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Isolation and Characterization of 2,4-Dichlorophenoxyacetic Acid-degrading Bacteria from Paddy Soils

Min-Jae Chung and Jong-Ok Ka*

Department of Agricultural Biology, Seoul National University, Suwon 441-744, Korea

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Nine numerically dominant 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria were isolated from rice field soils. Most of the isolates were identified as *Burkholderia* or *Sphingomonas* species by fatty acid methyl ester (FAME) analysis, and they exhibited diverse chromosomal DNA patterns in polymerase chain reaction (PCR) amplification of repetitive extragenic palindromic (REP) sequences. The isolates utilized 2,4-D as the sole source of carbon and two *Sphingomonas* species were capable of mineralizing both 3-chlorobenzoate (3-CB) and 4-chlorobenzoate (4-CB), in addition to 2,4-D. Plasmid DNAs were detected from all of the isolates, and conjugation analysis revealed that 2,4-D degradative genes were located on transferable plasmids in most of the isolates. PCR analysis with specific primers selected from *tfd* genes showed that 67% of the isolates had DNA sequences homologous to the five *tfd* genes of the 2,4-D degradative plasmid pJP4 of *Alcaligenes eutrophus* JMP134. Among the isolates, strain TFD7 appeared to be a new genotype in that it contained a transmissible 2,4-D degradative plasmid nonhomologous to the *tfd* genes. 2,4-D was persistent in natural paddy soils which contained no indigenous 2,4-D-degrading microorganisms, but the application of the 2,4-D-degrading isolates resulted in rapid decline of the soil 2,4-D residues.

Key words: Biodegradation, 2,4-dichlorophenoxyacetic acid, paddy soil, bacteria

Chlorinated phenoxyalkanoates are widely used herbicides in agriculture. Among them, 2,4-dichlorophenoxyacetic acid (2,4-D) is a selective systemic herbicide used in post-emergence control of broad-leaved weeds in cereals, grassland, and on non-crop land. Unlike many of the synthetic compounds released into the environment, 2,4-D is relatively easily mineralized by soil bacteria using it as a carbon source (2, 11, 21). The populations of microorganisms able to degrade 2, 4-D have been estimated by the most-probable-number method (3, 6, 10, 18). The isolated 2,4-D-degrading microorganisms have been reported to belong to a number of genera, including *Alcaligenes, Arthrobacter, Pseudomonas*, and *Sphingomonas* (5, 12, 16).

As a herbicide, 2,4-D has also been extensively used in wetland rice field soils. In contrast to upland fields, the wet rice fields are characterized by submergence during part or all of the cropping period. Flooding of rice fields affects soil pH, redox state, and nutrient availability, and thus results in the differentiation of diverse microhabitats that can support the growth of a variety of microorganisms. While a few recent studies have tried to analyze the microbial community of rice paddy soil (8, 15), little information is available about the populations, species diversity, and distribution of the microorganisms involved in chloroorganic compound degradation in flooded rice soils. Moreover, despite extensive use of chloroorganic herbicides, their fates in rice paddy soils have rarely been studied.

Recent studies describing the isolation and characterization of novel chloroorganic compound degraders attach great importance to the geographic location and habitat for the recovery of new genotypes. It has been reported that pristine soils contained new types of 2,4-D- or 3-CB-degrading bacteria and the distribution of the bacterial genotypes were geographically limited (7, 14). Most of the 2,4-D-degrading microorganisms, however, have been isolated from upland field soils, and thus few studies report about the phylogenetic and genetic properties of the indigenous 2,4-D-degrading microorganisms in wetland paddy soils.

In this study, we isolated nine numerically dominant 2,4-D-degrading bacteria from 34 rice field soils, and investigated their diversity, physiological and genetic properties. In addition, soil microcosms were used to study 2,4-D degradation patterns in natural paddy soils with and without the inoculated 2,4-D-degrading isolates.

* To whom correspondence should be addressed.
(Tel) 0331-290-2475; (Fax) 0331-294-8533
(E-mail) kagil@plaza.snu.ac.kr

Materials and Methods

Media and culture conditions

All isolates were maintained on SMB (standard mineral base) mineral medium (20) containing 2,4-D at a concentration of 300 ppm (μg/ml). Peptone-tryptone-yeast extract-glucose (PTYG) medium containing (per liter) 0.25 g of peptone (Difco), 0.25 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 0.5 g of glucose, 0.03 g of magnesium sulfate, and 0.003 g of calcium chloride was used for strain purification and colony production for the REP-PCR.

Chemicals

4-chlorophenoxyacetic acid (4-CPA), 2-chlorophenoxyacetic acid (2-CPA), 3-chlorobenzoic acid (3-CB), and 4-chlorobenzoic acid (4-CB) were obtained from Aldrich Chemical Co., and analytical grade 2,4-dichlorophenoxyacetic acid (2,4-D), and 2-methyl-4-chlorophenoxyacetic acid (MCPA) were obtained from Sigma Chemical Co.

Isolation of bacterial strains

Paddy soil samples were taken from diverse countrywide sites. Samples from the top 15 cm of paddy soil were taken and kept at 4°C prior to use. A 10 g soil sample from each site was homogenized with 95 ml of a sterilized 0.85% saline solution by shaking the preparation on a rotary shaker (200 rpm). Samples (0.1 ml) of appropriate 10-fold dilutions were inoculated into most-probable-number tubes containing 3 ml of 2,4-D mineral medium (SMB mineral medium containing 300 ppm of 2,4-D). The tubes were incubated at 30°C for 3 weeks and degradation of 2,4-D was analyzed by spectrophotometry. The culture of the terminal positive tube showing 2,4-D degradation was enriched by two additional transfers into fresh medium. Each enriched culture was streaked onto PTYG agar medium and single colonies were then tested for 2,4-D degradation in fresh 2,4-D mineral medium before strain purification.

FAME analysis

The isolates were cultured on tryptic soy agar medium at 28°C for 48 to 72 h, and then cells were harvested from the plates by scraping with a sterile glass loop and used for FAME analysis. Saponification, methylation, and extraction were performed as described in the MIDI manual (Microbial Identification, Inc.) (19).

Colony REP-PCR

The colony REP-PCR was performed, using BOXA 1R as a primer, as described previously (4, 22). Each isolate was grown on the PTYG agar medium for 24 to 48 h, and then a small amount of cells was resuspended in 25 μl of PCR mixture. After PCR amplification, 10 μl samples of the REP-PCR products were separated by electrophoresis on horizontal 1% agarose gels.

Axenic culture experiment

After growth in 2,4-D mineral medium or PTYG medium to produce cells induced or not induced for 2,4-D metabolism, cells were harvested, washed twice with an equal volume of 15 mM phosphate buffer (pH 7.0), and resuspended in the same buffer. Aliquots of suspended cells were inoculated into duplicate flasks containing 200 ml of 2,4-D (300 ppm) mineral medium at a final density of ca. OD_{550}=0.005 for the axenic growth experiment or ca. OD_{550}=0.5 for the resting cell experiment. All cultures were incubated at 30°C and were aerated by shaking at 200 rpm on a rotary shaker. Aliquots of cultures were regularly removed to determine cell growth and degradation of 2,4-D.

Degradation phenotype analysis

Each strain was cultured in 2,4-D mineral medium or PTYG medium. Cells were then harvested, washed, and prepared in sodium phosphate buffer as described above. Aliquots of suspended cells were inoculated into culture tubes, each of which contained SMB mineral medium supplemented with one of the structural analogs at a concentration of 300 ppm. After 3-week incubation, the cultures were centrifuged to remove the cellular material, and the UV absorption was measured to determine the degradation of chloroorganic compounds.

Plasmid detection and conjugation

For detection of plasmid DNA, cells were lysed using a modified form (9) of the procedure of Kado and Liu (13). To analyze the transferability of the 2,4-D^+ phenotype of the isolates, matings were performed on membrane filters as described by Willetts (23). Transconjugants were selected on 2,4-D mineral medium containing 300 ppm 2,4-D, appropriate antibiotics, and 1.5% Noble agar.

PCR amplification of *tfd* genes

The partial sequences of *tfd* genes were amplified by PCR with specific primers targeted for the *tfdA*, *B*, *C*, *D*, and *E* genes of the 2,4-D degradative plasmid pJP4. The PCR cycles and primers for the *tfdA*, *tfdB*, and *tfdC* genes were previously reported (1, 14, 17), and the primers for *tfdD* and *tfdE* were design-

Table 1. Identification of 2,4-D-degrading isolates by FAME analysis

Isolate	Soil site of isolation[a]	Identity as determined by FAME analysis
TFD1	Suwon, Kyungki-Do	*Burkholderia cepacia*
TFD2	Seochun, Chungchongnam-Do	*Burkholderia gladioli*
TFD3	Pohang, Kyungsangbuk-Do	*Sphingomonas paucimobilis*
TFD4	Milyang, Kyungsangnam-Do	*Sphingomonas paucimobilis*
TFD5	Kimje, Chollabuk-Do	*Burkholderia* sp.
TFD6	Naju, Chollanam-Do	*Burkholderia* sp.
TFD7	Naju, Chollanam-Do	*unidentifiable*[b]
TFD8	Naju, Chollanam-Do	*Burkholderia cepacia*
TFD9	Naju, Chollanam-Do	*Burkholderia cepacia*

[a] All soils were from rice fields.
[b] The isolate could not be identified because of its poor match with profiles in the MIDI library.

ed based on the respective gene sequences found in pJP4 of *Alcaligenes eutrophus* JMP134: *tfdD*, 5'-TGGAACTCATCGAACAGC-3' and 5'-CCTGCAGTTCGTAATCCC-3'; *tfdE*, 5'-GACTTTGACATGGAGGCA-3' and 5'-CAAATGAATGCCCCACTG-3'. The amplification of the *tfdD* and *tfdE* genes with the corresponding primers (30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min) were expected to produce 356-bp and 342-bp DNA fragments, respectively.

Degradation of 2,4-D in paddy soil

Two soil samples with or without the indigenous 2,4-D-degrading microorganisms were obtained from rice fields. The soil was stored at field moisture levels at 4°C until it was used. The isolate TFD6 (Table 1) was chosen as the inoculum strain, because it degraded 2,4-D quickly and had the highest tolerance to 2,4-D among the isolates. The strain TFD6 was grown at 30°C in PTYG medium, harvested, washed, and prepared in sodium phosphate buffer. Each of the paddy soil samples was inoculated with the strain TFD6 at a density of ca. 1.0×10^6 cells/g soil. The soil was thoroughly mixed, and 300 g was transferred to each of duplicate beakers; two other control replicates were not inoculated with the strain TFD6. Inoculated and uninoculated soils were treated with 2, 4-D dissolved in 0.1 M NaH_2PO_4 buffer (pH 7.0) to a concentration of 300 ppm and thoroughly mixed. The disappearance of 2,4-D from soil was monitored by high-performance liquid chromatography (11), and the soils were respiked with 2,4-D (300 ppm) after it was removed until a total of 5 cycles of degradation had been completed.

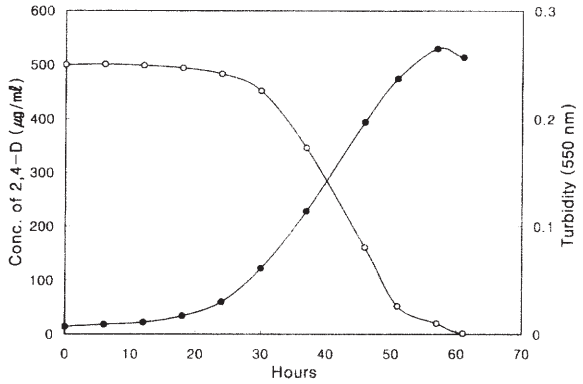

Fig. 1. Disappearance of 2,4-D (○) and growth of bacteria (●) during degradation of 2,4-D by strain TFD1.

Results and Discussion

Degradation of 2,4-D

2,4-D in mineral medium was completely degraded by the isolates and no intermediate products were detected by high-performance liquid chromatography analysis. Fig. 1 shows typical growth and degradation curves of strain TFD1 on 2,4-D medium. The isolates were able to utilize 2,4-D as a sole carbon and energy source, producing a significant biomass in 2,4-D mineral medium.

FAME and REP-PCR analysis

The isolates that could be reasonably identified

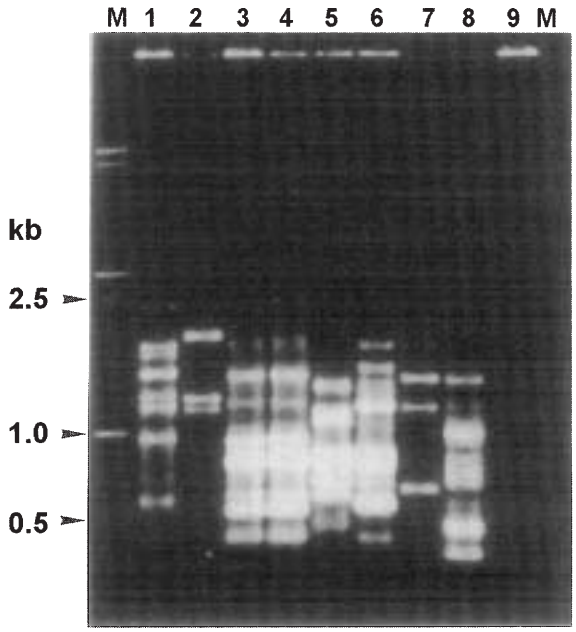

Fig. 2. REP-PCR patterns of the isolates. Lanes: M, 1 kb DNA ladder; 1, strain TFD1; 2, strain TFD2; 3, strain TFD3; 4, strain TFD4; 5, strain TFD5; 6, strain TFD6; 7, strain TFD 7; 8, strain TFD8; 9, strain TFD9.

by the FAME results are shown in Table 1. The isolates belonged to *Burkholderia* or *Sphingomonas* species, except strain TFD7 which could not be identified due to its poor match with profiles in the MIDI library.

A colony REP-PCR experiment was performed to study the genomic relatedness of the isolates (Fig. 2). The REP-PCR analysis of the isolates revealed that these bacteria produced 8 different DNA fingerprint patterns. Strains TFD3 and TFD 4, which were identified by the FAME analysis as *S. paucimobilis*, exhibited the same band pattern, suggesting that they are very closely related, although they were isolated from different locations. All of the other isolates had no significant common bands.

Growth of 2,4-D-degrading bacteria in axenic cultures

To understand axenic growth patterns of the 2,4-D degraders, some representative strains were inoculated into 2,4-D mineral medium under both induced and uninduced conditions (Fig. 3). Under the uninduced condition, strains TFD1 and TFD2 exhibited short lag periods (ca. 25 h) and thereafter began to grow exponentially. Strain TFD3 exhibited relatively longer lag periods (ca. 60 h). By contrast, the induced strains grew more quickly than the uninduced cells, suggesting that their 2,4-D degradative enzymes were inducible by the presence of the substrate 2,4-D. The inducibility of 2,4-D enzymes of the isolates was confirmed in resting cell experiments conducted with cells adapted or not adapted to 2,4-D metabolism. The adapted cells mineralized 2,4-D quickly without any lag time, while the unadapted cells could not degrade it during the incubation period.

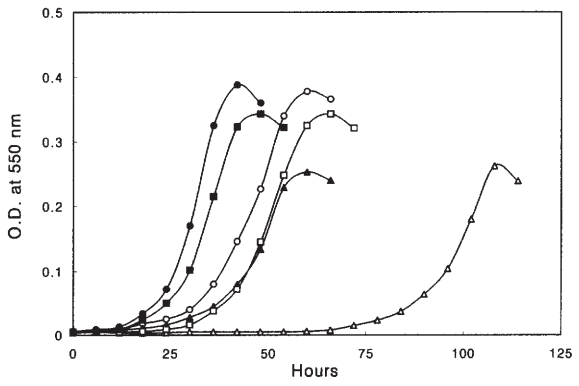

Fig. 3. Growth patterns of 2,4-D-degrading bacteria in axenic culture. Symbols: ● and ○, strain TFD1; ■ and □, strain TFD2; ▲ and △, strain TFD3. The bacteria were either adapted (solid symbols) or not adapted for 2,4-D metabolism (open symbols). Each point is the mean for two replicate liquid cultures. OD, optical density.

Degradative diversity analysis

The isolates were grown on 2,4-D mineral medium and PTYG medium and then examined for their ability to degrade other compounds related to 2,4-D (Table 2). The isolates exhibited diverse patterns in their substrate utilization abilities. Strains TFD6 and TFD7 vigorously utilized 2,4-D, 3-CB, and MCPA as sole carbon sources, as indicated by complete disappearance and by substantial cell growth. Strains TFD1 and TFD8 could degrade 4-CB. On the other hand, strain TFD9 was very restricted in its substrate utilization ability. Interestingly, strains TFD3 and TFD4, which were identified as *Sphingomonas paucimobilis* by FAME analysis, were able to degrade both 3-CB and 4-CB, in addition to 2,4-D. Since none of the previously described 2,4-D-degrading bacteria could degrade 4-CB (12), these strains appeared to have quite unique degradative enzyme systems among 2,4-D degraders. The degradation of 3-CB by the *Sphingomonas* strains only under 2,4-D-adapted conditions indicated that this compound was probably metabolized because of its structural similarity to 2,4-D or 2,4-D pathway intermediates.

Table 2. Patterns of utilization of 2,4-D-related compounds by 2,4-D-degrading isolates[a]

Isolate	Growth Condition	Substrate					
		2,4-D	3-CB	4-CB	4-CPA	MCPA	Salicylate
TFD1	A	+	−	−	+	−	+
	U	+	−	−	+	−	+
TFD2	A	+	−	−	−	−	+
	U	+	−	−	−	−	+
TFD3	A	+	+	+	−	−	+
	U	+	−	+	−	−	+
TFD4	A	+	+	+	−	−	+
	U	+	−	+	−	−	+
TFD5	A	+	−	−	−	−	+
	U	+	−	−	−	−	+
TFD6	A	+	+	−	−	+	−
	U	+	+	−	−	+	−
TFD7	A	+	+	−	−	+	+
	U	+	+	−	−	+	+
TFD8	A	+	+	−	+	−	−
	U	+	+	−	+	−	−
TFD9	A	+	−	−	−	−	+
	U	+	−	−	−	−	+

[a] The bacteria were grown on 2,4-D (A) or on PTYG (U) and then tested for substrate utilization capabilities. +, >90% reduction in peak height as determined by UV scanning and substantial growth (optical density at 550 nm, >0.25); −, <10% reduction in peak height and very scant growth (optical density at 550 nm, <0.01).

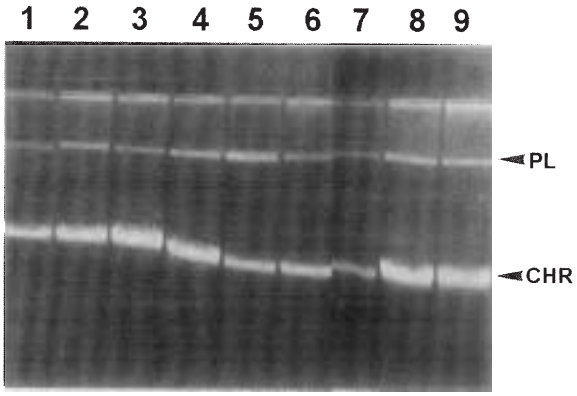

Fig. 4. Plasmid profiles of 2,4-D-degrading isolates. Lanes: 1, strain TFD1; 2, strain TFD2; 3, strain TFD3; 4, strain TFD4; 5, strain TFD5; 6, strain TFD6; 7, strain TFD7; 8, strain TFD 8; 9, strain TFD9. The positions of plasmid DNA (PL) and linear DNA (CHR) are indicated on the right of the gel.

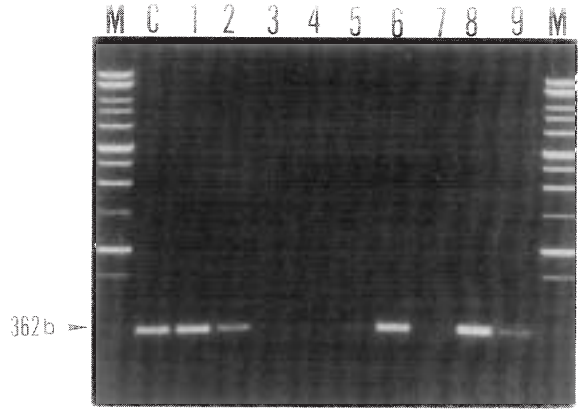

Fig. 5. Amplified DNA fragments obtained from the isolates with colony-PCR targeted for the *tfdA* gene. Lanes: M, 1 kb DNA ladder; C, *Alcaligenes eutrophus*/pJP4; 1, strain TFD1; 2, strain TFD2; 3, strain TFD3; 4, strain TFD4; 5, strain TFD5; 6, strain TFD6; 7, strain TFD7; 8, strain TFD8; 9, strain TFD9.

Transferability of 2,4-D degradation phenotype.

When the isolates were subjected to Kado's plasmid detection procedure, all of the isolates exhibited plasmid DNA bands (Fig. 4). To investigate whether the 2,4-D degradative genes were on the plasmids and transmissible to other bacteria, the isolates were mated with antibiotics-resistant recipients such as *Pseudomonas cepacia* and *Alcaligenes* sp. In most of the isolates, the 2,4-D$^+$ phenotype was easily transfered with average frequencies of 1.6×10^{-4} to 1.9×10^{-1} per donor colony formed. Plasmid bands exhibiting identical electrophoretic mobilities were observed in agarose gels containing the transconjugants and their respective donors (data not shown). On the other hand, the 2,4-D$^+$ phenotype was not transferred at a detectable frequency ($<10^{-9}$) from *Sphingomonas* strains TFD3 and TFD4, suggesting that their 2,4-D genes were located in the chromosomal DNA or on the untransmissible plasmid in these isolates. *S. paucimobilis* is a member of the alpha subdivision of the class *Proteobacteria* and has been reported not to have transmissible 2,4-D-degrading property (12, 14).

PCR amplification of *tfd* genes.

PCR amplification was performed for every isolate using specific primers selected from the internal sequences of the *tfdA*, *B*, *C*, *D*, and *E* genes. Fig. 5 shows typical PCR-amplified DNA bands (362 b in size) of the isolates when primers for the *tfdA* gene were used. Among nine isolates, strains TFD1, TFD 2, TFD5, TFD6, TFD8, and TFD9, which were identified as *Burkholderia* species, produced amplification products of the expected sizes from all the five *tfd* genes (data not shown). The other three strains did not produce any DNA bands with the selected *tfd* primers, indicating that these isolates carried no genes similar to the *tfd* genes. In recent study, Kamagata *et al.* described three classes of 2,4-D-degrading isolates based on their phylogenetic, physiological, and genetic properties (14). According to their classification, strains TFD1, TFD2, TFD5, TFD6, TFD8, and TFD9 belong to class I, and strains TFD3 and TFD4 belong to class II. On the other hand, strain TFD7, which is a fast-growing copiotroph and has a transmissible 2,4-D plasmid without any significant homology to the *tfd* genes, appears to be a new genotype, possibly constituting a new group, class IV. In the future study, apparently more attention needs to be placed on understanding this unknown group.

Degradation of 2,4-D in paddy soil

The patterns of degradation of 2,4-D in natural paddy soils were analyzed with and without 2,4-D degraders. 2,4-D was observed to be quickly degraded

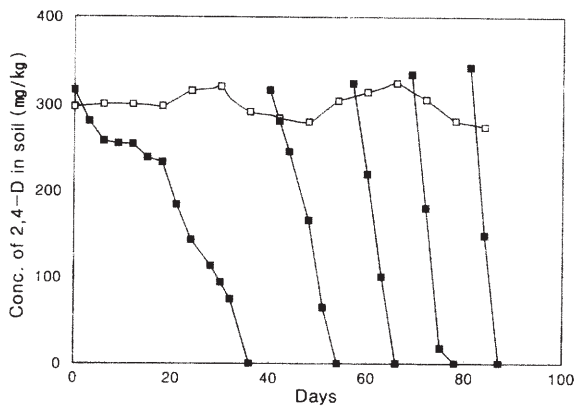

Fig. 6. Degradation of 2,4-D in Chungchongbuk-Do soil during 5 repeated additions in microcosms inoculated with strain TFD6 (■) or with only indigenous microbial population (□).

after short lag periods in paddy soils containing indigenous 2,4-D degraders (data not shown). However, 2,4-D was persistent for quite a long time in natural paddy soils containing no indigenous 2,4-D-degrading microorganisms (Fig. 6). The application of one of the isolates into this soil at a density of ca. 10^6 cells/g soil resulted in quick degradation of 2,4-D, taking about 1 week for the complete removal of 300 ppm of 2,4-D in adapted soils (Fig. 6). The result suggested that when used in agricultural practices, 2,4-D could persist in the soils lacking 2,4-D degraders, possibly leading to environmental pollution problem. The 2,4-D-degrading strains isolated in this study could thus help in removing the persistent 2,4-D residues through in situ bioremediation.

Acknowledgments

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