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Change of Sludge Consortium in Response to Sequential Adaptation to Benzene, Toluene, and *o*-Xylene

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Abstract Activated sludge was sequentially adapted to benzene, toluene, and *o*-xylene (BTX) to study the effects on the change of microbial community. Sludge adapted to BTX separately degraded each by various rates in the following order; toluene>*o*-xylene>benzene. Degradation rates were increased after exposure to repeated spikes of substrates. Eleven different kinds of sludge were prepared by the combination of BTX sequential adaptations. Clustering analyses (Jaccard, Dice, Pearson, and cosine product coefficient and dimensional analysis of MDS and PCA for DGGE patterns) revealed that acclimated sludge had different features from nonacclimated sludge and could be grouped together according to their prior treatment. Benzene- and xylene-adapted sludge communities showed similar profiles. The sludge profile was affected from the point of the final adaptation substrate regardless of the adaptation sequence followed. In the sludge adapted to 50 ppm toluene, *Nitrosomonas* sp. and bacterium were dominant, but these bands were not dominant in benzene and benzene after toluene adaptations. Instead, *Flexibacter* sp. was dominant in these cultures. *Dechloromonas* sp. was dominant in the culture adapted to 50 ppm benzene. *Thauera* sp. was the main band in the sludge adapted to 50 ppm xylene, but became vaguer as the xylene concentration was increased. Rather, *Flexibacter* sp. dominated in the sludge adapted to 100 ppm xylene, although not in the culture adapted to 250 ppm xylene. Two bacterial species dominated in the sludge adapted to 250 ppm xylene, and they also existed in the sludge adapted to 250 ppm xylene after toluene and benzene.

Keywords: Sequential adaptation, BTX, sludge consortium, DGGE, clustering analysis, dimensional analysis

Microbial degradation of benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene (BTEX) has been studied intensively. BTEX can be degraded by microbes aerobically [8] and anaerobically [12, 17]. Many bacterial strains that degrade BTEX, their degradation pathways, and related enzymes have been investigated [22]. Substrate interactions among BTEX have also been studied in order to better understand the behavior of BTEX in the environment [3, 9]. The constituents of BTEX usually coexist in the field, and the biodegradabilities of BTEX constituents are different among microbes. In addition to the various effects of substrates interactions among BTEX in the environment, the effects of methyl *tert*-butyl ether (MTBE) and phenolics on the microbial consortium biodegradation of BTEX have been investigated, because these compounds usually coexist with BTEX [3, 8, 10, 23, 32]. Many scientists have reported on the concurrent effects of BTEX on microbial consortia. However, the effects of subsequent changes of BTEX substrates on microbial consortia have not been reported to date. It is possible that each substrate could have a different toxic effect on the microbial consortium, resulting in a change in the community makeup. Many factors, such as the physicochemical properties of contaminants and the environment, may influence the rate of contaminant movement through soils and in an aquifer system [11]. Therefore, BTEX constituents could be migrated at different velocities and could affect the soil microorganisms in a different sequence, not necessarily at the same time.

In activated sludge reactors and bioremediation of contaminated sites where microbial communities are degrading and treating wastewaters and toxic chemicals, only a subset of the microorganisms are important for the transformation of hazardous materials [26]. Since pollutant fate is largely controlled by the native microbiota, a more complete understanding of community structure and activity should provide for better prediction and process control [17]. Since active microorganisms in the systems are in a dynamic state and most microbes cannot be cultured by conventional culture methods, the molecular biology techniques have been useful to monitor microbial community dynamics and to investigate their activities [17, 26].

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Many fingerprint methods, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism, or clone libraries have gained much recent attention as useful molecular techniques for the analysis of microbial communities [29]. DGGE is a quick and easy method that provides a useful tool for monitoring change in microbial communities, and it can be used to evaluate the generic diversity and dynamics of the community in question [17]. The change in structure of a microbial sludge community associated with BTEX degradation has been investigated using DGGE [1, 6, 7, 13, 17, 25, 33].

The object of this study was to better understand the dynamics of a sludge consortium when BTX substrates were applied in different sequences. After acclimating activated sludge to each BTX compound, it was then adapted to different substrates sequentially, and the community changes were analyzed by DGGE. Cluster analysis and dimensioning techniques were used to explain the DGGE profile. To our knowledge, this is the first time that the effect of subsequent feeding of BTX on a microbial community has been studied.

MATERIALS AND METHODS

BTX Degradation

Activated sludge operated in an aerobic state was obtained from the Jung-nang wastewater treatment plant (Seoul, Korea) and was washed twice with distilled water. Five ml of washed, activated sludge was inoculated to 250-ml flasks containing 100 ml of salt solution. The composition of salt solution was K_2HPO_4 1.55 g/l, $KH_2PO_4 \cdot H_2O$ 0.83 g/l, $(NH_4)_2SO_4$ 2 g/l, $MgCl_2 \cdot 6H_2O$ 100 mg/l, yeast extract 0.2 g/l, and 0.2% (v/v) of mineral salt solution. The mineral salt solution was composed of EDTA 5 g/l, $ZnSO_4 \cdot 7H_2O$ 1 g/l, $CaCl_2 \cdot 2H_2O$ 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 1.25 g/l, $Na_2MoO_4 \cdot 2H_2O$ 0.1 g/l, $CuSO_4 \cdot 5H_2O$ 0.1 g/l, $CoCl_2 \cdot 6H_2O$ 0.2 g/l, and $MnCl_2 \cdot 4H_2O$ 0.5 g/l [20]. The $FeSO_4 \cdot 7H_2O$ solution was added separately. Benzene, toluene, and *o*-xylene (BTX: Sigma-Aldrich Co., U.S.A.) were added to cultures to bring the concentration of each substrate to 50 ppm (w/v) and all cultures were incubated at 25°C, 150 rpm. Abiotic controls were prepared following the same procedure of corresponding cultures except inoculating activated sludge.

Incubating bottles were conical flasks with gas-permeable silicone stoppers for the cultures in an open system to make the condition aerobic during degradation, and cylindrical bottles with chlorobutyl rubber serum stoppers and aluminum caps for the cultures in a closed system to prevent volatile BTX from vaporizing and dissipating into the air. In the closed system, the caps were opened and the cultures were stirred twice a day to maintain the system in an aerobic state.

The analysis of BTX concentration was measured by gas chromatography (8610C for BTEX & Environmental, SRI Instruments, U.S.A.) equipped with a 60 m×0.53 mm capillary column of MXT-VOL, a "purge and trap" blank trap, and a Tenax-GR trap installed. Carrier gas was helium at a flow rate of 10 ml/min, and detected by FID detector (150°C, 25 ml/min hydrogen, and 250 ml/min air). The sample volume was 1 ml, and a linear temperature program from 40°C to 180°C at the rate of 10°C/min with a temperature program for purge and trap was applied.

Sequential Sludge Adaptation

Cultures inoculated by activated sludge were prepared, and BTX at concentrations of 50, 100, and 250 ppm were fed every day to each culture separately for two weeks. Activated sludge samples were taken from each culture for the analysis of the microbial community structure. Three sludge cultures adapted with toluene at different concentrations were transferred to benzene-containing media of the same concentration and adapted for two weeks. After sampling, these were adapted to xylene for another two weeks.

Isolation of DNA and PCR Amplification

Total community DNA from the batch activated sludge at each stage was obtained using an UltraClean Microbial DNA Kit (Mo Bio Laboratories, Inc., U.S.A.). This DNA preparation was used as a DNA template in a PCR performed with 10× PCR buffer (Promega Corp., Madison, U.S.A.) containing 15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100, a 200 μM concentration of each of the deoxynucleoside triphosphates, a 0.125 mM concentration of each of the primers (Bionics Co., Ltd, Seoul, Korea), and 2.5 U of *Taq* polymerase (Promega Corp., Madison, U.S.A.) in a final volume of 50 μl. For amplification of the 16S rRNA gene, the forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') were used. After that, the forward primer 341F (5'- CCTACGGGAGGCAGCAG-3') with a GC clamp (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3') [28] and the reverse primer 907R (5'-CCGTCAATTCCTTTAAGTTT-3') were used in the nested-PCR for DGGE. PCR was performed with a GeneAmp PCR Systems 9700 programmable thermal cycler (Applied Biosystems, U.S.A.) at the following program: an initial denaturation at 95°C for 5 min; 30 cycles of denaturation (45 sec at 95°C), annealing (45 sec at 55°C), extension (1 min at 74°C); and a final extension at 74°C for 5 min. Amplified DNA was verified by electrophoresis of 5 μl of the PCR product on a 1% agarose gel in 1× TAE buffer (20 mM Tris acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM disodium EDTA).

DGGE and Gel Image Analysis

DGGE was performed with the D-Gene system (Bio-Rad Laboratories, U.S.A.) at 70 V and 60°C for 720 min. Samples were loaded on a 6% (w/v) polyacrylamide gel

(37.5:1 acrylamide:*N*,*N*9-methylenebisacrylamide [Bio-Rad]) in 0.5× TAE buffer. The denaturing gradient in the gel was formed by mixing two stock solutions of 6% acrylamide containing 0% denaturing agent and 80% denaturing agent, respectively (5.6 M urea [Sigma Chemical Co., U.S.A.], 32% [v/v] formamide [Bio-Rad] deionized with AG501-X8 mixed-bed resin [Bio-Rad]). The separated DNA was visualized with ethidium bromide stain by the following procedure. First, the gel was removed from the glass plate. The gel was then placed into a dish containing 250 ml of running buffer and 25 μl of 10 mg/ml ethidium bromide (50 μg/ml) for 5 min. After staining, the gel was transferred into a dish containing 250 ml of 1× running buffer and was destained for 15 min. The gel was placed on a UV transilluminator and was photographed (Gel Doc 1000 system, Biorad).

Gel images were converted, normalized, and analyzed by a GelCompar II software package (Applied Maths, Kortrijk, Belgium). DGGE profiles were compared using a band assignment-independent method (Pearson product-moment correlation coefficient and unweighted-pair group clustering using arithmetic averages), as well as a method based on band presence/absence (Jaccard coefficient and Dice coefficient).

The Pearson product-moment correlation tests whether there is a relationship between two or more variables. It is widely represented as the "r factor" in regression and the definition is shown in Eq. (1)

The Pearson product moment=

$$r=\frac{\frac{\sum(X-\bar{X})(Y-\bar{Y})}{N}}{\sqrt{\frac{(X-\bar{X})^2}{N}}\sqrt{\frac{(Y-\bar{Y})^2}{N}}} \quad (1)$$

where X, Y=variables,
$\bar{X}$, $\bar{Y}$ =means of variables, and
N=total number of variables.

The Jaccard coefficient clustering is a similarity coefficient, and the definition is shown in Eq. (2)

$$\text{The Jaccard coefficient}=Nc/(Nq+Nt-Nc) \quad (2)$$

where Nq=the number of bands in the query line
Nt=the number of bands in the target line
Nc=the number of bands that are common to both the query and target lines [24].

The dendrogram was constructed using the unweighted pair group method with arithmetic means (UPGMA) tree building method [21].

In band assignment, a 1% band position tolerance (relative to the total length of the gel) was applied, which indicates the maximal shift allowed for two bands in different DGGE tracks to be considered as identical.

Multidimensional scaling (MDS) and principal components analysis (PCA) were performed with the GelCompar II software package.

Isolation, Cloning, and Sequencing of DGGE Bands

DNA bands in the DGGE gel were cut out with a razor blade, and the DNA of the excised bands was eluted overnight at 4°C in a 1.5-ml tube containing 100 ml of 13 TAE buffer. The DNA was amplified by PCR with primers of both 341F without GC-Clamp and 907R.

To obtain purified DNA, the cloning of the PCR product by the use of pGem-T vector System I (Promega, U.S.A.) and competent cells of ECOS 101, 9-5 (Yeastern Biotech Co. Ltd., Taiwan) was done as follows.

Five μl 2× rapid ligation buffer, 1 μl pGEM-T vector (50 ng), 2 μl PCR product or control insert DNA, 1 μl T4 DNA ligase (3 Weiss units/μl), and deionized water to a final volume of 10 μl were mixed by pipetting to set up ligation reactions. The solution was incubated overnight at 4°C to gain the maximum number of transformants.

The frozen competent cells were thawed at room temperature to obtain a thawed state of approximately 1/3, and the ligation mixture was added immediately to obtain a 5% (v/v) mixture. This solution was vortexed for 1 sec and incubated in a water bath at 42°C for 45 sec. Afterwards, the tube of mixture was vortexed again for 1 sec, and transferred onto chilled, dry selection medium (LB agar media containing 50 μg/ml ampicillin [Sigma, U.S.A.]) and spread with 20 μl 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal 50 mg/ml; Promega, U.S.A.) and 10 μl isopropyl-β-D-thiogalactopyranoside (IPTG, 839 mM, Promega, U.S.A.) by using 4 or 5 glass beads for plating (autoclaved, dried, chilled, 4.0 mm diameter). The plates were incubated at 37°C for 16 h, and the existence of transformed colonies was observed. After harvesting the white colonies from the plates, the colonies were incubated in 5 ml LB broth for 24 h. The plasmid was obtained from the cell using an UltraClean 6 Minute Mini Plasmid Prep Kit (MoBio Laboratories, Inc.). DNA sequences of plasmids were obtained from a forward direction by the biocompany Seoulin Bioscience (Korea).

Phylogenetic Analysis

The partial sequences obtained were compared with available 16S rRNA gene sequences in GenBank by use of the NCBI Blast program. Bacterial sequences closely related to those sequence types or representative sequences from different subclasses were used in the phylogenetic analysis. These sequences were first aligned by use of a sequence alignment editor, the Bioedit program (provided by Tom Hall, Ibis Therapeutics, U.S.A.) and the Clustal X program (provided by the National Center for Biotechnology Information, U.S.A.). A phylogenetic tree with 100 bootstrappings was constructed by use of the neighbor-joining method provided in the MEGA program.

The sequences obtained in this study have been deposited in the GenBank nucleotide sequence databases under accession numbers in parentheses: BTX 1 (EF488235), BTX 2 (EF488236), BTX 3 (EF488237), BTX 4 (EF488238), BTX 5 (EF488239), BTX 6 (EF488240), BTX 7 (EF488241), BTX 8 (EF488242), BTX 9 (EF488243), BTX 10 (EF488244), BTX 11 (EF488245), and BTX 12 (EF488246).

RESULTS AND DISCUSSION

Activated sludge adapted to each BTX at 50 ppm for two weeks. Although the adaptation periods were normally over a month, there was a report that the acclimated microbial consortium could degrade toluene and benzene

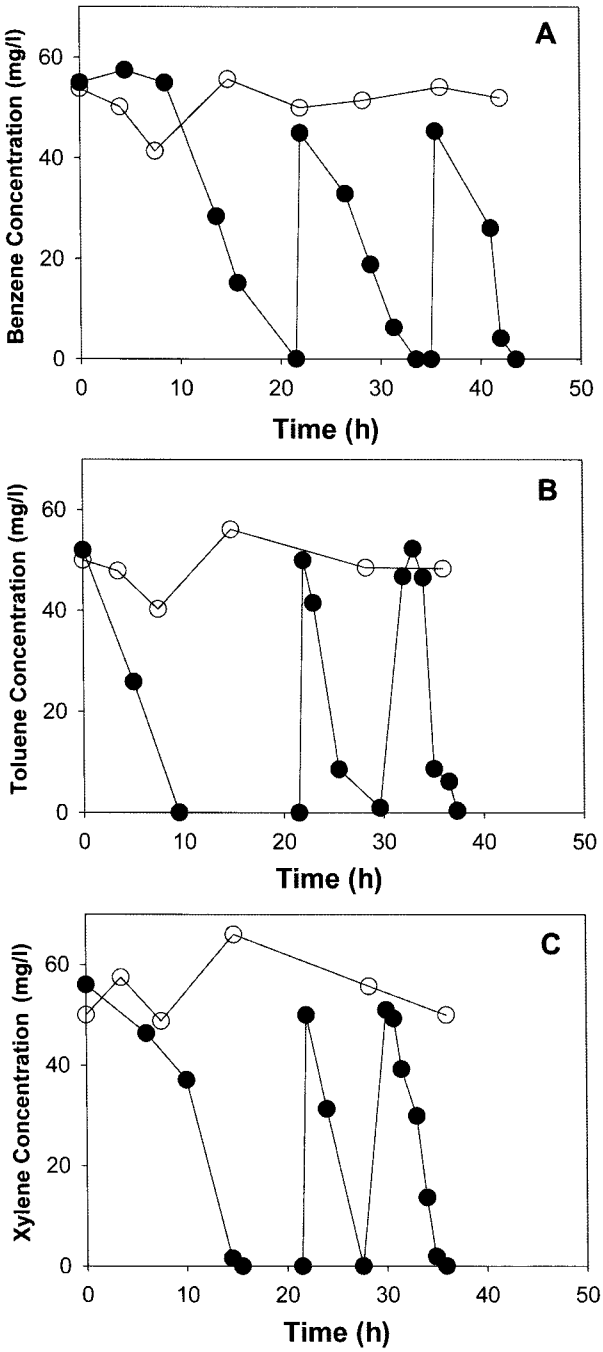

Fig. 1. Degradation of BTX by activated sludge in a closed system.
● Sludge culture; ○ abiotic control. **A**. Benzene; **B**. Toluene; **C**. *o*-Xylene.

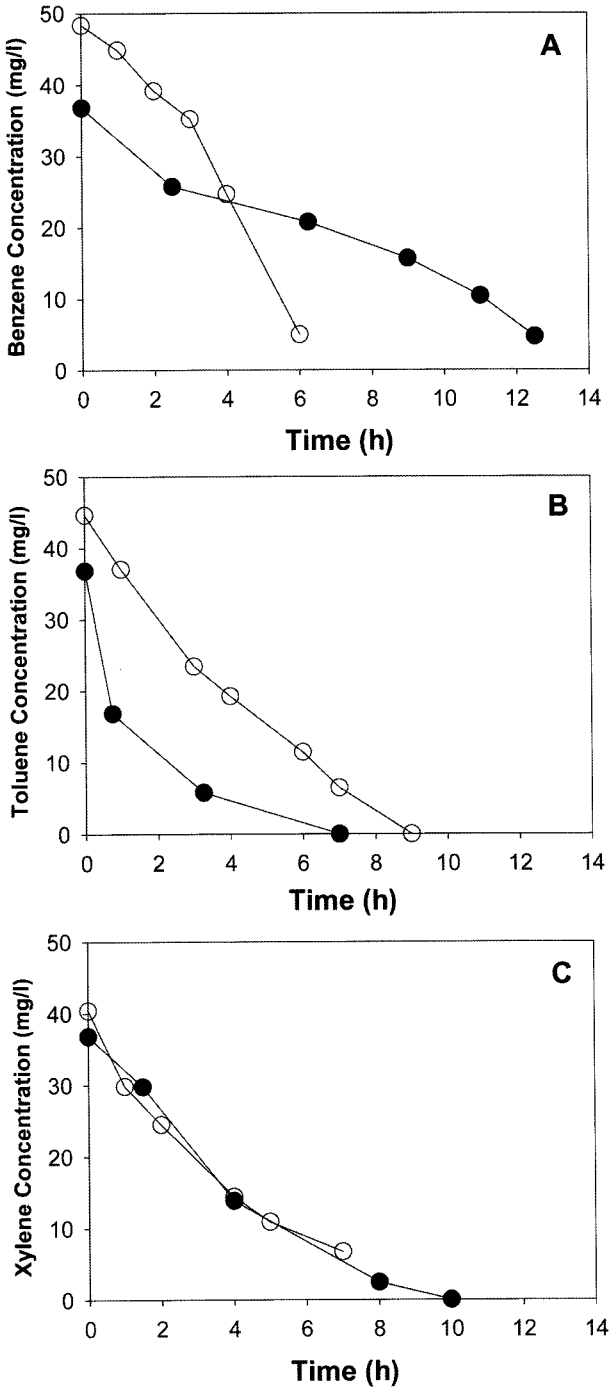

Fig. 2. Degradation of BTX by activated sludge in an open system.
● Sludge culture; ○ abiotic control. **A**. Benzene; **B**. Toluene; **C**. *o*-Xylene.

within a few days after adaptation, even in an anaerobic system [19]. Because our sludges were obtained from a wastewater treatment plant, and therefore had been adapted fully to wastewater, we decided for the adaptation period to be two weeks. The resultant degradation profiles (Figs. 1 and 2) showed that BTX were degraded within a few hours, which confirmed that the adaptation state had been reached. Each BTX was fed as a sole carbon source to enhance the community change. The degradation rates of BTX were measured after two weeks.

As was illustrated in Figs. 1 and 2, the difference in degradation rate between the abiotic control and sludge culture were not distinct in the open system. During degradation of benzene, benzene disappearance in the sludge culture was slower than the abiotic control. The same phenomenon was observed after repeated experiments (data not shown). The reason was thought to be that dispersed sludge inhibited BTX from evaporation by decreasing the contact area with air, and it delayed the time of volatilization in the sludge culture. Moreover, benzene had a long lag time at the first spike, which was shown in the closed system. Benzene was not degraded for the initial few hours by the sludge, and therefore, the reducing rate was slower than for the abiotic control. Toluene had no lag time and its degradation rate was fast. Therefore, the degradation rate of the sludge culture was faster than that of the abiotic control. Since the xylene degradation rate was between that of benzene and toluene the concentration profiles between biotic and abiotic cultures in the open system were almost similar.

In the closed system, toluene was degraded faster than benzene and *o*-xylene. This is a general phenomenon considering the microbial BTX pathway [15]. The first step of aerobic degradation pathways of BTX is the oxidation of the benzene ring to attach a hydroxyl group by monooxygenase and dioxygenase. This reaction changed BTX to catechol-like chemicals, and this catechol-like metabolite catabolized further to enter the TCA cycle. Toluene has one methyl group, and this side group slightly distorted the electron cloud of the benzene ring to become reactive to enzymatic attack. No other long side group that could give steric hindrance to the reaction exists. Besides, benzene has no other side chain, so the ring conformation is perfectly stabilized by resonance. Therefore, ring attack of benzene by an enzyme is very difficult compared with toluene. Xylene has two side methyl groups and these side groups can give rise to the effect of steric hindrance [14, 30]. In the closed system, benzene degradation was the slowest reaction among them. Toluene degradation was completed after 5.5 h at the third successive spike, and xylene and benzene were completed after 6 and 8.5 h, respectively. The completion times were reduced as the number of spikes of substrate was increased for all three compounds. The lag time of benzene degradation was dramatically reduced by the successive spike of substrate. The acceleration by the accumulated enzymes in the liquid media was thought to be the main reason. Toluene and xylene degradation also showed the same phenomena of reduction in lag time.

The nested-PCR was used to obtain a large amount and high concentration of DNA for DGGE. The whole region of the 16S rRNA gene from genomic DNA was amplified and small region PCR with GC-clamp primers using the 16S rRNA gene as template was conducted. The nested-PCR product easily obtained large amounts of DNA and gave a clear DNA band after amplification compared with the direct-PCR from chromosomal DNA.

Eleven different kinds of DNA samples were extracted; original sludge (Sludge), 50 ppm benzene-adapted sludge (Benzene 50), 100 ppm benzene-adapted sludge (Benzene 100), 250 ppm benzene-adapted sludge (Benzene 250), 50 ppm toluene-adapted sludge (Toluene 50), 50 ppm xylene-adapted sludge (Xylene 50), 100 ppm xylene-adapted sludge (Xylene 100), 250 ppm xylene-adapted sludge (Xylene 250), 100 ppm benzene-adapted sludge after 100 ppm toluene adaptation (TB 100), 250 ppm benzene-adapted sludge after 250 ppm toluene adaptation (TB 250), and 250 ppm xylene-adapted sludge after 250 ppm toluene adaptation followed by 250 ppm benzene adaptation (TBX 250).

As is illustrated in Fig. 3, many conspicuous bands were observed. The arrows in Fig. 3 indicate excised bands to identify species, whose results are shown in Table 1.

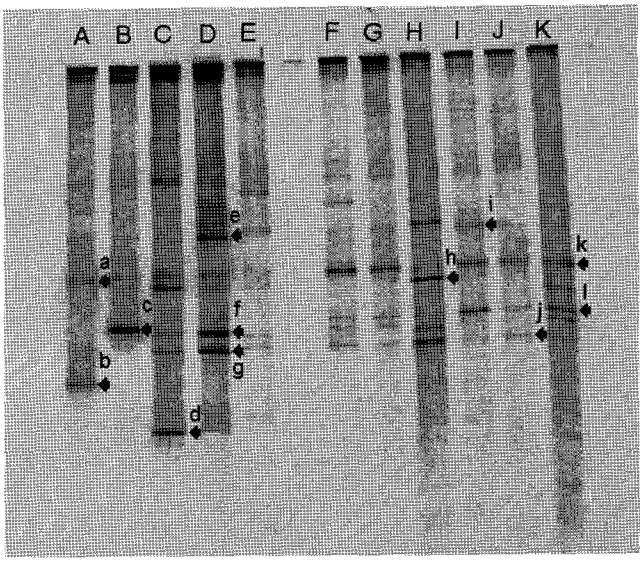

Fig. 3. DGGE profile of BTX-adapted sludges. Experiment conditions were as follows. Denaturing agents: 30%–60%; primer: 341f-GC, 907r.
A. Sludge; B. Xylene 50; C: Benzene 50; D: Toluene 50; E: TBX 250; F: TB 250; G: TB 100; H: Xylene 250; I: Xylene 100; J: Benzene 250; K: Benzene 100. a, BTX1; b, BTX2; c, BTX3; d, BTX4; e, BTX5; f, BTX6; g, BTX7; h, BTX8; i, BTX9; j, BTX10; k, BTX11; l, BTX12.

Table 1. BLAST analysis of 16S rRNA gene sequences from the BTX-adapted sludge samples.

Band name	Lane where band was excised	Most closely related strain (% sequence identity)	Accession Nos. in GenBank of the related strain
BTX 1	Sludge	Uncultured sludge bacterium H10 (98%)	AF234693
BTX 2	Sludge	Uncultured bacterium clone AKIW750 (98%)	DQ129441
BTX 3	Xylene 50	*Thauera* sp. DNT-1 (97%)	AB066262
BTX 4	Benzene 50	*Dechloromonas* sp. PC1 (98%)	AY126452
BTX 5	Toluene 50	*Bacillus* sp. C117 (98%)	DQ091011
BTX 6	Toluene 50	*Nitrosomonas europaea* ATCC 19718 (99%)	BX321856
BTX 7	Toluene 50	Bacterium rJ13 (93%)	AB021331
BTX 8	Xylene 250	Uncultured bacterium clone HP1B33 (99%)	AF502222
BTX 9	Xylene 100	Uncultured bacterium clone J-94 (91%)	AY600930
BTX 10	Benzene 250	Uncultured alpha-proteobacterium clone AKY1687 (93%)	AY921972
BTX 11	Benzene 100	Uncultured *Flexibacter* sp. (93%)	AB076886
BTX 12	Benzene 100	*Bacillus* sp. SA Ant14 (97%)	DQ079058

Clustering analyses were made to ascertain the correlation and similarity between bands.

The binary coefficient analyses of Jaccard and Dice are shown in Fig. 4. In the Jaccard analysis, three categories of DGGE bands were divided around a similarity level of 50%. Although the similarity level of 50% was not so low in clustering bands, it was hard to find common or similar treatment conditions for the communities within each category. From the Dice result, the same groups were found, with a higher similarity level of 70%. Since both methods used similar analysis methods for measuring the similarity based upon common and different bands, the resultant cluster groups were exactly the same.

A few researchers declared that the Pearson product-moment correlation coefficient analysis was affected much less than band-based similarity coefficients by the amount

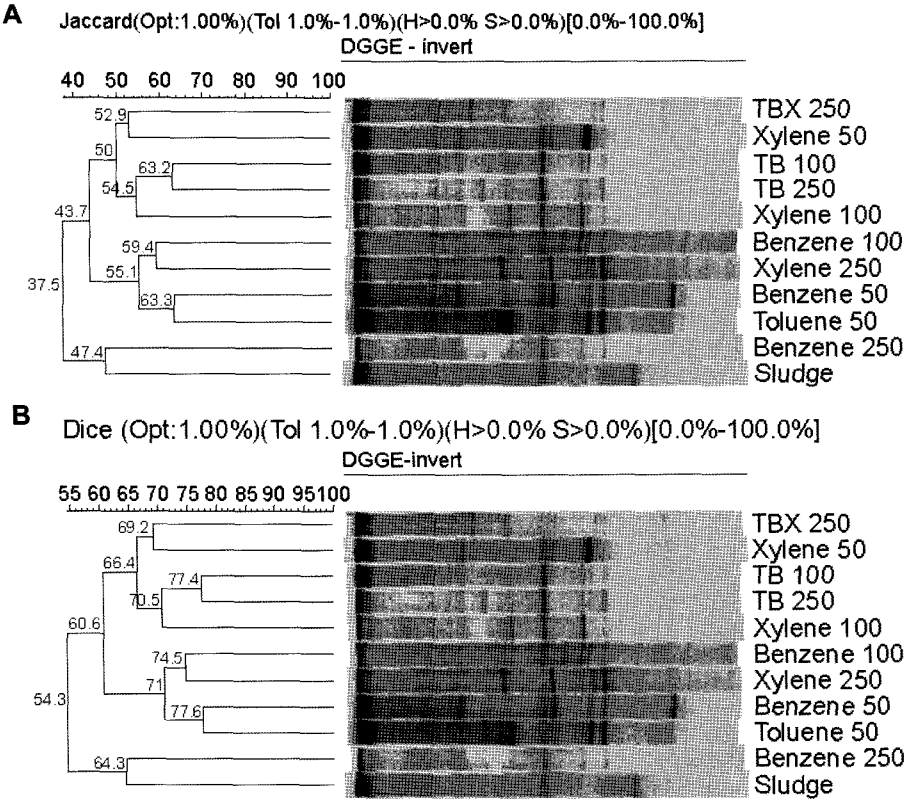

Fig. 4. Cluster analysis of the DGGE gel using GelCompar II.
Band-based similarity coefficient. **A**. Jaccard coefficient; **B**. Dice coefficient. Dendrogram by UPGMA.

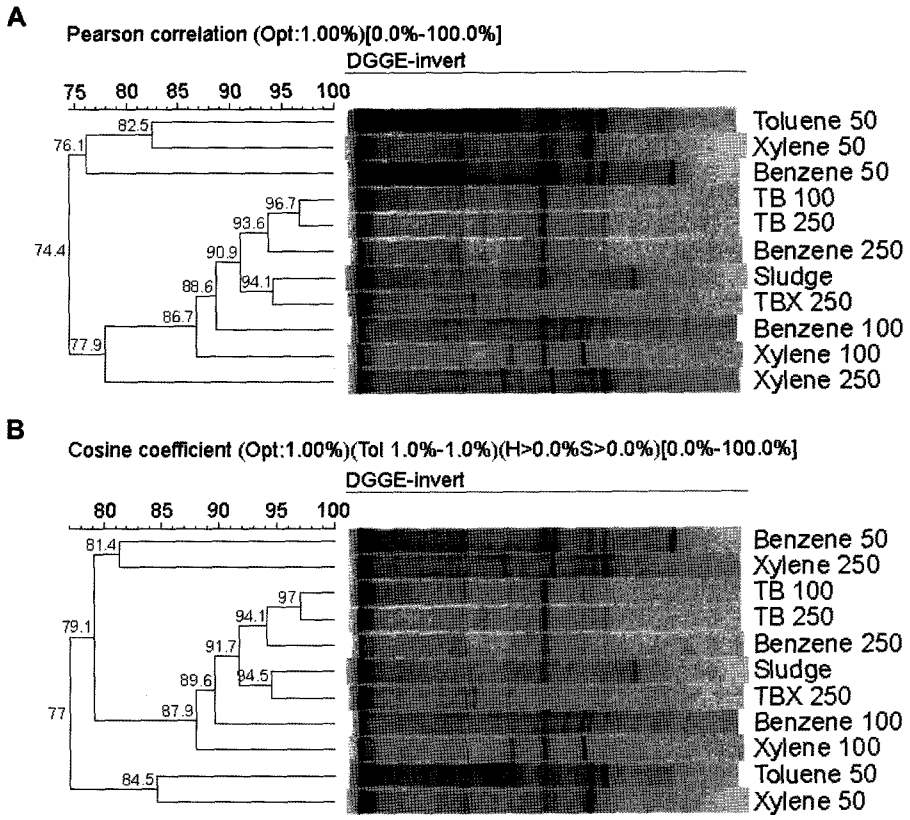

Fig. 5. Cluster analysis of the DGGE gel using GelCompar II.
Densitometric curve-based similarity coefficient. **A**. Pearson product-moment coefficient; **B**. Cosine coefficient. Dendrogram by UPGMA.

of PCR products loaded onto the gel and was a fast, objective method to compare microbial community profiles [16, 18]. This was confirmed by the present research that a higher similarity value and more reasonable clusters were obtained by using the Pearson correlation coefficient and the cosine correlation coefficient, compared with the cluster analyses based on band absence or presence only, such as the Jaccard and Dice coefficients. In the Pearson correlation coefficient in Fig. 5, two different clusters were revealed. One mainly consisted of the communities that had been adapted at low BTX concentration (50 ppm), and the other consisted of the communities at high (over 100 ppm) BTX concentration. In the lower part cluster of Fig. 5, the communities acclimated to benzene gave profiles with similarity values over 90%. From this analysis, the microbial communities adapted to benzene directly, and the one adapted to benzene after toluene adaptation could be grouped together, although Benzene 50 was not so closely related with the others. The communities from xylene adaptation also showed high similar values of over 85% similarity. However, the hierarchical structures by the Pearson product coefficient and by the cosine product coefficient were different for the xylene-related consortia in the positions of Xylene 50 and Xylene 250. The community profiles themselves in DGGE also gave a different configuration of bands. The intense band in DGGE represents numerical dominance in the microbial community. Although numerical dominance does not necessarily reflect catabolic activity dominance [24], these intense bands presumably represented active species catabolically in this study, since each community had been started from the same sludge and affected by various selection pressures.

MDS and PCA are dimensioning techniques, which produce two- or three-dimensional plots in which the entries are spread according to their relatedness (GelCompar II manual, Applied Maths BVBA, Sint-Martens-Latem, Belgium). By using MDS and PCA analyses, the different data of the complex DGGE patterns of one sample could be reduced to one point in a three-dimensional space. MDS does not analyze the original data set but the matrix of similarities obtained using a similarity coefficient. The bars shown within MDS in Fig. 6 were drawn according to the dendrogram of the Dice coefficient, because the similarity coefficient matrix used in MDS was the same as the one used in the Dice calculation. However, the relationships between each lane were different. The consortia from benzene adaptations were closely related to each other, except for the one acclimated to 250 ppm benzene, and xylene-adapted ones were close in the space, although they did not hold or share a position on a point in the space. The

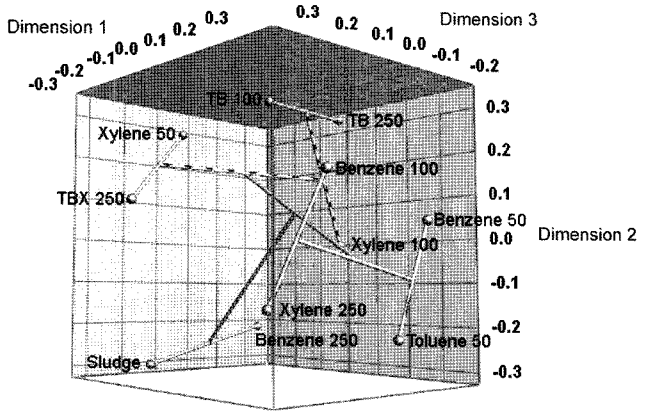

Fig. 6. Multidimensional Scaling (MDS) of the DGGE gel lane.
Similarity coefficient-based dimensional analysis.

toluene-adapted sludge was slightly far from both the benzene and xylene ones, and the original sludge was located far away from the others. This analysis was hard to interpret because of its characteristics of complexity. However, the relation between each component was more accurate compared with the dendrogram analysis, because a dendrogram often oversimplifies the data available in a similarity matrix, and tends to produce overestimated hierarchies (GelCompar II manual, Applied Maths BVBA, Sint-Martens-Latem, Belgium), and these characteristics were revealed in our study.

A PCA analysis is different from MDS, in that the data are directly analyzed. Fingerprint types can only be processed by generating a band-matching table first in PCA [4]. The table is made with information of band presence and absence, together with a quantitative value such as band intensity, which was done previously in the clustering

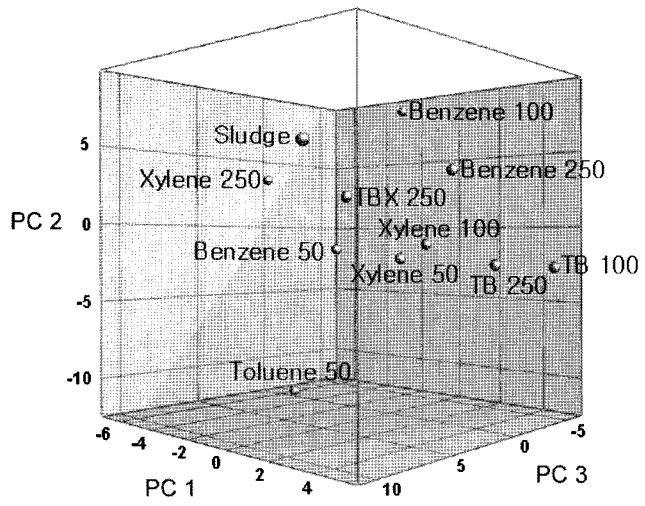

Fig. 7. Principal Components Analysis (PCA) of DGGE gel lane.
Relative contribution of PCs. PC 1=20.5%, PC 2=16.7%, PC 3=13.9%.

analysis in our case. As is illustrated in Fig. 7, the PCA result was similar to the MDS result. The sludge consortium was located far from the others, which was the same result as the cluster analysis and MDS. Toluene 50 was located very far from the others. Consortia that were acclimated only to benzene at different concentrations were separated at one corner in the space. Moreover, Xylene 50, Xylene 100, Xylene 250, and TBX 250 were closely related along X axis (primary PCA axis), and TB 100 and TB 250 were also closely related and close to Benzene 250 in positions already clustered at Pearson analysis.

All clustering analyses and dimensioning techniques did not show the same results for one image. However, some common results could be derived from these analyses. A similar consortium could be obtained if the sludge followed the same adaptation procedure, which was obtained from TB 100 and TB 250. The final adaptation substrate gave a similar consortium that was deduced from the similarity between benzene-adapted consortia, between xylene-adapted ones and TBX 250, and between benzene ones, TB 250, and TB 100. Toluene-adapted ones were not similar to benzene or xylene ones. We thought that xylene ones could be very different from benzene ones, but this conclusion was hard to be derived because xylene ones were not correlated closely and sometimes were located close to benzene or toluene ones. Benzene 250 and TBX 250 were vague in band clarity, which changed positions in a few analyses.

DGGE data need to be complemented by phylogenetic analysis, as was done in this study. DNA sequencing from the excised band was accomplished by direct sequencing or T-vector cloning sequencing. The result of the band sequencing analysis is shown in Table 1.

In the Sludge lane, many bands were observed and two conspicuous bands were sequenced as a sludge bacterium and an uncultured bacterium. After adaptation to benzene, the BTX 4 band that was closely related to *Dechloromonas* sp. was dominant at 50 ppm and became vague at a high concentration. BTX 11 was closely related to *Flexibacter* sp. and was seen at almost every lane as a main active microbe, especially in the lanes of Benzene, TB, and Xylene 100. BTX 12 was revealed as a *Bacillus* sp. and was seen in the lanes of benzene and xylene and was dominant in the Benzene 100 and Xylene 100 lanes.

BTX 6 and BTX 7 were both considered as main microbes in the Toluene 50 lane. These two bands were also shown at other benzene-related lanes, but were less distinct than the toluene one. These *Nitrosomonas* sp. (BTX 6) and bacterium (BTX 7) could be considered as toluene-degrading species and have potential to degrade benzene or metabolite of benzene. BTX 10 in Benzene 100 positioned at the same migration length as BTX 7 was revealed as an *alpha*-proteobacterium.

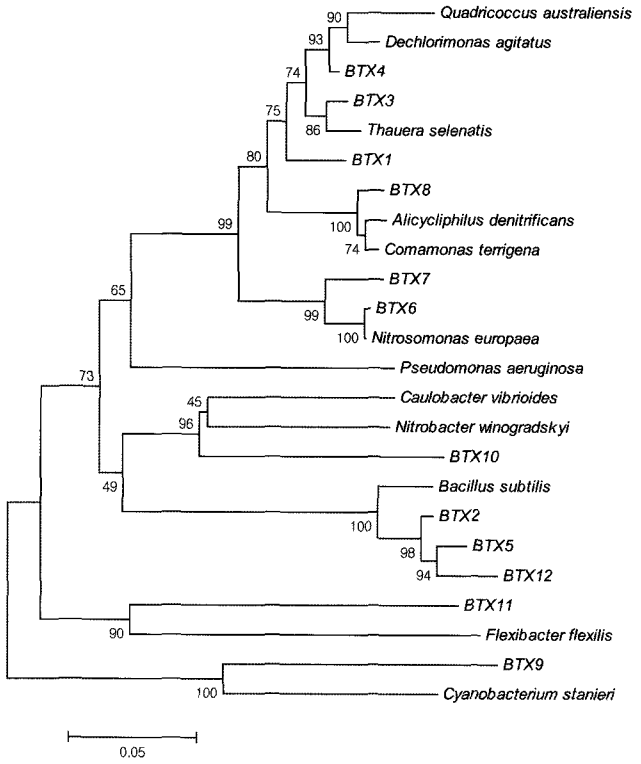

Fig. 8. A parsimony tree with bootstrapping. Bootstrap numbers are written on the tree.
The scale bar indicates the number of nucleotide substitutions per site.

Considering xylene-related lanes, BTX 3, which was closely linked to *Thauera* sp., was dominant at the Xylene 50 lane and could be seen at the Xylene 100 lane. Since *Thauera* sp. had been reported previously to be able to degrade toluene at denitrifying conditions [31], this species might have the ability to degrade xylene. At the Xylene 250 lane, two bacteria (BTX 8 and the band corresponding to BTX 7), were becoming dominant, and these species were also shown at the TBX 250 lane. It was uncertain whether these species were xylene degrading microbes, because they are not dominant microbes in low xylene concentration. The consortium change from 50 ppm to 250 ppm was obvious but abrupt, although the same bands were still observable in the TBX 250 lane.

The phylogenetic tree using these sequential analyses is shown in Fig. 8.

According to general ecological principles, a more extreme environment is expected to be less diverse. Therefore, the number of different groups is willing to decrease after adapting to toxic chemicals, and to produce similar and closely related sequences (microdiversity). However, these sequences may easily result in heteroduplex formation [2, 5]. Several heteroduplexes were revealed in our experiment and they were discarded.

From this study, bacterial communities are acclimated differently by each BTX and presumably acclimated to the similar community when the same substrate was fed irrespective of the concentration. Moreover communities having different adapting paths to the same substrate showed the similar microbial consortium.

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