S8-3

Molecular Analysis of Bacterial Community in Activated Sludge Enriched with 2,4-Dichlorophenolxyacetic Acid (2,4-D) under Different Cultural Conditions

Tae Ho Lee
Institute for Environmental Technology and Industry, Pusan National University
30 Jangjeon-dong, Geumjeong-gu, Pusan, Korea

The herbicide 2,4-D has frequently been used as a model chemical to investigate the evolution and diversity of catabolic genes involved in the degradation of anthropogenic contaminants in the environment. Many of the 2,4-D degrading bacteria isolated from heterogeneous environments such as agricultural soils, sediments, and waste treatment facilities have been obtained by using batch enrichment cultures. The repeated subculturing in 2,4-D rich medium is highly selective, so it often results in the isolation of copiotrophic fast-growing microbial species. Currently it is not clear if isolates obtained using this approach is representative of the phylogenetic and functional diversity of 2,4-D degrading bacterial strains originally residing in the environment. To understand the factors important in microbial degradation of anthropogenic compounds in the environment it is essential to analyze microbial diversity and to detect and quantify the responsible functional genes.

In this study, we employed three different cultural conditions to reflect different scenarios that may occur in the environment using activated sludge as the initial inoculum. (1) A culture sequentially fed a high concentration (300 mg/L) of 2,4-D (HS). (2) A culture continuously fed a low concentration (10 mg/L) of 2,4-D (LC). (3) A serial batch culture in which 1% (v/v) of culture was transferred to a fresh medium containing a high concentration (300 mg/L) of 2,4-D (HB). The HS and LC bioreactors were operated for 3 months and HB was repeatedly transferred for 1 month. The 2,4-D was stably degraded under all the cultural conditions tested. Differential emergence and diversity of bacterial communities from the three different cultures were investigated by coupling molecular analyses based on 16S rDNA with functional genes.

Bacterial communities that developed in the three cultures were examined using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rDNA fragments amplified by PCR (Fig. 1). The richness of 2,4-D degrading isolates obtained by cultivation was also determined and the isolates were compared to the community from which they were isolated by PCR-DGGE. DGGE analysis showed low similarity coefficient values ($\leq$0.35) when compared to the original activated sludge, suggesting that 2,4-D amendment caused a drastic change in the bacterial community. Particularly, HB showed only 6 bands (16-18 bands in the other cultures) and very low similarity coefficient values when compared to the other communities (0.10 to HS, 0.17 to LC, and 0.0 to original sludge). All the PCR-DGGE profile of the 2,4-D degrading isolates had corresponding bands in the community fingerprints from which they were isolated. However, majority of bands in the DGGE profiles did not have a corresponding isolate.

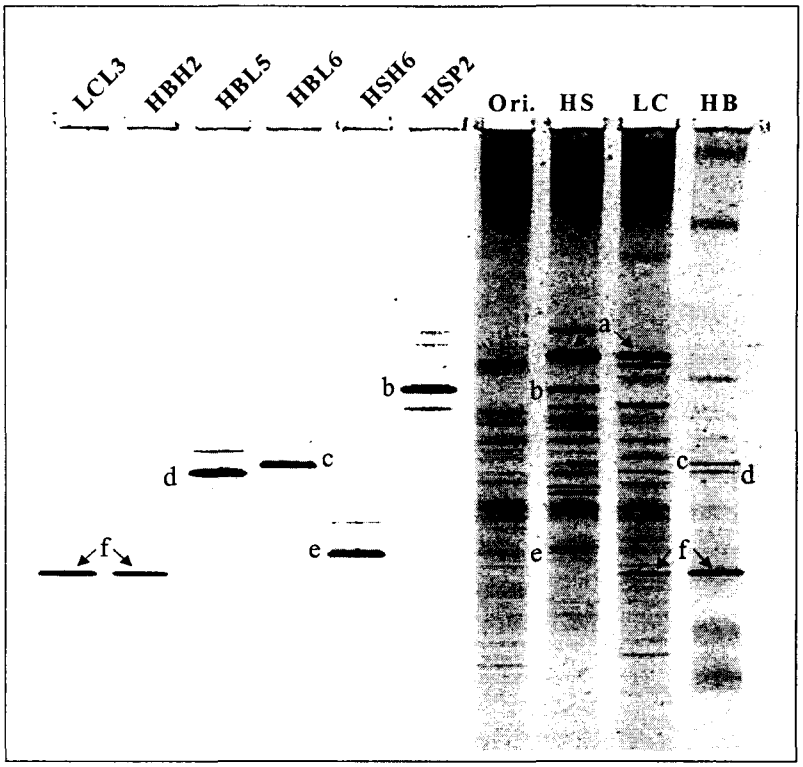

Fig. 1 DGGE profiles (negative image) of PCR amplified 16S rDNA fragments of 2,4-D degrading bacterial isolates (lane 1-6) and bacterial community in the original activated sludge (Ori.) and the three different cultures (HS, LC, and HB).

Currently there are four genes that have been found that can carry out the first step in the degradation pathway. Two are α-ketoglutarate dependent 2,4-D dioxygenases, *tfd*A from *Ralstonia eutrophus* JMP134 and *tfdA* from *Burkholderia* sp. strain RASC. Very recently, *cadAB* genes have been found in *Bradyrhizobium* sp. strain HW13, which is a slow-growing bacterium in the α-subdivision of Proteobacteria isolated from a pristine environment. The *cadAB* genes have no sequence similarity with the *tfdA* gene but has 46% and 44% deduced amino acid sequence identity to the *tftAB* genes, respectively, from a 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) degrading bacterium *Burkholderia cepacia* AC1100. PCR amplification and cloning-based analysis of functional genes using community DNAs from the three cultures revealed five different oxygenase genes that may be involved in the initial step of 2,4-D degradation. All five gene-types were present in HS, while one of the five genes, type V (*tftA*) was not detected in LC. Quantitative PCR analysis showed that in HS, *R. eutropha* JMP134 type-*tfdA* (type I) was the most abundant in copy number ($2.0\pm0.1\times10^7$ copies/μg DNA) followed by RASC type-*tfdA* (type II) ($1.8 \pm 1.0\ 10^6$ copies/μg DNA), putative *cadA*-like gene (type IV) ($2.6 \pm 0.8 \times 10^5$ copies/μg DNA), *cadA* gene (type III) ($1.3 \pm 1.0\times10^4$ copies/μg DNA), and *tftA* gene (type V) ($3.5 \pm 1.1\times10^3$ copies/μg DNA). Similar results were obtained in LC. In contrast, HB contained only type I and type III genes, and the type I gene was five orders of magnitude greater in copy number than the type III gene.

These results indicated that serial batch culturing (HB) resulted in a phylogenetically limited number of 2,4-D degrading bacteria carrying limited catabolic genes whereas more diverse 2,4-D

degraders and catabolic genes were present in HS and LC. Therefore, the approach used for monitoring should be taken into account when one evaluates the population dynamics of contaminant-degrading bacteria at bioremediation sites.

This research was supported by the Japanese Society for the Promotion of Science and conducted by cooperation with Dr. Yoichi Kamagata (National Institute of Advanced Industrial Science and Technology, Japan), Dr. Sinya Kurata (Kankyo Engineering Corporation, Japan), and Cindy H. Nakatsu (Department of Agronomy, Purdue University, US).

References

1. Danganan CE, Ye RW, Daubaras DL, Xun L, Chakarabarty AM (1994) Nucleotide sequence and functional analysis of the genes encoding 2,4,5-trichlorophenoxyacetic acid oxygenase in *Pseudomonas cepacia* AC1100. Appl Environ Microbiol 60:4100-4106
2. Fukumori F, Hausinger RP (1993) *Alcaligenes eutrophus* JMP134 "2,4-dichlorophenoxyacetic acid monooxygenase" is an α-ketoglutarate-dependant dioxygenase. J Bacteriol 175:2083-2086
3. Kamagata Y, Fulthorpe RR, Tamura K, Takami H, Forney LJ, Tiedje JM (1997) Pristine environments harbor a new group of oligotrophic 2,4-dichlorophenoxy acid-degrading bacteria. Appl Environ Microbiol 63:2266-2272
4. Kitagawa W, Takami S, Miyauchi K, Masai E, Kamagata Y, Tiedje JM, Fukuda M (2002) Novel 2,4-dichlorophenoxyacetic acid degradation genes from oligotrophic *Bradyrhizobium* sp. strain HW13 isolated from a pristine environment. J Bacteriol 184:509-18
5. Kurata S, Kanagawa T, Yamada K, Torimura M, Kamagata Y, Yokomaku T, Kurane K (2001) Fluorescent quenching-based quantitative detection of specific DNA/RNA using a BODIPY FL-labeled probe or primer. Nucleic Acids Res 29: e34