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Characteristics of Bacterial Communities in Shallow and Thin Heavy Oil Reservoir

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ABSTRACT

Revealing the characteristics of microorganisms that inhabit oil reservoirs is important in the effective application of microbial enhanced oil recovery (MEOR) technique. Plenty of studies have been conducted to discover microbial communities in light oil reservoirs, but investigations on the characteristics of bacterial communities in shallow and thin heavy oil reservoirs are limited. The aim of this study is to investigate bacterial communities in shallow and thin heavy oil reservoir, an oilfield in Henan (China) was taken as an example, and the 16S rDNA clone library approach was adopted to analyze the composition, abundance, and distribution of bacterial communities. A total of 682 sequences obtained from the four clone libraries were assigned to 84 operational taxonomic units (OTU) and 11 bacterial groups were identified in the oil reservoir. Results demonstrate the following: (1) The heavy oil reservoir has low bacterial diversity. (2) Differences exist in the bacterial community structures of the clone libraries. (3) The distribution of bacterial communities is consistent with the temperature, salinity, and oil properties of the oil reservoir. The findings of this study can provide basic theoretical guidance for the application of MEOR in shallow and thin heavy oil reservoirs.

INTRODUCTION

Heavy oil is characterized by high viscosity and density relative to light oil (Nazina et al. 2017). With the depletion of light oil resources and the increasing global energy demand, considerable attention has been paid to the development of inexpensive recovery technologies for heavy oil resources (Bachmann et al. 2014). General forms of enhanced oil recovery techniques for heavy oil reservoirs include chemical and physical methods. Chemical methods include the use of solvents and surfactants. Thermal methods involve the treatment of production wells with hot fluids and steam injection, which is currently the most effective technique for improving heavy oil recovery. However, thermal methods require high initial investment for equipment, and they cause water and soil pollution during application, which can lead to the rapid decline of oil production (Wang et al. 2012). Therefore, researchers have increasingly focused on the application of microbial enhanced oil recovery (MEOR) due to the simplicity, wide applicability, and economic and environmental benefits of this technique (Safdel et al. 2017).

MEOR is a cost-effective technique for exploiting crude oil in light oil reservoirs, and many successful field trials have been reported (Youssef et al. 2009). MEOR uses biosurfactants to reduce the viscosity of heavy oil, or convert heavy oil into light oil through bacterial degradation (Sen 2008). Reducing the viscosity of crude oil is the key step in improving oil recovery in heavy oil reservoirs. Therefore, before MEOR can be applied for heavy oil exploitation, the ecological distribution and metabolic characteristics of microorganisms in an oil reservoir should be fully investigated. Nearly 99% of the microorganisms in nature cannot be identified through culture experiments. The 16s rDNA sequencing, a breakthrough technique in molecular biology, has been used to study microorganisms in oil reservoirs (Sherry et al. 2017). However, although microbial communities in light oil reservoirs have been widely reported, the studies related to heavy oil reservoirs, especially shallow and thin oilfields, are limited. A thorough analysis of bacterial diversity in shallow and thin heavy oil reservoirs is therefore needed to provide important theoretical reference for the adoption and application of microbial oil recovery techniques in crude oil production in similar oil reservoir.

The rest of this study is organized as follows. Section 1 gives the relevant background. Section 2 discusses the collection of experimental samples, construction of 16S rDNA clone libraries, and data analysis of clone libraries. Section

3 analyzes the composition, abundance, and distribution of bacterial communities in the shallow and thin oilfields heavy oil reservoir, and potential bacteria for MEOR. Finally, Section 4 presents the conclusions of this study.

STATE OF THE ART

The investigation of microbial populations in oil reservoirs is an important strategy in understanding microorganisms that inhabit heavy crude oil reservoirs. In general, the research methods of studying microorganisms in oil reservoirs include culture, 16S rDNA clone library, polymerase chain reaction (PCR)/denaturing gradient gel electrophoresis, real-time PCR, fluorescence in situ hybridization, and 454 high-throughput sequencing. The techniques used for biological analyses have surpassed the limitations of traditional cultivation methods; consequently, the microbial communities in oil reservoirs have since been objectively revealed (Youssef et al. 2009). Researchers widely use 16S rDNA clone library technology due to its simplicity, time efficiency, and inexpensiveness. This method has been used to investigate microbial communities in light oil reservoirs with temperatures of 20-50°C, and it has promoted the successful implementation of MEOR in field trials (Sherry et al. 2017). However, few reports on microbial communities in heavy oil reservoirs have been documented. Zhang (2012) investigated the microbial communities in the heavy oil reservoir located in Xinjiang (China) with a temperature of 30°C based on the 16S rDNA clone library. The results have shown that hydrocarbon-degrading, surfactant-producing, fermentative, nitrate-reducing, and sulfate-reducing bacteria coexisted in the oilfield environment. Nazina (2017) analyzed the metabolic diversity of bacterial communities in in Dagang (China) with a temperature of 50°C and reported the presence of several microbial communities with varying characteristics.

The success of MEOR application depends on the activity of microorganisms and the synergistic effect of their metabolites in oil reservoirs (Varjani 2017). Additionally, MEOR involves many complex physiological, biochemical, and physical processes. An investigation of microbial communities in oil reservoirs is a prerequisite of MEOR. In particular, the proper characterization of bacteria in oil reservoirs can help identify measures to improve the mobility of crude oil and enhance oil production (i.e., by decreasing crude oil viscosity through bacterial biodegradation or bioemulsification for MEOR) (Al-Sayegh 2017, Liu et al. 2017, Shibulal et al. 2017). The majority of the studies have focused on bacteria that can enhance oil recovery in laboratories. The dominant groups of bacteria for MEOR belong to the genera of *Bacillus*, *Pseudomonas*, *Acinetobacter* and *Clostridium*, which can metabolize hydrocarbon and produce surfactants/emulsifiers, organic solvents, acids, and gases (Gao et al. 2017, Shibulal et al. 2014, Xu et al. 2009). The results of physical simulations in laboratories have shown that the above-mentioned strains can significantly improve oil recovery (Youssef et al. 2009). In recent years, a considerable number of bacteria for MEOR have been reported to degrade or emulsify heavy oil. For example, the strains of bacteria affiliated with *Bacillus subtilis*, *Brevibacillus brevis*, *Petrobacter*, *Marinobacter*, *Geobacillus stearothermophilus*, *Geobacillus pallidus*, and *Paenibacillus ehimensis* have the potential to exploit crude oil or increase heavy oil recoverys (Kopytov et al. 2014, Li et al. 2017, Liang et al. 2004, She et al. 2011, Sherry et al. 2017, Vila et al. 2017, Wentzel et al. 2007, Zhou et al. 2017).

Studies on the characteristics of bacterial communities in shallow and thin heavy oil reservoirs remain limited. Therefore, the composition, abundance, and distribution of bacterial communities in the Henan oilfield were elucidated by using the 16S rDNA clone library. The results can provide theoretical values for the application of MEOR and contribute to the exploration of crude oil or the enhancement of heavy oil recovery in shallow and thin oil reservoirs.

MATERIALS AND METHODS

Sample Collection

Oil-water samples were collected directly from the wellhead of four production wells in October 25, 2017 and stored in sterile 500 mL serum bottles. The four samples were transported to the laboratory as soon as possible for further analysis. The four production wells of the Henan oilfield are labeled as G1235, G2204, G2708 and L3721.

The Henan oilfield is located in Nanyang in Henan Province (China) (Fig. 1). The reservoir temperature range is 23-30°C. The salinity content of the oil reservoir is 6.2 g/L. The average buried depth is 100m-400m with 5m-15m of single-layer thickness (oil layer). The oil viscosity is 3070 mPa.s-80000mPa.s in 30°C conditions.

Construction of 16S rDNA Clone Libraries

The bacterial communities in the shallow and thin heavy-oil reservoir in Henan were analyzed on the basis of the 16S rDNA clone library (Fig. 2). The experimental procedures included bacterial cell collection from samples, genomic DNA extraction, 16S rDNA fragment amplification, positive clone selection, amplified ribosomal DNA restriction analysis, and 16S rDNA sequence analysis.

Collection of bacterial cells and genomic DNA extraction from samples: After the four oil-water samples were well shaken, all 500 mL samples were used to collect pellet cells by centrifugation at 12,000 rpm in a high-speed centrifuge

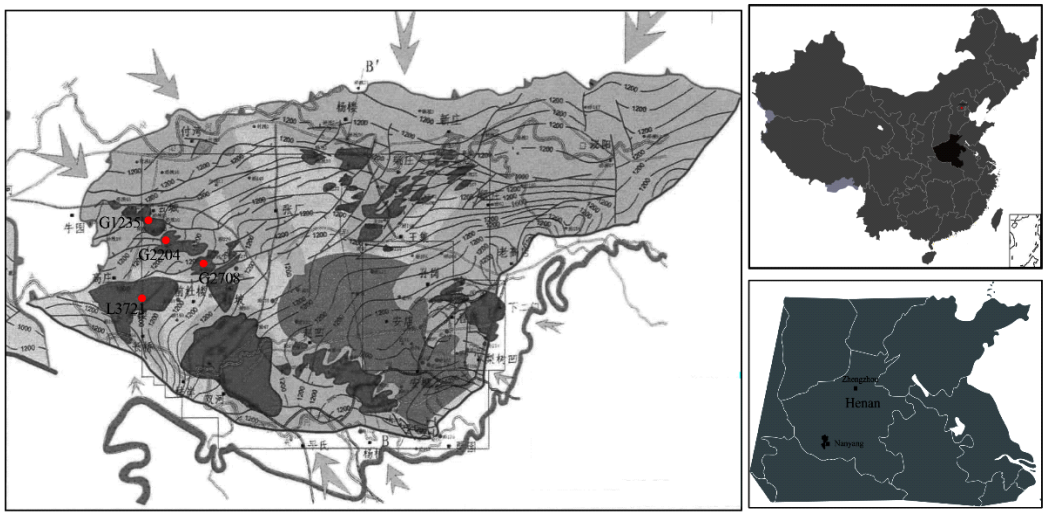

Fig. 1: Location of the Henan shallow and thin heavy oil reservoir (China).

(5424R, Eppendorf, Germany) for 10 min. Following the manufacturer's instructions for the TIANamp Micro DNA Kit (DP316, Tiangen Biotech (Beijing) Co., Ltd., China), genomic DNA was extracted in triplicate to avoid bias and the DNAs were mixed.

Amplification of 16S rDNA fragment: The 16S rDNA fragments of genomic DNA were amplified by using the universal bacterial primers 27F/1492R, and the amplified fragments were approximately 1500 bp (Zhang et al. 2012). The PCR reacting system (25μL) contained 2.5μL of PCR buffer (Mg^{2+}), 10 nmol of deoxynucleotide triphosphate, 1 U Taq DNA polymerase (TaKaRa, Japan), 10 pmol of each primer, and 1μL of genomic DNA. The thermal cycling conditions were as follows: an initial denaturation at 94°C for 5 min; 40 cycles at 94°C for 30 s, 56°C for 60 s, and 72°C for 90 s; and a final extension step at 72°C for 10 min.

Construction of TA clone and selection of positive clones: After purification with the Agarose Gel DNA Purification Kit (Tiangen Biotech (Beijing) Co., Ltd., China) following manufacturer's instructions, the amplicons of 16S rRNA genes were cloned into Trans1-T1 competent cells (Tiangen Biotech (Beijing) Co., Ltd., China) by using the PGEM-T Easy Vector (Promega, Madison, WI, USA). Two hundred putative clones (white) from each clone library were randomly chosen, transferred to a labelled Luria-Bertani (LB) plate with ampicillin (100μg/mL), and cultured at 37°C overnight. A re-amplification was performed with sets of vector-specific primers (T7/SP6) to determine positive clones. During the re-amplification procedure, a small quantity of cells of putative clones was used as template DNA in the reaction mixtures. The PCR products of positive clones were classified into different operational taxonomic units (OTUs) by using amplified ribosomal DNA restriction analysis with *Hinf*I and *Hae*III (Fermentas, Lithuania) (Lagacé et al. 2004).

Data analysis of clone libraries: The representative clones were inoculated at 37°C for 24 h in LB medium with ampicillin (100μg/mL) and then were selected for 16S rDNA sequencing. The sequencing was conducted using the ABI PRISM 3730 DNA sequencer (SinoGenoMax Co. Ltd., Beijing, China). The obtained sequences were manually checked and edited with DNAMAN (version 5.2.2.0). Then, by using BLAST, a representative sequence from each OTU was compared with sequences in the GenBank database to determine the most closely related sequences of bacteria. Sequences with more than 97% similarity were considered to be of the same bacterial genus. The evenness of OTU distribution of clone libraries was statistically analyzed with the Shannon index. The coexisting OTU analysis was calculated with R (version 2.15.3). The correlation analysis between bacterial communities and samples was evaluated with Canoco (version 4.5) (Caporaso et al. 2010).

RESULT ANALYSIS

Statistical analysis of 16S rDNA clone libraries: To characterize the bacterial communities within shallow and thin heavy oil reservoirs, the four oil-water samples retrieved directly from the Henan shallow and thin heavy oil reservoir were used to construct all four clone libraries. A total of 682 positive clones were obtained from the four clone libraries (Table 1). The 16S rDNA sequences of the positive clones were digested by *Hae*III and *Hinf*I. According to the results of the amplified ribosomal DNA restriction analysis,

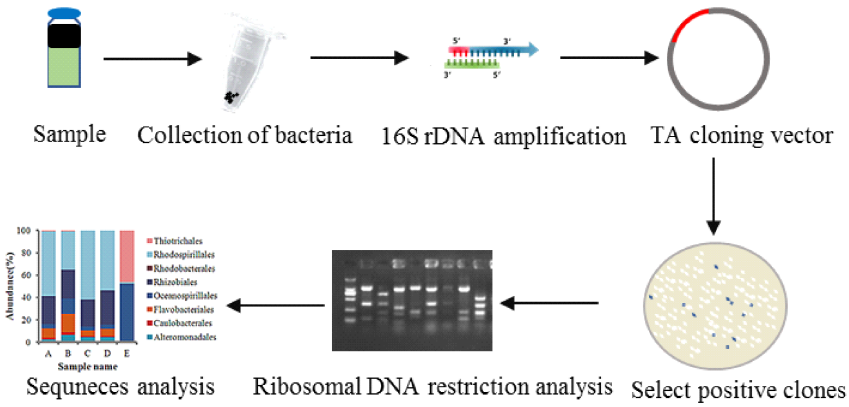

Fig. 2: Diagram illustrating the experimental procedures for the construction of 16S rDNA clone library.

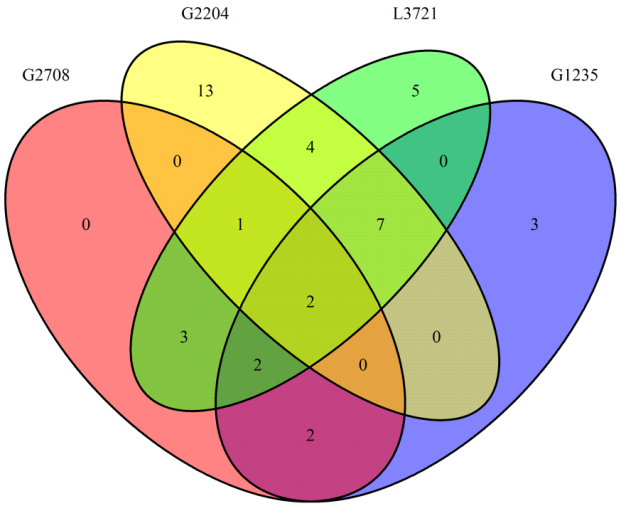

Fig. 3: The coexisting OTUs of the four clone libraries using Venn diagram.

the positive clones in the G1235, G2204, G2708, and L3721 clone libraries numbered 16, 27, 17 and 24 OTUs, respectively. The coverage values of the four clone libraries range from 80.49% to 92.59%, which indicate that the positive clones obtained from the four clone libraries highly reflect the composition of bacterial communities of the four production wells.

The Shannon index of bacterial diversity was calculated to assess the evenness of the OTU distribution. The four samples markedly differed in terms of their Shannon indices (Table 1). The L3721 sample has obtained the highest Shannon index (2.21), indicating the highest bacterial diversity among the four samples. The Shannon indices of the G1235 and G2708 sample are 1.36 and 1.51, respectively. By contrast, the G2204 sample has obtained the lowest Shannon index at 0.72.

Data on coexisting OTUs can be used to derive the relationships of microbial communities in different environments. In the study, two OTUs coexist among the four clone libraries (Fig. 3). G2204-G270-L3721 share two OTUs that are similar to G1235-G2204-G2708. G2204-G2708 and G1235-G2708 share nine and six OTUs, respectively, which indicate much closer relationships of microbial communities in the above two samples than G2204 and G2708 with three OTUs.

Composition and abundance of bacterial communities in the shallow and thin heavy oil reservoir: The composition and relative abundance of bacterial communities of the four clone libraries from Henan the shallow and thin heavy oil reservoir are shown in Fig. 4. Similarities are found in the composition of bacterial communities at the genus level. First, the groups of *Pseudomonas* are identified in all the four clone libraries, which accounted for 19% (G1235), 8% (G2204), 44% (G2708) and 6% (L3721), respectively. Second, unclassified bacteria are observed in the four clone libraries, and they account for 42% (G1235), 69% (G2204), 87% (G2708) and 49% (L3721), respectively. Third, *Acinetobacter* and *Shewanella* coexist in the G1235 and L3721 clone libraries. *Aeromonas* is also found to coexist in the G2204 and L3721 clone libraries.

More bacterial communities have been in L3721 than other three clone libraries. The G1235, G2204 and G2708 clone libraries have 3, 4 and 3 genera, respectively. Meanwhile, the L3721 clone library has 10 genera. The remaining bacterial sequences of the L3721 clone library mainly include the genera *Psychrobacter*, *Geobacter*, *Desulfovirgula*, *Desulfotomaculum* and *Rahnella*. Additionally, *Nitrincola* is only found in the G1235 clone library.

Table 1: Statistical analysis of four clone libraries.

Production well	G1235	G2204	G2708	L3721
Number of positive clones	168	172	158	184
OTUs	16	27	17	24
Coverage (%)	92.59	82.14	85.71	80.49
Shannon index	1.36	0.72	1.51	2.21
Simpson index	0.48	0.24	0.62	0.69

Additionally, the genus *Pseudomonas* predominates the G1235, G2204 and G2708 clone libraries with 19%, 8%, and 44%, respectively. By contrast, the genus *Shewanella* accounts for 24% and predominates in L3721 clone library. The correlation between bacterial communities and samples was evaluated by using Canoco. The results show differences in the structure of bacterial communities of the four samples. Relative to L3721, the degree of differences between the bacterial communities and samples has the order of G1235>G2708>G2204 (Fig. 5).

Analysis of the distribution of bacterial communities in the shallow and thin heavy oil reservoir: Geological characteristics, such as temperature, salinity and oil properties, affects microbial diversity and distribution in oil reservoirs (Sherry et al. 2017). The habitat requirements of the genera closely related to *Pseudomonas*, *Acinetobacter*, *Psychrobacter* and *Enterobacter*, which are all observed in the Henan oilfield, matches with the 30°C temperature of the oil reservoir. Halotolerant groups of *Nitrincola* and *Marinobacterium* are detected in the highly saline (6.2 g/L) of oil-water samples collected from the Henan oil reservoir. Moreover, the strains of *Pseudomonas*, which account for 6% to 44%, are identified in all four clone libraries (i.e., these strains grow in heavy oil), which is consistent with Bacosa's report (Bacosa et al. 2013).

Only a few types of bacteria are identifiable because of the general complexity of oilfield environments and the limitations of the present work (Magot et al. 2000). Nonetheless, this study can be able to identify several 16S rDNA sequences with less than 97% similarity to the bacteria mentioned in previous studies, which indicates that new bacterial species exist in the Henan oilfield. For example, sequences of G2708-8 and G1235-5 OTUs are closely related to *Pseudomonas* (96% and 95% identities, respectively). Additionally, unclassified bacteria accounting for 42%-87% are obtained from the four clone libraries, which indicates that the oil reservoir harbors a unique community of novel bacterial species or genera.

Potential bacteria for microbial enhanced heavy oil recovery: Effective MEOR application generally involves the combination of bacteria with different physiological and metabolic abilities (Youssef et al. 2009). According to the alignment analysis of the 16S rDNA sequences of the G1235, G2204, G2708 and L3721 with those in GenBank, the groups of hydrocarbon-degrading, biosurfactant-producing, and fermentative bacteria for MEOR can improve oil recovery in the Henan shallow and thin oilfield.

First, the groups of thermophilic hydrocarbon-degrading bacteria have been identified in the shallow and thin heavy oil reservoir. *Pseudomonas stutzeri* identified in the G1235, G2708 and L3721 clone libraries, can degrade aromatic hydrocarbons of crude oil (Sen 2008). *Pseudomonas aeruginosa* which is detected from the four clone libraries, reportedly can use heavy oil to grow (Bacosa et al. 2013).

Second, the groups of biosurfactants-producing bacteria have been identified. Groups of *Pseudomonas* detected in the four clone libraries was reported to be able to produce rhamnolipids that could emulsify heavy oil and could be successfully applied in oil reservoir (Amani et al. 2010). The strains of *Acinetobacter lwoffii* are detected in G1235, G2708 and L3721 clone libraries. Studies have shown that *A. lwoffii* can produce bioemulsifiers, and emulsify crude oil to improve oil recovery (Rosenberg et al. 1999).

Additionally, the strains of fermentative bacteria detected in the oil reservoir can produce gases or biopolymers. *Enterobacter*, which is identified in the samples of G1235 and L3721, can produce gas and biopolymer that can also be used as functional bacteria for microbial oil recovery (Nagase 2001, Rabiei et al. 2013).

CONCLUSIONS

To investigate the characteristics of bacterial communities in shallow and thin heavy oil reservoirs, as illustrated by the example of the Henan oilfield in this study. In particular, the 16S rDNA clone library approach was performed to analyze the composition, abundance, and distribution of the bacterial communities. The conclusions of this study are as follows:

1 The diversity of the bacterial communities in the shallow and thin heavy oil reservoir is low. Eleven groups of bacteria were identified in this reservoir, and unclas-

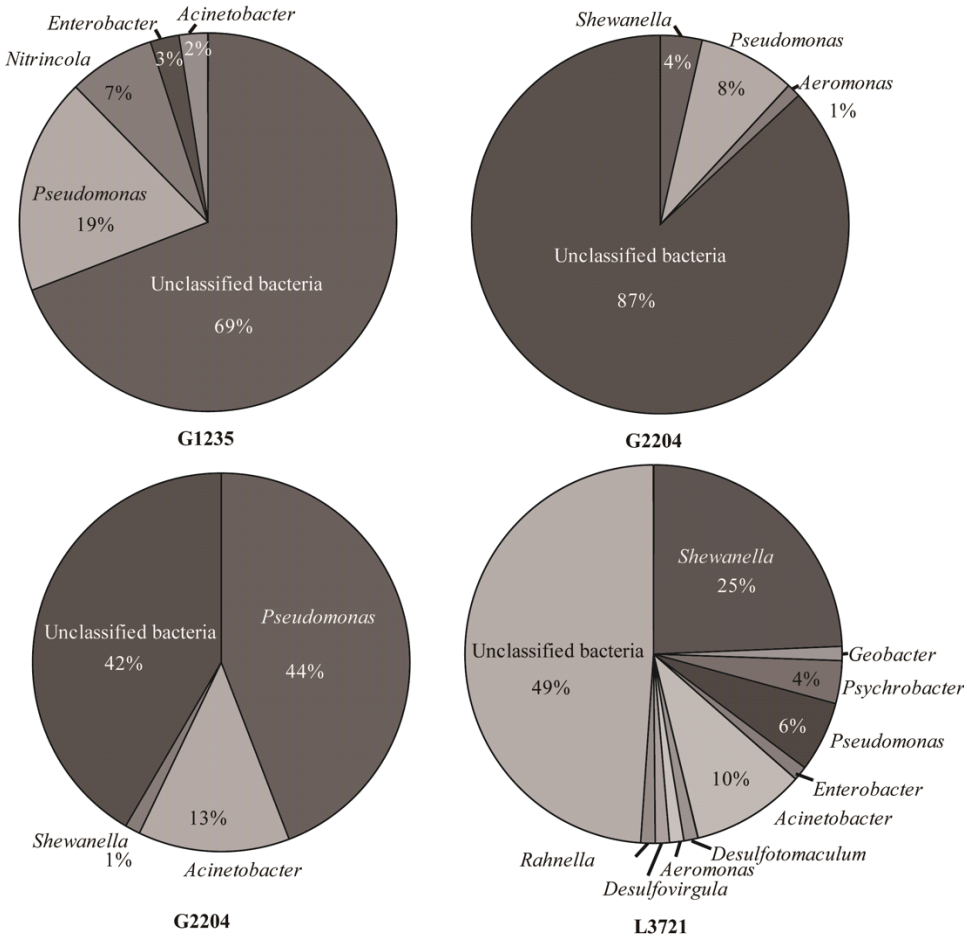

Fig. 4. Composition and relative abundances of bacterial communities in G1235, G2204, G2708 and L3721 clone libraries.

sified bacteria dominate the analytical results. Differences exist in the bacterial community structures of the clone libraries.

2 The distribution of bacterial communities is consistent with the temperature, salinity, and oil properties of the oil reservoir. *Pseudomonas*, *Acinetobacter*, *Psychrobacter* and *Enterobacter*, as well as unclassified bacteria and halotolerant bacterial species (*Nitrincola* and *Marinobacter*) are observed in the Henan shallow and thin oilfield.

3 The potential bacteria for MEOR have been identified in the shallow and thin heavy-oil reservoir. The identified hydrocarbon-degrading and biosurfactants-producing bacteria (*Pseudomonas* and *Acinetobacter*) and fermentative bacteria (*Enterobacter*) can reduce the viscosity of heavy oil and improve oil recovery.

On the one hand, although fewer sequences could be detected compared with 454 high-throughput sequencing, the 16S rDNA clone library approach used in this study could effectively reveal the characteristics of bacterial communities with different physiological and metabolic abilities, particularly in the shallow and thin heavy-oil reservoir in Henan. The results of this study provide a theoretical reference value for the application of MEOR in shallow and thin heavy oil reservoirs. On the other hand, the study was delimited by the time-point sampling approach used to analyze the bacterial communities, and the change law of the bacterial communities in the oil field has not been established. Therefore, collecting a large number of samples from shallow and thin heavy-oil reservoirs is needed to further analyze and fully understand the characteristics of bacterial communities in special oil reservoir environments.

The successful application of microbial oil recovery technology often requires the synergy of various oil-displacing bacteria. A possible direction of future research is the comprehensive evaluation of bacterial interactions for MEOR in heavy oil reservoirs.

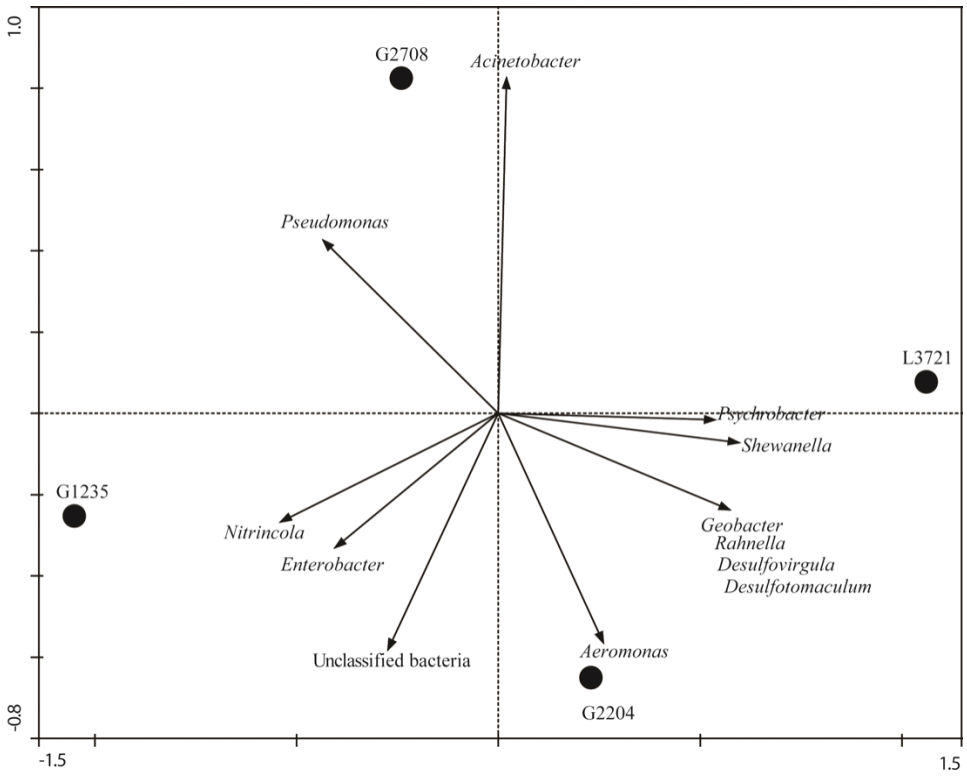

Fig. 5: Correlation analysis of bacterial communities and samples.

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