Isolation of Effective 3-Chlorobenzoate-Degraders in Soil Using Community Analyses by PCR-DGGE

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The screening of pollutant degraders by relying solely on cultivation techniques such as liquid enrichment often fails to isolate the actual degraders in the environment. Community analyses by PCR-denaturing gradient gel electrophoresis (DGGE) were performed to isolate bacteria that can degrade 3-chlorobenzoate (3CB) effectively in soil. A forest soil sample was repeatedly dosed with 3CB (500 mg kg⁻¹) to enrich it with indigenous 3CB-degraders, and changes in the bacterial community were monitored by PCR-DGGE of the 16S rRNA gene and benzoate 1,2-dioxygenase alpha subunit gene (*benA*). Initially, it required about 3 weeks to degrade 3CB in the soil, whereas it took only 3 days after the third dose. With this accelerated degradation, several intensified bands appeared in the DGGE profiles of both 16S rRNA gene and *benA*. We succeeded in isolating five 3CB-degrading *Burkholderia* strains corresponding to these bands by direct plating, while most of them were eliminated by liquid enrichment. Inoculation of the strains into the soil demonstrated that the five strains could degrade 3CB effectively in the soil. This study clearly shows significant bias during the liquid enrichment process and the advantage of using PCR-DGGE in screening effective degraders under environmental conditions.

Key words: PCR-DGGE, 3-chlorobenzoate degrading bacteria, soil, isolation

Discovering microorganisms capable of degrading target pollutants effectively is the most important step in bioremediation. To isolate such degraders, conventional cultivation techniques, typically liquid enrichment, have often been employed. This technique is a simple and efficient way to find target degraders in highly complex microbial communities in environmental samples. However, the degraders obtained by this method do not necessarily represent the actual degrader populations under environmental conditions, which are quite different from culture conditions^{7,39}. In other words, most degraders that function effectively under natural conditions would be overlooked if the screening strategy relied only on such culture methods.

In the past decade, “culture-independent methods” based on analyses of molecular markers such as 16S rRNA genes have been used extensively in the field of environmental microbiology^{1,26,36}. These methods enable one to understand microbial communities under natural conditions more directly without critical cultivation bias. PCR-denaturing gradient gel electrophoresis (DGGE) and PCR-temperature gradient gel electrophoresis (TGGE) are among the most established methods for molecular community profiling²⁷. Until now, the impacts of various pollutants on microbial communities in environmental samples have been studied by PCR-DGGE/TGGE, and many studies indicated changes in the community or dominance of certain members following pollution^{8,23,32,37}. Such observations are helpful to find key members involved in the degradation of pollutants under environmental conditions. However, to demonstrate the actual contribution of certain members found in the DGGE/

TGGE profiles, it is necessary to isolate them as substantial culture strains. In some studies, such key strains were isolated from relatively simple microbial communities by DGGE/TGGE profiling with culture methods^{18,34,38,39}. However, from highly complex communities such as those in soils, very few studies have succeeded in isolating such strains based on DGGE/TGGE profiles.

The main objective of this study is to isolate 3-chlorobenzoate (3CB)-degrading bacteria that can work effectively in soil, using a combination of PCR-DGGE and culture methods. Chlorobenzoates can be generated from the biodegradation of polychlorinated biphenyls¹¹, and 3CB has been most frequently used to study the bacterial degradation of this group of compounds. Up to now, 3CB-degrading bacteria have been isolated from environmental samples based on their 3CB-degrading ability in culture media^{4,6,12–16,20,29,31}, but there have been few attempts to isolate these degraders based on their ability in the environment. Previously, we showed that several *Burkholderia*-like bacteria became dominant in a forest soil after 3CB was added, by PCR-DGGE targeting the 16S rRNA gene and benzoate 1,2-dioxygenase alpha subunit gene (*benA*)²⁵. This time we aimed to isolate such bacteria in order to demonstrate their contribution to the degradation of 3CB in soil. For this purpose, the soil was repeatedly dosed with 3CB to enrich it with indigenous 3CB-degraders, and 3CB-degrading bacteria were collected from the soil by direct plating, which was expected to have less bias than liquid enrichment. In parallel, changes in the bacterial community following the 3CB treatments were monitored by PCR-DGGE. Based on the DGGE profiles, the bacterial collection was screened to identify the enriched strains in the 3CB-dosed soil. Finally, the strains identified as the enriched populations were inoculated into the soil to evaluate their 3CB-

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degrading ability in the soil.

Materials and Methods

Soil

Soil was obtained from the surface horizon of the conserved forest in the National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki prefecture. Before the experiments, the field-moist soil was passed through a 2-mm sieve and pre-incubated at room temperature for 3 days to acclimatize the microbial communities to the experimental conditions. The physicochemical properties of the soil were described in our previous report²⁵.

Accumulation of 3CB-degrading bacteria in the soil

To concentrate the 3CB-degrading bacteria, 292.5 g of the soil was put into a glass jar (900 mL capacity) and mixed with 7.5 g of Celite[®] (Wako Pure Chemical Industries, Osaka, Japan) that had absorbed 15 mL of 3CB stock solution (10 mg mL⁻¹ in methanol), resulting in a final concentration of 500 mg kg⁻¹ 3CB in the soil. Celite[®] was used to ensure even mixing of 3CB with the soil, and the methanol in the Celite[®] was completely evaporated before the mixing. The soil was incubated at 28°C for 38 days with a moisture content of 40%. Soil subsamples were collected at days 0, 7, 14, 21, 28, 35, and 38 for the quantification of 3CB and extraction of DNA. Based on the consumption of 3CB during the incubation, additional 3CB (500 mg kg⁻¹) was added at days 21 and 35. As controls, autoclaved soil (at 121°C for 1 h) with 3CB (a single dose) and soil without 3CB were also prepared in the same manner as above.

HPLC analysis for quantification of 3CB

Soil subsamples (2 g in triplicate) from each microcosm were suspended in 18 mL of 0.025 M NaOH. The suspensions were subjected to sonication for 5 min and shaking on a horizontal shaker for 1 h. The resulting slurry was centrifuged at 9,800×g for 5 min. Then, the supernatant was filtered with a 0.2-μm pore-size membrane filter and subjected to HPLC using an HP1100 system (Agilent Technologies, Palo Alto, CA, USA) with an XDB-C18 column (Agilent Technologies). The mobile phase was an isocratic mixture of acetonitrile: 0.1% H₃PO₄ (1:1, v/v) delivered at a flow rate of 1 mL min⁻¹. The column temperature was held constant at 36°C, and the absorption was measured at a wavelength of 200 nm.

Media

Soil extract agar was prepared as follows. The forest soil mixed with distilled water (five times the weight of soil) was autoclaved at 121°C for 20 min and left overnight for the soil particles to precipitate. The supernatant was filtered using filter paper No. 131 (Toyo Roshi Co. Ltd., Tokyo, Japan) and adjusted to pH 6.8 with KOH solution. To make the agar medium, K₂HPO₄ and Bacto-Agar (Difco Lab., Detroit, MI, USA) at a final concentration of 0.02% and 2%, respectively, were added to the extract. After autoclaving at 121°C for 20 min, the medium was supplemented with the filter-sterilized 3CB stock solution (100× concentrated and neutralized with NaOH) to a final concentration of 500 mg L⁻¹.

The basal salts medium²⁹) consisted of the following components (per liter of distilled water): (NH₄)₂SO₄, 1.1 g; K₂HPO₄, 2.29 g; KH₂PO₄, 0.9 g; MgSO₄·7H₂O, 0.1 g; MnSO₄·4-6H₂O, 0.025 g; FeSO₄·7H₂O, 0.005 g; and L-ascorbic acid, 0.005 g. As a sole carbon source, 3CB was added to the medium to a final concentration of 500 mg L⁻¹ as described above. For preparing the agar plates, 2% purified agar (Difco) was added to the medium.

Isolation of bacteria from the 3CB-dosed soil

Direct plating on the soil extract agar (see Media) was used to isolate bacteria from the 3CB-dosed soil. We sampled 5 g of the soil after 38 days and made a 10-fold dilution series with 10 mM sodium phosphate buffer (pH 6.8). Triplicate soil extract agar plates were inoculated with 100 μL from each of the 10⁻⁵ and 10⁻⁶ dilutions and incubated at 28°C for 10 days. The resulting single colonies were

picked at random and transferred onto new plates to ensure their purity.

Liquid enrichment was also used. Triplicate flasks containing 99 mL of the basal salts medium (see Media) were inoculated with 1 g of the 3CB-dosed soil and incubated with rotary shaking (180 rpm, 28°C). After the first incubation for 2 days, 1 mL of culture taken from each flask was transferred to 99 mL of the new medium and incubated for a further 2 days. After the second incubation, 0.5 mL of the culture was serially diluted with the sodium phosphate buffer. Bacterial isolates were collected from the dilutions by plating on the soil extract agar as described above. During the liquid enrichment, a small portion from each culture was sampled to extract DNA for PCR-DGGE.

The isolates obtained by these methods were incubated on R2A agar (Difco) supplemented with 500 mg L⁻¹ of 3CB at 28°C for 4 days. After the incubation, the bacterial cells were collected from the plates for DNA extraction (see DNA extraction). In addition, the isolates were inoculated onto the basal salts agar (see Media) to briefly examine their ability to utilize 3CB.

DNA extraction

For PCR-DGGE of the soil microcosms, DNA was extracted from 0.4 g of duplicate soil subsamples using a FastDNA[®] spin kit for soil (Qbiogene, Inc., Irvine, CA, USA) and FastPrep[®] instrument (Qbiogene), for 30 sec at a speed setting of 5.5. To improve DNA recovery efficiency, 16 mg of skim milk (Difco) was added to 978 μL of sodium phosphate buffer (included in the kit) before 0.4 g of soil was suspended in the buffer¹⁷). Further procedures followed the manufacturer's instructions, and DNA was eluted using 80 μL of the DNA elution buffer included in the kit. To remove the remaining humic substances from the DNA solution, the samples were further purified using a DNA Clean & ConcentratorTM-25 (Zymo Research Corp., Orange, CA, USA). DNA extraction from the liquid enrichment culture was performed using a standard miniprep procedure⁴⁰). DNA extraction from the bacterial isolates was performed using a Puregene DNA Isolation Kit (Gentra Systems, Inc. Minneapolis, MN, USA) according to the instruction manual.

PCR conditions

All PCR amplifications were performed in an iCyclerTM (Bio-Rad Laboratories, Hercules, CA, USA) with KOD-Plus (Toyobo, Osaka, Japan) DNA polymerase. Two primer sets were used for PCR-DGGE of bacterial communities in the soil and liquid enrichment cultures. The same sets were also used for the analyses of bacterial isolates.

The first set targeting the V3–V5 region of bacterial 16S rRNA genes consisted of GC338f (5'-ACTCCTACGGGAGGAGCAG-3', attached with a GC clamp³⁰) and 907r (5'-CCGTCAATTC-CTTTRAGTTT-3')²¹). The PCR mixtures contained 5 μL of 10×PCR buffer for KOD-Plus, 0.2 mM of each dNTP, 1.5 mM of MgSO₄, 1 μL of dimethyl sulfoxide (DMSO), 0.2 μM of each primer, 1.0 U of KOD-Plus DNA polymerase, and 1 μL of DNA template (ca. 100 to 200 ng) in a total volume of 50 μL. The reaction conditions were as follows: initially 94°C for 2 min; 35 cycles of 30 sec each at 94°C, 50°C, and 68°C; and finally 68°C for 1 min.

The second set consisted of semi-nested primers designed from the conserved regions of the genes encoding the alpha subunit of benzoate 1,2-dioxygenase (*benA*) derived from various known isolates. The primers were described in detail in our previous report²⁵). The first PCR mixtures consisted of the same composition as those for the PCR of 16S rRNA genes but with the primers, BAF1 (5'-CGTTYCAYGGCTGGACVTTC-3') and BAR1 (5'-CGGAAYT-CYTCSAGRTCGTC-3'). The conditions for the first PCR were as follows: initially 94°C for 2 min; 30 cycles of 30 sec each at 94°C, 54°C, and 68°C; and finally 68°C for 1 min. In the second PCR, 0.5 μL of the first PCR product was transferred to the second reaction mixture containing the same constituents as the first but with the primers, BAF1-GC (BAF1 with a GC clamp attached to the 5' end) and BAR2 (5'-GTCGCGGCGTAGTTCCAGTG-3'). The second

PCR was performed as follows: initially 94°C for 2 min; 20 cycles of 30 sec each at 94°C, 56°C, and 68°C; and finally 68°C for 1 min.

All PCR products were electrophoresed on a 1.0% agarose gel to ascertain that they exhibited sizes close to what was expected: approximately 630 bp (16S) and 398 bp (*benA*). The products were purified using a PCR Clean-Up™ Kit (MO BIO Laboratories, Solana Beach, CA, USA).

DGGE analyses

DGGE was performed using a D-Code universal mutation detection system (Bio-Rad Laboratories), according to the instruction manual. The conditions for separation of the 16S rRNA gene fragments and *benA* fragments were as follows: running at 65 V for 21 h in a 6% polyacrylamide gel with the denaturing gradient from 45% to 65% at 60°C, and running at 60 V for 24 h in a 10% polyacrylamide gel with the denaturing gradient from 55% to 70% at 60°C, respectively. In both analyses, about 200 ng of each PCR product was loaded per lane. After electrophoresis, gels were stained with SYBR® Green I nucleic acid gel stain (Molecular Probes, Leiden, The Netherlands), and the bands were visualized with a Molecular Imager FX (Bio-Rad Laboratories). Bands of interest were excised for DNA sequencing. Each excised band was washed twice with 1 mL of distilled water. A small chip (less than 1 mm³) of each piece was used as a template for PCR to recover the DNA fragments. Conditions were the same as those for the original PCR (see above) except that the initial denaturing step was extended to 5 min and the number of cycles was changed to 28–40 based on the intensity of each band. The DNA fragments were subjected to DGGE again to confirm their purity and identity. The confirmed fragments were used as templates for direct sequencing. When it was difficult to make a target band homogeneous, the fragments were cloned using a PCR-Script™ Amp cloning kit (Stratagene, La Jolla, CA, USA). The clone containing the target fragment was screened by DGGE and then sequenced.

DNA sequencing and phylogenetic analyses

DNA sequencing reactions were performed using a BigDye™ Terminator v3.1 (Applied Biosystems, Foster City, CA, USA). The reaction products were analyzed with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). The phylogenetic analyses, including a BLAST search of the GenBank database and construction of neighbor-joining trees, were performed using the MEGA 3.1 program (available at URL: <http://www.megasoftware.net>).

Inoculation of bacteria into soil

Bacterial isolates having the same 16S rRNA gene or *benA* as the intensified DGGE bands were selected based on the nucleotide sequences obtained from each isolate and band. To evaluate their ability to degrade 3CB in the soil, the selected isolates were inoculated independently or together into the 3CB-dosed soil, which was newly prepared for this inoculation experiment as follows. The forest soil sample was divided into glass bottles (450 mL capacity), each containing 92.5 g. Each soil was mixed with 2.5 g of 3CB-containing Celite® (see Experimental design) to a final concentration of 500 mg kg⁻¹ 3CB, and then inoculated with 5 mL of bacterial cell suspension. The cell suspension was prepared as follows. Each selected isolate was incubated overnight at 28°C in 10-fold diluted tryptic soy broth (Difco) containing 500 mg L⁻¹ 3CB, with rotary shaking at 180 rpm. Cells were collected from the culture by centrifugation, washed with 10 mM sodium phosphate buffer (pH 6.8), and resuspended in the sodium phosphate buffer at a concentration of OD₆₀₀=0.4 (corresponding to a cell density of ca. 10⁸–10⁹ cells mL⁻¹). When several isolates were inoculated together, the same volume of each cell suspension was mixed and 5 mL of the mixture was used as an inoculum. After the inoculation, the soils were incubated for 14 days at 28°C with a moisture content of 40%. During the incubation, triplicate subsamples were collected temporally from each soil and subjected to the quantification of 3CB and

extraction of DNA, as described above.

Nucleotide sequence accession numbers

The sequences obtained in this study have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB303625 to AB303643.

Results

Degradation of 3CB in the soil

The concentration of 3CB in the soil decreased to less than 3% of the initial value after 3 weeks of incubation (Fig. 1). This decrease was because of indigenous degraders, because it was not observed in the autoclaved soil. Moreover, the rate of decrease was accelerated by repeated addition of 3CB. That is, it took only 3 days to degrade 500 mg kg⁻¹ 3CB after the third dose. These results suggest that indigenous 3CB-degraders were made more abundant in the forest soil by repeated doses of 3CB.

PCR-DGGE analyses

There was no change in the DGGE profiles of the soil without 3CB throughout the incubation period (only the profiles of day 0 and 38 are shown in Fig. 2). However, for the soil dosed with 3CB, the profiles of both 16S rRNA gene and *benA* clearly showed that several bands were intensified. There were differences in when each band intensified. For example, bands Sr1, 2, and 3 intensified at day 7 in the profiles of 16S rRNA gene, whereas bands Sr5, 6, and 7 intensified after the second dose at day 21. Similarly, band Sb1 intensified more quickly than other bands (Sb2, 3, 4, and 5) in the profiles of *benA*.

The DGGE profiles of liquid enrichment cultures showed very similar patterns in the triplicate batches; therefore, only the profiles derived from one of the batches are shown in Fig. 2. The profiles of both 16S rRNA gene and *benA* indicated that one of the intensified bands (Sr6 and Sb4, respectively) in the profiles of the 3CB-dosed soil was particularly strong in the liquid enrichment culture (Lr5 and Lb2, respectively). In other words, most of the intensified bands in the profiles of the 3CB-dosed soil were eliminated during the process of liquid enrichment. For example, bands Sr1, Sr3, and Sr4 in the profile of 16S rRNA gene, and Sb1, Sb2, and Sb5 in that of *benA* disappeared from the profiles of the liquid enrichment.

Fig. 1. Degradation of 3CB in raw soil and autoclaved soil. Bold arrows indicate when the 3CB was added, but the second and third arrows are only for raw soil. Error bars represent the standard deviation of triplicate determinations.

Fig. 2. DGGE profiles of PCR-amplified 16S rRNA gene fragments (A) and *benA* fragments (B) from soil and liquid enrichment cultures. Control is the soil without 3CB. Liquid enrichment culture was inoculated with the 3CB-dosed soil collected at day 38. Bold arrows indicate when the 3CB was added. Sequenced bands were named and indicated with arrows in the profiles. The bands having identical sequences were as follows: Sr3 and Lr3; Sr4 and Lr4; Sr6 and Lr5; Sb2 and Lb1; Sb4 and Lb2.

ment culture at day 4. We sequenced DGGE bands indicated by arrows in Fig. 2 for further analyses.

Isolation of bacteria from the 3CB-dosed soil

In total, 31 isolates were picked from hundreds of colonies obtained from the 3CB-dosed soil by direct plating or liquid enrichment. Partial fragments of the 16S rRNA gene and *benA* were amplified from each isolate using the same primers as those for the PCR-DGGE analyses, and then the nucleotide sequences of the fragments were determined for comparison with the sequences of the DGGE bands shown in Fig. 2. It was found that 28 of the 31 isolates had the same 16S rRNA or *benA* sequences as those of the DGGE bands. We grouped these 28 isolates into six strains based on their 16S rRNA and *benA* sequences. The six strains and their corresponding DGGE bands are shown in Table 1.

One of the strains, ASS7, was isolated with high frequency by both direct plating and liquid enrichment. This was consistent with the finding that the DGGE bands (Sr6 and Lr5 in the profiles of the 16S rRNA gene, and Sb4 and Lb2 in the profiles of *benA*) corresponding to ASS7 were prominent in the profiles of both the soil and the liquid enrichment culture. The bands corresponding to ASS7 (Sr6, Lr5, Sb4, and Lb2) nearly overlapped with the bands corresponding to ASS11 (Sr5 and Sb3). Although these bands looked to be single bands in the profiles of both 16S rRNA gene and *benA*, direct sequencing and cloning revealed that they were derived from the two strains (ASS7 and ASS11), and mainly from ASS7 especially in the liquid enrichment profiles. Strains ASS3, 8, 11, and 14 had the same sequences as those of the DGGE bands that intensified in the profiles of the 3CB-dosed soil but declined in those of the liquid enrichment culture. Although strain ASS8 presented an intensified band (Sr3) in the profile of 16S rRNA gene, we could not identify the band

corresponding to this strain in the profile of *benA*. However, it was deduced that a faint band between Sb2 and Sb3 corresponded to strain ASS8 because the *benA* fragments amplified from this strain formed a band at the position in the profile of *benA* (data not shown). The five strains mentioned above could grow on the basal salt agar containing 3CB as a sole carbon source.

Strain ASLS14 isolated from the liquid enrichment culture had the same 16S rRNA sequence as that of band Lr2, which appeared only in the profile of the liquid enrichment culture. Although we determined the *benA* sequence of the strain using the PCR product amplified from its genomic DNA, we could not identify the band corresponding to this strain in the profile of *benA*. This strain could not grow on the basal salt agar containing 3CB.

Phylogenetic analyses

Neighbor-joining trees were constructed using partial nucleotide sequences of 16S rRNA gene or *benA* derived from the above-mentioned strains and DGGE bands, and those obtained from GenBank (Figs. 3 and 4).

The tree of 16S rRNA genes showed that all strains and bands with two exceptions (bands Sr8 and Sr9) belonged to the family *Burkholderiaceae*. In particular, all the bands intensified in the 3CB-dosed soil belonged to the cluster of *Burkholderia* spp. Strain ASS14, which appeared as intensified bands in the profiles of 3CB-dosed soil but disappeared from those of liquid enrichment culture, was closely related to an uncultured bacterium MeBr20, which had been found in a soil treated with methyl bromide²⁴). Strains ASS3, 7, and 11, corresponding to bands Sr7, 6, and 5, respectively, were genetically similar to each other. Strain ASS7 formed prominent bands in the profiles of both the soil and liquid enrichment culture and was most closely related to *Burkholderia*

Strain	Genus ^a	Corresponding DGGE band		3CB utilization ^b	Number of homologus isolates	
		16S rRNA gene	<i>benA</i>		Direct plating ^c	Liquid enrichment ^d
ASS3	<i>Burkholderia</i> sp.	Sr7	Sb6	+	7	1
ASS7	<i>Burkholderia</i> sp.	Sr6, Lr5	Sb4, Lb2	+	6	6
ASS8	<i>Burkholderia</i> sp.	Sr3, Lr3	not identified	+	3	
ASS11	<i>Burkholderia</i> sp.	Sr5	Sb3	+	2	
ASS14	<i>Burkholderia</i> sp.	Sr4, Lr4	Sb2, Lb1	+	2	
ASLS14	<i>Ralstonia</i> sp.	Lr2	not identified	–		1

Phylogenetic tree showing the relationships between various bacterial species, primarily within the family *Burkholderiaceae*. The tree is rooted at the bottom left with a scale bar of 0.02. The *Burkholderiaceae* clade is highlighted in green. Bootstrap values are indicated at the nodes.

Species and associated identifiers (from top to bottom):

- Sr2** [AB303626]
- environmental clone "16S-2"* [AB207828]
- Burkholderia* sp. RP007* [AF061872]
- Burkholderia xenovorans* LB400* [CP000270]
- environmental clone "16S-3"* [AB207829]
- Sr3, Lr3**, [ASS8] [AB303627]
- 2,4-D-degrading bacterium TFD2* [AF184927]
- Sr4, Lr4**, [ASS14] [AB303628]
- uncultured bacterium MeBr20 [AY439198]
- Sr7**, [ASS3] [AB303631]
- Burkholderia* sp. tpg4.4 [AY691396]
- Burkholderia* sp. EN-B3* [AF074711]
- Sr5**, [ASS11] [AB303629]
- Sr6, Lr5**, [ASS7] [AB303630]
- environmental clone "16S-7"* [AB207833]
- Sr1** [AB303625]
- Burkholderia glathei* Hg18° [AY154378]
- environmental clone "16S-1"* [AB207827]
- Burkholderia* sp. NK8° [AB208548]
- Lr1** [AB303634]
- Lr2**, [ASLS14] [AB303635]
- Ralstonia* sp. PHD-2° [DQ227340]
- Ralstonia eutropha* JMP134° [CP000091]
- Cupriavidus necator* NH9° [AB208547]
- Sr8** [AB303632]
- Mycobacterium* sp. JS621 [AF498659]
- Sr9** [AB303633]
- Mycobacterium palustre* E846 [AJ308603]

The tree of *benA* showed that all of the genes of our strains were closely related to those of *Burkholderiaceae*, and this was consistent with the findings from the tree of 16S rRNA genes. Although the strain corresponding to band Sb5 was not isolated, the tree indicated that this band was also derived from a *Burkholderia* sp. closely related to strains ASS3, 7,

and 11. Band Sb1 was identical to the environmental clone ben-1, which had been obtained from the 3CB-dosed soil in our previous study²⁵⁾. The tree suggested that this band had a quite different origin from those of the other intensified bands.

3CB-degrading ability of the strains in soil

The bacterial strains listed in Table 1 were inoculated into the soil with 3CB to evaluate their 3CB-degrading ability in the soil. The degradation of 3CB was clearly accelerated in all soils inoculated with each of the five *Burkholderia* strains (ASS3, 7, 8, 11, and 14) in comparison with that in the raw soil without inoculation (Fig. 5a–e). In addition, the DGGE profiles of each soil indicated that these strains quickly adapted to the soil after the inoculation. Co-inoculation of the five strains increased the rate of degradation in the soil to the same extent as the independent inoculations (Fig. 5f).

No acceleration of 3CB degradation was observed in the soil inoculated with strain ASLS14 corresponding to band Lr2, which had appeared only in the profiles of the liquid enrichment culture. The DGGE profile of the soil suggested that this strain could not adapt to the soil (Fig. 5g). From these findings, and also that this strain could not grow on the

basal salts agar containing 3CB, we concluded the strain did not relate to the degradation of 3CB in the soil.

Based on above results, we concluded that the five *Burkholderia* strains (ASS3, 7, 8, 11, and 14) were effective 3CB-degraders in soil.

Discussion

In this study, we succeeded in isolating five *Burkholderia* strains that could effectively degrade 3CB in soil using a combination of culture methods and PCR-DGGE. Although a few interesting bands (e.g., Sr1 and Sb1), which did not correspond to any of our isolates, remained in the DGGE profiles, bands corresponding to the five *Burkholderia* strains (ASS3, 7, 8, 11, and 14) comprised most of the prominent bands that intensified following the repeated application of 3CB. Therefore, these strains likely represent key members comprising the 3CB-degrading populations in the

Fig. 5. Effects of isolated strains on 3CB degradation in soil. Figure (a), inoculated with ASS3; (b), ASS7; (c), ASS8; (d), ASS11; (e), ASS14; (f), coinoculated with the preceding five strains; (g), inoculated with ASLS14. Each DGGE profile consists of 16S rRNA gene fragments amplified from the soil immediately after the inoculation and after 3 days. The DGGE bands corresponding to the inoculated strains are indicated with arrows. Error bars represent the standard deviation of triplicate determinations.

soil. Although 3CB-degrading *Burkholderia* spp. have been isolated from various soils^{12,14,15,32}, it had not been confirmed whether this group of bacteria contributes to the degradation of 3CB in the soil environment. Our results suggest that several *Burkholderia* strains actually function as effective 3CB-degraders in soil.

We employed not only 16S rRNA gene but also a functional gene, *benA*, as a molecular marker. This gene encodes the alpha-subunit of benzoate 1,2-dioxygenase which is necessary for the conversion of 3CB to 3-chlorocatechol³³. It has been reported that this gene is essential for 3CB-degradation in *Burkholderia xenovorans* LB400⁵. On the other hand, some 3CB-degrading bacteria (e.g. *Alcaligenes* sp. L6) do not use a metabolic pathway involving 3-chlorocatechol but use a pathway involving gentisate or protocatechuate as the intermediate^{19,28}. The PCR-DGGE of *benA* was thought to be inadequate to detect this type of 3CB-degrader. This time, however, the PCR-DGGE of *benA* could detect the greater part of the enriched populations that were detected in the profiles of 16S rRNA genes. These results suggested that the bacteria using a pathway involving 3-chlorocatechol were the major 3CB-degrading populations in the soil.

Several studies reported that bacteria introduced into a microbial community for bioaugmentation were hardly detected by PCR-DGGE^{2,3,22}. In general, bacterial populations that make up 1% or more of the total target community can be detected by PCR-DGGE²⁶. Thus, the difficulty in detection of the introduced bacteria probably suggests that the bacteria could not proliferate to such levels in the community. In our experiment, the initial density of the inoculated strains was calculated at about 10^7 – 10^8 cells per gram of soil. Despite such a high population density, the intensity of DGGE bands corresponding to the strains was not so high immediately after the inoculation (day 0). Therefore, it is most likely that at least 10^7 – 10^8 cells per gram of soil were required to detect the inoculated strains as the bands in the DGGE profiles of the soil. On the other hand, 3 days after the inoculation, five 3CB-degrading strains (ASS3, 7, 8, 11, and 14) came to be detected as prominent bands. These results suggested that the five strains could increase their populations to a level which largely exceeded their initial population densities. The DGGE profile of the co-inoculated soil showed that strains ASS8 and ASS14 intensified more quickly than the others. This result suggested that the five strains competed with each other to utilize 3CB, and that ASS8 and ASS14 were superior to the other strains, at least at the point in time immediately after their introduction into the soil. This also seems to be one of the reasons for why bands corresponding to ASS8 and ASS14 appeared earlier than those of other strains in the DGGE profiles of the 3CB-dosed soil.

It has been often shown that the results of bacterial community analyses based on culture-independent methods are not consistent with those based on culture-dependent methods^{9,10}. In contrast, in the present study, more than 90% of the isolates had the same sequences as any of those of the intensified bands in the DGGE profiles. This good correspondence between the isolates and the intensified bands might be because the repeated application of 3CB acted as a strong selective pressure, and because the soil extract agar

was appropriate to detect the 3CB-degrading bacteria.

It has been recognized that liquid enrichment has a significant bias in the isolation of pollutant degraders. Dunbar *et al.*⁷ assessed the diversity of 2,4-dichlorophenoxyacetate-degrading bacteria obtained from soil by direct plating and by liquid enrichment. They found that the diversity of the isolates obtained from liquid enrichment culture was significantly lower than that of the isolates obtained by direct plating. Watanabe *et al.*³⁹ identified dominant phenol-degrading bacteria in activated sludge using PCR-TGGE and reported that the dominant populations could not be isolated from liquid enrichment cultures. Also in our study, the PCR-DGGE analyses revealed that liquid enrichment selectively concentrated only a small proportion of the 3CB-degrading bacteria in the soil. Strains ASS8 and ASS14, which accumulated in the 3CB-dosed soil but disappeared in the liquid enrichment culture, showed good 3CB-degrading ability and especially good adaptability to the soil. For the bioaugmentation of polluted sites, it is essential that the introduced bacteria adapt smoothly to the environment and degrade the pollutant effectively under the expected conditions. Our results clearly illustrated that such bacteria were missed throughout the liquid enrichment process. In addition, the results showed that direct plating on soil extract agar is more suitable for isolating the degraders under environmental conditions.

One of the major advantages of using molecular community profiling such as PCR-DGGE for the study of pollutant-degrading bacteria is that the method enables one to understand their dynamics in the environment. Of course, because the method is strongly dependent on PCR, the resultant profile might be biased by several factors in the PCR, especially by primer design. Therefore, to draw more reliable conclusions, several primer sets should be used as in the present study. Our results suggest that a combination of PCR-DGGE and culture methods greatly helps in isolating useful bacteria capable of degrading a target pollutant effectively under the expected environmental conditions.

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