J. Microbiol. Biotechnol. (2005), 15(2), 376–383

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Analysis of Plasmid pJP4 Horizontal Transfer and Its Impact on Bacterial Community Structure in Natural Soil

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Received: July 13, 2004
Accepted: September 8, 2004

Abstract *Alcaligenes* sp. JMP228 carrying 2,4-dichlorophenoxyacetic acid (2,4-D) degradative plasmid pJP4 was inoculated into natural soil, and transfer of the plasmid pJP4 to indigenous soil bacteria was investigated with and without 2,4-D amendment. Plasmid pJP4 transfer was enhanced in the soils treated with 2,4-D, compared to the soils not amended with 2,4-D. Several different transconjugants were isolated from the soils treated with 2,4-D, while no indigenous transconjugants were obtained from the unamended soils. Inoculation of the soils with both the donor *Alcaligenes* sp. JMP228/pJP4 and a recipient *Burkholderia cepacia* DBO1 produced less diverse transconjugants than the soils inoculated with the donor alone. Repetitive extragenic palindromic-polymerase chain reaction (REP-PCR) analysis of the transconjugants exhibited seven distinct genomic DNA fingerprints. Analysis of 16S rDNA sequences indicated that the transconjugants were related to members of the genera *Burkholderia* and *Pandoraea*. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA genes revealed that inoculation of the donor caused clear changes in the bacterial community structure of the 2,4-D-amended soils. The new 16S rRNA gene bands in the DGGE profile corresponded with the 16S rRNA genes of 2,4-D-degrading transconjugants isolated from the soil. The results indicate that introduction of the 2,4-D degradative plasmid as *Alcaligenes* sp. JMP228/pJP4 has a substantial impact on the bacterial community structure in the 2,4-D-amended soil.

Key words: Gene transfer, plasmid pJP4, bacterial community structure

Recent developments in biotechnology have led to the construction of genetically engineered microorganisms (GEMs) which can be used for a variety of commercial purposes, including biocontrol and bioremediation. Some of these GEMs will be applied to the open environment, and therefore, are apt to escape from research laboratories. The release of these GEMs into the environment may have an impact on indigenous soil microorganisms. Previous studies have focused on conjugative transfer of plasmid from an introduced donor to microbial recipients in sterile soils [21, 28]. More recently, some studies have been performed with indigenous soil microorganisms as potential recipients in nonsterile soils [5, 16, 22, 23, 29]. Even if a fair amount of work have been carried out to understand the fate of the GEMs released into environment and their impact on environment, the risk assessment of the GEMs still awaits for more elegant studies. Potential hazards include the survival and persistence of introduced microorganisms and genes, the probability and rate of horizontal gene transfer, and the impact of released GEMs on the microbial community structure in soil.

Soil microbial populations substantially increase their rates of evolution and adaptation through gene transfer. Plasmid-borne genes, specifically, can be disseminated rapidly between diverse microorganisms in soil through cell-to-cell conjugation. In the present study, we used the 2,4-D degradative plasmid pJP4 as a model for gene transfer among indigenous soil microbial populations. The plasmid pJP4, a broad-host-range IncP1 plasmid, contains genes encoding 2,4-D degradation and confers mercury resistance [7, 8]. The herbicide 2,4-D is known to be a growth substrate for a number of different soil microorganisms capable of degrading the compound [3, 10, 11]. Thus, the 2,4-D degradative plasmid pJP4 allows the plasmid harboring microbial populations to increase under 2,4-D selection, making it easy to detect and purify transconjugants in natural soil.

In this study, we have investigated the transfer of the 2,4-D degradative plasmid pJP4, from an introduced donor strain, *Alcaligenes* sp. JMP228, to the indigenous bacteria

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and an inoculated recipient, *Burkholderia cepacia* DBO1, in natural soils. The fate of microbes released into the soil was monitored, using their antibiotics-resistant properties. In addition, we examined the persistence of the *tfdA* gene with competitive PCR, the phylogenetic diversity of transconjugants by 16S rDNA sequence analysis, and the impact of released GEMs on the microbial community structure by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes.

MATERIALS AND METHODS

Bacterial Strains and Plasmid

The bacterial strain *Alcaligenes* sp. JMP228, which contains plasmid pJP4 encoding degradation of 2,4-D and resistance to mercuric ions [7, 8], was used as a donor organism, and the strain *Burkholderia cepacia* DBO1 was inoculated as a recipient organism. *Alcaligenes* sp. JMP228 is resistant to rifampicin (100 µg/ml) through spontaneous mutation, but the strain is sensitive to streptomycin, trimethoprim, nalidixic acid, polymixin B, and neomycin, each at concentration of 20 µg/ml, respectively. *B. cepacia* DBO1 contains Tn5 transposon and is resistant to kanamycin (75 µg/ml) and bacitracin (50 µg/ml) [13], but it is susceptible to trimethoprim (20 µg/ml).

Media and Growth Conditions

Alcaligenes sp. JMP228/pJP4 was grown on peptone-tryptone-yeast extract-glucose (PTYG) medium [2] supplemented with 2,4-D (500 µg/ml) and rifampicin (100 µg/ml). *B. cepacia* DBO1 was grown on PTYG medium with kanamycin (75 µg/ml) and bacitracin (50 µg/ml). The numbers of the inoculated strains were determined by plating appropriate dilutions of soil suspensions onto PTYG agar containing the respective antibiotics. The most probable numbers for 2,4-D degraders were determined by inoculating the serial dilutions of soil suspensions into 2,4-D mineral medium [27]. Total counts of bacteria, actinomycetes, and fungi were measured with tryptic soy agar, sodium caseinate agar, and malt extract agar medium, respectively [31]. All cultures were incubated at 28°C for 4-7 days.

Microcosm Design and Treatments

Alcaligenes sp. JMP228/pJP4 and *B. cepacia* DBO1 were grown at 28°C, harvested by centrifugation at 10,000 ×*g* for 10 min at 4°C, washed twice with an equal volume of 0.85% sterile saline, kept on ice, and enumerated by a counting chamber.

Microcosms consisted of 150 g (dry weight) of natural soil, which was obtained from the Agricultural Research site at Suwon, Gyunggido, South Korea and characterized as a clay loam with a pH of 6.3, in polyethylene wide-mouth bottles. In preliminary experiments, 2,4-D applied to the soil was not degraded even after 60 days, therefore, there were no indigenous 2,4-D-degrading bacteria in this soil, making it easy to detect and purify any transconjugants arising from plasmid pJP4 transfer. Soil was sifted through a 2-mm-pore-size sieve, and inoculated with the donor and/or the recipient at a density of about 8.0×10^6 cells/g soil, and then the soil was thoroughly mixed. Experimental groups of microcosms were treated with 2,4-D dissolved in distilled water (10 mg/ml, pH 7.0) to a final concentration of 500 µg/g soil and thoroughly mixed. The microcosms were treated as two replicates in each of the following five experimental sets: microcosm I was inoculated with the donor and recipient strains and amended with 2,4-D; microcosm II was inoculated with the donor and recipient strains but not amended with 2,4-D; microcosm III was inoculated with the donor strain and amended with 2,4-D; microcosm IV was inoculated with the donor strain but not amended with 2,4-D; microcosm V was an uninoculated, unamended control. Soil samples were removed from each microcosm at intervals of 7 days for a 35-day period to analyze total microbial counts, 2,4-D concentrations, 2,4-D-degrading populations, putative transconjugants, and soil DNA. The 2,4-D in soil was analyzed with a Shimadzu LC-10 series high-performance liquid chromatography (Shimadzu Co., Tokyo, Japan) as described previously [14].

Isolation, Identification, and Characterization of Transconjugants

Putative 2,4-D-degrading transconjugants were purified by inoculating appropriate dilutions of soil suspensions onto 2,4-D mineral plate media containing streptomycin, trimethoprim, nalidixic acid, polymixin B, and/or neomycin at 20 µg/ml concentration, respectively. Presumptive transconjugants were subjected to REP-PCR procedure to distinguish different strains by comparison of their genomic DNA fingerprints [6, 30], and the presence of plasmid in transconjugants was confirmed by the procedure of Kado and Liu [15]. For taxonomic identification of the transconjugants, total genomic DNA was extracted from each strain with a Wizard Genomic DNA Purification Kit (Promega, Madison, U.S.A.). PCR amplification of nearly full-length 16S rRNA genes was performed with 27f and 1492r (*E. coli* 16S rRNA gene sequence numbering) as previously described [4, 18]. Sequencing was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, U.S.A.), according to the manufacturer's instructions. Sequences from nearest relatives were identified from the Ribosome Database Project (RDP), using the Similarity-Rank program of the RDP [20].

Competitive PCR Analysis for *tfdA* Gene

For PCR analysis, a subsample of 0.50 g was removed from each microcosm and stored at -70°C. DNA was extracted

from soil by using a FastDNA Spin Kit (Qbiogene, Carlsbad, U.S.A.). DNA samples were subjected to competitive PCR amplification with a forward primer *tfdA*-858f (5' GGACGAGAAGTCGGTGCTGGT 3') and a reverse primer *tfdA*-1387r (5' GCGCGCCGATGAAGAGAAACT 3') designed based on the DNA sequence of the *tfdA* gene. The expected PCR products are approximately 530 bp in size. A DNA competitive internal standard (CIS) was constructed by the looped template method [32]. Briefly, PCR amplification of the *tfdA* gene with a deletion forward primer (5' GGACGAGAAGTCGGT GCTGGTCATCAA-GGTCAATCAAAGA 3') and the reverse primer *tfdA*-1387r produced a PCR product 80 bp shorter than the corresponding *tfdA* gene region. This shortened *tfdA* gene fragment was cloned into the pGEM-T Easy Vector (Promega, Madison, U.S.A.). A miniprep of the recombinant plasmid was linearized by using *NaeI* and used as the DNA CIS in competitive PCR assays. The concentration of the CIS was determined by comparison with a known amount of pGEM-T Easy Vector DNA. The dilution factor of the soil DNA, yielding a 1:1 ratio of band densities of the *tfdA* and CIS amplicons, was calculated from a plot of dilution factor versus the ratio. PCR amplification was performed in 25-μl reaction mixtures containing 10× PCR buffer (200 mM Tris/HCl, 500 mM KCl, pH 8.4), 100 ng of template DNA, 25 pmol each of primer, 200 μM each dNTP (Boehringer Mannheim, Indianapolis, U.S.A.), and 2 U of *Taq* polymerase. PCR cycles consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 68°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. After PCR amplification, 10 μl samples of the PCR products were separated by electrophoresis on horizontal 1.5% agarose gels at 43 V for 7 h. After the agarose gels were stained with ethidium bromide (0.5 μg/ml), images of the gels were analyzed by using an image analyzer BIO-PROFIL V.97 (Vilber Lourmat, France).

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

Soil bacterial community DNA was extracted by using a FastDNA Spin Kit (Qbiogene). For pure culture, total genomic DNA was extracted from each strain with a Wizard Genomic DNA Purification Kit (Promega). PCR amplification of the 16S rRNA genes was performed with primers 1070f and 1392r (*E. coli* 16S rRNA gene sequence numbering) as previously described [9]. The PCR product contains a GC clamp of 40 bases, added to the reverse primer, and has a total length of 323 bp, including the highly variable V9 region. PCR reaction mixtures were prepared as previously described [24]. PCR cycles consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were subjected to DGGE with the Dcode™ Universal Mutation Detection System (BIO-RAD, Hercules, U.S.A.), according to the previous procedure [1, 9]. PCR samples were applied directly onto 8% (wt/vol) polyacrylamide gels with a denaturing gradient ranging from 40% to 70% (where 100% denaturant contains 7 M urea and 40% formamide). The gels were electrophoresed for 17 h at 60°C and a constant voltage of 40 V. After electrophoresis, the gels were stained with SYBR Green I, rinsed for 10 min, and photographed with UV transillumination (302 nm).

Nucleotide Sequence Accession Numbers

The full 16S rDNA sequences of 6 transconjugants have been deposited in the GenBank under the following accession numbers: TC62, AY677087; TC81, AY677090; TC82, AY677088; TC83, AY677091; TC84, AY677092; TC85, AY677089.

RESULTS AND DISCUSSION

Biodegradation of 2,4-D in Soil

The applied 2,4-D was quickly degraded after a short lag period of 4 days in microcosms I and III, which were inoculated with 2,4-D-degrading bacteria, *Alcaligenes* sp. JMP228/pJP4 (Fig. 1). It took about 2 weeks or less for 500 μg/g of 2,4-D to be degraded completely in soil, whereas 2,4-D applied to the uninoculated soil was not degraded even after 60 days of incubation (data not shown), indicating no indigenous 2,4-D-degrading microorganisms in the soil.

In microcosms I and III inoculated with *Alcaligenes* sp. JMP228/pJP4, the initial populations of 2,4-D degraders were 8.4×10^6 cells/g soil and 8.7×10^6 cells/g soil, respectively,

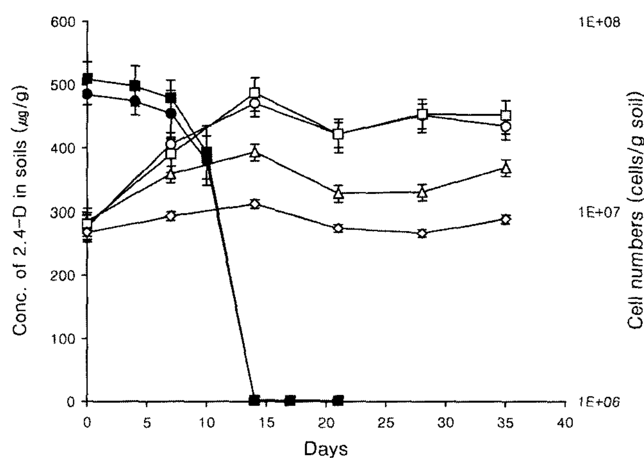

Fig. 1. Biodegradation of 2,4-D (solid symbol) and MPN counts of 2,4-D-degrading microorganisms (open symbol) in microcosm soils.

Symbols: ●, microcosm I; ■, microcosm III; ○, microcosm I; △, microcosm II; □, microcosm III; ◇, microcosm IV. Bars represent the standard deviations from duplicate microcosms.

as measured by most probable number (MPN) counting (Fig. 1). By day 14 after the 2,4-D amendment, the population densities had markedly increased to 3.7×10^7 and 4.2×10^7 cells/g soil, respectively, and these densities were stably maintained throughout the experiment. In the microcosms II and IV, which were inoculated with *Alcaligenes* sp. JMP228/pJP4 but not treated with 2,4-D, the initial population densities of 2,4-D degraders were 9.0×10^6 and 7.8×10^6 cells/g soil, respectively. After 14 days, the values increased to 2.0×10^7 and 1.1×10^7 cells/g soil, respectively, and then were more or less maintained at these levels throughout the experiment.

Population Changes of *Alcaligenes* sp. JMP228/pJP4 and *B. cepacia* DBO1

The initial populations of *Alcaligenes* sp. JMP228/pJP4 and *B. cepacia* DBO1 in the microcosm soils ranged from 8.2×10^6 to 9.5×10^6 cells/g soil (Fig. 2). The population of *Alcaligenes* sp. JMP228/pJP4 greatly increased to 2.1×10^7 cells/g soil by day 14 in microcosm III amended with 2,4-D. After 21 days, this population decreased to 1.1×10^7 cells/g, and then was more or less maintained at this level throughout the experiment. The population densities of *Alcaligenes* sp. JMP228/pJP4 in the unamended microcosms II and IV ranged from 8.2×10^6 to 1.4×10^7 cells/g soil over the experiment (Fig. 2). The numbers of *B. cepacia* DBO1 inoculated into microcosms I and II slightly increased on day 7 and day 14, and then declined to the initial population levels from day 21 (Fig. 2). The observations are consistent with the previous result of Hong *et al.* [12], who showed that *B. cepacia* DBO1/pJP4 and *Alcaligenes* sp. JMP228/pJP4 remained stable in a field soil during the early phase of the long-term survival experiment. 2,4-D is known to be a good carbon source for microorganisms capable of degrading the herbicide [19, 25]. As shown in Fig. 2, the 2,4-D amendment stimulated growth of *Alcaligenes* sp. JMP228/pJP4 in microcosm III; however, its population was only slightly increased in microcosm I where *B. cepacia* DBO1 was also inoculated together. It is quite likely that the transconjugants, including *B. cepacia* DBO1/pJP4, coming from gene transfer might have competed with *Alcaligenes* sp. JMP228/pJP4 for 2,4-D, as suggested by Hong *et al.* [12]. Our results indicate that the two inoculated strains more or less increased in numbers until day 14 and their levels remained relatively stable throughout the experiment.

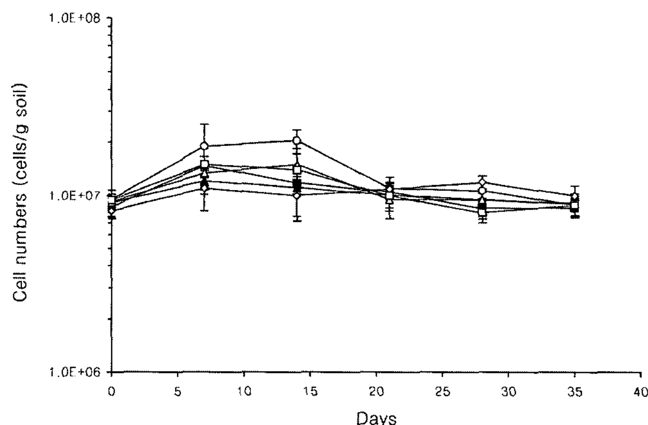

Fig. 2. Population levels of *Alcaligenes* sp. JMP228/pJP4 and *B. cepacia* DBO1 inoculated into microcosm soils.
Symbols: ▲, *Alcaligenes* sp. JMP228/pJP4 of microcosm I; △, *B. cepacia* DBO1 of microcosm I; ■, *Alcaligenes* sp. JMP228/pJP4 of microcosm II; □, *B. cepacia* DBO1 of microcosm II; ○, *Alcaligenes* sp. JMP228/pJP4 of microcosm III; ◇, *Alcaligenes* sp. JMP228/pJP4 of microcosm IV. The data represent the means and standard deviations of duplicate microcosms.

Competitive *tfdA* PCR

To quantify the *tfdA* gene copy number in the microcosm soils, diluted soil DNA samples and CIS with known copy number were subjected to competitive amplification in a series of PCR tubes. Competitive PCR analyses of soil DNAs indicated that the *tfdA* gene was stably maintained in the soils, inoculated with *Alcaligenes* sp. JMP228/pJP4, over the course of the experiment (Fig. 3). The estimated *tfdA* concentration in the microcosms ranged from 1.0×10^6 to 2.3×10^7 copies/g soil throughout the 35-day incubation period. The stable *tfdA* density observed is consistent with the persistence of *Alcaligenes* sp. JMP228/pJP4, which has the *tfdA* gene in plasmid pJP4, in the microcosm soils.

Changes of Total Microbial Populations

The total counts of bacteria, actinomycetes, and fungi in soils during this study were in the range of about 3.8×10^7–2.2×10^8 cells/g soil, 1.6×10^6–3.9×10^6 cells/g soil, and 4.4×10^4–3.4×10^5 cells/g soil, respectively. The changes of soil microbial populations in the representative microcosms are shown in Fig. 4. In microcosms I and III amended with 2,4-D, the initial bacterial and fungal populations ranged

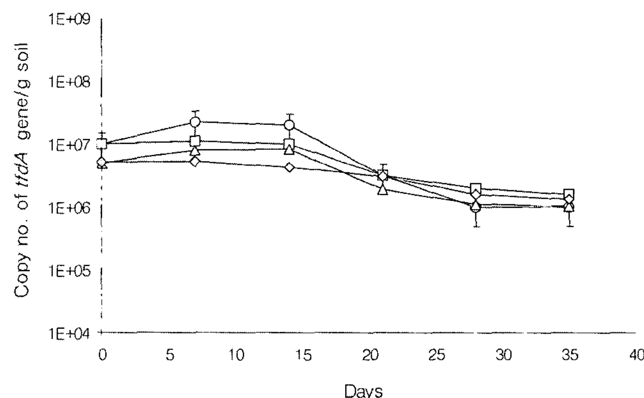

Fig. 3. *tfdA* gene copies in microcosm soils.
Symbols: □, microcosm I; △, microcosm II; ○, microcosm III; ◇, microcosm IV. Bars represent the standard deviations from duplicate microcosms.

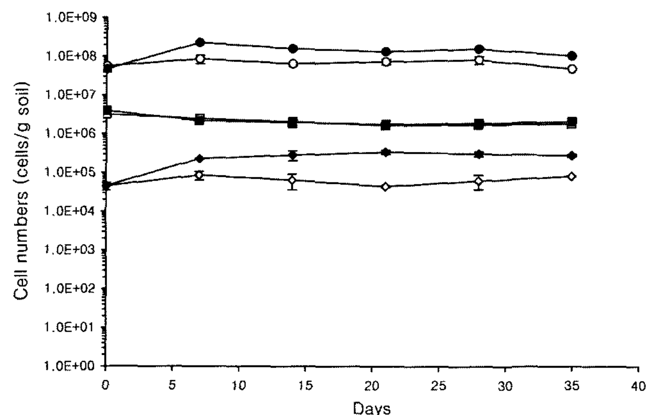

Fig. 4. Population changes of bacteria, fungi, and actinomycetes in representative microcosms.

Symbols: ●, bacteria in microcosm I; ○, bacteria in microcosm V; ■, actinomycetes in microcosm I; □, actinomycetes in microcosm V; ◆, fungi in microcosm I; ◇, fungi in microcosm V. The data represent the means and standard deviations of duplicate microcosms.

from 5.6×10^7 to 2.2×10^8 cells/g soil and from 4.5×10^4 to 3.4×10^5 cells/g soil, respectively. The initial values were increased about twice by day 7 and then maintained at these levels throughout the experiment. By contrast, the bacterial and fungal populations of microcosms II, IV, and V, which were not amended with 2,4-D, were not changed significantly during the experimental period. The actinomycetes population also remained quite stable regardless of 2,4-D amendment during the experiment. It has been reported that the number of bacteria increased slightly in 2,4-D-treated soils, showing 2,4-D degradation activity [17, 22], and that fungal population was significantly stimulated by application of 500 μg 2,4-D/g soil [26]. The above result indicates that degradation of the applied 2,4-D by the 2,4-D-degrading microorganisms stimulates growth of other bacteria and fungi as well as themselves, while it does not significantly affect the population of the actinomycetes in the soil used.

Transfer of Plasmid pJP4

Plasmid pJP4 transfer from *Alcaligenes* sp. JMP228/pJP4 to indigenous microbial populations was analyzed in the inoculated and control microcosms with and without 2,4-D amendment. Various colonies of presumptive transconjugants were purified and then characterized by genomic DNA fingerprints and 16S rDNA sequences. The numbers of transconjugants isolated varied with time and amendment of 2,4-D. The most diverse transconjugants were isolated from the microcosm III, which was inoculated with *Alcaligenes* sp. JMP228/pJP4 and amended with 500 μg/g soil of 2,4-D. By day 7, two indigenous transconjugants (TC81 and TC82) were isolated from the microcosm III at the levels of 10^6 cells/g soil. The species diversity of transconjugants increased with time, giving four transconjugants

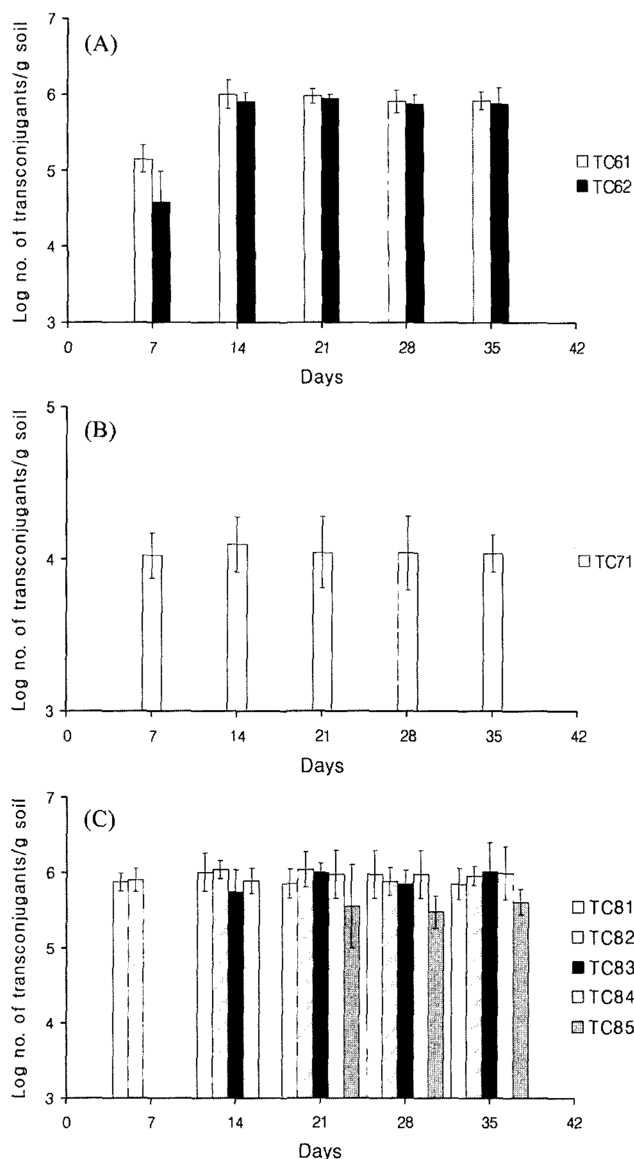

Fig. 5. Occurrence and distribution of the transconjugants in microcosm I (A), microcosm II (B), and microcosm III (C).

on day 14 and five on day 21, and then these five transconjugants were persistently detected during the remainder of the study (Fig. 5C). In microcosm I, which was amended with 2,4-D and inoculated with *Alcaligenes* sp. JMP228/pJP4 and *B. cepacia* DBO1, two transconjugants (TC61 and TC62) were found at 10^5 cells/g soil by day 7 (Fig. 5A). The populations of the transconjugants increased to 10^6 cells/g soil by day 14 and then remained stably at those levels over the experiment. In microcosm II, which was not treated with 2,4-D but inoculated with *Alcaligenes* sp. JMP228/pJP4 and *B. cepacia* DBO1, one transconjugant (TC71) was purified at day 7 (Fig. 5B). In contrast, no transconjugants were found from microcosms IV, which was not treated with 2,4-D but inoculated with *Alcaligenes*

sp. JMP228/pJP4, during the experiment. In previous studies, the frequency of plasmid pJP4 transfer was observed to vary with soil type and level of 2,4-D [21-23]. It is of note that the diversity of transconjugants in the microcosm III was increased with time from day 7 to day 21. The donors and indigenous microorganisms may have become metabolically active for the first 21 days due to growth carbons coming from 2,4-D degradation. For this period, successive gene transfer could occur between indigenous microbial populations, in addition to ongoing transfer from *Alcaligenes* sp. JMP228/pJP4 to indigenous recipients. The population level of each transconjugant detected in this study represented approximately 1% of the viable counts (ca. 1×10^8 cells/g soil) of the soil. The results of this study demonstrate that significant populations of transconjugants can arise through natural transfer of a large catabolic plasmid to indigenous microorganisms in the soil used.

Characterization of Transconjugants

The transconjugants isolated from the microcosm soils were further characterized by performing genomic DNA fingerprint, plasmid profile, *tfdA* PCR, and 16S rDNA sequence analyses. REP-PCR analysis revealed that each genomic DNA fingerprint pattern of two transconjugants (TC61 and TC71) was identical to that of *B. cepacia* DBO1, indicating that the two transconjugants originated from *B. cepacia* DBO1 (Fig. 6). The other transconjugants (TC62, TC81, TC82, TC83, TC84, and TC85) showed six distinct genomic DNA fingerprinting patterns, indicating that these transconjugants were indigenous soil microorganisms which acquired pJP4 through gene transfer in the soil. All of the transconjugants degraded 2,4-D and contained 80-kb plasmid similar in size to *Alcaligenes* sp. JMP228 plasmid pJP4 (Fig. 7, indicated by an arrow). When PCR amplification was performed for every transconjugant by using specific primers selected from the internal sequence of the *tfdA* gene, the transconjugants produced the expected positive bands of 530 bp (Fig. 8).

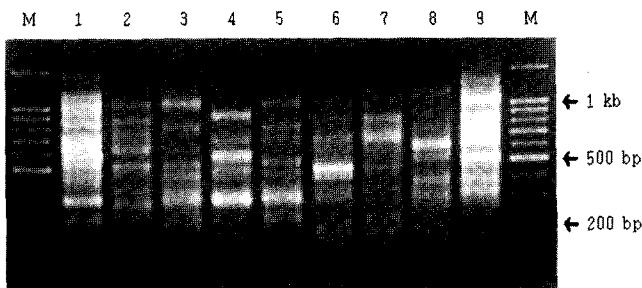

Fig. 6. Genomic DNA fingerprints of the donor, representative transconjugants, and recipient.
Lanes: 1, *Alcaligenes* sp. JMP228/pJP4; 2, TC61; 3, TC62; 4, TC81; 5, TC82; 6, TC83; 7, TC84; 8, TC85; 9, *B. cepacia* DBO1/pJP4; M, 100 bp ladder marker.

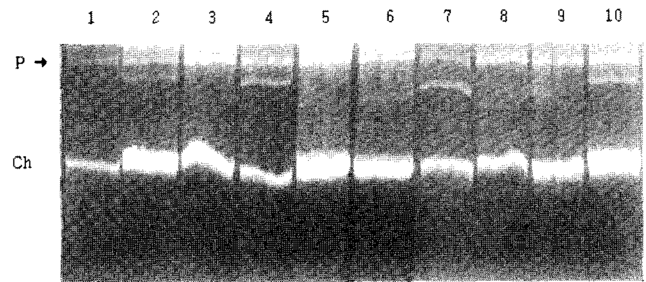

Fig. 7. Plasmid profiles of the donor and transconjugants.
Lanes: 1, *Alcaligenes* sp. JMP228/pJP4; 2, TC61; 3, TC62; 4, TC71; 5, TC82; 6, TC84; 7, TC83; 8, TC81; 9, TC85; 10, *B. cepacia* DBO1/pJP4. P, plasmid band; Ch, chromosomal band. The arrow indicates plasmid pJP4 band.

Analyses of the 16S rDNA sequences revealed that the transconjugants were related to members of the genera *Burkholderia* and *Pandoraea* (Table 1). All of the transconjugants were Gram-negative and belonged to the beta subgroup of Proteobacteria. *Burkholderia* populations were dominant transconjugants, with six different strains being identified. Other studies also reported that dominant transconjugant populations belonged to the genus *Burkholderia* [22, 23], suggesting that these populations may be somehow apt to receive plasmid DNA through conjugation in soil.

DGGE Analysis

To investigate the impact of microbe inoculation and 2,4-D treatment on the structure of the microbial community, total soil microbial DNAs extracted from microcosm soils were analyzed by DGGE after PCR amplification of the variable V9 region of the 16S rRNA gene. For the uninoculated and unamended microcosm V, very similar DGGE banding patterns were observed throughout the experiment, indicating that no apparent changes occurred in the soil microbial community of this control soil (Fig. 9A). Similarly, no changes were detected in microcosms II and IV, which were not treated with 2,4-D (data not shown). On the other hand, substantial changes in DGGE band patterns were observed in microcosms I and III, which were inoculated with microorganisms and amended with 2,4-D (Figs. 9B

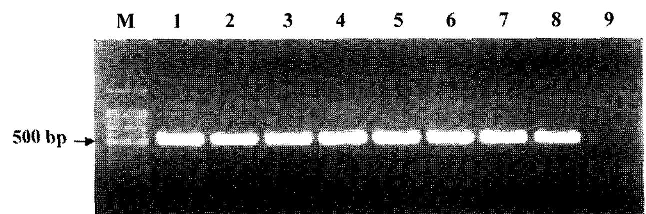

Fig. 8. PCR-amplified DNA fragments obtained from the transconjugants with the primers targeted for the *tfdA* gene.
Lanes: M, 100 bp ladder marker; 1, *Alcaligenes* sp. JMP228/pJP4; 2, TC61; 3, TC62; 4, TC81; 5, TC82; 6, TC83; 7, TC84; 8, TC85; 9, *B. cepacia* DBO1.

Table 1. Identification of the transconjugants by 16S rDNA sequence analysis.

Transconjugant	GenBank accession no.	Nearest relative	Similarity
TC61	Nd[a]	*Burkholderia cepacia*	100
TC62	AY677087	*Burkholderia cepacia*	99
TC71	Nd	*Burkholderia cepacia*	100
TC81	AY677090	*Burkholderia pyrrocinia*	99
TC82	AY677088	*Burkholderia cepacia*	99
TC83	AY677091	*Burkholderia pyrrocinia*	100
TC84	AY677092	*Pandoraea sputorum*	99
TC85	AY677089	*Burkholderia cepacia*	99

Based upon full sequences of the 16S rRNA gene.
[a]Not deposited.

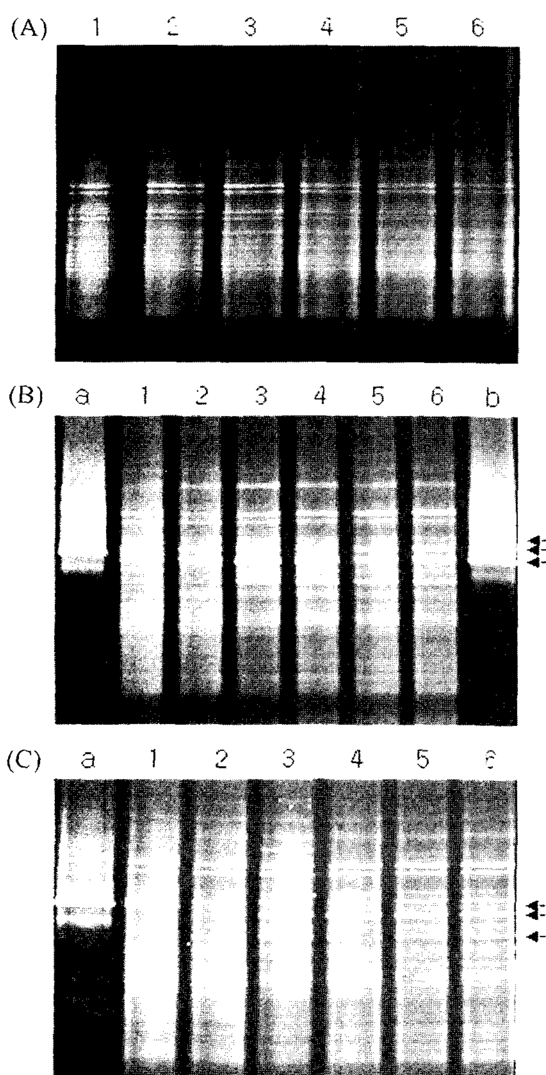

Fig. 9. DGGE analyses of 16S rDNA fragments obtained after PCR amplification with eubacterial primers 1070F and 1392R.
DGGE profiles for control microcosm V (A), microcosm I (B), and microcosm III (C) soils of day 0 (lane 1), day 7 (lane 2), day 14 (lane 3), day 21 (lane 4), day 28 (lane 5), and day 35 (lane 6). a, *Alcaligenes* sp. JMP228/pJP4; b, *Burkholderia cepacia* DBO1. Arrows point to the products corresponding with the transconjugants.

and 9C). Specifically, clear changes were observed in microcosm III, which gave the most diverse transconjugants during the experiment. When the purified transconjugants were analyzed together by DGGE, it was observed that the new 16S rDNA bands in the DGGE profile corresponded with those of 2,4-D-degrading transconjugants, such as *B. cepacia*, *B. pyrrocinia*, and *P. sputorum*, isolated from the soil (Fig. 9, indicated by arrows). The microbial communities in the soils were potentially complex, but there were obvious differences in DGGE band profiles with time and between the control microcosm and the treated microcosms.

This work shows that plasmid pJP4 was well transferred from the introduced donor to indigenous microbial populations, especially under 2,4-D selection. 2,4-D-degrading microbial populations were significantly increased with 2,4-D amendment, and their degradation activity stimulated the growth of other indigenous bacterial and fungal populations. The inoculated microbes and the *tfdA* gene remained quite stable throughout the experiment. The formation of transconjugants through plasmid pJP4 transfer to indigenous populations and subsequent growth on 2,4-D caused clear changes in the microbial community structure of the soil used in this study.

Acknowledgments

This study was supported by a research grant from LMO Environmental Risk Assessment Program, National Institute of Environmental Research, Ministry of Environment, and the BioGreen 21 Project, Rural Development Administration, Republic of Korea. Mr. Kim was supported by a grant from the Grop Functional Genomics Center, Korea (CG1520).

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