteraceae* 18.6%; Fig. 2).

3.2 *Identification of oil-degrading bacteria*

A total of 15 strains (8 genera in *Proteobacteria*) were isolated and identified (Table 2, Fig. 3). All the strains belonged to the phylum of *Proteobacteria* and *Alphaproteobacteria* accounted for 80% of the 15 strains. One strain, C3, may be a novel strain due to its relatively low similarity (98.37%) with the type strain (*Thioclava pacifica* DSM 10166^T) (Stackebrandt and Ebers, 2006). All the 16S rRNA gene sequences of the 15 strains were submitted to the National Center for Biotechnology Information (GenBank accession numbers in Table 2).

The strains belonging to genus *Thioclava* showed high similarity in 16S rRNA gene sequence. In order to discriminate the six strains, five house-keeping genes were used for the phylogenetic analysis. In comparison with 16S rRNA gene tree, the phylogenetic trees of Strains C3, C4, and C5 showed deep branches on the basis of the house-keeping genes (Fig. 4). The other three strains (D3, D4 and E5) were cluster to the same clade with high similarities. Then the five house-keeping genes were concatenated for

Table 2. Matches of sequences from isolated bacterial strains compared against the GenBank database

Strain No.	Accession No.	Type strain (accession No.)	Similarity/%	Affiliation
A4	KT715762	*Marinobacter adhaerens* HP15^T (CP001978)	99.87	*Gammaproteobacteria*
A3	KT715761	*Roseovarius nubinhibens* ISM^T (AALY01000002)	99.87	*Alphaproteobacteria*
A5	KT715763	*Roseovarius nubinhibens* ISM^T (AALY01000002)	99.86	*Alphaproteobacteria*
C1	KT715765	*Labrenzia alba* CECT 5094^T (AJ878875)	99.74	*Alphaproteobacteria*
C2	KT715766	*Thalassospira mesophila* MBE#74^T (AB786711)	99.87	*Alphaproteobacteria*
C3	KT715767	*Thioclava pacifica* DSM 10166^T (AUND01000024)	98.37	*Alphaproteobacteria*
C4	KT715768	*Thioclava pacifica* DSM 10166^T (AUND01000024)	98.25	*Alphaproteobacteria*
C5	KT722757	*Thioclava pacifica* DSM 10166^T (AUND01000024)	98.13	*Alphaproteobacteria*
D2	KT722759	*Celeribacter baekdonensis* L-6^T (HM997022)	100	*Alphaproteobacteria*
D3	KT722760	*Thioclava dalianensis* DLFJ1-1^T (JHEH01000075)	99.74	*Alphaproteobacteria*
D4	KT722761	*Thioclava dalianensis* DLFJ1-1^T (JHEH01000075)	99.74	*Alphaproteobacteria*
E1	KT722762	*Catenococcus thiocycli* DSM 9165^T (HE582778)	100	*Gammaproteobacteria*
E2	KT722763	*Celeribacter baekdonensis* L-6^T (HM997022)	99.87	*Alphaproteobacteria*
E4	KT722765	*Alcanivorax borkumensis* SK2^T (AM286690)	100	*Gammaproteobacteria*
E5	KT722766	*Thioclava dalianensis* DLFJ1-1^T (JHEH01000075)	99.74	*Alphaproteobacteria*

Note: A*n* (C*n*, D*n*, E*n*) represents the screened nst isolate from the A (C, D, E) oil-degrading consortium solution processed by crude oil as the sole source.

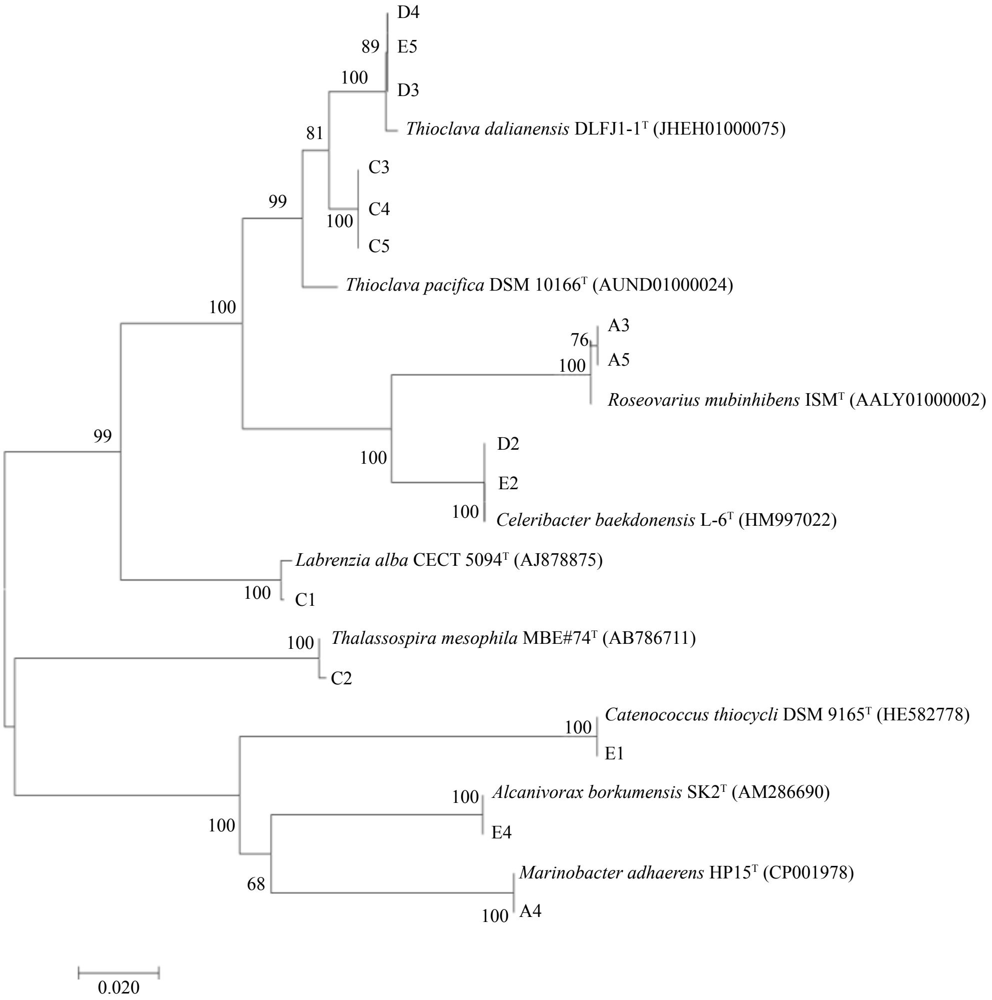

Fig. 3. Phylogeny of the isolated oil-degrading strains, based on 16S rRNA gene sequences. A, C, D and E denote the community from which the strains were isolated.

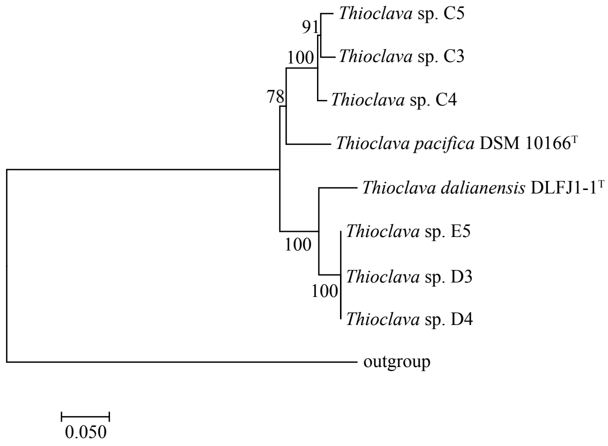

Fig. 4. Phylogeny of the isolated oil-degrading strains based on the five concatenated housekeeping genes sequences.

the MLSA analysis. The topology of the MLSA tree was similar to most of the single house-keeping gene trees (Fig. A2). The MLSA similarities ranged from 86.9% to 100%, with a mean value of 94.0%. The similarities of Strains C3, C4 and C5 ranged from 97.1%–97.3%, which were less than or equal to the soft species threshold (Liu et al., 2017). Strains D3, D4 and E5 were the same species and Strains C3, C4 and C5 would be different species belonging to genus *Thioclava*.

3.3 *Oil degradation rates of the bacterial consortia and isolates*

An oil-degrading test was performed within two weeks with the crude oil as the sole carbon resource. All the four consortia apparently showed good degrading ability, like emulsifying and turning brown, with no floating oil visible on the medium surface. The degradation rate was evaluated by gravity method (Fig. 5a). Consortia A and C both showed degradation rates of around 20% (24% and 22%, respectively) while Consortia D and E both showed similar higher rates around 40% (49% and 46%, respectively; Fig. 5a).

Furthermore, nine of the isolates with different 16S rRNA gene sequences were selected for degradation analysis. The degrading rates of the isolates varied widely (Fig. 5b). Three strains exhibited low degrading abilities (<15%), which were lower than those of the consortia in which they were found. The other six strains showed degradation rates of 25.8%–40.3%. Strain C3 showed the highest degradation rate (40.3%) among all screened bacteria.

3.4 *Degradation of different oil components*

Similar to the results of gravimetric method, the degradation

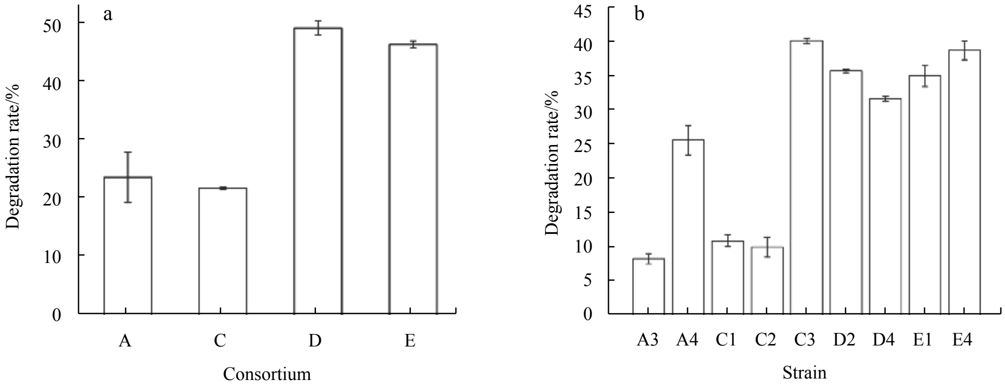

Fig. 5. The oil degradation rates of the enriched consortia (a) and the isolated strains (b). A, C, D and E denote the community from which the strains were isolated.

rates of four strains (A3, A4, C1 and C2) were below 40% on alkanes (two weeks at 25°C; Fig. 6). The other five strains present much higher degrading ability. The degrading rates were very close to each other, ranging from 50.1% to 63.0%. Strain D2 showed the highest degrading ability. Most strains did not differ from each other in degradation of short-chain (<C15) alkanes. Strains A3, A4, C1 and C2 showed much lower degrading ability in different n-alkanes. Strains C3, D2, D4, E1 and E4 which showed good degrading ability in total alkanes were mainly degrading the 15–29 carbon atom alkanes (Table A3). Strain C3 exhibited broader degradation ability, affecting chain lengths from C15 to C39. The utilization rate of the alkane from C15 to C29 (except C16) ranged from 51.7% to 82.3%, and the degrading rate of C15 was especially higher than 80%. The middle-chain (C15 to C25 except C16, higher than 60%) alkane utilization of Strain D2 was higher than Strain C3.

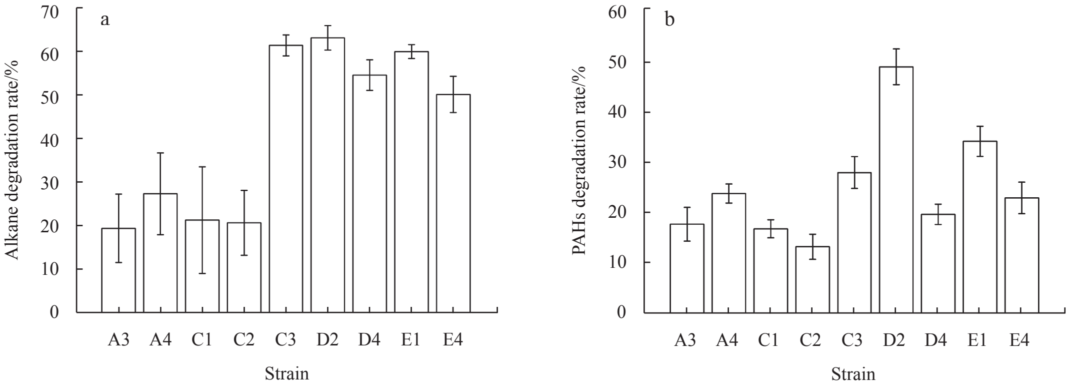

Fig. 6. Percentage of biodegradation of total alkanes (a) and PAHs (b) degraded by isolated bacterial strains generated by GC/MS. A, C, D and E denote the community from which the strains were isolated.

The isolated strains showed much lower degrading abilities to degrade PAHs than the n-alkanes. Most of the bacteria show very low ability in PAHs degradation, with only Strain D2 showing a PAHs degrading rate above 40% (Fig. 6). Most strains were markedly less able to degrade PAHs than n-alkanes, especially Strains D4 and E4, which degraded less than 30% of the PAHs (Table A4). Strain D2 degraded the broadest range of PAHs, affecting all compounds examined (22 PAH compounds, two- to four-ring PAHs), with degrading rates of 34.9%–77.5%.

The degrading potential of the nine isolates was determined with seven types of carbon source (Table A5). Hexadecane and Dotriacontane represent middle chain and long chain alkanes, respectively. The other five carbon sources represent the PAHs with different rings. Strains A4 and E4 were found to have the ability to degrade Hexadecane and Dotriacontane. Strain D2 was able to utilize a variety of carbon sources and all the seven types of carbon source could be degraded.

4 Discussion

In this study, we examined the bacterial characteristics of degradation in recently-spilled oil. The age of an oil spill likely played an important role in defining the bacterial diversity. Indeed, isolation of different strains has been shown to depend on succession within the sampled bacterial community (Atlas et al., 2015; Dubinsky et al., 2013; Mishamandani et al., 2016). Until now, most samples of bacterial communities associated with oil spills had been obtained at least 10 d after the spill (Kostka et al., 2011; Ruberto et al., 2003; Thomas et al., 2014). Functional consortia, with the potential to be utilized in bioremediation of oil spills, would exhibit substantial successional changes within this period (Dubinsky et al., 2013). Thus, strains of *Alcanivorax, Marinobacter*, *Pseudomonas*, *Roseovarius*, *Thalassospira*, *Thioclava*, etc. had been studied the ability of oil-degradation (Bacosa et al., 2015; Chronopoulou et al., 2015; Liu et al., 2007; Wang et al., 2014). Here, we collected oil-contaminated sediments 7 d after the oil-spill. Accordingly, we found several differences, including isolating the potentially novel strain *Thioclava* sp. C3. The strain belonging to *Catenococcus* was found to be the first time as an oil-degrading bacteria. Whereas previously isolated strains showed a good ability to degrade both alkanes and PAHs (Gao et al., 2015), most strains isolated here (except D2) degraded alkanes well, but not PAHs. This suggested that alkane-degrading bacteria, particularly obligate alkane degraders, were most easily isolated from freshly oil-polluted samples.

Bioaugmentation contributed importantly to the biodegradation of hydrocarbon contaminants in oil polluted environment (Ruberto et al., 2003; Simons et al., 2013). To improve the biodegrading efficiency, studies of suitable oil-degrading strains focused on isolating bacteria that showed high degradation efficiency. However, this approach ignored the potential of environmental factors in limiting degrading efficiency (Boopathy, 2000). The dominant bacteria were least limited to the environment. The predominant microorganisms with high oil-degrading ability would exhibit their ecological functions (Hazen et al., 2016). Using both high-throughput sequencing and 16S rRNA sequences from isolated strains, we found that *Proteobacteria* were the dominant bacterial phylum, in keeping with many previous studies (Hassanshahian et al., 2012; Hazen et al., 2010; King et al., 2015). Genus belonging to *Alcanivoracaceae* was a dominant genus at two of four sampling sites. The 16S rRNA gene sequence of Strain E4 was aligned with the representative OUT sequence of the dominant consortia. It showed a 99.56% similarity between the two sequences (Fig. A3). This means that Strain E4 was one of the most dominant strains in Consortium E. This research confirmed that the dominant strains could be discovered by the direction of the consortia structural analysis. This isolation method would provide time-saving way to screen dominant bacteria with high oil-degrading ability. Hence, Strain E4 might be more useful in field applications. This hypothesis could productively be investigated in future research.

5 Conclusions

The current study investigated the enriched oil-degrading consortia via high-throughput sequencing, and the isolation procedure was also performed in the same enrichment. We found that the obligate alkane degraders were most easily isolated from freshly oil-polluted samples. Strain C3 would be a novel strain according to the low similarity to the type strain (98.37%). *Alcanivorax borkumensis* SK2 was one of the dominant taxon in Consortium E. Under the direction of the sequencing analysis, Strain E4 should be the dominant strain with high oil-degrading ability. Strain E4 was deposited in China General Microbiological Culture Collection Center with the number CGMCC 1.16160.

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Appendix:

Table A1. The sampling information for this research

Site	Corresponding consortia	Oil concentration/g·(100 g sediments)$^{-1}$	Type of oil
S1	A	1.12	heavy oil
S2	C	0.92	heavy oil
S3	D	1.02	heavy oil
S4	E	1.40	heavy oil

Table A2. The accession numbers of the gene sequences for the isolates

Strain	*rpoD*	*dnaK*	*trpB*	*recA*	*gyrB*
C3	MG892027	MG892033	MG892039	MG892045	MG892051
C4	MG892026	MG892032	MG892038	MG892044	MG892050
C5	MG892029	MG892035	MG892041	MG892047	MG892053
D3	MG892025	MG892031	MG892037	MG892043	MG892049
D4	MG892024	MG892030	MG892036	MG892042	MG892048
E5	MG892028	MG892034	MG892040	MG892046	MG892052

Table A3. The concentration of the main n-alkanes of diffferent chain lengths degraded by each isolated bacterial strain

Alkane	Concentration/μg·g^{-1}									
	A3	A4	C1	C2	C3	D2	D4	E1	E4	Control
C15	95.16	73.05	98.93	107.61	21.13	27.86	50.90	22.56	29.60	119.60
C16	37.15	44.84	37.24	42.33	29.26	21.25	44.65	28.56	32.96	45.24
C17	119.38	94.46	121.51	115.00	37.59	38.26	65.95	31.24	43.13	145.07
C18	111.50	87.55	112.21	115.71	36.12	32.39	57.16	35.13	44.45	134.33
C19	125.38	102.94	125.33	109.05	45.65	38.74	70.16	44.85	60.64	149.55
C20	151.71	126.63	149.38	130.04	60.06	47.19	85.36	57.76	79.06	178.37
C21	174.95	144.40	168.70	154.63	70.69	55.56	85.34	60.21	84.60	201.21
C22	206.83	159.83	185.50	191.74	78.93	62.35	79.31	69.05	95.31	220.63
C23	247.55	198.71	225.12	218.59	95.19	78.60	80.87	78.30	112.27	258.71
C24	244.64	193.34	216.79	212.69	90.67	79.91	75.17	77.25	108.54	241.77
C25	268.35	227.92	243.16	229.58	108.70	101.93	100.00	105.91	140.86	268.43
C26	213.64	185.11	193.57	214.31	91.99	91.43	90.29	93.17	123.02	213.58
C27	192.03	168.46	171.88	177.82	78.32	84.41	80.29	94.11	118.32	187.23
C28	144.05	133.77	132.20	141.20	64.72	74.66	71.64	82.36	102.98	144.29
C29	125.81	116.86	113.79	109.47	58.88	73.18	69.63	76.10	96.31	121.90
C30	85.94	83.12	78.55	92.23	44.48	58.24	55.40	56.89	71.20	83.25
C31	63.96	63.11	59.82	68.46	34.10	47.68	44.23	44.38	56.54	63.03
C32	37.93	38.28	36.02	37.59	21.51	30.43	28.91	28.29	35.82	37.53
C33	24.17	21.98	23.04	23.81	12.11	20.06	19.08	18.52	23.66	21.10
C34	17.37	17.91	16.68	16.05	10.40	14.62	14.87	13.65	17.11	17.06
C35	10.53	10.74	9.79	10.58	6.28	8.77	8.75	8.36	10.52	10.09
C36	0.72	0.71	0.63	0.62	0.46	0.50	0.54	0.59	0.63	0.58
C37	4.70	4.54	4.74	4.63	2.63	4.01	4.04	3.72	4.55	4.60
C38	0.17	0.41	0.20	0.21	0.06	0.06	0.02	0.15	0.23	0.14
C39	3.62	3.38	3.63	4.19	1.91	3.06	2.94	2.81	3.63	3.37

Note: C*n* indicates alkanes with *n* carbon atom, alkanes (<15 carbon) were not shown in the table; and A*n* (C*n*, D*n*, E*n*) the isolate from the A (C, D, E) oil-degrading consortium.

Table A4. The concentration of the main PAHs degraded by isolated bacteria

PAH	Concentration/μg·g^{-1}									
	A3	A4	C1	C2	C3	D2	D4	E1	E4	Control
C1-NAP	326.44	385.42	463.30	404.88	325.02	224.41	251.62	286.73	340.97	427.44
C2-NAP	425.56	318.96	510.83	548.64	493.39	335.97	521.18	450.48	542.94	633.76
C3-NAP	144.29	153.65	169.88	206.16	176.35	121.39	198.99	151.03	171.10	215.47
C4-NAP	23.96	25.49	22.20	25.97	18.45	13.01	20.59	15.52	15.60	25.35
Flu	8.49	11.36	8.33	11.64	6.34	5.11	8.57	5.86	7.24	12.07

to be continued

Continued from Table A4

PAH	Concentration/$\mu g\cdot g^{-1}$									
	A3	A4	C1	C2	C3	D2	D4	E1	E4	Control
C1-FLU	186.10	121.77	176.11	162.06	144.37	104.02	169.64	125.53	148.82	195.28
C2-Flu	24.05	22.16	17.93	19.53	12.79	9.60	20.53	16.02	17.81	25.03
C3-Flu	4.40	4.97	2.83	4.47	3.22	2.44	4.76	2.73	2.97	5.35
C4-flu	5.68	10.73	8.67	10.58	7.36	5.59	8.65	6.46	7.77	12.34
DBT	23.96	26.49	22.20	25.97	18.45	13.01	20.59	15.52	15.60	26.35
C1-DBT	45.06	42.82	39.14	48.79	24.52	18.72	31.31	22.37	27.68	50.18
C2-DBT	24.76	52.76	21.47	51.15	35.32	25.97	44.49	33.66	38.06	55.11
C3-DBT	15.64	10.37	18.97	10.41	7.73	5.78	9.48	6.86	8.26	19.65
C4-DBT	12.50	9.38	11.62	14.09	4.26	3.24	4.69	4.15	5.63	11.83
Phe	10.45	12.37	9.82	12.36	8.50	6.49	10.42	7.45	8.86	15.25
C1-Phe	1 271.68	1 164.77	1 155.72	1 119.87	972.19	730.16	1 179.28	903.04	1 031.58	1 298.25
C2-Phe	87.35	63.08	74.72	88.87	61.99	46.51	71.72	55.15	64.70	71.42
C3-Phe	83.03	74.91	85.66	99.09	65.42	50.27	85.85	65.42	78.00	85.29
C4-Phe	1.32	2.84	2.69	2.49	0.91	1.07	2.42	1.10	1.42	4.76
Chr	58.59	34.54	54.57	65.50	40.82	34.85	53.49	41.06	47.70	67.71
C1-Chr	37.22	21.60	28.62	39.04	26.04	20.98	37.35	30.11	33.25	37.16
C2-Chr	0.39	0.46	0.18	1.50	0.23	1.08	1.57	1.00	0.36	1.71

Note: NAP, Flu, DBT, Phe and Chr were Napthalene, Fluorene, Dibenzothiopene, Phenanthrene and Chrysene, respectively; C1(2, 3, 4) alkylated PAHs with 1(2, 3, 4) carbon straight-chains; and A*n*(C*n*, D*n*, E*n*) the isolate from the A(C, D, E) oil-degrading consortium.

Table A5. Degrading capability of alkanes and PAHs by the isolates

Strain	Degradation						
	C16	C32	Nap	Dbt	Flu	Phe	Pyr
A3	–	+	–	–	+	–	–
A4	+++	+++	–	+	+	–	–
C1	–	++	–	+	+	+	+
C2	++	+	+	+	+	+	–
C3	+	+	+	+	+	+	–
D2	+	+	++	++	++	+	+
D4	+	+	+	+	+	+	–
E1	–	–	–	+	+	+	–
E4	+++	+++	–	–	+	+	–

Note: The degrading ability was measured by optical density at 600 nm. +++, ++, + and +/– indicate the capability with alkane or PAHs as sole carbon and energy source. +++ means $OD_{600}>0.6$, ++ $0.6>OD_{600}>0.1$, and + $0.1>OD_{600}>0$.

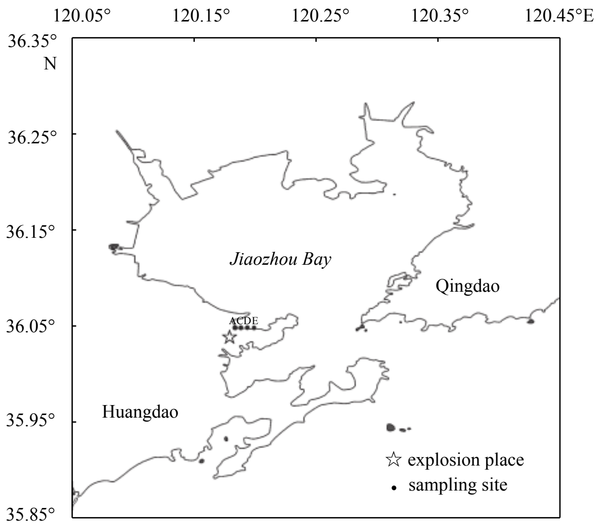

Fig. A1. Sendimental sampling sites in Huangdao District, Qingdao. The consortia that caused most visible degradation of the oil from Sites A, C, D and E were defined as Consortia A, C, D and E, respectively.

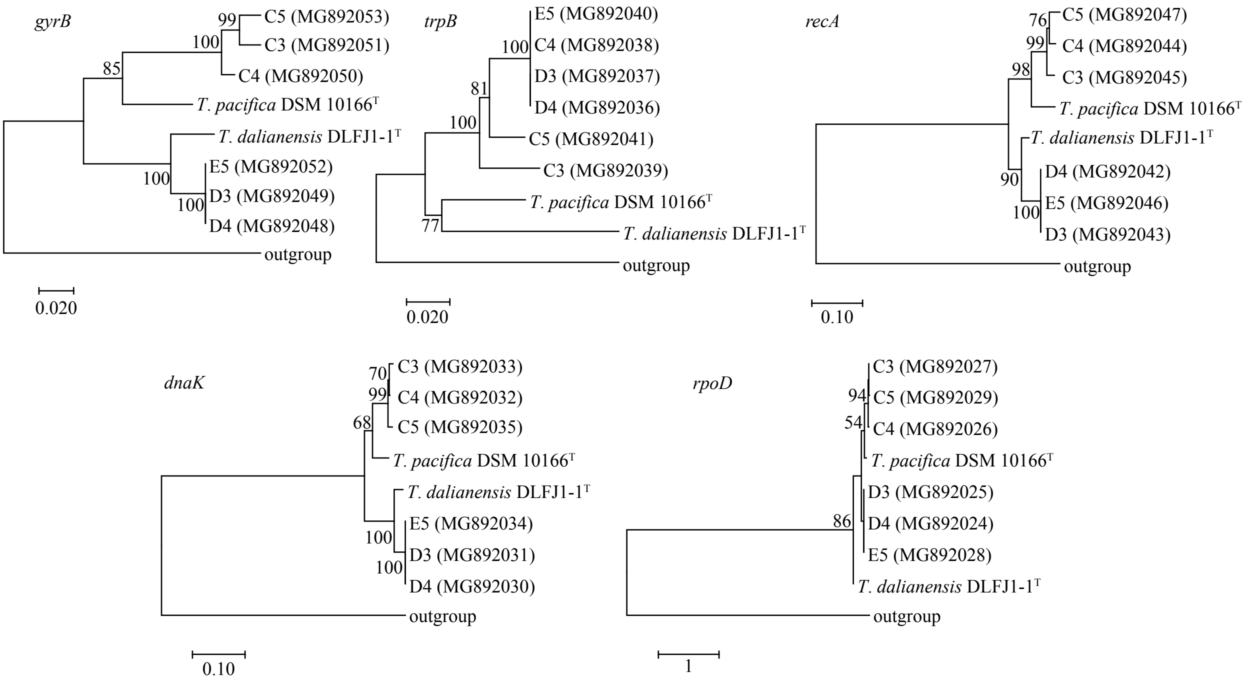

Fig. A2. Phylogenetic tree based on *gyrB*, *rpoD*, *dnaK*, *trpB* and *recA* gene sequences.

```
>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   GGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT   40
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   GGACTACTAGGGTTTCTAATCCTGTTTGCTCCCCACGCTT   40
Consensus                                                         ggactac agggt tctaatcctgtttgctccccacgctt

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   TCGCACCTCAGCGTCAATGTCAGTCCAGGAGGCCGCCTTC   80
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   TCGCACCTCAGCGTCAATGTCAGTCCAGGAGGCCGCCTTC   80
Consensus                                                         tcgcacctcagcgtcaatgtcagtccaggaggccgccttc

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   GCCACTGGTGTTCCTTCCGATCTCTACGCATTTCACCGCT   120
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   GCCACTGGTGTTCCTTCCGATCTCTACGCATTTCACCGCT   120
Consensus                                                         gccactggtgttccttccgatctctacgcatttcaccgct

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   ACACCGGAAATTCCACCTCCCTCTACTGCATTCTAGCCTG   160
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   ACACCGGAAATTCCACCTCCCTCTACTGCATTCTAGCCTG   160
Consensus                                                         acaccggaaattccacctccctctactgcattctagcctg

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   CCAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGGGCTTT   200
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   CCAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGGGCTTT   200
Consensus                                                         ccagtttcaaatgcagttcccaggttgagcccggggcttt

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   CACATCTGACTTAGCAAACCGCCTACGCGCGCTTTACGCC   240
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   CACATCTGACTTAGCAAACCGCCTACGCGCGCTTTACGCC   240
Consensus                                                         cacatctgacttagcaaaccgcctacgcgcgctttacgcc

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   CAGTAATTCCGATTAACGCTCGCACCTTTCGTATTACCGC   280
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   CAGTAATTCCGATTAACGCTCGCACCTTTCGTATTACCGC   280
Consensus                                                         cagtaattccgattaacgctcgcacctttcgtattaccgc

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   GGCTGCTGGCACGAAATTAGCCGGTGCTTCTTCTGTAGGT   320
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   GGCTGCTGGCACGAAATTAGCCGGTGCTTCTTCTGTAGGT   320
Consensus                                                         ggctgctggcacgaaattagccggtgcttcttctgtaggt

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   AACGTCAAGTACTCATCCGTATTAAGGATAAGCCTTCCTC   360
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   AACGTCAAGTACTCATCCGTATTAAGGATAAGCCTTCCTC   360
Consensus                                                         aacgtcaagtactcatccgtattaaggataagccttcctc

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   CCTACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACA   400
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   CCTACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACA   400
Consensus                                                         cctactgaaagtgctttacaacccgaaggccttcttcaca

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   CACGCGGCATGGCTGGATCAGGGTTGCCCCCATTGTCCAA   440
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   CACGCGGCATGGCTGGATCAGGGTTGCCCCCATTGTCCAA   440
Consensus                                                         cacgcggcatggctggatcagggttgcccccattgtccaa

>E41_________GGACTACCAG_GGTATCTAAT_CCTGTTTGCT_CCCCACGCTT_TCGCAC   GATTCCCCA                                  449
>OTU108_6_69705_6000+GTGGGGAATCTTGGACAATGGgggCAACCCTGATCCAGCCA   GATTCCCCA                                  449
Consensus                                                         gattcccca
```

Fig. A3. The alignment between 16S rRNA gene sequence and the representative OUT sequence.