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Isolation of Xenobiotic-degrading Bacteria and Analysis of Bacterial Community Variation in Soil Microcosm Contaminated with Xenobiotics

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A natural forest soil was contaminated artificially with mixture of tetraethyleneglycol (TEG), 1-amino-2-propanol (APOL), diethylene glycolmonomethylether (DGMME), diethyleneglycolmonoethylether (DGMEE), tetrahydrothiophene-1,1-dioxide (THT), 1-methyl-2-pyrrolidinone (MP), and tetramethylammoniumhydroxide (TMAH) but was not for control test. Both contaminated and uncontaminated soil microcosms were cultivated at 20°C for 10 weeks under humid conditions, which were named the xenobio microcosm (XM) and conventional microcosm (CM), respectively. DNA was directly extracted from both the microcosms every 2 weeks from 2nd to 14th week during incubation. Temperature gradient gel electrophoresis (TGGE) patterns for variable region (V3) of 16S-rDNA amplified with DNA extracted from the XM were changed more variably than those from the IM. The mineralization of xenobiotics in the XM was analyzed every 2 weeks. TEG, DGMME, DGMEE and TMAH were mineralized completely in 16 weeks, and APOL and MP were in 20 weeks. However, THT was not mineralized completely for 20 weeks. TEG-, APOL-, DGMME-, DGMEE-, THT-, MP-, TMAH-degrading bacterium isolated from the XM was *Burkholderia* sp. SK100101, *Burkholderia* sp. SK100102, *Arthrobacter* sp. SK100103, *Dyella* sp. SK100104, *Phyllobacterium* sp. SK100105, *Burkholderia* sp. SK100106, *Acidovorax* sp. SK100107, respectively. These results indicated that the bacterial species that had not been exposed to the specific xenobiotics used in this research could adapt metabolically to the xenobiotics in 14 weeks.

Key words: Bacterial community, Forest soil, Soil microcosm, TGGE, Xenobiotics

1. Introduction

Some xenobiotic compounds are degraded or mineralized by various soil bacterial community[1,2] and the organic compounds added to the bacterial habitat functions as driving force for change of the bacterial community.[3,4] Prokaryotic community composition may be changed in coupling with nutrient variation or resource supply in natural ecosystem.[5-7] The prokaryotic community composed of rich and balanced species is predicted to follow a unimodal curve with respect to resource supply in plant and animal communities.[8] Plant-associated habitats shaped by abiotic and biotic factors may be the causes to modify composition and activity of microbial community.[9] Wieland *et al.*[10] represented that specific species of plants had the greatest effect in plant-associated habitats and also affected soil patterns; however, a plant development had a minor habitat-dependent effect. Nutrient-excessive unrestricted growth, nutrient-limited growth and starvation of microorganisms may be regularly cycled in natural ecosystem.[11] Difference of nutrient availability, growth rate and substrate affinity among microbes can affect microbial community structure.[12]

Microbiological investigation focusing on laboratory microcosm and selective enrichment experiments are useful to analyze bacterial community degrading aromatic hydrocarbons (xenobiotic compounds) under

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redox conditions ranging from nitrate-reducing to methanogenic bacteria.[13,14] Shi *et al.*[15] demonstrated that alteration of microbial communities resulting from anthropogenic perturbation can be inferred from microcosm studies. Soil microcosms allow direct studies of the microbial community diversity and variation in forest soil under experimental condition3). The capacity of bacterial community in forest soil to degrade xenobiotic compound may be induced by long-term adaptation.[16,17] Wagner-Döbler *et al.*[18] found that the microorganisms isolated from the microcosm after 6 months of enrichment showed little taxonomic diversity and moreover, isolates were dominant organisms in all samples.

In this study, we isolated the xenobiotics-mineralizing bacteria by enrichment culture of contaminated forest soil with xenobiotics that are TEG, APOL, DGMME, DGMEE, THT, MP and TMAH, and analyzed the effect of xenobiotics on the bacterial community variation in the microcosm.

2. Materials and Methods

2.1. Xenobiotics

DGMME, TEG, DGMEE, APOL, MP, THT and TMAH were purchased from the Korean branch of Sigma-Aldrich (Yongin-city, Korea). Other chemicals also were purchased from same one.

2.2. Microcosms

Forest soils were obtained from Youmyung mountain national park located in Gapyung-gun (Kyunggi province, Korea). Soil was collected from outer layer containing composted fallen leaves ranging from surface to 15 cm depth in May 23, 2009. Soils collected from 10 points were mixed uniformly using a tilt head mixer (5QT model, kitchen Aid, USA) for 30 min. Each 30 mM of 7 xenobiotics was added to the forest soil based on soil weight. Approximate 100 g (dry weight base) of the uniformly mixed soil was put into a 500 mL-volume of medium bottle, which was plugged with cotton and incubated at 20°C under 65~70% of humidity using a control chamber (CTHC-150P, Biocryogen, Korea). DNA was extracted directly from the CM and XM every two weeks using an extraction kit (PowerSoil DNA isolation kit, MoBio laboratories, USA) according to the manual presented by manufacturer.

2.3. Isolation of xenobiotic-degrading bacteria

Autoclaved cotton swab (length 6 inches) pricked into the soil in CM and XM was smeared on mineral-based agar medium containing an individual xenobiotic every two weeks. The agar plate medium was cultivated at 20°C under 65~70% of humidity to check bacterial colony development for 5 days. Bacterial colonies grown on the agar plate were transferred in mineral-based broth medium containing an individual xenobiotic. The mineral-based medium was composed of 2 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 2 mL/L trace mineral stock solution and 50 mM individual xenobiotic. Medium pH was adjusted to 7 before autoclave. The trace mineral stock solution contained 0.01 g/L of $MnSO_4$, 0.01 g/L of $MgSO_4$, 0.01 g/L of $CaCl_2$, 0.002 g/L of $NiCl_2$, 0.002 g/L of $CoCl_2$, 0.002 g/L of $SeSO_4$, 0.002 g/L of WSO_4, 0.002 g/L of $ZnSO_4$, 0.002 g/L of $Al_2(SO_4)_3$, 0.0001 g/L of $TiCl_3$, 0.002 g/L of $MoSO_4$, and 10 mM EDTA.

2.4. TGGE

The 16S-rDNA amplified from DNA extracted from soil cultures was used as a template for amplification of V3 of 16S-rDNA. V3 of 16S-rDNA was amplified with forward primer (eubacteria, V3 region) 341f 5'-CCTACGGGAGGCAGCAG-3' and reverse primer (universal, V3 region) 518r 5'-ATTACCGCGGCTGCTGG-3'. GC clamp (5-CGCCCGCCGCGCGCGGCGGGCG-GGGCGGGGGCACGGGGGGCCTACGGGAGGC-AGCAG-3') was attached to the 5'-end of the GC341f primer.[19] The procedures for PCR and DNA sequence were same to the 16S-rDNA amplification condition except annealing temperature of 53°C. The TGGE system (Bio-Rad, Dcode™, Universal Mutation Detection System, US) was operated as specified by the manufacturer. Aliquots (45 mL) of PCR products were electrophoresed in gels containing 8% acrylamide, 8 M urea, and 20% formamide with a 1.5×TAE buffer system at a constant voltage of 100 V for 12.5 hr and

then 40 V for 0.5 hr, applying a thermal gradient of 39 to 52°C. Before electrophoresis, the gel was equilibrated to the temperature gradient for 30 to 45 min.

2.5. Amplification and identification of TGGE band

DNA was extracted from TGGE band and purified using a DNA gel purification kit (Accuprep, Bioneer, Korea). The purified DNA was amplified with the same primers and procedures used for TGGE sample preparation, in which the GC clamp was not attached to the forward primer. Species-specific identity of the amplified V3 of 16S-rDNA was determined based on the sequence homology in GenBank database system.

2.5. Mineralization of xenobiotics by isolated bacteria

Individual bacterial strain isolated by using a specific xenobiotic was cultivated in mineral-based medium containing individual xenobiotic. Each bacterial strain was cultivated previously in the medium used for isolation, which was used as an inoculum. Initial xenobiotic concentration was adjusted to 20 mM and bacterial culture was incubated at 25°C for seven days under 150 rpm-shaking condition. Residual xenobiotics in each bacterial culture were analyzed after 7 days-incubation.

2.6. Analysis

Xenobiotic compounds were extracted directly from 5 g of wet soil using 15 g of double-distilled water. Mixture of soil and distilled water was placed in a screw-capped glass tube and shaken at 200 rpm and room temperature for 180 min. Soil was separated by centrifugation at 10,000×g and 4°C for 60 min. The soil separated by centrifugation was dried at 110°C for 48 hr to determine dry weight, which was used to correct concentration of xenobiotic compounds. One sample was analyzed repeatedly three times to correct sampling error based on very small amounts of the sampling volume (1 and 10 μL).

DGMME, DGMEE, NMP, and Sulfolane were quantitatively and qualitatively analyzed by GC-MS based on the modified procedures, which was adapted from technique developed by Shin and Jung.[20] 100 mL of the diluted xenobiotic extraction was placed in a 250-mL separating funnel. About 30 g of NaCl and 100 μL of Ethylene glycol butyl ether internal standard solution (1,000 mg/L) were added to this solution, and the sample was extracted with 10 mL of methylene chloride by mechanical shaking for 20 min. Then the solvent layer was recovered in a flask. One thousand μL of the solvent phase was transferred to a GC vial. At appropriate times, a 1 μL-sample of the solution was analyzed by GC. GC-MS analyses were performed using a PerkinElmer Clarus 600 gas chromatography (GC) interfaced with a mass spectrometer detector (PerkinElmer Clarus 600T mass spectrometer). The GC separation was carried out with Agilent DB-WAXETR capillary column (30 m by 0.32 mm; film thickness, 1 μm) using helium as the carrier gas (flow rate, 1.0 mL min^{-1}). The following GC temperature program was used: injector temperature, 250°C; initial oven temperature, 80°C; rate of temperature increase, 10°C min^{-1} up to 250°C; holding time, 2 min. Samples were injected in a splitless injection mode. The injector was switched to split mode in 2 min after sample was injected.

A high performance liquid chromatography (HPLC; HP1200, Agilent, USA) coupled with ESI-ion trap MS was adopted for the analysis of TEG and APOL. The HPLC apparatus consisted of an Rx-SIL column (3×50 mm, 1.8 μm; Agilent, USA) connected to an LXQ ion trap mass spectrometer (Thermo Scientific, USA) through its ESI interface. A small aliquot of the diluted xenobiotic extract (10 μL) was injected into the chromatographic system. The mobile phase consisted of Methanol/H_2O/Formic acid (80/20/0.05, V/V/V) with flow rate of 0.1 mL/min. The LXQ spectrometer, completely controlled by the Xcalibur software (Thermo Scientific, USA), was operated in the positive ion mode; selected ion monitoring (for an *m/z* of 195.0 and 76.0, respectively, corresponding to the charged ion $(M+H)^+$ of TEG and APOL) modes were recorded for each sample.

3. Results

3.1. Analysis of TGGE patterns

TGGE patterns for DNA extracted from the CM were not greatly changed as shown in Fig. 1A. Unusual DNA bands (number 8, 9, 12 and 13) observed at 6th and 14th week were homologous with bacterial species belong to *Burkholderia* genus, *Bradyrhizobium* sp., *Nitrobacter* sp. and *Deinococcus* sp.. The TGGE patterns for DNA extracted from the XM, meanwhile, were significantly changed as shown in Fig. 1B. Unusual DNA bands (number 3, 4, 8, 9, 11, 13, 14 and 15) observed at 4th, 6th, 8th and 14th week were homologous with bacterial species belong to *Streptomyces* sp., *Beijerinckiaceae* family, *Arthrobacter* sp., *Dyella* sp., *Phyllobacterium* sp., *Burkholderia* sp. and *Acidovorax* sp..

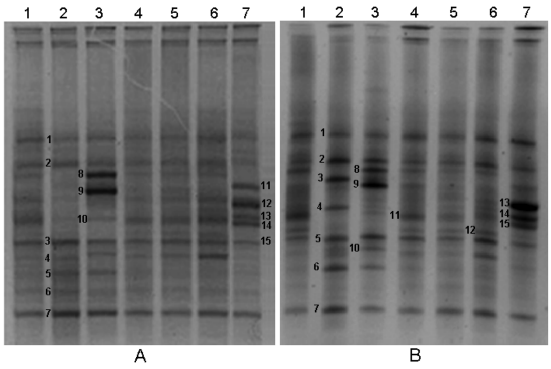

Fig. 1. TGGE pattern of 16S-rDNA variable region (341st bp to 518th bp) amplified with DNA that was extracted from soil culture without (A) and with xenobiotics (B) at 2nd (lane 1), 4th (lane 2), 6th (lane 3), 8th (lane 4), 10th (lane 5), 12nd (lane 6), and 14th (lane 7) week, respectively. The sequence of each DNA extracted from the numbered band in TGGE gel (A) was more than 97% homologous with the bacterial species as follows: 1. Uncultured *Bradyrhizobiaceae*; 2. Uncultured *Rhizobium* sp.; 3. *Chelatococcus* sp.; 4. *Balneimonas* sp.; 5. *Methylobacterium* sp.; 6. *Acetobacter* sp.; 7. *Streptomyces* sp.; 8. *Burkholderia* sp.; 9. *Bradyrhizobium* sp.; 10. *Acetobacter* sp.; 11. *Bradyrhizobium* sp.; 12. Uncultured Nitrobacter sp.; 13. Deinococcus sp.; 14. Acetobacter sp.; 15. Chelatococcus sp. Meanwhile, the sequence of each DNA extracted from the numbered band in TGGE gel (B) was more than 97% homologous with the bacterial species as follows: 1. Uncultured *Bradyrhizobiaceae*; 2. Uncultured *Rhizobium* sp.; 3. *Streptomyces* sp.; 4. *Beijerinckiaceae*; 5. *Chelatococcus* sp.; 6. *Kitasatospora* sp.; 7. *Streptomyces* sp.; 8. *Burkholderia* sp.; 9. Uncultured *Bradyrhizobiaceae*; 10. *Arthrobacter* sp.; 11. *Dyella* sp.; 12. *Phyllobacterium* sp.; 13. *Perenniporia* sp.; 14. *Burkholderia* sp.; 15. *Acidovorax* sp.

3.2. Isolation of xenobiotic-degrading bacteria

When the cotton swabs pricked into soil in CM was smeared on mineral-based agar plate containing an individual xenobiotic from 2nd to 14th week, bacterial colony was not grown but some fungi were grown. Various bacterial colonies, on the other hand, were grown on mineral-based agar plate containing an individual xenobiotic by smearing the cotton swabs pricked into soil in the XM from 2nd to 14th week. However, most of the colonies were not grown when transferred to mineral-based broth medium containing an individual xenobiotic. By more than 10 times-repeated trials, the bacterial species actively grown in the MBBX was isolated. As shown in Table 1, the isolate capable of degrading a specific xenobiotic was registered in GenBank database system.

3.3. Mineralization of xenobiotics by growing cells

All xenobiotic compounds were analyzed separately by GC-MS or LC-MS. No metabolic intermediates were detected from the soil extraction prepared for analysis. Minimal detection concentration of GC-MS and LC-MS for xenobiotic compounds used in this study was

Table 1. Xenobiotic-mineralizing bacteria isolated from the XM during incubation from 4th to 12nd week

Xenobiotics	Bacterial strain of isolates	GenBank Accession number
TEG	*Burkholderia* sp. SK100101	GU552464
APOL	*Burkholderia* sp. SK100102	GU552465
DGMME	*Arthrobacter* sp. SK100103	GU552466
DGMEE	*Dyella* sp. SK100104	GU552467
THT	*Phyllobacterium* sp. SK100105	HM358808
MP	*Burkholderia* sp. SK100106	HM055910
TMAH	*Acidovorax* sp. SK100107	GU552470

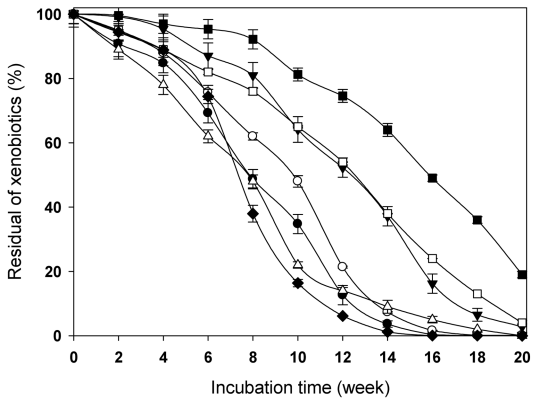

Fig. 2. Time-coursed mineralization of TEG (△), APOL (□), DGMME (◆), DGMEE (●), THT (■), MP (▼), and TMAH (○) in soil microcosm. Each xenobiotic compound was extracted directly from wet soil. Concentration of xenobiotic compound was determined by three times analysis and corrected based on the dry weight of soil samples every time whenever sampled.

0.2 mg/L. All xenobiotic compounds were water-soluble, of which boiling point was minimal 79°C to maximal 314°C. Accordingly, the xenobiotic compounds were not possible to be evaporated at 20°C under humidity-controlled condition. As shown in Fig. 2, most of DGMME, DGMEE, TEG, and TMAH were mineralized for 14 weeks and approximate 80% of APOL and MP were mineralized for 16 weeks. However, about 80% of THT was mineralized for 20 weeks.

3.4. Mineralization of xenobiotics by Isolates

Individual bacterial strain mineralized more than 95% of xenobiotic that was used for bacterial isolation except THT as shown in Table 2 (bold letters). However, *Arthrobacter* sp., *Dyella* sp., *Phyllobacterium* sp., and *Acidovorax* sp. mineralized less than 90% of xenobiotics or did not mineralize. These results show that most of bacterial strains adapted limitedly to specific xenobiotic but *Burkeholderia* sp. adapted broadly to more various xenobiotics than other bacterial strains.

4. Discussion

The xenobiotic compounds can be mineralized completely by microbial consortium but not by a single bacterial population in wastewater treatment reactor,[21-23] which causes bacterial species to be more diverse than the chemical species of xenobiotic compounds.[24] For example, TMAH was mineralized biologically by microbial community adapted to the specific xenobiotic compounds.[25,26] In order to isolate a single bacterial species capable of mineralizing a xenobiotic compound, enrichment of microbial community adapted to the specific xenobiotic compounds is required.[19] The enrichment culture is a useful technique to induce some bacterial species to acclimate to xenobiotic compounds. Acclimation of bacteria on a xenobiotic compound is the result of several mechanisms-inductions of enzymes, mutation of genetic exchange, multiplication of the initially small populations of degrading organisms, and

Table 2. Mineralization activity of bacterial species isolated from soil culture for other xenobiotics that were not used in the process of enrichment and isolation. Initial concentration of each xenobiotic was adjusted to 20 mM and bacterial cultures were incubated for 1 week

Bacterial strains	Mineralization of Xenobiotics (%) (Growth, OD_{660})						
	TEG	APOL	DGMME	DGMEE	THT	MP	TMAH
Burkholderia sp. SK100101	**98±2**	93±1	98±1	93±2	78±2	84±4	98±1
Burkholderia sp. SK100102	98±2	**98±2**	96±2	98±1	64±3	91±2	98±1
Arthrobacter sp. SK100103	98±1	89±3	**99±1**	96±1	X	X	X
Dyella sp. SK100104	99±1	X	98±1	**99±1**	68±4	X	98±2
Phyllobacterium sp. SK100105	93±1	88±4	X	X	**89±1**	63±5	X
Burkholderia sp. SK100106	98±1	91±1	97±1	95±2	73±5	**99±0**	96±2
Acidovorax sp. SK100107	99±1	X	95±2	98±2	X	88±3	**99±0**

X, bacterium was not grown in the xenobiotic-defined medium

syntrophism among bacterial populations.[27-31]

The forest soil may contain various organic compounds originated from fallen leaves, organic compounds extruded from plant roots, various metabolites produced by microbial communities. Plant body, animal body and animal faces may also be accumulated over the forest soil with the lapse of time,[32] by which more stable nutritional condition may be generated and more various bacterial communities may be developed than other natural ecosystem.[33,34] Soil bacterial community was reported not to be changed significantly by exposure to *p*-nitrophenol,[17] benzene-toluene-xylene,[35] 6-aminonaphthalene-2-sulfonates,[36] 2,4,6-trichlorophenol,[37] and isoprene[38] but was changed significantly by shift of soil environment from forest to pasture vegetation.[39] Plant harvesting may also be a cause to change forest soil bacterial community,[40] of which physicochemical effects on forest ecosystems was reported to be resemble the effect of wild fire on the bacterial community variation.[41] Addition of the structurally different xenobiotic compounds that are TEG, APOL, DGMME, DGMEE, THT, MP or TMAH to forest soil may be a physicochemical factor to change bacterial community structure or diversity in general viewpoint. Conversion of forest to pasture, plant harvesting and wild fire cause organic compounds to remove from forest soil; however, the addition of xenobiotic compounds to the forest soil causes soil organic compounds to be diverse.[35] Practically, total TGGE band number for V3 region of 16S-rDNA amplified with DNA extracted from the CM was a little increased; however, TGGE band pattern for XM was different significantly from that for the CM (Fig. 1), which may be caused by the syntrophism[34] in bacterial community multiplied or adapted to catabolize new carbon sources (xenobiotics) in the XM.

5. Conclusion

A pure culture of a bacterial species in the mineral-based medium containing a xenobiotic compound was obtained via the adaptation of the bacterial species to a specific xenobiotic, and the adaptation was accomplished using an enrichment culture of forest soil in the microcosm. The actual diversity of the bacterial community grown in the microcosms cannot be determined via the TGGE technique. The TGGE band number cannot be considered truly reflective of the bacterial population number and community diversity in the microcosm; however, the variation in TGGE band patterns over the course of incubation time and according to the difference in xenobiotics may prove useful in understanding bacterial community variations owing to exposure to xenobiotic compounds. In the current study, we determined that the xenobiotic compounds--APOL, DGMME, DGMEE, THT, MP, and TMAH--can be mineralized by a forest soil bacterial community originating from a natural ecosystem using a microcosm enrichment culture.

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