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Change of bacterial community in oil-polluted soil after enrichment cultivation with low-molecular-weight polyethylene

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ABSTRACT

Enrichment cultivation was performed after a seashore soil polluted by crude oil was inoculated into a basal medium with low-molecular-weight polyethylene (LMWPE) powder as the sole carbon source. From the whole DNAs, alkane monooxygenase gene (*alkB*), which has been reported to participate in the degradation of polyethylene, was measured with quantitative real-time PCR to monitor the abundance of polyethylene degrading bacteria in the culture. The change in the ratio of *alkB*/16S rRNA in the culture broth as a function of enrichment cultivation time was measured and used as an index for the ratio of microbes with *alkB* to those in the entire bacterial community in the culture broth. Through 16S rRNA sequence analysis, the bacterial community was analyzed at the genus level. With this technique, the changes in the community of soil microbes and their diversity as a function of enrichment cultivation time were examined. In addition, improvement in the LMWPE degradation ability of the bacterial community due to LMWPE enrichment cultivation was analyzed through biodegradability testing under controlled compost conditions.

1. Introduction

Individual microbes are constantly adapting themselves to their surrounding ecosystem (Kang et al., 2005). The soil is the most complex and vast habitat of microbes on earth. The diversity of prokaryotes in the soil is much higher than in other environments, as there are thousands of microbe species in 1 g of soil with about 2000–18,000 different DNA units (Stefanis et al., 2013). The microbes that have been isolated and identified up to now are very few in number and most of the microbes in nature have not been identified yet (Kang et al., 2005). Most of the microbes in the soil are hard to isolate and to cultivate in the laboratory, but they are very useful in our practical life.

The metagenome method is a very useful technique for the discovery of new microbes and genes that can barely be detected through the conventional isolation method in the laboratory (Singh et al., 2009). With the introduction of next-generation sequencing (NGS) technology, a large amount of DNA sequences can be analyzed, which provides important biological information for the metabolism and phylogenetics of microbes.

Enrichment cultivation is conducted to efficiently search for genes and enzymes with special functions through the metagenome from natural samples such as chemically-polluted environments or habitats exposed to extreme conditions. The process promotes the specific metabolism of microbes in the culture medium as if in the natural environment by providing microbes that are hard to cultivate in the laboratory with special substrates (Davis et al., 2005). Microbes require specific nutrients and chemicals for growth. Therefore, if the microbes are exposed to the necessary nutrients such as carbon or nitrogen sources, they can grow faster, and consequently, specific genes and enzymes can be isolated more efficiently.

Enrichment cultivation can be used to increase the activity and abundance of specific genes, but it may decrease the diversity of microbes in the sample community (Vartoukian et al., 2010). Therefore, enrichment cultivation is used more frequently than direct extraction of DNA from the natural sample in order to search the genes and to isolate bacteria efficiently.

De Vasconcellos et al. (2010) performed enrichment cultivation for 30 days by adding hexadecane as a sole carbon source and selected 5 clones with excellent degradability against hexadecane among 31,000 clone libraries. They reported that the 5 clones degraded hexadecane by more than 70% over 21 days. Tanase et al. (2013) transferred soil polluted by oil into a minimal salts

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cultivation medium (MSM) containing hexadecane or crude oil and performed enrichment cultivation for 21 days. They inoculated it into a medium containing different kind of *n*-alkane (decane, dodecane or hexadecane) and assessed the bacterial growth for 21 days. They observed 4 kinds of bacteria (*Acinetobacter baumanii* MH102, *Achromobacter xylosoxidans* MH18, *Rhodococcus ervthropolis* MH15 and *Burkholderia cepacia* FH103) to grow in the medium and all these bacteria were proved to possess *alkB* (Tanase et al., 2013).

Polyethylene is used for a variety of applications due to its high durability, flexibility, excellent mechanical properties, easy processing and low price. It has the same chemical structure as *n*-alkane and thereby it is highly hydrophobic. The hydrophobicity inhibits microbes to attach to polyethylene surface and thus impedes clustering of microbes and biofilm formation (Orr et al., 2004). Consequently, polyethylene is extremely recalcitrant to degradation in the natural environment, and thereby discarded polyethylene is accumulated in the nature to pollute the ecosystem seriously.

Harshvardhan and Jha (2013) isolated *Kocuria palustris* M16, *Bacillus pumilus* M27 and *B. subtilis* H1584 which can degrade low-density polyethylene (LDPE) from pelagic water. Usha et al. (2011) also isolated 6 bacteria (*Pseudomonas* sp. *Bacillus* sp. *Staphylococcus* sp. *Aspergillus nidulans, Aspergillus flavus*, and *Streptomyces* sp.) which can degrade polyethylene from garbage soil.

Though microbial strains which can degrade polyethylene are very scarce, 16S rRNA sequencing analysis or the metagenome technique can be used efficiently to search for microbial strains that have never been isolated in the laboratory.

Alkane monooxygenase, which is well-known as an alkane degrading enzyme, generates alcohol radicals at the terminal carbon of alkane to induce the first reaction for alkane degradation. It is reported that alkane monooxygenase is manifested by *alkB*, and plays a more important role in the degradation of chlorinated hydrocarbons or other diverse additives (Van Beilen and Funhoff, 2007). The enzymes that degrade polyethylene have not been identified yet, but Jeon and Kim (2015) cloned *alkB* from *P. aeruginosa* E7 isolated as a polyethylene degrading bacterium to confirm that *alkB* actually participates in the degradation of polyethylene. They found that the bacterial strain devoid of *alkB* was inactive at all toward polyethylene degradation. In sharp contrast, the recombinant strain with cloned *alkB* showed high polyethylene biodegradation ability to confirm that *alkB* is an enzyme indispensable to the degradation of polyethylene (Jeon and Kim, 2015).

In this study, the seashore soil from Malipo, Tae-An, Korea, where an oil spill accident occurred 8 years ago was selected as the source of microbes. Enrichment cultivation was performed using LMWPE as the sole carbon source to analyze the change in the bacterial community following exposure to LMWPE. Through quantitative real-time PCR for the universal 16S rRNA of bacteria, the influence of enrichment cultivation on the bacterial community was examined. The change in the bacterial community was also observed through 16S rRNA library construction. In addition, the change in the LMWPE degradability of the soil sample was measured during the enrichment cultivation to check the effect of the bacterial community on the degradation of polyethylene.

2. Materials and methods

2.1. Soil sample

Soil sample was collected 10 cm beneath the seashore soil at Malipo, Taean-gun, Korea, where occurred a serious crude oil spill accident because of collision of a super oil tanker with another vessel 8 years ago.

2.2. LMWPE

LMWPE powder was prepared by thermal degradation of high-density-polyethylene (HDPE) in a strict nitrogen atmosphere. A well shielded reactor was used for the thermal degradation. Weight-average-molecular weight was lowered from 120,000 to 1700. The peaks at ~1650 cm^{-1} and at 1025–1260 cm^{-1} were not observed on the FTIR spectrum of LMWPE confirming the absence of oxidation of PE during the thermal degradation.

2.3. Enrichment cultivation with LMWPE

Changes in the microbial community was monitored after exposing the microbial source soil sample to LMWPE to perform enrichment cultivation according to the method described by Tanase et al. (2013).

15 g of soil sample and 100 ml of basal medium (K_2HPO_4, 2.34 g l^{-1}; KH_2PO_4, 1.33 g l^{-1}; $MgSO_4$ $7H_2O$, 0.2 g l^{-1}; $(NH_4)_2SO_4$, 1 g l^{-1}; NaCl, 0.5 g l^{-1}; yeast extract, 0.06 g l^{-1} and trace element solution ($CoCl_2$, 11.9 mg l^{-1}; $NiCl_2$, 11.8 mg l^{-1}; $CrCl_2$, 6.3 mg l^{-1}; $CuSO_4$, 15.7 mg l^{-1}; $FeCl_3$, 0.97 g l^{-1}; $CaCl_2$, 0.78 g l^{-1} and $MnCl_2$, 10.0 mg, l^{-1}), 1 ml l^{-1}; pH 7.0) were placed in a 500 ml glass bottle along with 3% (v/v) or 5% (v/v) LMWPE for cultivation. For each broth, shake culture was carried out at 30°Cand 200 rpm. Then, 10 ml of existing broth was inoculated into 100 ml of new basal medium containing LMWPE at the same concentration at an interval of 7 days. After 30, 60 and 90 days of cultivation, 10 ml of the culture broth was collected and centrifuged at 15,000 rpm for 10 min to obtain a pellet from which DNAs were extracted with a Power DNA SPIN kit for soil (MP bio). As the control, soil without enrichment cultivation was used, from which the soil DNAs were extracted according to the same method described as above. Three replications were performed for each experiment.

2.4. Quantification of alkB and 16S rRNA in enrichment culture

To identify the change in total bacterial number and amount of *alkB* in the broths before and after the enrichment cultivation, quantitative real-time PCR was carried out with CFX96 (Bio-Rad). GoTaq® qPCR Master Mix (Promega) was used in triplicate according to manufacturer's instruction. The PCR for *alkB* was carried out with primer alkBFd and alkB-r. For the primer, PCR on 16S rRNA was conducted with 518F and 800R. The detailed sequence is shown in Table 1.

PCR program for *alkB* was performed as: the initial denaturation occurred at 95°C for 2 min followed by 35 cycles of denaturation, annealing and elongation at 95 °C for 15 s, at 50 °C for 30 s, and at 72°C for 30 s, respectively. And 15 s at 83°C for data acquisition. Melting curves were checked with a 0.5% heating rate in the range of 60–95 °C. In the real-time PCR program for 16S rRNA, the initial denaturation occurred at 95 °C for 2 min followed by 35 cycles of denaturation, annealing and elongation at 95 °C for 30 s, at 55°C for 40 s, and at 72 °C for 30 s, respectively. And 10 s at 80°C for data acquisition. Melting curves were checked with a 0.5% heating rate in the range of 60–95 °C. The standard curve was drawn according to the method described by Powell et al. (2006) using *P. aeruginosa* E7, in which we already have demonstrated the existence of *alkB* (Jeon and Kim, 2015).

2.5. 16S rRNA library construction

For the extracted soil DNAs, PCR was carried out with 27F, 1492R (Table 1). PCR was performed in the order of initial denaturation at 95°C for 2 min, 35 cycles of denaturation, annealing and elongation at 95°C for 30 s, at 52°C for 40 s and at 72°C for 30 s, respectively,