

# Monitoring exogenous and indigenous bacteria by PCR-DGGE technology during the process of microbial enhanced oil recovery

Jun Wang · Ting Ma · Lingxia Zhao · Jinghua Lv ·  
Guoqiang Li · Hao Zhang · Ben Zhao ·  
Fenglai Liang · Rulin Liu

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**Abstract** A field experiment was performed to monitor changes in exogenous bacteria and to investigate the diversity of indigenous bacteria during a field trial of microbial enhanced oil recovery (MEOR). Two wells (26–195 and 27–221) were injected with three exogenous strains and then closed to allow for microbial growth and metabolism. After a waiting period, the pumps were restarted and the samples were collected. The bacterial populations of these samples were analyzed by denaturing gradient gel electrophoresis (DGGE) with PCR-amplified 16S rRNA fragments. DGGE profiles indicated that the exogenous strains were retrieved in the production water samples and indigenous strains could also be detected. After the pumps were restarted, average oil yield increased to 1.58 and 4.52 tons per day in wells 26–195 and 27–221, respectively, compared with almost no oil output before the injection of exogenous bacteria. Exogenous bacteria and indigenous bacteria contributed together to the increased oil output. Sequence analysis of

the DGGE bands revealed that *Proteobacteria* were a major component of the predominant bacteria in both wells. Changes in the bacteria population in the reservoirs during MEOR process were monitored by molecular analysis of the 16S rRNA gene sequence. DGGE analysis was a successful approach to investigate the changes in microorganisms used for enhancing oil recovery. The feasibility of MEOR technology in the petroleum industry was also demonstrated.

**Keywords** Microbial enhanced oil recovery (MEOR) · Exogenous bacteria · Denaturing gradient gel electrophoresis (DGGE) · Diversity · Oil field

## Introduction

Microbial enhanced oil recovery (MEOR) processes employ microbial metabolites to achieve several aims, including lowering interfacial tension between brine and oil, reducing oil viscosity and enhancing permeability in limestone reservoirs [25]. Compared to other EOR processes, MEOR processes have several advantages: lower energy consumption, no thermal processes, and no chemical processes [1, 15, 16]. However, the technical performances in many field trials have been inconsistent [36]. It is difficult for microbiologists to monitor and control the conditions for optimal performance during the process of MEOR. First, the injected exogenous strains are hard to track. Many studies have been done in oil reservoirs [32], however, few report the monitoring the changes of injected strains for MEOR [36]. The relationship between the microbial operation and oil yields is still very vague. Therefore, microbial processes are generally viewed with considerable skepticism.

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J. Wang · T. Ma · L. Zhao · J. Lv · G. Li · F. Liang · R. Liu  
Key Laboratory of Molecular Microbiology and Technology,  
Ministry of Education, Tianjin 300071,  
People's Republic of China

J. Wang · T. Ma (✉) · L. Zhao · J. Lv · G. Li · F. Liang ·  
R. Liu  
Tianjin Key Laboratory of Microbial Functional Genomics,  
College of Life Sciences, Nankai University, 94, Weijin Road,  
Tianjin 300071, People's Republic of China  
e-mail: meor@nankai.edu.cn

H. Zhang · B. Zhao  
The Drilling and Production Research Institute,  
Liaohe Oilfield Branch, PetroChina, Panjin,  
Liaoning 124010, People's Republic of China

Fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP) analysis, offer the best compromise between the number of samples processed and the information obtained. DGGE, in particular, provides both rapid comparison data for many communities and specific phylogenetic information derived from excised bands [5]. DGGE has recently become a common technique to detect bacterial population ecology and dynamics in environmental samples, especially multiple samples [12, 17–19, 27]. As for oil reservoirs, there have been studies mainly on microbial diversity in oil wells and bacterial changes during their exploitation [7, 8, 13, 21].

The aim of this work was to assess the efficacy of MEOR field trials via exogenous bacterial injection, monitor bacterial population changes in the oil wells and prepare a time course of the diversity profile using molecular fingerprinting to demonstrate if the exogenous bacteria and their indigenous counterparts effect oil recovery in the Jin45 zone at the Liaohe Oil Field, Liaoning Province, China [11]. The Jin45 zone has been in primary production since November 1984. Steam stimulation was used from January 1986 to November 1991. Then, filling pattern technology was adopted twice. From May 2006, we employed an MEOR approach to replace steam stimulation.

## Materials and methods

### Strains and media

Three strains, LJ-1, LJ-2 and LJ-3, were isolated from an oil-polluted soil sample in the Liaohe Oil Field. The mineral medium for oil utilization contained ( $\text{g l}^{-1}$ ):  $\text{Na}_2\text{HPO}_4$  1.5,  $\text{KH}_2\text{PO}_4$  3.48,  $(\text{NH}_4)_2\text{SO}_4$  4,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.7, yeast extract 0.01 and 0.5% (w/v) crude oil, pH 7.2. The medium was incubated at 37 °C [35]. Surface tension was measured with an interfacial tensiometer (JK99B, Powereach, Shanghai) at room temperature. The crude oil-degrading ability was measured by infrared spectrophotometry (M-22A, Midwest group, Beijing). Experiments were repeated at least twice, and consistent results were obtained.

### Preparation of the inocula

Three bacteria, LJ-1, LJ-2 and LJ-3, were used as the inoculum. They were separately cultivated in 200 ml of mineral medium with 2% (v/v) liquid paraffin as the sole carbon source. When the culture reached the late exponential growth phase ( $7.2 \times 10^8$  cells  $\text{ml}^{-1}$ ), it was used to inoculate a 10-l carboy of the same medium, which was incubated at 37 °C for 48 h with agitation and aeration

until the culture reached the late exponential phase of growth. One liter of cell concentrate was used to inoculate a 132-l tank. The medium was prepared aseptically due to the lack of facilities on site, and tanks were incubated for approximately 48 h at ambient temperature.

### Field experiment

A field expedition was conducted on two oil production wells 26–195 and 27–221 in the Jin45 zone of Liaohe Oil Field. First, 5,000 l of formation water was pumped out from each well (flow rate, 500  $\text{l min}^{-1}$ ) before injecting exogenous bacteria. Then, the two wells each received two separate inoculations with the three strains (consortium LJ) on 16 June and 26 July 2006, including 374 l of strain LJ-1 ( $6.7 \times 10^7$  cells  $\text{ml}^{-1}$ ), 293 l of strain LJ-2 ( $7.8 \times 10^8$  cells  $\text{ml}^{-1}$ ) and 333 l of strain LJ-3 ( $7.7 \times 10^8$  cells  $\text{ml}^{-1}$ ). After inoculation, 15,000 l of mineral medium (as nutrient) and 5,000 l of formation water were injected successively (flow rate, 500  $\text{l min}^{-1}$ ). Finally, production was stopped for 10 days to allow time for bacterial growth and metabolism. After this incubation period, the pumps were restarted and water samples were collected over time. Flow meters were attached to the tubing of the wells to measure the volume of fluids produced. The total volume produced was recorded when each sample was collected.

### Sampling

Production water samples were collected every week from the two oil production wells. Samples were collected in bottles that had previously been autoclaved and rinsed with water from each sample. All samples were stored at 4 °C and then analyzed within 48 h.

### DNA extraction

DNA was extracted from water samples using a modified method [33]. Each water sample (1,000 ml) was centrifuged at  $10,000 \times g$  for 20 min. The integrity of the extracted DNA was checked by agarose gel electrophoresis and the concentration was measured by the absorbance at 260 nm. DNA was stored at  $-20$  °C until analyzed.

### PCR amplification of 16S rRNA V9 region

PCR amplification was performed in a 50  $\mu\text{l}$  PCR reaction mixture containing 1.5 U Taq DNA polymerase,  $10 \times$  buffer, and 50 pmol of each primer, 200  $\mu\text{mol l}^{-1}$  dNTP and 100 ng of purified DNA extract. PCR was

conducted with primers 1,055F and 1,406R. A 40 bp GC-clamp was attached to the 5' end of the 1,406R primer [6, 17]. Theoretically, these primers should amplify a 323-bp product, including the highly variable V9 region. A modified Touch-down PCR program was employed. DNA was first incubated at 94 °C for 10 min, followed by cycles consisting of 1 min at 94 °C, 1 min at X °C, 3 min at 72 °C (X represents annealing temperature which was decreased by 2 °C from 66 °C to 52 °C and 3 cycles were run for each annealing temperature), then 15 cycles comprising 1 min at 94 °C, 1 min at 55 °C, 3 min at 72 °C, and finally 10 min of extension at 72 °C. For each set of reactions, a negative control, in which the template was replaced by an equivalent volume of sterile deionized water, was included. The PCR products were validated by agarose gel electrophoresis and then purified to eliminate the ssDNA and heteroduplexes [30, 37].

## DGGE

DGGE analysis of the amplified sequences was performed on 16 × 16 cm 6% polyacrylamid gels with a denaturing gradient of 40 to 60%. The PCR amplicon (200 ng) was loaded in each well and electrophoresis was performed at a constant voltage of 160 V for 200 min at 60 °C in the DGGE tank (Biorad, USA). Gels were stained with ethidium-bromide and photographed under UV light by a Bio-Rad gel Doc XR system [17].

## Cloning of DGGE bands sequences

DGGE bands were cut from the gels and DNA was recovered using a Biospin Polyacrylamide Gel Extraction Kit (Japan). The fragments were amplified with the V9 region primers without GC clamp. The fragments were separated by electrophoresis with 1.0% agarose gels, recovered by a DNA Recovery Kit, ligated to pGEM-T cloning vector and transformed into *E. coli* DH5 $\alpha$ .

## Sequences analysis

Three positive clones from each DGGE band were selected randomly for sequence analysis. If the sequence of the three clones was not identical, more clones from the same band were sequenced to estimate the number of sequences co-migrating on the same DGGE band. The clones were sequenced on an automated ABI 377 sequencer using a T7 primer. DNA sequences were submitted to NCBI to determine their closest phylogenetic relatives. Sequences that differed by less than 3% were considered to be the

same phylotype [28], and each phylotype was represented by a sequence type. Sequence alignments were performed by Clustal X [29]. A phylogenetic tree was constructed by Mega 3.1 based on Kimura Two-parameter model [10] and neighbor-joining algorithm [25].

## Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been deposited in the NCBI nucleotide sequence databases under accession numbers EU009936 to EU009951 and EU333951 to EU333966.

## Results

### Characteristics of the injected strains

Three strains were isolated from an oil-polluted soil sample in the Liaohe Oil Field. According to the physiological-biochemical characterizations and 16S rRNA gene sequences analysis, strains LJ-1, LJ-2 and LJ-3 were identified as belonging to the genera *Brevibacillus*, *Rhizobium* and *Nocardia*, respectively. Strain LJ-1 grew well with strains LJ-2 and LJ-3, and it accounted for 40% of the total cell concentration in the consortium. The LJ-2 and LJ-3 strains degraded 65.1 and 46.7% of the crude oil in 100 ml mineral medium with 0.5 g crude oil after being cultivated at 37 °C for 5 d. Strain LJ-3 was also able to produce a biosurfactant and reduce the surface tension to 34.7 mN m<sup>-1</sup>, about half of the control 70.2 mN m<sup>-1</sup>. The consortium LJ, which contained all three of these strains, degraded 75.0% of the crude oil and reduced the surface tension to 28.8 mN m<sup>-1</sup>.

### Chemical characteristics of the Liaohe Oil Field

According to analyses by atomic absorption spectrophotometry, the formation water was classified as a bicarbonated sodic type with an NaCl concentration of approximately 0.52 g l<sup>-1</sup>, and a pH of 7.2 (Table 1). The average gross chemical composition (in weight %) of the crude oil was around 33.12% saturates, 22.42% aromatics, 35.97% resins and 8.49% asphaltenes. Low saturate and high resin and asphaltene contents are characteristic of biodegraded heavy oils.

### Oil and liquid production

Yields of oil and liquid production from wells 26–195 and 27–221 were measured by the staff at the Liaohe Oil Field

**Table 1** Physicochemical characteristics of the samples obtained from the two wells

Parameters	26–195	27–221
Temperature (°C)	20	20
pH	6.9	7.2
Chemical characteristics of production water (mg l <sup>-1</sup> ) <sup>a</sup>		
Na <sup>+</sup>	495	520
K <sup>+</sup>	2.7	3.0
NH <sub>4</sub> <sup>+</sup>	8.0	20
Cl <sup>-</sup>	109.0	168.5
Mg <sup>2+</sup>	8.7	5.0
Ca <sup>2+</sup>	10.4	12.4
SO <sub>4</sub> <sup>2-</sup>	nd	15.8
Total iron	0.05	0.1
Nitrate	nd	nd
Phosphate	nd	nd

<sup>a</sup> The compounds not detected (nd) were under the detection limits

and the data are shown in Fig. 1. In well 26–195 (Fig. 1a), there was almost no crude oil output by water flooding before injection with bacteria on June 16. From June 17 to June 25, the well was stopped to allow for microbial growth and metabolism. On June 26, pumping was resumed. The liquid and oil production increased. The average oil production was 1.57 tons per day. In the well 27–221 (Fig. 1b), the yields were higher. After pumping on August 5, the liquid and oil production increased during 1 week reaching 14.6 and 5.7 tons per day, respectively, at which point they leveled off until October 29. Oil production was maintained at a relatively high yield (averaging 4.52 tons per day). The data suggested that the MEOR approach played an active role in increasing oil production in the oil wells.

#### Monitoring changes in injected strains

Twenty water samples were collected from the two oil wells. Total DNA was extracted from each sample and amplified by the V9 region primers. V9 regions of three injected strains were also amplified. To monitor the changes in the injected strains and to compare bacterial populations during MEOR, PCR-DGGE analysis was conducted using the injected strains LJ-1, LJ-2 and LJ-3 as markers. The banding patterns were shown in Fig. 2.

In both wells, there were obvious bands with identical migration to those of the injected strains LJ-1, LJ-2 and LJ-3. In samples from 26–195 (Fig. 2a), band n was identical to LJ-1 and band f was identical to LJ-2, and they were the major band in all samples. There was no band that corresponded to LJ-3. In samples from well 27–221 (Fig. 2b),

bands A, E and LJ-1 had the same migration pattern, as did bands B, F and LJ-2, and bands N and LJ-3. With DGGE, the relative intensity of each band most likely represents the relative abundance of a particular species in the population [17]. Our results showed that the presence of exogenous strains in the oil wells could be detected using PCR-DGGE analysis. Meanwhile, three injected bacteria were isolated by agar plate dilution method from these water samples. Morphological and 16S rRNA gene sequence analysis further proved their existence in the formation.

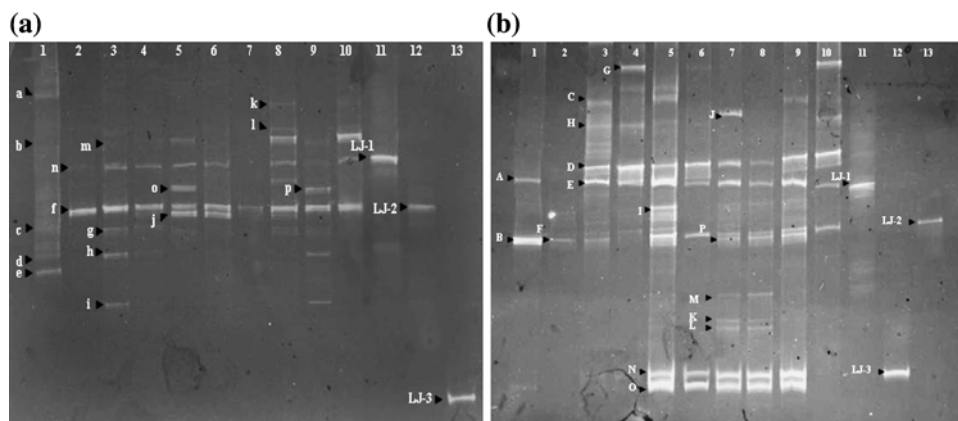
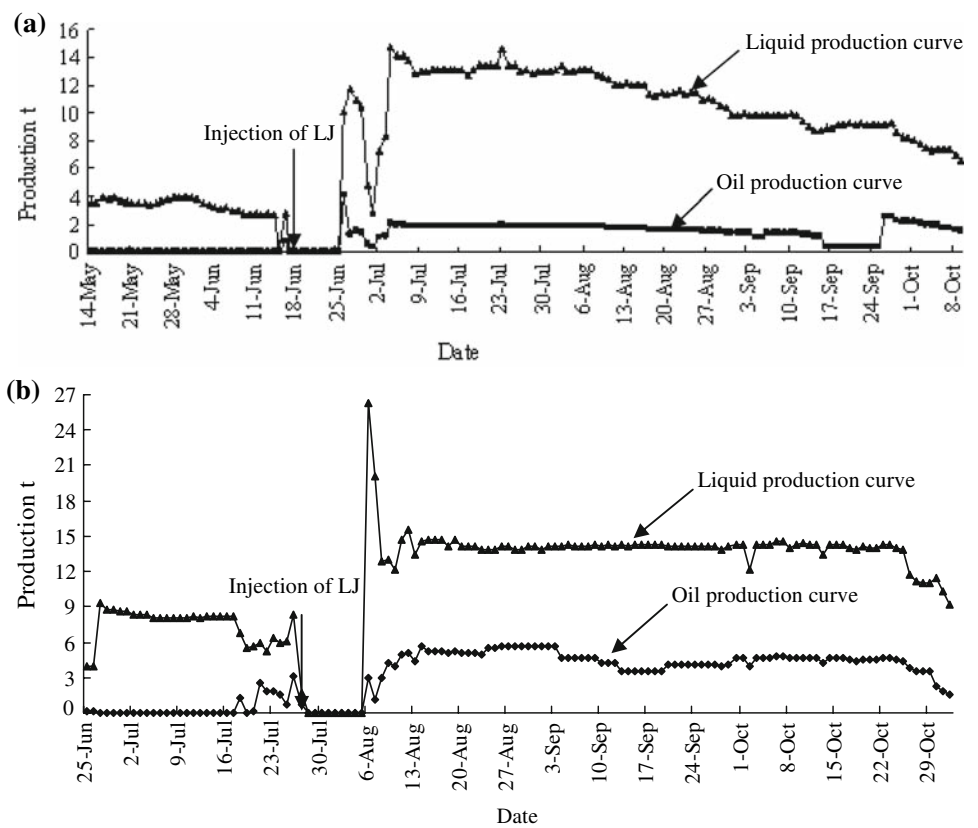
#### Sequence analyses of the DGGE bands

In order to determine the microbial community structure and analyze changes in the microbial community in the wells during oil recovery, major DNA bands marked in Fig. 2 were excised from the DGGE gels and sequenced. Thirty-two DNA sequences were compared with those in the NCBI database and related sequences are listed in Tables 2 and 3. Phylogenetic trees are shown in Figs. 3 and 4.

In well 26–195, 5 bands (a–e) present in the water sample before injecting extraneous bacteria on June 15 were sequenced. All five sequences belonged to the group *Proteobacteria*, including *Betaproteobacteria* (a, b, d and e) and *Gammaproteobacteria* (c). During the MEOR process, ten new bands appeared. Bands m and b had the same sequence and were related to *Brachymonas petroleovorans* (100%, similarity). This strain was simulated by the injection of nutrients at the end of the trial. Band n was identical with the injected strain LJ-1, and was very similar to *Brevibacillus* sp. (99.0%, similarity). Band f was the same as the strain LJ-2, and was clearly related to *Rhizobium* sp. (97%, similarity). Band g was similar to *Dictyoglomus thermophilum* (94%, similarity) and it became the major band during the experiment. Ten novel bands from the samples represented three genera. Bands d, h and p were related to *Comamonas* sp. (98–100%, similarity) and band h to *Dictyoglomus thermophilum* (94%, similarity). The remaining bands (i, j, k, l and o) were most closely related to uncultured bacteria at similarity levels in the 87–100% range. Strain LJ-3 was not detected in the water samples.

All stimulated indigenous strains found in well 27–221 belonged to the group *Proteobacteria*, including *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Epsilonproteobacteria*. Bands A and E had the same sequences as injected strain LJ-1 and were most similar to *Brevibacillus* sp. (99.0%, similarity). Bands B and F were identical with strain LJ-2 and appeared related to *Rhizobium* sp. (97%, similarity). Band G was most closely related to *Pseudomonas* sp. (88% similarity), band I to

**Fig. 1** Oil and liquid productions of the wells 26–195 (a) and 27–221 (b)



**Fig. 2** DGGE patterns of liquid samples DNA from two wells. 26–195 (a) Lane 1 Before injection (June 15), lane 2 July 26, lane 3 August 10, lane 4 August 26, lane 5 September 4, lane 6 September 11, lane 7 September 18, lane 8 September 25, lane 9 October 3, lane 10 October 9, lane 11 LJ-1, lane 12 LJ-2, lane 13 LJ-3, 27–221 (b)

lane 1 start of the exploitation (August 28), lane 2 September 4, lane 3 September 11, lane 4 September 18, lane 5 September 25, lane 6 October 3, lane 7 October 9, lane 8 October 16, lane 9 October 24, lane 10 October 30, lane 11 LJ-1, lane 12 LJ-3, lane 13 LJ-2

*Acinetobacter* sp. (84%, similarity), band K to *Comamonas* sp. (90%, similarity), band L to *Mesorhizobium plurifarium* (100%, similarity), and band M to *Agrobacterium tumefaciens* (100%, similarity). The sequence of band N was the same as that of strain LJ-3 and both were related to *Nocardia* sp. (99.0%, similarity). The rest of the bands (C, H, J, O and P) were similar to uncultured bacteria. Percent similarities ranged from 86 to 99%.

**Discussion**

In our trial, the efficacy of an MEOR approach was tested at Liaohe Oil Field, which was at the end of the secondary phase of oil production. For injection, we used a bacterial mixture, “LJ”, containing three microorganisms. Two oil wells, 26–195 and 27–221, in the Jin45 zone of the Liaohe Oil Field were inoculated with the strains and nutrients.

**Table 2** Alignment of 16S rRNA V9 region DGGE sequences from well 26–195

Band name	Sequence size (bp)	Accession numbers	Closest relatives (accession numbers)	Alignment, similarity %	Score
a	320	EU333953	Uncultured bacterium clone (DQ327694)	297/304, 97	513
			<i>Azospira oryzae</i> (DQ863512)	297/304, 97	513
b	310	EU333955	<i>Brachymonas petroleovorans</i> (AY275432)	299/299, 100	593
c	297	EU333952	Uncultured <i>Pseudomonas</i> clone (AF529093)	216/240, 90	285
			<i>Pseudomonas</i> sp. SB3 (DQ981458)	192/208, 92	278
d	307	EU333958	<i>Comamonas</i> sp. Y14B (EF175877)	290/290, 100	575
e	309	EU333954	Beta proteobacterium d8-2 (EF158391)	286/295, 96	513
			<i>Dechloromonas</i> sp. EMB 50 (DQ413149)	284/295, 96	498
f	311	EU333962	<i>Rhizobium</i> sp. CCNWTB701 (EF370483)	302/311, 97	545
h	307	EU333961	<i>Comamonas</i> sp. Y14B EF175877	290/290, 100	575
g	316	EU333959	<i>Dictyoglomus thermophilum</i> (X69194)	290/306, 94	440
i	302	EU333957	Uncultured bacterium clone(DQ088755)	290/290, 100	575
			<i>Hydrogenophaga</i> sp. a9 (EF179863)	288/290, 99	559
j	310	EU333964	Uncultured bacterium(DQ497777)	282/296, 95	444
			<i>Acinetobacter</i> sp. WAB1950 (AM184289)	281/296, 94	440
k	308	EU333965	Uncultured bacterium (AB286521)	307/307, 100	609
			<i>Acinetobacter radioresistens</i> (AF526908)	307/307, 100	609
l	350	EU333966	Uncultured bacterium (AB176243)	341/350, 97	615
			<i>Bacteriovorax</i> sp. EPC3 (AY294222)	341/350, 97	615
m	310	EU333956	<i>Brachymonas petroleovorans</i> (AY275432)	299/299, 100	593
n	311	EU333963	<i>Brevibacillus</i> sp. Lm-2–2 (EF025349)	309/311, 99	601
o	302	EU333952	Uncultured bacterium clone (DQ803248)	207/237, 87	232
			<i>Pseudomonas</i> sp. (D87100)	207/237, 87	232
p	309	EU333960	<i>Comamonas</i> sp. PD3 (EF373535)	306/310, 98	567

PCR products were analyzed by PCR-DGGE analysis. On the DGGE patterns, bands of the three injected strains were detected and it appeared that these strains had become the predominant population in the oil well during the process of recovery.

Indigenous microbes were also stimulated in the oil wells during the test. The most related species identified by DNA sequencing of DGGE bands are listed in Tables 2 and 3. In well 26–195, band b was detected in the production water sample before injection; during the MEOR process, it became the major band. DNA sequence analysis showed that this strain was similar to *Brachymonas petroleovorans* (similarity 100%). *Brachymonas petroleovorans* is a cyclohexane-degrading bacterium isolated from a wastewater plant of petroleum refinery [22]. The *Pseudomonas* genus represented by bands c and o is also a group that contains many oil-degrading bacteria [26, 31]. Bands d and p were identified as *Comamonas* sp. which is a genus that contains many bacteria with the ability to degrade organic compounds [2]. Band g was simulated during the MEOR process. It was similar to *Dictyoglomus thermophilum* (similarity 94%) which is a chemoorganotrophic, anaerobic, extremely thermophilic bacterium [24]. Other bands were not predominant and frequent in the water samples,

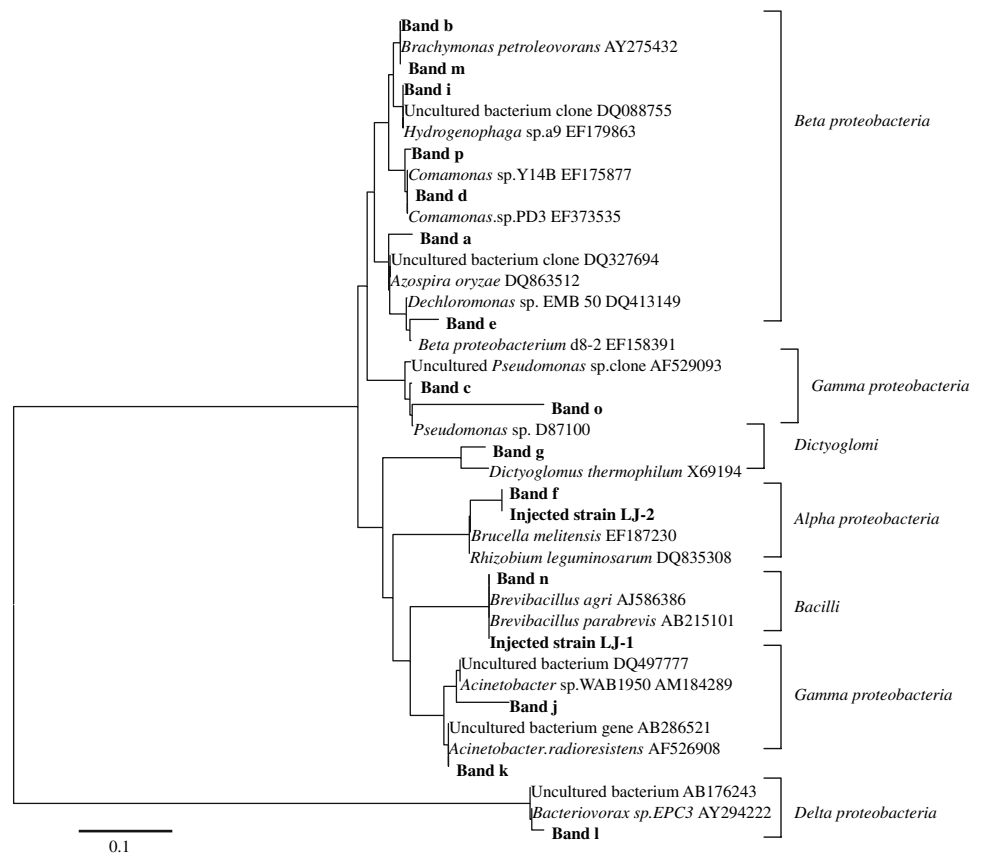
such as i, k and l, which were matched to *Hydrogenophaga* sp. a9 (similarity 99%), *Acinetobacter radioresistens* (similarity 100%) and *Bacteriovorax* sp. EPC3 (similarity 97%), respectively. These genera are also important environmental bacterial groups [9, 14, 20].

In well 27–221, all the detected indigenous bacteria belonged to the group *Proteobacteria* including *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Epsilonproteobacteria*. On the DGGE pattern, band D was novel and present at a high concentration in the production water samples. The sequence analysis of this band indicated that it was related to an uncultured gamma *Proteobacterium* (similarity 86%). The results suggested that it might represent a new species. Band O emerged at the later stage of treatment and became a major band. DNA sequence analysis showed that it was similar to an uncultured strain and the closest relative was *Burkholderia* sp. NIUS7 (similarity 99%). Species of the *Burkholderia* genus, including *B. cepacia* and *B. fungorum*, have been isolated from different environmental samples [4, 23] and are well-known degraders of chlorinated pesticides and polychlorinated biphenyls. Bands C, G, H and P belonged to the genus *Pseudomonas* and shared more than 97% similarity, with the exception of band G which was only 88% similar. DNA

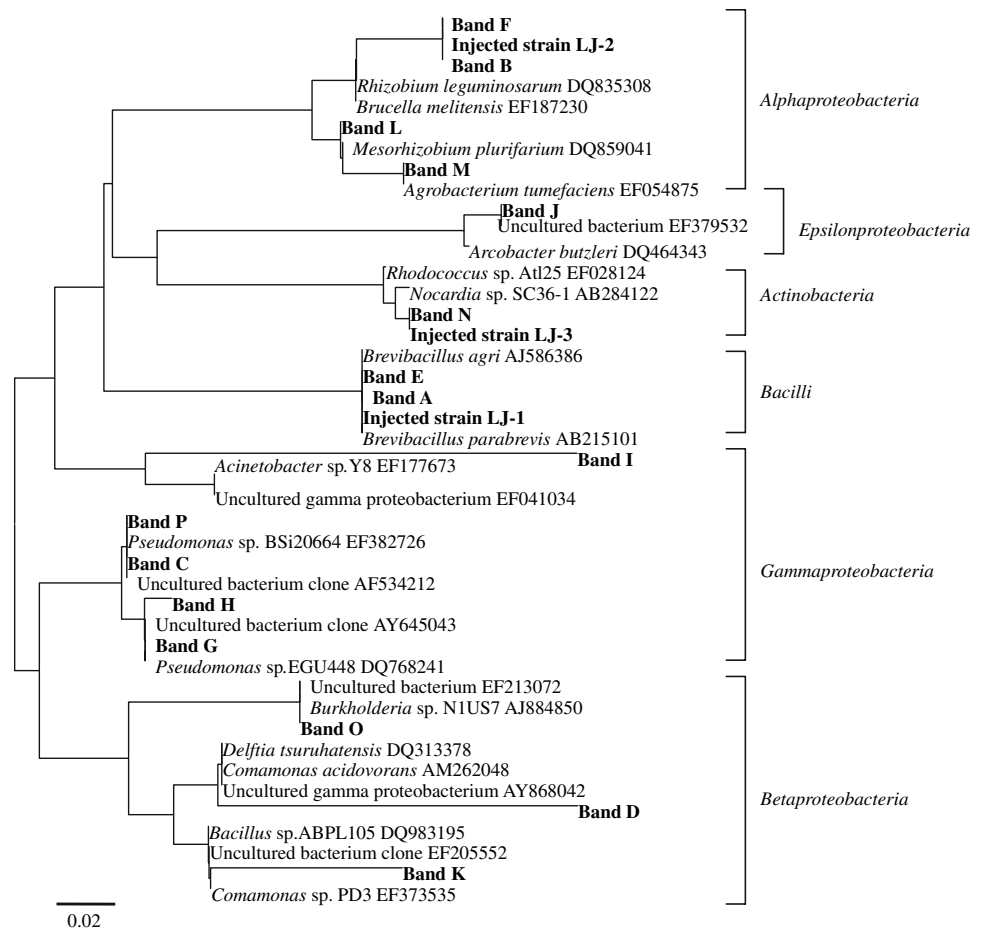
**Table 3** Alignment of 16S rRNA V9 region DGGE sequences from the well 27–221

Band name	Sequence size (bp)	Accession numbers	Closest relatives (accession numbers)	Alignment, similarity %	Score
A	311	EU009936	<i>Brevibacillus</i> sp. Lm-2-2 (EF025349)	309/311, 99	601
B	311	EU009937	<i>Rhizobium</i> sp. CCNWTB701 (EF370483)	302/311, 97	545
C	308	EU009938	Uncultured alpha proteobacterium (DQ463739)	305/306, 99	601
			<i>Pseudomonas</i> sp. EGU448 (DQ768241)	305/306, 99	599
D	309	EU009939	Uncultured gamma proteobacterium (AY868042)	251/291, 86	252
			<i>Delftia tsuruhatensis</i> (DQ313378)	251/291, 86	250
E	308	EU009940	<i>Brevibacillus</i> sp. Lm-2-2 (EF025349)	309/311, 99	601
F	311	EU009941	<i>Rhizobium</i> sp. CCNWTB701 (EF370483)	302/311, 97	545
G	313	EU009942	<i>Pseudomonas</i> sp. BSi20664 (EF382726)	263/296, 88	325
H	319	EU009943	Uncultured bacterium clone (AY645043)	290/296, 97	541
			<i>Pseudomonas</i> sp. EGU448 (DQ768241)	290/296, 97	539
I	309	EU009944	<i>Acinetobacter</i> sp. Y8 (EF177673)	184/219, 84	157
J	312	EU009945	Uncultured bacterium clone (EF379532)	303/304, 99	595
			<i>Arcobacter butzleri</i> (DQ464343)	300/304, 99	571
K	314	EU009946	<i>Comamonas</i> sp. PD3 (EF373535)	251/276, 90	349
L	314	EU009947	<i>Mesorhizobium plurifarium</i> (DQ859041)	304/304, 100	603
M	311	EU009948	<i>Agrobacterium tumefaciens</i> (EF054875)	304/304, 100	603
N	311	EU009949	<i>Nocardia</i> sp. SC36-1 (AB284122)	309/311, 99	601
O	315	EU009950	Uncultured bacterium (EF213072)	314/315, 99	617
			<i>Burkholderia</i> sp. NIUS7 (AJ884850)	313/315, 99	609
P	319	EU009951	Uncultured bacterium clone (AF534212)	315/317, 99	605
			<i>Pseudomonas</i> sp. yged132 (EF419339)	312/314, 99	599

**Fig. 3** Phylogenetic analysis of DGGE bands from the 26–195 water samples. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branching points. The scale bar represents the number of changes per nucleotide position. Accession numbers of the sequences retrieved from the database are shown in parentheses



**Fig. 4** Phylogenetic analysis of DGGE bands from the liquid samples of the well 27–221. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branching points. The scale bar represents the number of changes per nucleotide position. Accession numbers of the sequences retrieved from the database are shown in parentheses



sequences of bands L and M showed 100% similarity with *Mesorhizobium plurifarum* and *Agrobacterium tumefaciens*, respectively, both of which belong to *Rhizobiales*. Bacteria represented on the DGGE pattern were well-known environmental microorganisms and some of them were related to oil-utilizing bacteria. However, our investigation of the existing diversity was certainly limited and biased. First, PCR-DGGE profiles could only detect the predominant population in the environmental samples [3]. Second, the reported sensitivity of DGGE was 1% of the template DNA [17]. Third, many problems might arise during the sample collection, DNA extraction, PCR amplification or DGGE steps (gel resolution, gel staining) [34]. Only microorganism populations with high concentrations could be detected by DGGE fingerprinting.

Combining the time courses shown in Figs. 1 and 2, we conclude that the oil yield was related to the quantity and density of the bacterial bands. For example, in well 26–195, the oil yield was low on September 18 and the DGGE pattern bands were fewer and lower in density. On September 25, more bands were detected and the oil yield had increased correspondingly. The same result was observed in the well 27–221. After October 29, the oil yield began to drop and the

DGGE bands had decreased correspondingly; particularly the LJ-3 band was missing. The exogenous bacterial group, LJ, and the indigenous strains may have all contributed to the oil yields. In a subsequent experiment, we plan to inject the bacterial group LJ with lower and higher proportions of LJ-3 in the wells 26–195 and 27–221, respectively.

Many factors influence oil recovery during the MEOR process, such as formation conditions and microbial community structures. However, our results confirm the feasibility of the MEOR technique. After injection of exogenous bacteria and nutrients, the indigenous bacteria were simulated and together with the injected exogenous bacteria formed a new microbial community that contributed to the increased oil yield. PCR-DGGE fingerprints monitored the changes in the exogenous and indigenous microorganism populations. This technology was informative for detection of the injected strains and provided guidance for isolation of useful strains from the production water samples.

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