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Metagenomic Analysis of BTEX-Contaminated Forest Soil Microcosm

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Abstract A microcosmal experiment using a metagenomic technique was designed to assess the effect of BTEX (benzene, toluene, ethylbenzene, and xylenes) on an indigenous bacterial community in a Daejeon forest soil. A compositional shift of bacterial groups in an artificial BTEX-contaminated soil was examined by the 16S rDNA PCR-DGGE method. Phylogenetic analysis of 16S rDNAs in the dominant DGGE bands showed that the number of *Actinobacteria* and *Bacillus* populations increased. To confirm these observations, we performed PCR to amplify the 23S rDNA and 16S rDNA against the sample metagenome using *Actinobacteria*-targeting and *Bacilli*-specific primer sets, respectively. The result further confirmed that a bacterial community containing *Actinobacteria* and *Bacillus* was affected by BTEX.

Keywords: BTEX, 16S rDNA PCR-DGGE, class-specific PCR

Volatile benzene, toluene, ethyl benzene, and xylenes, commonly referred to as BTEX, are important monocyclic aromatic environmental contaminants. Main sources of BTEX contamination are gasoline leakage from storage tanks, surface spills, and pipeline leaks. Released BTEX tend to volatilize, dissolve in groundwater, attach to soil particles, or degrade biologically. When organic pollutants like BTEX are released into the environment, the function and structure of the pristine microbial communities are generally affected. It is well known that microbial BTEX biodegradation occurs under both aerobic and anaerobic conditions [2, 18]. Therefore, it is reasonable to assume that a microbial species able to degrade BTEX contaminants should flourish in the polluted site.

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To successfully achieve microbial biodegradation in a BTEX-polluted site, it is necessary to understand the microbiological characteristics within the area, such as the indigenous microbial community and its biodegradability [9, 19, 22]. Traditional methods, which are based on microbial cultivation, underestimate considerably the real microbial diversity, whereas modern molecular methods (PCR, cloning, and sequencing) have provided a greater insight into the extent of prokaryotic diversity [4, 10, 16]. The molecular analysis of the 16S rDNA gene is now essential for studies examining the diversity of microorganisms in the environment [7, 13, 17]. For example, amplified PCR products have been applied to denaturing gradient gel electrophoresis (DGGE) [23]. The DNA fragments, which are the same in length but different in nucleotide sequences, are separated on the gel according to their differential melting behavior. Application of the 16S rDNA PCR-DGGE method to the analysis of microbial communities present in a variety of habitats is considered to be a popular tool in microbial diversity studies [12, 23, 24].

Recently, an interest in monitored natural attenuation (MNA) in a chemical-polluted site has been increasing among environmental microbiologists. An adequate characterization and a long-term monitoring plan for the contaminated site are essential for both *in situ* microbial bioremediation and MNA [11]. Although soil contaminated accidentally with BTEX has been used in many case studies as experimental materials [5, 11, 15], examination using uncontaminated forest soil has not adequately been carried out. Thus, the aim of our study was to sequentially monitor the microbial community in artificial BTEX-contaminated soil using molecular techniques, and to assess a BTEX effect on the indigenous community during the study.

A forest around the Korea Research Institute of Bioscience and Biotechnology was selected as a sampling site for construction of a lab-scale microcosm. Soil samples were

Table 1. PCR primers used in this study.

Primer	Sequence	References
968F	5'-AAC GCG AAG AAC CTT AC-3'	[12]
1401R	5'-CGG TGT GTA CAA GGC CCG GGA ACG-3'	[12]
968F-GC clamp	5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCA ACG CGA AGA ACC TTA C-3'	[23]
23S rDNA insert Forward	5'-CCG ANA GGC GTA GBC GAT GG-3'	[6]
23S rDNA insert Reverse	5'-CCW GWG TYG GTT TVS GGT A-3'	[6]
BLS342F	5'-CAG CAG TAG GGA ATC TTC-3'	[3]
907R	5'-CCG TCA ATT CMT TTG AGT TT-3'	[3]

collected in depth of 10 cm at three different locations, mixed together, and homogenized by sieving (2-mm mesh). To mimic a spill of BTEX, 100 g of the soil was treated with 5 ml of BTEX (benzene:toluene:ethyl benzene:*m*-xylene:*p*-xylene=1:1:1:0.5:0.5), and the soil-BTEX mixture was carefully homogenized using a sterile spatula. One microcosm was left untreated as the control. The artificial microcosm was placed in a 100-ml glass beaker, incubated in the dark at 20°C, and sampled periodically after 1, 4, 7, 14, 21, and 28 days.

Sampled soil (5 g) was subjected to direct molecular analysis. Total DNAs were extracted from the soil as previously described [14], and further purified by a Wizard DNA cleanup kit (Promega, U.S.A.). The PCR reaction of bacterial 16S rDNA was performed in 20 μl of reaction mixture (AccuPower HL PCR premix, Bioneer, Korea) containing the purified DNA (10 ng), and 10 pmole each of 968F-GC clamp primer [23] and 1401R primer (Table 1), which target the 16S rDNA 968–1,401 nucleotides, including the variable regions of V6 through V8 [12]. As described by Rosado *et al.* [26], a touch-down temperature cycling program was used for PCR, in which the annealing temperature was initially set at 65°C and decreased by 2°C every second cycle until 55°C, at which temperature 20 additional cycles were carried out. An initial denaturation was carried out at 95°C for 10 min. Melting was carried out at 94°C for 1 min, primer annealing was performed according to the above scheme for 1 min, and primer extension was at 72°C for 3 min. A final extension was carried out at 72°C for 10 min. DGGE of the PCR products was performed using a 16×16 cm 7% (w/v) polyacrylamide gel on the Dcode System (Bio-Rad, U.S.A.) in 7 l of 0.5× TAE buffer. The gradient gel was prepared with 30% to 60% gradients of urea and formamide solution [100% is 7 M urea plus 40% (v/v) formamide]. The gel was run at 60°C and 200 V for 6 h [27], and stained with a Silverstar staining kit (Bioneer).

When the PCR amplicons in size of ~470 nt were separated on DGGE gel and thoroughly examined, a decrease in the band number was unexpectedly observed in BTEX-treated soil in 4 days. After that, however, six bands (BT1 to BT6) detectable by the naked eye newly appeared in 7-day soil, which were not detected at all in the BTEX-untreated control during the entire incubation period (1 to 28 days). In BTEX-treated soil, these band patterns and the DNA concentration of each band remained consistent for up to 28 days (Fig. 1A). The six dominant DNA bands in the DGGE profiles of BTEX-treated soil were purified, re-amplified by PCR under the same conditions as above, and cloned into a sequencing vector. Randomly selected clones were sequenced and analyzed by BLAST programs [1] and the Similarity-Rank program of the Ribosome Database Project (RDP) [20]. The analysis revealed that the 16S rDNA of each of the 6 DNA bands showed high

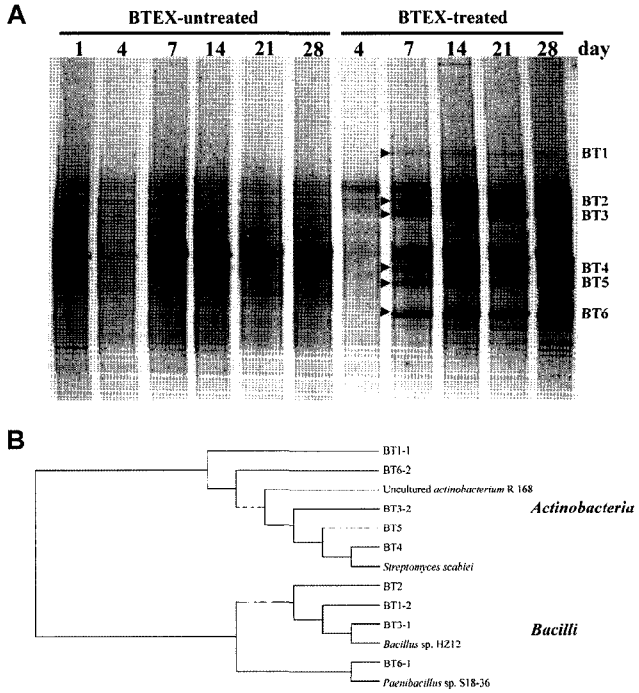

Fig. 1. A. DGGE profiles of bacterial 16S rDNA PCR products (base 968 to 1,401 relative to the *E. coli* rDNA sequence) amplified against the metagenome from BTEX-untreated and BTEX-treated forest soils. **B.** Cladogram tree, which was constructed by the ClustalW program, showing the phylogenetic relationship between bacterial strains in the dominant 6 DGGE bands (BT1 to BT6) from BTEX-treated soil samples.

identity with that of bacterial strains in class *Actinobacteria* (clone BT1-1, BT3-2, BT4, BT5, and BT6-2) and class *Bacilli* (clone BT1-2, BT2, BT3-1, and BT6-1) (Fig. 1B), indicating that these bacterial groups containing *Actinobacteria* and *Bacilli* might be affected by the BTEX mixture.

In order to confirm the DGGE profiles and sequence analysis, we amplified bacterial 23S rDNA and 16S rDNA with two corresponding class-specific PCR primer sets, respectively, against the total DNAs previously extracted from the soil samples. The PCR reaction was performed in 20 μl of reaction mixture (AccuPower HotStart PCR premix, Bioneer) containing the purified DNA (10 ng) and 10 pmole each of primers. For PCR using *Actinobacteria*-targeting primers (23S rDNA insert Forward and 23S rDNA insert Reverse, Table 1), the temperature/time profile used was as follows: an initial denaturation step at 97°C for 15 min, 30 cycles of 30 sec of denaturation at 97°C, 30 sec of hybridization at 55°C, 1 min of elongation at 72°C, and a final 7 min of extension at 72°C. The PCR temperature/time profile used with a *Bacilli*-specific primer set (BLS342F and 907R, Table 1) was an initial denaturation step at 95°C for 10 min, 10 cycles of 30 sec of denaturation at 94°C, 30 sec of hybridization at 58°C, 1 min of elongation at 72°C, additionally 20 cycles of 30 sec of denaturation at 94°C, 30 sec of hybridization at 53°C, 1 min of elongation at 72°C, and finally, an extension step for 15 min at 72°C.

It is generally known that, between positions 4009 and 4120 (*E. coli* numbering) of 23S rRNA genes from high DNA G+C content Gram-positive bacteria, there is an additional 90 to 110-bp insertion [25]. Thus, the insertion (~340 bp) within the central part of the 23S rDNA might be considered as a phylogenetic marker for the detection of Gram-positive bacteria with high G+C contents, similar to *Actinobacteria*. Indeed, as shown in Fig. 2A, a positive control, *Streptomyces coelicolor* KCTC 9005 genomic DNA, produced ~340-bp PCR amplicon in the PCR reactions using the *Actinobacteria*-targeting primers, whereas two negative controls from *Escherichia coli* K-12 and *Bacillus subtilis* KCTC 1021 were able to produce ~240-bp products. As expected, the BTEX-treated soil samples (4 to 28 days) produced ~240- and ~340-bp PCR amplicons, although the 4-day soil sample was able to produce a very weak ~340-bp band. However, all the control samples (1 to 28 days) produced only ~240-bp PCR amplicons. These data indicate that the ~340-bp PCR product is due to the presence of the ~100-bp DNA insert detectable among Gram-positive *Actinobacteria*.

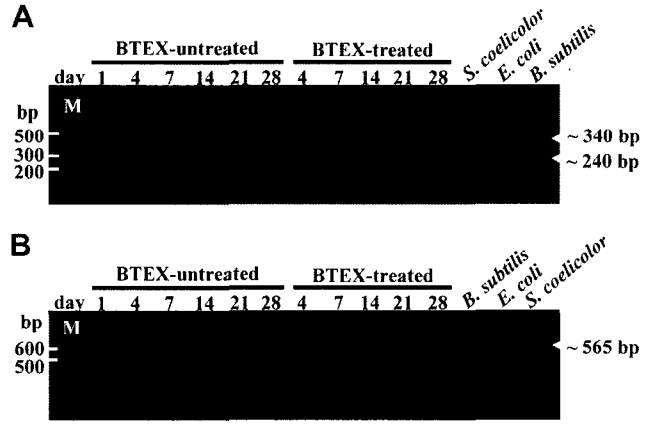

Fig. 2. Agarose gel showing the PCR products amplified against the metagenome from BTEX-untreated and BTEX-treated forest soils, using *Actinobacteria*-targeting (**A**) and *Bacilli*-specific (**B**) primer sets, respectively.

Class *Bacilli* has been known to have a signature region identified between positions 354 and 371 (*E. coli* numbering) in its 16S rDNA sequence [21]. Thus, it is expected that the *Bacilli*-specific signature region can be amplified only in the genomic DNAs from *Bacilli* spp. As seen in Fig. 2B, a positive control, *B. subtilis* KCTC 1021 genomic DNA produced the ~565-bp region in the PCR reaction when the *Bacilli*-specific primers BLS342F and 907R were used, whereas the two negative controls *E. coli* K-12 and *S. coelicolor* KCTC 9005 did not at all. By PCR, we confirmed the results of the DGGE profiles and sequence analysis by showing that the growth of *Bacilli* strains was stimulated by the BTEX mixture, and therefore, the ~565-bp products were amplified in only the BTEX-treated soil samples (4 to 28 days). On the other hand, all the control samples (1 to 28 days) did not produce any PCR amplicons.

Previously, Greene *et al.* [8] characterized the structure of the bacterial community responsible for aromatic biodegradation in an aromatic hydrocarbon-contaminated site, which included high G+C content Gram-positive bacteria, such as *Actinobacteria*, and low G+C content Gram-positive *Bacillus* spp. Wilcoxson [28] has also described a variety of *Actinobacteria* strains present in a petroleum hydrocarbon-contaminated site. Our results obtained through 16S rDNA PCR-DGGE and class-specific PCR methods were in good accordance with those previous experimental data, in that Gram-positive *Actinobacteria* and *Bacillus* spp. are widely distributed in the natural environment and involved in the remediation of soils polluted with hydrocarbons. Although we could not understand any on-going interactions between the microbial members of the soil consortia, we suggest that soilborne Gram-positive bacteria such as *Actinobacteria* and *Bacillus* are related to the BTEX biodegradation in soil.

One of the great challenges in microbiology is to find an efficient way to monitor, and thereby understand, the microbial diversity and community dynamics in a natural environment. If the microbial diversity and dynamics can be adequately monitored, then the direction and rate of processes catalyzed by the environmental microbial communities could be better understood. Our knowledge obtained through these laboratory-

scale experiments is expected to expand the understanding of the *in situ* microbial degradation processes at BTEX-contaminated sites.

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