

Culture-independent-based Analyses Identified Site-specific Microbial in Two Oil-wells from Malaysia

(Analisis Bebas Kultur berasaskan Mikrob Khusus Tapak Dikenal Pasti
di Dua Buah Telaga Minyak dari Malaysia)

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ABSTRACT

Microbial communities of two oil reservoirs from Malaysia, denoted as Platform Bo and Platform Pe were studied using culture-independent approach. Environmental DNA was extracted and the universal amplified ribosomal region (UARR) was target amplified for both prokaryotes and eukaryotes. The amplified products were purified and cloned into pTZ57R/T vector to construct the 16S/18S rDNA library. Restriction endonucleases HhaI and MspI were used to screen the library. From that, 125 and 253 recombinant plasmid representative clones from Platform Bo and Platform Pe, respectively, were sent for DNA sequencing. Twenty-six operational taxonomic units (OTUs) consist of 20 genera detected at Platform Bo and 17 OTUs consist of 13 genera detected at Platform Pe. Marinobacter and Acinetobacter species co-occurred in both platforms whereas the rest are site-specific. Gammaproteobacteria accounted for 86.0% of the microbial community in Platform Bo, where OTUs affiliated to Marinobacter, Pseudomonas and Marinobacterium that were the most abundant. The major OTUs in the Platform Pe were with affinities to Achromobacter, followed by Stenotrophomonas and Serratia. The only archaeal isolates were detected in Platform Pe, which affiliated to Thermocodium. The singletons and doubletons accounted for about 50.0% of the OTU abundance in both platforms, which considerably significant despite their rare occurrence.

Keywords: Microbial community; operational taxonomic unit; petroleum reservoir; 16S/18S rRNA

ABSTRAK

Komuniti mikrob di dua telaga minyak dari Malaysia, ditandakan sebagai Platform Bo dan Platform Pe telah dikaji menggunakan pendekatan bebas kultur. DNA alam sekitar diekstrak dan rantau ribosom umum teramplifikasi (UARR) adalah sasaran diperkuatkan untuk kedua-dua prokaryotes dan eukaryotes. Produk yang diperkuatkan telah dituliskan dan diklon ke vektor pTZ57R/T untuk membina perpustakaan rDNA 16S/18S. Sekatan endonucleases HhaI dan MspI telah digunakan untuk menyaring perpustakaan tersebut. Daripada itu, 125 dan 253 wakil plasmid rekombinan klon masing-masing dari Platform Bo dan Platform Pe, telah dihantar untuk penjujukan DNA. Dua puluh enam unit taksonomi operasi (OTUs) terdiri daripada 20 genus dikesan di Platform Bo dan 17 OTUs terdiri daripada 13 genus dikesan di Platform Pe. Spesies Marinobacter dan Acinetobacter hadir bersama-sama di kedua-dua platform manakala selebihnya adalah khusus tapak. Gammaproteobacteria menyumbang sebanyak 86.0% komuniti mikrob di Platform Bo, manakala gabungan OTUs Marinobacter, Pseudomonas dan Marinobacterium adalah yang paling banyak. OTUs utama di Platform Pe adalah gabungan Achromobacter, diikuti oleh Stenotrophomonas dan Serratia. Satu-satunya penciran archaeal dikesan di Platform Pe iaitu bergabung dengan Thermocodium. Tunggalan dan duaan yang mewakili kira-kira 50.0% daripada kelimpahan OTU di kedua-dua platform agak ketara walaupun keadaan ini jarang berlaku.

Kata kunci: Komuniti mikrob; takungan petroleum; unit taksonomi operasi; 16/18S rRNA

INTRODUCTION

The harsh conditions of an oil reservoir in terms of pressure, temperature, pH, nutrients and salinity does not make it a hotbed for the vast majority of microorganisms to survive. Those that successfully colonized, which may be benign or harmful to the oil production do possess diverse physiological and metabolic capabilities. There are continual efforts in identifying and characterizing the microorganisms indigenous to oil fields. This knowledge would be useful to stimulate the growth of beneficial

microbial consortia while suppress those deleterious for maximal oil production or recovery.

Under the extreme habitats in a reservoir, some microbes are capable to metabolize hydrocarbons and excrete useful biomolecules such as biosurfactants and biopolymers. Biosurfactants reduce interfacial tension between oil and water to promote oil flow whereas biopolymers stabilize oil-in-water emulsions to enhance oil recovery. Several studies demonstrated the application of these byproducts in enhancing or recovering oil production

at laboratory scale (Al-Bahry et al. 2013; Gudiña et al. 2013; Soudmand-asli et al. 2007; Thavasi et al. 2011) as well as in field tests (Li et al. 2002; Nagase et al. 2003; Youssef et al. 2007). So far, *Bacillus* and *Pseudomonas* strains are the mostly studied biosurfactant- and biopolymers-producers in oil fields.

In fact, not all types of microbial activities in an oil field produce favorable consequences. Oil reservoir souring due to sulfate-reducing microorganisms (SRM) appears as a notorious issue in oil production. Increasing concentrations of hydrogen sulfide (H₂S) produced by SRM turn the crude into sour oil that can be toxic and corrosive that eventually causing poor quality of produced oil, corrosion of wellbore and plugging of the conduits and injection wells. Involvements of prokaryotic *Desulfovibrio*, *Desulfobulbus*, *Desulfobacter*, *Desulfoglaeba*, *Desulfotomaculum*, *Thermodesulforhabdus*, *Thermodesulfobacterium* (Davidova et al. 2006; Feio et al. 2004; Guan et al. 2013; Jeanthon et al. 2002; Tanaka et al. 2002) and eukaryotic *Archaeoglobus* (Beeder et al. 1994; Gittel et al. 2009) in sulfate reduction in oil fields have been identified. In response to biogenic H₂S production, nitrate injection into oil reservoirs encourages growth of nitrate-reducing microorganisms (NRM) to outcompete SRM has been practiced (Grigoryan et al. 2009; Hubert & Voordouw 2007; Zhuang et al. 2011). Continuous nitrate injection at low concentration stably inhibited SRM activity in Veslefrikk and Gullfaks oil fields (North Sea) over the years (Bødtker et al. 2008).

Traditional culture-dependent method has been used in accessing microbial diversity for decades and its limitations are well acknowledged. Majority of the microorganisms that colonized extreme environments are uncultivable on synthetic media. A comparison study reported only less than 0.1% subset of the soil microbial community has been accounted by culturing method (Hill et al. 2000). Even if cultivable microbes do exist, comprehensive, costly and laborious cultivation strategies may involve. The drawbacks of culture-dependent methods in recovering diverse microbial populations have popularized the culture-independent approaches in exploring microbial diversity. Culture-independent may be use in complement with or rather in favor to culture-dependent methods for resolving larger fraction of the total microbial population. The present study exploits culture-independent methods to stretch out the microbial communities at two distantly located oil reservoirs.

MATERIALS AND METHODS

SAMPLING

The present study targeted the microbial communities in two locational distinct petroleum reservoirs, denoted as Platform Bo and Platform Pe, located at offshore East Malaysia and offshore Peninsular Malaysia, respectively. Oil-water emulsions sampled from three oil wells of each platform were contained in sterile laboratory bottles and transported at ambient temperature to the laboratory.

DNA EXTRACTION

The formation water and oil phases were separated. For formation water phase, a 50 mL water sample was centrifuged at 8000 × g for 10 min to collect the pelleted materials. As for the oil phase, 50 mL of warmed oil sample (65°C) was added with equal volume of sterile distilled water and shook vigorously. The samples were left for separation into two phases at 65°C. Then, the aqueous phase was transferred into new centrifuge tube and centrifuged at 8000 × g for 10 min. The pelleted materials collected from formation water and oil phases were subjected to DNA extraction using GF-1 Soil DNA Extraction Kit (Vivantis, Malaysia) according to the kit manufacturer's instructions.

PCR AMPLIFICATION AND 16S/18S rDNA LIBRARY CONSTRUCTION

U n i v e r s a l p r i m e r s U 1 F (5'-CTYAAAKRAATTGRCGGRSSC-3') and U1R (5'-CGGGCGGTGTGTRCAARRSSC-3') (Rivas et al. 2004) at a final concentration of 0.6 μM were used to amplify UARR of both prokaryotes and eukaryotes. The 25 μL-PCR reaction contained 3.0 mM of MgCl₂, 0.2 mM of dNTP mix, 1 U of *Taq* DNA polymerase and 2.0 μL of DNA template. The PCR amplifications were done by initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 30 s and final extension at 72°C for 10 min.

PCR products were visualized in 1% agarose gel. UARR DNA fragments (495 bp for prokaryotes; 508 bp for eukaryotes) were gel purified using GF-1 Gel DNA Recovery Kit (Vivantis, Malaysia) and cloned into pTZ57R/T vector using the InsTAclone™ PCR Cloning Kit (Thermo Scientific, US) following the manufacturer's instructions.

AMPLIFIED RIBOSOMAL DNA RESTRICTION ANALYSES (ARDRA)

Colony-PCR amplifications using M13/pUC forward and M13/pUC reverse primers were performed on randomly selected clones from 16S/18S rDNA library of Platform Bo and Platform Pe. Amplified products from positive clones were digested with *Hha*I (GCG[^]C) and *Msp*I (C[^]CGG) restriction endonucleases (Vivantis, Malaysia) followed by electrophoresis on 3% agarose gel. The resulting ARDRA patterns were analyzed and grouped. The representative clones were selected from each ARDRA group. The 16S/18S rDNA of a total of 125 clones from Platform Bo and 253 clones from Platform Pe were sequenced.

SEQUENCE ANALYSES

The 16S/18S rRNA gene sequences were analyzed using BLASTn program (<http://blast.ncbi.nlm.nih.gov/>) and chimeric sequences were filtered using DECIPHER (Wright et al. 2012). By using RDP's Pyrosequencing Pipeline (<http://pyro.cme.msu.edu/>), the non-chimeric sequences were used for multiple sequence alignment and clustered

into operational taxonomic units (OTUs) with >97% identity as the cutoff. One representative sequence of each OTU group was submitted to GenBank under the accession numbers KC206311 to KC206336 for OTU-Bo-p01 to OTU-Bo-p26 (prokaryotes from Platform Bo), KC206337 to KC206353 for OTU-Pe-p01 to OTU-Pe-p17 and KC206354 for OTU-Pe-a01 (prokaryotes from Platform Pe).

OTUs' representative sequences were assigned to the phylogenetically consistent higher-order bacterial and fungal taxonomy using RDP Classifier at 95% threshold (Wang et al. 2008). DNA sequences of OTU representatives and two closest matches for each OTU group obtained from BLASTn were used to construct the neighbor-joining phylogenetic trees using PHYLIP software (version 3.6) with bootstrap values calculated from 100 replicate runs.

RESULTS AND DISCUSSION

MICROBIAL COMPOSITIONS OF TWO CRUDE OIL SAMPLES

A total of 100 and 211 non-chimeric clones containing 16S/18S rDNA were obtained from Platform Bo and Platform Pe, respectively. All the 26 OTUs detected in Platform Bo and 17 OTUs detected in Platform Pe were bacterial origin (Table 1). Notably, only OTU-Pe-a01 from Platform Pe was archaeal origin. Unrooted neighbor-joining trees based on the bacterial 16S rRNA genes of each OTU and their nearest neighbors were constructed (Figures 1 and 2). Phylogenetic analysis illustrates the relationships of the clones with their closest affiliates and identifies bacterial members at the species level based on the 16S rDNA sequences.

The microbial community of Platform Bo consisted 86.0% of Gammaproteobacteria and OTUs with affinities to *Marinobacter* species (38.0%), *Pseudomonas* species (13.0%) and *Marinobacterium* species (12.0%) were dominant. Other detected Proteobacteria were Betaproteobacteria and Deltaproteobacteria but in significantly lower populations. Other phyla detected included Bacteroidetes (OTU-Bo-p23), Firmicutes (OTU-Bo-p15, OTU-Bo-p22 and OTU-Bo-p26) and Spirochaetes (OTU-Bo-p21).

On the other hand, both Gammaproteobacteria (47.8%) and Betaproteobacteria (45.0%) were detected in proportion in the microbial community of Platform Pe. Nevertheless, the most dominant OTUs in the community were with affinities to *Achromobacter* species (43.1%) of Betaproteobacteria, followed by *Stenotrophomonas* species (29.4%) and *Serratia* species (12.8%) of Gammaproteobacteria. Alphaproteobacteria detected at 1.0%. Only one Firmicutes OTU (Pe-p10) and one Crenarchaeota OTU (Pe-a01) were observed in the community.

The common OTUs with affinities to *Marinobacter* species and *Acinetobacter* species co-occurred in both platforms. There was lack of observations of eukaryotes in both sampling sites. Thirty-one genera were identified in two crude oil samples from two petroleum reservoirs. The

archaeal and most bacterial groups were site-specific with exceptions on *Marinobacter* and *Acinetobacter* species that co-occurred in both reservoirs, with *Marinobacter* being the most abundant genus in Platform Bo. *Marinobacter* species are omnipresent in marine environment and a number of them are recognized as hydrocarbon degraders, such as *M. hydrocarbonoclasticus* (Gauthier et al. 1992), *M. aquaeolei* (Nguyen et al. 1999), *M. alkaliphilus* (Takai et al. 2005), and the most recently characterized *M. nanhaiticus* (Gao et al. 2013). Members of the genus *Acinetobacter* are ubiquitous and known as biosurfactant producers by utilizing hydrocarbons (Bao et al. 2014; Chen et al. 2012; Pirog et al. 2012). The next abundant genera in Platform Bo after *Marinobacter* were followed by *Pseudomonas* and *Marinobacterium*. Their distribution in proportion was somehow contrast to the findings of Li et al. (2012), where *Pseudomonas* reduced in abundance when *Marinobacterium* present and vice versa.

Microbial community at Platform Pe was dominated by *Achromobacter*, *Stenotrophomonas* and *Serratia* which are more inscrutable in oil productivity although a few of these particular species are known hydrocarbons-utilizing bacteria and involved in bioremediation. The singletons and doubletons accounted for about 50.0% of the OTU abundance in both platforms, which considerably significant despite their rare and low existence. The only Gram-positive bacteria present were *Enterococcus* together with endospore-forming *Bacillus* and *Clostridium*. Endospore-forming microbes can survive under stressful reservoir conditions hence emerge as the commonly used species for microbial enhanced oil recovery (MEOR) applications (Gudiña et al. 2013; Jang et al. 1983).

Upon succeed in accessing microbial system of an oil reservoir and with such information in hands, corrective interventions can be implemented. Nutrient injection to stimulate beneficial indigenous microorganisms has been showing promising incremental oil recovery under laboratory settings (Yao et al. 2011) as well as in field pilots (Feng et al. 2012; Raiders et al. 1985; Yao et al. 2011). In the absence of beneficial indigenous microbial population, injection of exogenous microorganisms with suitable nutrients, known as microbial plugging can be applied instead. Wang et al. (2008) demonstrated encouraging biopolymer production and enhanced oil production by microbial plugging in a series of simulation experiments.

However, field trial showed that the survival rate of injected exogenous microorganisms is depressed (Zhang et al. 2010). Another approach is via injection of *ex situ* produced biomolecules such as biosurfactant and biopolymer, which expected to enhance oil recovery positively (Armstrong & Wildenschild 2012). Regardless of which approach is adopted or used in combination, continuous monitoring of microbial activities and oil productivity should be evaluated to avoid destructive impacts on oil yield.

The only archaeal isolates were detected in Platform Pe, which affiliated with *Thermocodium*. It grows anaerobically or microaerobically in extremely

TABLE 1. Clone abundance and RDP classification of OTU groups recovered from two locational distinct petroleum reservoirs

Affinity			Platform Bo (100 clones)		Platform Pe (211 clones)		
Kingdom	Phylum	Class; Order; Family; Genus	Clone abundance (%)	OTU-	Clone abundance (%)	OTU-	
Bacteria	Bacteroidetes	Flavobacteria; Flavobacteriales; Flavobacteriaceae; <i>Chryseobacterium</i>	2.0	Bo-p23			
	Firmicutes	Bacilli; Bacillales; Bacillaceae 1; <i>Bacillus</i>	2.0	Bo-p15,p26			
		Bacilli; Lactobacillales; Enterococcaceae; <i>Enterococcus</i>				1.4	Pe-p10
	Proteobacteria	Clostridia; Clostridiales; Clostridiaceae 1; <i>Clostridium sensu stricto</i>	1.0	Bo-p22			
		Alphaproteobacteria; Caulobacterales; Caulobacteraceae; <i>Phenylobacterium</i>				0.5	Pe-p15
		Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; <i>Bradyrhizobium</i>				0.5	Pe-p16
		Betaproteobacteria; Burkholderiales; Alcaligenaceae; <i>Achromobacter</i>				43.1	Pe-p01, p02,p03
		Betaproteobacteria; Burkholderiales; Alcaligenaceae; <i>Parapusillimonas</i>				0.9	Pe-p09
		Betaproteobacteria; Burkholderiales; Burkholderiaceae; <i>Ralstonia</i>				0.5	Pe-p17
		Betaproteobacteria; Burkholderiales; Comamonadaceae; <i>Acidovorax</i>				0.5	Pe-p14
		Betaproteobacteria; Burkholderiales; Comamonadaceae; <i>Comamonas</i>	1.0	Bo-p25			
		Betaproteobacteria; Burkholderiales; Comamonadaceae; <i>Schlegelella</i>	4.0	Bo-p14			
		Deltaproteobacteria; Desulfuromonadales; Pelobacteraceae; <i>Pelobacter</i>	2.0	Bo-p20			
		Deltaproteobacteria; Syntrophobacteriales; Syntrophaceae; <i>Syntrophus</i>	1.0	Bo-p19			
		Gammaproteobacteria; Alteromonadales; Alteromonadaceae; <i>Marinobacter</i>	38.0	Bo-p01, p02,p16	3.8	Pe-p12	
		Gammaproteobacteria; Alteromonadales; Alteromonadaceae; <i>Marinobacterium</i>	12.0	Bo-p07,p08			
	Gammaproteobacteria; Alteromonadales; Shewanellaceae; <i>Shewanella</i>	5.0	Bo-p05				
	Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Escherichia/Shigella</i>			0.9	Pe-p13		
	Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Pectobacterium</i>	1.0	Bo-p17				
	Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Serratia</i>			12.8	Pe-p04		
	Gammaproteobacteria; Oceanospirillales; Halomonadaceae; <i>Halomonas</i>	3.0	Bo-p06				
	Gammaproteobacteria; Pseudomonadales; Moraxellaceae; <i>Acinetobacter</i>	2.0	Bo-p11	0.9	Pe-p08		
	Gammaproteobacteria; Pseudomonadales; Moraxellaceae; <i>Enhydrobacter</i>	2.0	Bo-p10				
Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; <i>Azomonas</i>	1.0	Bo-p13					
Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; <i>Pseudomonas</i>	13.0	Bo-p09,p18					
Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; <i>Serpens</i>	5.0	Bo-p03,p04					
Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; <i>Pseudoxanthomonas</i>	1.0	Bo-p24					
Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; <i>Stenotrophomonas</i>			29.4	Pe-p05- p07,p11			
Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; <i>Thermomonas</i>	3.0	Bo-p12					
Spirochaetes	Spirochaetes; Spirochaetales; Spirochaetaceae; <i>Spirochaeta</i>	1.0	Bo-p21				
Archaea	Crenarchaeota	Thermoprotei; Thermoproteales; Thermoproteaceae; <i>Thermocladium</i>			4.7	Pe-e01	

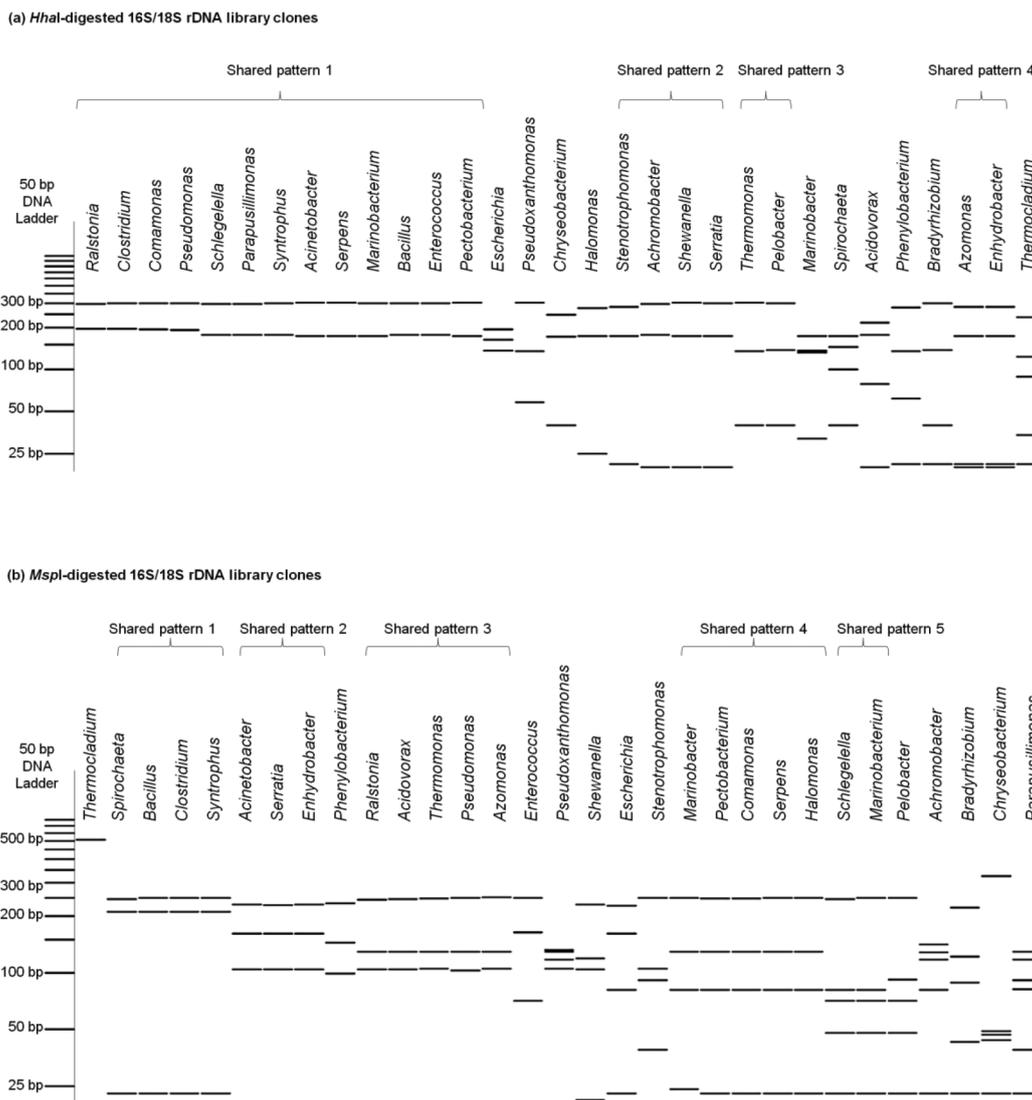


FIGURE 3. Schematic presentation of ARDRA patterns for (a) *HhaI*-digested and (b) *MspI*-digested 16S/18S rDNA library clones. Fragments shorter than 20 bp were omitted. The shared ARDRA patterns produced by either *HhaI* or *MspI* digestion disallowed inter-genera differentiation

of these microorganisms, whether beneficial or deleterious to oil production. This information is critical for deciding corrective interventions in MEOR attempts.

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