International Biodeterioration & Biodegradation 85 (2013) 400–406

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Phylogenetic diversity of bacterial communities associated with bioremediation of crude oil in microcosms

Hui Wang [a, b, 1], Caixia Wang [a, 1], Meng Lin [a], Xiangnan Sun [a], Chuanyuan Wang [a], Xiaoke Hu [a, *]

[a] Yantai Institute of Costal Zone Research, Chinese Academy of Sciences, 264003 Yantai, China
[b] State Environmental Protection Key Laboratory of Microorganism Application and Risk Control (MARC), Tsinghua University, 100084 Beijing, China

ARTICLE INFO

Article history:
Received 14 January 2013
Received in revised form
8 July 2013
Accepted 11 July 2013
Available online 1 October 2013

Keywords:
Bioremediation
Crude oil
Bacterial community
Microcosm

ABSTRACT

Bioremediation, mainly by indigenous bacteria, has been regarded as an effective way to clean up oil pollution after an oil spill. In order to obtain a systematic understanding of the succession of bacterial communities associated with oil bioremediation, sediments collected from the Penglai 19-3 oil platform were co-incubated with crude oil. Oil biodegradation was assessed on the basis of changes in oil composition monitored by GC–MS. Changes in the bacterial community structure were detected by two 16S rRNA gene based culture-independent methods, denaturing gradient gel electrophoresis (DGGE) and clone library. The results suggested that crude oil was rapidly degraded during the 30-day bioremediation period. Bacteria affiliated with the genus *Pseudomonas* dominated all three clone libraries. But dramatic changes were also detected in the process of biodegradation of crude oil. The "professional hydrocarbonocastic bacteria" (e.g., *Alcanivorax*) became abundant in the two samples during the bioremediation period. Meanwhile, δ-proteobacteria was only detected in the two samples. Information on the bacterial community revealed in this study will be useful in developing strategies for bioremediation of crude oil dispersed in the marine ecosystem.

1. Introduction

Oil pollution originating from both natural and anthropogenic sources constitutes a significant detriment to the environment. Vast amounts of oil entering marine locations, groundwater, soil, have a great impact on resident organisms. Recent disastrous oil spills, e.g., the Deepwater Horizon oil spill in the Gulf of Mexico in 2010 and the Penglai 19-3 oil spill in the Bohai Bay, China, 2011, have been major sources of oil pollution. Cost-effective and environmentally benign strategies are urgently needed for spilled oil remediation. Conventional physical and chemical methods could rapidly remove the major portion of leaked oil, but in most cases, these methods merely transfer contaminants from one environmental medium to another, even producing toxic byproducts (Gan et al., 2009; Gavrilescu, 2010). Most important is the fact that oil cannot be completely cleaned up by physical and chemical methods.

Bioremediation is the technique utilizing biological organisms to aid in removal of hazardous substances from a polluted area (Head et al., 2006). Microorganisms, especially bacteria, are key players in the process of bioremediation of most organic pollutants. The ability to degrade hydrocarbon components of crude oil has been widely detected among different bacteria, and microorganism-related bioremediation has proven to be an effective method for cleaning up residual oil in various environments (Atlas, 1995; Margesin, 2000; Röling et al., 2004; Zhao et al., 2011). Hydrocarbon-degrading bacteria from various phyla, including the Alpha-, Beta-, and Gamma-proteobacteria; Flexibacter-Cytophaga-Bacteroides, Firmicutes, Actinobacteria, and Cyanobacteria, have been successfully cultured in the laboratory (Yakimov et al., 1998; Hennessee et al., 2009; Zhao et al., 2010; Szabó et al., 2011; Luo et al., 2012). Other studies have indicated that many microorganisms usually considered to be incapable of degrading hydrocarbon contain genes homologous to alkane-hydroxylase genes (Harayama et al., 2004). Some bacterial strains affiliated with genera *Burkholderia*, *Pseudomonas*, *Legionella*, *Mycobacterium*, *Mycobacterium*, and *Silicibacter* were found to contain homologues of alkane-hydroxylase genes, and several of these species could produce proteins encoded with functional alkane hydroxylases (Van Beilen et al., 2003; Van Beilen et al., 2004).

However, laboratory-cultured functional bacteria play only limited roles in indigenous bioremediation of crude oil, for these

* Corresponding author. Tel.: +86 535 2109127.
E-mail addresses: xkhu@yic.ac.cn, huxiaoke@gmail.com (X. Hu).
[1] These two authors contributed equally to this work.

0964-8305/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibiod.2013.07.015

Table 1
Microcosm assemblage setups for experiments and samples used for DGGE and 16S rRNA gene based clone library analysis. S, R1 and R2 represent samples used for constructing clone libraries from sediment, experiment 1 and experiment 2 respectively. "–" and " √" represent non-existence and existence in the sample.

Experimental setup	Sediment/S	Experiment 1/R1	Experiment 2/R2
Crude oil	–	√	√
Mineral solution	–	√	√
Surfactant (Tween 20)	–	–	√
Sediment from Bohai sea	√	√	√

reasons: (1) Environmental parameters, especially nutrients (nitrogen and phosphorus), vary dramatically between the in-situ and laboratory environments, which could be an important restrictive factor for the growth of functional bacteria; and (2) the application of alien bacteria in the environment is always restrained due to ecological concerns. By contrast, the natural biodegradation processes of indigenous bacteria were believed to play a dominant role in removing oil from polluted environments. Based on the old microbiological tenet "Everything is everywhere, but the environment selects" (De Wit and Bouvier, 2006), oil bioremediation-related bacteria should exist at the home position of each oil spill. These hydrocarbon-degrading microorganisms usually exist in very small amounts in indigenous environments (Margesin and Schinner, 2001; Harayama et al., 2004). However, crude oil pollution could promote the growth of functional microorganisms and thus cause the succession of microbial communities in the contaminated field. Studies done after the Deepwater Horizon oil spill indicated dramatic changes of the in-situ microbial communities (Redmond and Valentine, 2011). One month after the spill, the first report indicated that an uncultured bacterial group of *Oceanospirillales* was seen to dominate in plume samples (Hazen et al., 2010). In June, two month after the spill, the dominant bacterial groups switched to Gamma-proteobacteria, *Colwellia*, *Cycloclasticus* (Valentine et al., 2010). These bacterial communities have been proven to play large roles in the process of hydrocarbon degradation (Hazen et al., 2010; Valentine et al., 2010). After the well was capped, previously dominant bacterial groups were replaced by *Methylococcaceae*, *Methylophaga*, *Methylophilaceae*, Flavobacteria, and Rhodobacterales (Kessler et al., 2011). The succession of bacterial communities during the monitoring period revealed the response of functional bacteria to the oil pollution.

In June 4, 2011, well blowout at the Penglai 19-3 platform in the Bohai bay, China. The incident released large amounts of crude oil (equivalent of 1500 barrels of oil) into the Bohai Bay. Although different strategies have been applied to clean up oil pollution, a substantial portion of the oil is still trapped in the marine ecosystems. Bioremediation using intrinsic microorganisms could be an optional strategy to remediate residual oil. Therefore, the aims of this study were to investigate: (1) the crude oil bioremediation capability of indigenous bacterial communities; and (2) changes of bacterial communities in the process of oil bioremediation.

2. Materials and methods

2.1. Experimental setup for bioremediation of crude oil

Two different microcosm assemblages were set up in this study to conduct the oil bioremediation experiment. For experiment 1, aliquots (2% w/v) of crude oil and 10-g sediments were co-incubated in 100 ml mineral solution (5.6 mM K_2HPO_4, 4 mM $MgSO_4$, 0.5 mM $CaCl_2$, 18.6 mM NH_4Cl, 7 mM $NaNO_3$, 340 mM NaCl, and 2 μM $FePO_4$). To evaluate the effects of surfactant on bioremediation, another assemblage (experiment 2) with additional Tween-20 (1%, v/v) was simultaneously performed (Table 1). Each experiment was carried out in triplicate. Crude oil used in this study was kindly provided by Ocean University of China, while sediments collected from an area adjacent to the Penglai 19-3 oil platform were kindly supplied by the North China Sea Branch of The State Oceanic Administration. Mixtures were incubated at 170 rpm, at 30 °C, for 30 days. Original sediments (S) and samples collected at the end of the incubation for both remediation experiments 1 (R1) and 2 (R2) were kept at −80 °C for further analysis.

2.2. Analysis of crude oil composition

The components of extracted oil hydrocarbons were analyzed by GC–MS (Agilent 7890-5975c) equipped with an HP-5 capillary column (60 m × 0.25 mm × 0.25 μm, Agilent Technologies, USA) (Wang et al., 1995). High-purity helium (99.999%) was applied as the carrier gas (1 ml min^{-1}). The temperature program was conducted as follows: 50–120 °C at 20 °C min^{-1}, 120–250 °C at 4 °C min^{-1}, 250–310 °C at 3 °C min^{-1}, and held at 310 °C for 30 min. Injector and transfer line temperatures were both set at 300 °C.

2.3. DNA extraction and DGGE analysis of bacterial communities

Aliquots (5 ml) of samples collected in the process of bioremediation were centrifuged at 6000*g* to collect microbial flora in the samples. Pellets were then applied for extracting total genomic DNA by using the Mobio Soil DNA Extraction Kit (MoBio Laboratories, Solana Beach, CA). Bacterial communities were analyzed both before and after bioremediation by two culture-independent methods, denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene based clone library community analysis, as described by Wang et al. (Mohamed et al., 2008; Wang et al., 2012).

Briefly, a variable region (~195-bp) corresponding to positions 341 and 534 in the 16S rRNA gene of *Escherichia coli* was PCR amplified from total genomic DNA using primers P2 (5′-ATTACCGCGGCTGCTGG-3′) and P3 (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGG CAGCAG-3′). The PCR amplification was conducted in a 50-μl reaction system that includes 37.8 μl of sterilized distilled water, 5 μl of 10× high-fidelity PCR buffer, 2 μl $MgSO_4$ (50 mM), 1 μl of a mixture of dNTP (2.5 mM each), 1 μl of 100 μM of P2 and P3 primer, 0.2 μl of Platinum® Taq DNA (Invitrogen Life Technologies, Carlsbad, CA) and 2 μl of template DNA. The PCR amplification was conducted as follows: A 5 min initial denaturing period at 97 °C was followed by 29 cycles at 92 °C for 30 s, 52 °C for 2 min, 72 °C for 90 s, and a final extension of 72 °C for 30 min. The DCode system (Bio-Rad, Hercules, CA) was applied for DGGE analysis of bacterial communities. The 6% (w/v) polyacrylamide gel with denaturing gradient from 40 to 70% in 1× Tris-acetate-EDTA was used for separating different bacterial amplicons. Electrophoresis was carried out at 60 V at 60 °C for 16 h. Gel was then stained with SYBR green II for 20 min and visualized with a Molecular Imager® Gel Doc™ XR+ System (BioRad, Hercules, CA). All triplicate samples from each set of experimental conditions were analyzed by DGGE, the results of which indicated that the bacterial communities were identical to each other among triplicate samples in each set of experimental conditions (data not shown). One sample from each set (sediment, R1, and R2) was then selected to run a simple and clear DGGE profile (S&C DGGE) to show the changes of bacterial communities.

2.4. Construction of 16S rRNA gene based clone library

Samples selected for performing the S&C DGGE analysis mentioned above (sediment, R1, and R2) were also applied for