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Isolation and Characterization of 4-(2,4-Dichlorophenoxy)Butyric Acid-Degrading Bacteria from Agricultural Soils

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Abstract Eight numerically dominant 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB)-degrading bacteria and three pairs of bacteria showing syntrophic metabolism of 2,4-DB were isolated from soils, and their phylogenetic and phenotypic characteristics were investigated. The isolates were able to utilize 2,4-DB as a sole source of carbon and energy, and their 2,4-DB degradative enzymes were induced by the presence of 2,4-DB. Analysis of 16S rDNA sequences indicated that the isolates were related to members of the genera, *Variovorax*, *Sphingomonas*, *Bradyrhizobium*, and *Pseudomonas*. The chromosomal DNA patterns of the isolates obtained by polymerase-chain-reaction (PCR) amplification of repetitive extragenic palindromic (REP) sequences were distinct from each other. Four of the isolates had plasmids, but only one strain, DB1, had a transmissible 2,4-D degradative plasmid. When analyzed with PCR using primers targeted to the *tfdA*, *B*, and *C* genes, only strains DB2 and DB9a produced DNA bands of the expected sizes with the *tfdA* and *C* primers, respectively. All of the isolates were able to degrade 2,4-D as well as 2,4-DB, suggesting that the degradation pathways of these compounds were closely related to each other, but respiratory activities of many isolates adapted to 2,4-DB metabolism were quite low with 2,4-D.

Key words: 4-(2,4-Dichlorophenoxy)butyric acid, bacteria, biodegradation

Large amounts of man-made chlorinated organic chemicals have been used as pesticides in agriculture. Among them, chlorinated phenoxyalkanoates are widely used, and thus many studies have focused on their persistence and fate in the natural environments [32, 33, 34]. Some phenoxyacid herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA), are readily mineralized by a number of soil microorganisms and stimulate substantial growth of the corresponding microbial population [5, 12, 27]. In contrast, other closely related pesticides, such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), are not easily degraded by soil microorganisms, and organisms able to degrade them are very rare in the environment [1].

2,4-DB is one of the chlorinated phenoxy alkanoic herbicides and is closely related to 2,4-D in its structure and action mechanism. 2,4-DB was developed for use in agriculture as a selective herbicide against broad-leaved weeds in several leguminous crops that are sensitive to 2,4-D [37]. 2,4-DB itself has no effect as a herbicide, but when it is exposed to the plant, which is capable of beta-oxidation, it is converted to phytotoxic 2,4-D and kills the plant.

Soil microorganisms are the most active agents in detoxifying pesticide residues and industrial chemicals exposed to the environment, thus many studies have focused on the isolation and application of microorganisms capable of degrading pesticides and industrial organic pollutants [7, 17, 22, 26]. 2,4-DB reaches the soil mainly during application process and by transfer from plants to the soil by rainwater. In soil, 2,4-DB is suggested to undergo beta-oxidation to 2,4-D, which is further degraded by other soil microflora [14]. However, unlike other phenoxyalkanoate herbicides such as 2,4-D and MCPA, very few microorganisms able to degrade 2,4-DB have been isolated from soils and thus little information is available on their metabolic, genetic, and physiological properties. It is presumed that 2,4-DB could be degraded by 2,4-D-degrading bacteria either containing beta-oxidation systems or that are able to cleave the ether linkage. However, since most of the previously reported 2,4-D-degrading microorganisms were not able to attack 2,4-DB [8, 19], it appears that a new class of microorganisms different from the previous 2,4-D-degrading bacteria isolated under 2,4-D selection are expected to be involved in 2,4-DB degradation in natural soils.

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In this study, eight 2,4-DB-degrading bacteria and three pairs of syntrophic bacteria capable of co-mineralizing 2,4-DB were isolated from 64 agricultural soils. Their species diversity was investigated by 16S rDNA sequence analysis and REP-PCR patterns of chromosome, and their physiological and genetic properties related to 2,4-DB biodegradation are described.

MATERIALS AND METHODS

Media and Culture Conditions

Peptone-tryptone-yeast extract-glucose (PTYG) medium contained (in grams per liter) 0.25 g of peptone (Difco Laboratories, Detroit, MI, U.S.A.), 0.25 g of tryptone (Difco), 0.5 g of yeast extract (Difco), glucose (0.5), magnesium sulfate (0.03), calcium chloride (0.003), and agar (for plates only, 15). The 2,4-DB mineral medium used for enrichment, isolation, and maintenance of isolates contained (in grams per liter) 2,4-DB (0.3), KH_2PO_4 (1.36), Na_2HPO_4 (1.41), $(NH_4)_2SO_4$ (0.3), $MgSO_4 \cdot 7H_2O$ (0.05), $CaCl_2 \cdot H_2O$ (0.0058), trace metal solution (5 ml per liter), and agar (for plates only, 15). The trace metal solution contained (in grams per liter) $FeSO_4 \cdot 7H_2O$ (0.55), $ZnSO_4 \cdot 7H_2O$ (0.23), $MnSO_4 \cdot 7H_2O$ (0.34), $Co(NO_3)_2 \cdot 6H_2O$ (0.075), $CuSO_4 \cdot 5H_2O$ (0.047), and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.025). Each of the bacteria showing syntrophic degradation of 2,4-DB was maintained on PTYG medium. PTYG medium was also used for strain purification and colony production for the repetitive extragenic palindromic PCR (REP-PCR). All cultures were incubated at 30°C and liquid cultures were aerated by shaking at 200 rpm on a rotary shaker (Vision Co., Bucheon, Korea).

Chemicals

4-(2,4-Dichlorophenoxy)butyric acid (2,4-DB), 4-(2-methyl-4-chlorophenoxy)butyric acid (MCPB), 4-chlorophenoxyacetic acid (4-CPA), 4-chlorocatechol (4-CC), and chlorohydroquinone (CHQ) were obtained from Aldrich Co., Milwaukee, U.S.A., and analytical grade 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenol (2,4-DCP), and 2-methyl-4-chlorophenoxyacetic acid (MCPA) were obtained from Sigma Co., St. Louis, U.S.A. 3,5-Dichlorocatechol (3,5-DCC) was kindly donated by Dr. W. Reineke (Bergische Universitat-Gesamthochschul Wuppertal, Germany).

Enumeration and Isolation of 2,4-DB-Degraders

Agricultural soil samples were taken from upland fields in various locations in South Korea. The agricultural fields selected in this study have been under normal agricultural practices, growing dry crops, such as legumes and barley, for more than 10 years. Samples from the top 15 cm of soil were taken, sifted through a 2-mm-pore-size sieve, and kept at 4°C prior to use. A 10-g soil sample from each site was homogenized with 95 ml of sterile 0.85% saline solution by shaking the preparation at 200 rpm on a rotary shaker. Samples (0.1 ml) of appropriate 10-fold dilutions were inoculated into five replicate MPN (most-probable-number) tubes containing 3 ml of 2,4-DB medium (300 ppm). The tubes were incubated at 30°C for 4 weeks and degradation of 2,4-DB was analyzed by a Shimadzu LC-10 series high-performance liquid chromatography (Shimadzu Co., Kyoto, Japan) on μBondapack C18 column (3.9 by 300 mm; Waters, Milford, PA, U.S.A.) and a UV detector set at 278 nm; methanol-0.1% phosphoric acid (70:30) was used as the eluant. The culture of the terminal positive tube showing substantial cell growth and less than 20% of the 2,4-DB remaining 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-DB degradation in fresh 2,4-DB medium before strain purification. The population density of 2,4-DB degraders was estimated from MPN tables [2].

16S rDNA Sequence Analysis

Total genomic DNA was isolated from the isolates and PCR amplification of 16S rRNA genes was performed with 27f and 1492r as previously described [24]. The amplified 16S rRNA genes were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, WI, U.S.A.) according to the manufacturer's instructions with the sequencing primers 27f and 519r [16]. Approximately 400 unambiguous nucleotide positions were used for comparison to the data in GenBank using Basic Local Alignment Search Tool (BLAST) [3]. Sequences from nearest relatives were identified from the Ribosome Database Project (RDP) using the SIMILARITY-RANK program of the RDP [30].

Colony REP-PCR

The colony REP-PCR was performed using BOXA1R as a primer as described previously [9, 36]. 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.

Degradative Phenotype Analysis

Each strain was grown in PTYG medium. Cells were then harvested by centrifugation at 10,000 ×g for 10 min at 4°C, 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 culture tubes, each of which contained the mineral medium supplemented with one of the structural analogs at a concentration of 300 μg/ml. After 4 week's incubation, the

cultures were centrifuged to remove the cellular material, and the UV absorption was measured to determine the degradation of phenoxyacetates.

Axenic Culture Experiment

After growth in 2,4-DB medium or PTYG medium, cells were harvested, washed, and resuspended in sodium phosphate buffer. Aliquots of suspended cells were inoculated into duplicate flasks containing 200 ml of 2,4-DB medium at a final density of OD_{550}=0.005. All cultures were incubated at 30°C and were aerated by shaking at 200 rpm on a rotary shaker. Aliquots of the cultures were regularly removed to determine cell growth and degradation of 2,4-DB.

Plasmid Detection and Conjugation

For detection of plasmid DNA, cells were lysed using a modified form [18] of the procedure of Kado and Liu [20]. To analyze the transferability of the 2,4-DB degradative phenotype of the isolates, matings were performed on membrane filters as described by Willetts [39], using *Alcaligenes eutrophus* JMP228 and *Pseudomonas cepacia* DBO1 as the recipients. Transconjugants were selected on 2,4-DB or 2,4-D mineral medium containing appropriate antibiotics, 1.5% Noble agar, and 300 µg/ml of 2,4-DB or 2,4-D, respectively.

PCR Amplification of the *tfd* Genes

The partial gene sequences specific to the 2,4-D degradation pathway were amplified by PCR, with specific primers targeted for the *tfdA*, *B*, and *C* genes of the 2,4-D degradative plasmid pJP4. The PCR cycles and primers for the *tfd* genes were previously reported [4, 21, 35]. The amplification of the *tfdA*, *B*, and *C* genes with the corresponding primers is expected to produce a 362 bp, 205 bp, and 361 bp DNA fragment, respectively.

Respirometric Experiments

Each strain was grown in 2,4-DB medium. Cells were then harvested at 4°C, washed twice, and resuspended in mineral medium. The resuspended cells were kept in ice prior to being added to the respiratory chambers. The initial rates of oxygen uptake were measured with Clark type polarographic oxygen probes of the YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) according to the manufacturer's manual. The sample chamber contained 100–300 ppm substrate and the equivalent of about 0.4 mg dry wt. bacteria in a total volume of 3.0 ml. The experiments were carried out at 28°C in duplicates, and the indicated rates of oxygen uptake have been corrected for indigenous respiratory rates measured in the absence of any added substrates. Protein content was measured by the method of Lowry *et al.* [28], using bovine serum albumin as a standard and activities were expressed as µl O_2/h/mg protein.

RESULTS AND DISCUSSION

Distribution and Isolation of 2,4-DB-Degrading Bacteria

The numbers of 2,4-DB-degrading microorganisms in different agricultural soils were estimated with the MPN procedure. The 2,4-DB-degrading populations ranged from 0 to 18.8 cells/g soil in 64 different agricultural soils, and 82% of the soil samples did not show any detectable degradation of 2,4-DB during the incubation time.

Eight 2,4-DB-degrading bacteria were isolated and purified from the positive MPN tubes (Table 1). 2,4-DB in mineral medium was completely degraded by the isolates, and no dead-end products were accumulated during biodegradation of 2,4-DB, when analyzed with high-performance liquid chromatography. Since 2,4-DB is highly water-soluble and not volatile, its disappearance is due to microbial degradation. Figure 1 shows typical growth and degradation curves of strain DB3 on 2,4-DB medium. However, some enriched cultures failed to produce purified strains able to degrade 2,4-DB. When the different colony types from each of these cultures were combined on 2,4-DB medium, some mixed cultures were able to grow and mineralize 2,4-DB. From these mixed cultures, three pairs of presumably syntrophic bacteria (denoted by a and b) capable of degrading 2,4-DB were isolated from three different soils (Table 1). For each of three syntrophic pairs, both organisms were able to grow together on 2,4-DB medium, but neither organism alone was able to grow on 2,4-DB (Fig. 2). It has been suggested that synergistic degradation of agricultural pesticides in soil could be the important mechanism and pathway of detoxification of environmental contaminants. In the case of organophosphate insecticides, an *Arthrobacter* sp. and a *Streptomyces* sp. have been shown to degrade diazinon synergistically [13]

Table 1. Nearest relatives of the 2,4-DB-degrading isolates based upon 16S rDNA sequence analysis.

Isolate	Nearest relative	% Similarity*
DB1	*Variovorax* sp. WFF52	94
DB2	*Variovorax* sp. WFF52	96
DB3	*Sphingomonas macrogoltabidus*	94
DB4	*Sphingomonas macrogoltabidus*	96
DB5	*Bradyrhizobium japonicum*	98
DB6	*Bradyrhizobium japonicum*	97
DB7	*Bradyrhizobium japonicum*	95
DB8	*Bradyrhizobium japonicum*	96
DB9a	*Sphingomonas* sp. JSS-54	98
DB9b	*Pseudomonas* sp. Isolate CRE 12	99
DB10a	*Variovorax* sp. WFF52	97
DB10b	*Bradyrhizobium japonicum*	97
DB11a	*Variovorax* sp. WFF52	95
DB11b	*Pseudomonas* sp. Isolate CRE 12	96

*Based upon approx. 400 nucleotide positions at the 5' end of the 16S rRNA gene.

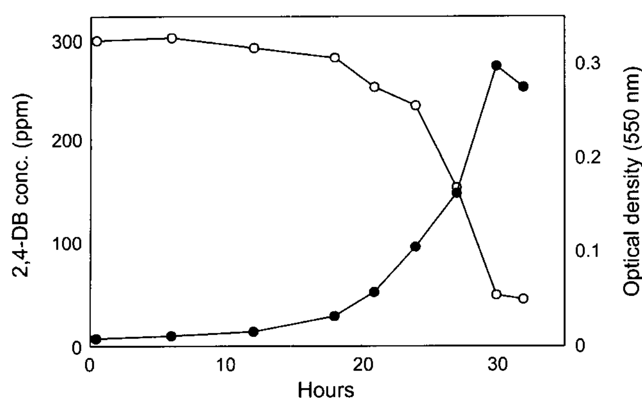

Fig. 1. Disappearance of 2,4-DB (○) and growth of strain DB3 (●) during its growth in mineral medium.
Each point is the mean for two replicate liquid cultures.

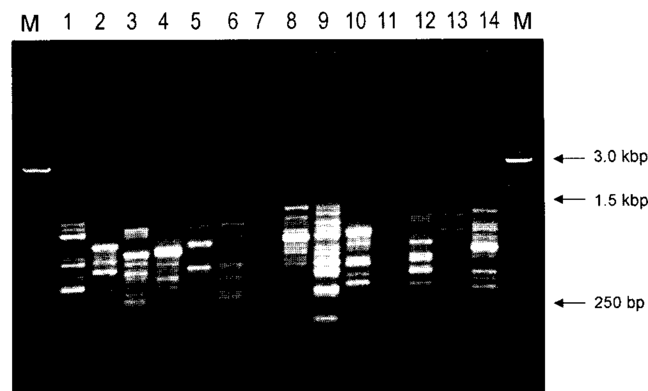

Fig. 3. REP-PCR band patterns of the isolates.
Lanes : 1, DB1; 2, DB2; 3, DB3; 4, DB4; 5, DB5; 6, DB6; 7, DB7; 8, DB8; 9, DB9a; 10, DB9b; 11, DB10a; 12, DB10b; 13, DB11a; 14, DB11b; M, DNA size marker.

and mixed cultures were able to grow on parathion [6, 31]. For the phenoxyalkanoate pesticides, complex enriched cultures have been reported to be able to degrade mecoprop [15, 25]. The results showed that 2,4-DB could also be syntrophically mineralized by two-component cultures.

16S rDNA Sequence and REP-PCR Analyses

When analyzed by 16S rDNA sequences, the isolates were found to be related to members of the genera *Variovorax*, *Sphingomonas*, *Bradyrhizobium*, and *Pseudomonas* (Table 1). All of the isolates were Gram-negative and belonged to the alpha, beta, and gamma subgroups of *Proteobacteria*. Although the 2,4-DB-degrading strains were isolated from different soil locations, some isolates were most closely related to the same species, such as *Variovorax* sp. WFF52 and *Bradyrhizobium japonicum*. A REP-PCR experiment was performed to study the genomic relatedness among the closely related isolates by 16S rDNA sequence analysis. It was revealed that not only did the eight isolates capable of degrading 2,4-DB alone exhibit distinct REP-PCR DNA patterns, but the three pairs of syntrophic bacteria also produced six different DNA fingerprint patterns (Fig. 3), suggesting that each isolate is a distinct strain.

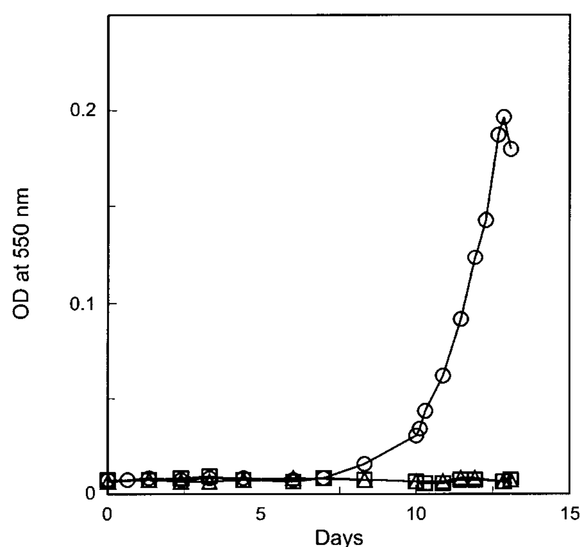

Fig. 2. Syntrophic growth of strains DB11a and DB11b in 2,4-DB mineral medium.
Symbols: ○, DB11a and DB11b; □, DB11a; △, DB11b. OD, optical density.

Growth of 2,4-DB-Degrading Bacteria in Axenic Cultures

To understand axenic growth patterns of the 2,4-DB degraders, the strains were inoculated into 2,4-DB medium after growth on 2,4-DB (inducing) or on PTYG (non-inducing). The growth curves of some representative strains are shown in Fig. 4. Among the isolates capable of degrading 2,4-DB alone, strains DB1 to DB4 grew quickly on 2,4-DB medium, showing doubling times ranging from 3.8 h (strain DB3) to 11.5 h (strain DB1), but strains DB5 to DB8 grew very slowly, taking about 20-30 days for degrading 300 μg/ml of 2,4-DB completely. Under an uninduced condition, the strains DB3 and DB4 exhibited a short lag period (ca. 2 days). Strains DB1 and DB2 exhibited relatively longer lag periods (ca. 5-7 days). By contrast, when adapted to 2,4-DB metabolism, the isolates degraded 2,4-DB much more quickly, suggesting that the 2,4-DB degradative enzymes were inducible by the presence of 2,4-DB. The inducibility of 2,4-DB degradative enzymes of the isolates by 2,4-DB was further confirmed by respirometry experiments conducted with adapted or non-adapted cells: Respiratory activities on 2,4-DB ranged from 8.0 (strain DB6) to 259.1 (strain DB3) μl O_2/h/mg protein for the isolates grown on 2,4-DB (Table 3), but the cells cultivated on glucose as the sole carbon source oxidized the herbicide very slowly, or not at all (data not shown).

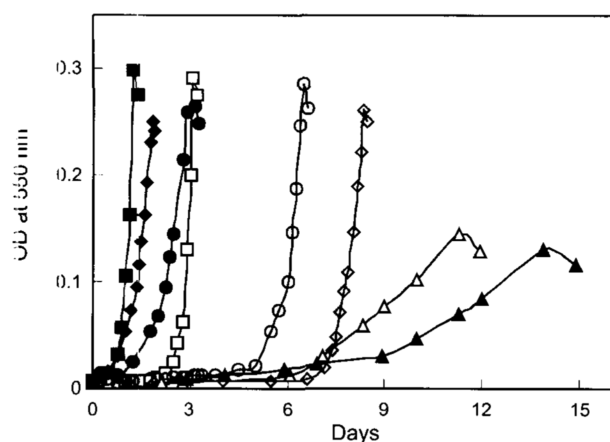

Fig. 4. Growth of 2,4-DB-degrading bacteria on 2,4-DB. Symbols: ○ and ●, strain DB1; ◇ and ◆, strain DB2; □ and ■, strain DB3; △ and ▲, strain DB11ab. The inoculated bacteria were either adapted (solid symbol) or not adapted (open symbol) to 2,4-DB metabolism. Each point is the mean for two replicate liquid cultures. OD, optical density.

For the syntrophic bacteria, DB9ab showed a growth pattern similar to the strain DB3, but the other syntrophic groups exhibited longer lag periods (ca. 7 days) under uninduced conditions (Fig. 4). When induced with 2,4-DB metabolism, the syntrophic groups (DB10ab and DB11ab) still exhibited slow growth on 2,4-DB medium. From plate counts of each member of the syntrophic pair DB11ab, it was observed that the density of DB11b, which was initially inoculated at the ratio of 1:1 from the non-inducing PTYG medium, was about 4-fold higher than DB11a at the end of exponential growth on 2,4-DB medium. When these adapted cells were washed and transferred into fresh 2,4-DB medium, the density of DB11b declined slightly (ca. half-fold) at the early phase (for the first 9 days) before it resumed its growth, while the other member, DB11a, showed steady increase in plate counts during growth on 2,4-DB. These results may explain the observed slow growth of the adapted syntrophic pair DB11ab on 2,4-DB medium and suggest that DB11a attacks 2,4-DB initially, followed by DB11b which begins to degrade the accumulated intermediate from the middle phase of 2,4-DB degradation.

Degradative Diversity Analysis

The isolates were grown on PTYG medium, and then examined for their ability to degrade other compounds related to 2,4-DB. All of the isolates capable of degrading 2,4-DB independently were also able to mineralize 2,4-D (Table 2), suggesting that the degradation pathways for these two compounds were closely related to each other in these isolates. Strains DB2 and DB3 could utilize MCPB, in addition to 2,4-DB and 2,4-D, and strain DB1 was the most versatile in substrate utilization, using 2,4-DB, 2,4-D, MCPB, MCPA, and 4-CPA as sole carbon sources. By

Table 2. Patterns of utilization of substrates by the isolates.

Isolate	Substrate*				
	2,4-DB	2,4-D	MCPB	MCPA	4-CPA
DB1	+	+	+	+	+
DB2	+	+	+	-	-
DB3	+	+	+	-	-
DB4	+	+	+	-	-
DB5	+	+	-	-	+
DB6	+	+	-	-	+
DB7	+	+	-	-	+
DB8	+	+	-	-	-
DB9a	-	-	-	-	-
DB9b	-	-	-	-	-
DB9ab	+	+	+	+	-
DB10a	-	-	-	-	-
DB10b	-	-	-	-	-
DB10ab	+	+	-	-	-
DB11a	-	-	-	-	-
DB11b	-	-	-	-	-
DB11ab	+	+	-	-	-

*Test for the utilization of substrates. +, >80% reduction in peak height as determined by UV scanning and substantial growth (optical density at 550 nm >0.13); -, <15% reduction in peak height and very scant growth (optical density at 550 nm <0.01). 2,4-DB, 4-(2,4-dichlorophenoxy)butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MCPB, 4-(2-methyl-4-chlorophenoxy)butyric acid; MCPA, 2-methyl-4-chlorophenoxyacetic acid; 4-CPA, 4-chlorophenoxyacetic acid.

contrast, none of the isolates were able to degrade mecoprop or dichloroprop, which has a propionic acid side chain.

Each of the two-member communities was also able to degrade 2,4-DB and 2,4-D syntrophically, while none of those pure cultures could degrade any of the compounds (Table 2). Interestingly, the DB9a and DB9b pair was capable of synergistically mineralizing MCPB and MCPA, in addition to 2,4-DB and 2,4-D. It is not yet clear which strain attacked the herbicide initially and what kinds of intermediates were produced during syntrophic metabolisms. In future studies, more attention will be placed on understanding the detailed mechanisms of 2,4-DB as well as other herbicides and co-degradation by these syntrophic bacteria.

Respirometric Experiments

To investigate the diversity of 2,4-DB degradation pathways among the isolates, each strain and the syntrophic pairs were grown in 2,4-DB medium. They were then analyzed for oxygen uptake in the presence of various substrates, including the assumed intermediates of 2,4-DB, such as 2,4-D, 2,4-DCP, 3,5-DCC, and chlorohydroquinone (Table 3). 2,4-DB-grown cells of strain DB1 rapidly oxidized 2,4-DB, 2,4-D, 2,4-DCP, and 3,5-DCC, suggesting that this strain initially beta-oxidized 2,4-DB to 2,4-D, which was

Table 3. Respiratory activities (μl O_2/h/mg protein) of whole cells of 2,4-DB-degrading bacteria grown on 2,4-DB*.

Substrate[†]	Bacterial cells										
	DB1	DB2	DB3	DB4	DB5	DB6	DB7	DB8	DB9ab	DB10ab	DB11ab
2,4-DB	171.6	109.1	259.1	211.4	72.1	8.0	31.8	111.0	151.7	55.4	171.3
2,4-D	387.2	18.2	39.5	36.9	55.0	10.8	<1.0	30.7	43.5	7.0	171.7
2,4-DCP	495.8	160.1	95.4	71.5	113.1	<1.0	28.3	114.8	92.4	66.0	30.2
3,5-DCC	367.8	38.4	67.8	44.2	<1.0	<1.0	13.2	102.9	52.3	81.3	70.2
CHQ	24.7	24.4	<1.0	<1.0	37.5	9.6	2.8	74.9	30.9	<1.0	12.9
Glucose	36.4	21.5	<1.0	7.0	<1.0	19.3	<1.0	<1.0	20.6	3.1	10.6

*The values are the mean of two replicate assays of two independent cultures.
[†]2,4-(2,4-dichlorophenoxy)butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; CHQ, chlorohydroxyquinone; 4-CC, 4-chlorocatechol.

then further mineralized through the previously described 2,4-D degradative pathway of *Alcaligenes eutrophus* JMP134(pJP4) [10]. In contrast, 2,4-DB-grown cells of strain DB2 had much lower oxygen consumption with 2,4-D, while this strain rapidly oxidized 2,4-DCP. These results suggest that strain DB2 might initially have attacked 2,4-DB by cleavage of the ether linkage of the side chain, which has been reported as an alternative pathway for 2,4-DB in a *Flavobacterium* species [29]. Strain DB7 also showed similar patterns to strain DB2 in oxygen consumption with 2,4-D and 2,4-DCP. Among the syntrophic bacteria, group DB10ab showed much higher oxygen consumption with 2,4-DCP than 2,4-D, while group DB11ab had higher oxygen consumption with 2,4-D than 2,4-DCP. It has been suggested that 2,4-DCP could be degraded through chlorohydroxyquinone in *Streptomyces* species [40]. Among the isolates, strains DB5 and DB6 consumed oxygen at a faster rate in the presence of chlorohydroxyquinone as compared to 3,5-DCC, suggesting that chlorohydroxyquinone rather than 3,5-DCC may be the prefered route for these strains. All of the other strains, including three syntrophic pairs, exhibited lower oxygen consumption with chlorohydroxyquinone than with 3,5-DCC, indicating that these isolates degrade 2,4-DB via the formation of 3,5-DCC instead of chlorohydroxyquinone. Most of the cells grown on 2,4-DB showed relatively low oxygen consumption with glucose.

Plasmid Detection and Transferability of 2,4-DB Degradation Phenotype

When the isolates were subjected to Kado's plasmid detection procedure, four strains of the 14 isolates exhibited one or two plasmid DNA bands (Fig. 5). These plasmids were stably maintained in cells cultivated for two months with repeated transfers in Luria-Bertani medium and were not cured with sodium dodecyl sulfate. To investigate whether the 2,4-DB degradative genes are on the plasmid and transmissible to other bacteria, the isolates containing plasmid were mated with two antibiotic-resistant recipients, *Pseudomonas cepacia* DBO1 and *Alcaligenes eutrophus* JMP228. The 2,4-DB degradation phenotype was not transferred to any of the two recipients at a detectable frequency (<10^{-9}) from any of the isolates, except strain DB1 which was identified as *Varivorax* sp. by 16S rDNA sequence analysis. The plasmid of DB1 was transferred to *Alcaligenes eutrophus* JMP228, but not to *Pseudomonas cepacia* DBO1, at an average frequency of 1.5×10^{-5}. The transconjugant was able to mineralize 2,4-D, but it could not degrade other phenoxyalkanoates, such as 2,4-DB, MCPB, MCPA, and 4-CPA, while the donor strain DB1 utilized them as sole carbon sources. Such results suggest that in DB1, chromosomal beta-oxidation systems convert 2,4-DB to 2,4-D, which is further degraded by 2,4-D degradative enzymes encoded from the plasmid, and that its chromosome contains indispensable genes for degradation of the other herbicides.

It has been reported that the aliphatic side chains of phenoxyalkyl carboxylic acids, such as 4-(4-chlorophenoxy)-butyric acid and 4-CPA, are metabolized by beta oxidation in certain pure cultures [38]. In the case of 2,4-DB, some plants and soil microflora have been shown to possess beta oxidation systems to convert 2,4-DB to 2,4-D [11, 14], but no pure cultures of bacteria have been reported to carry out beta oxidation for the biodegradation of 2,4-DB. Thus, strain DB1 may use the chromosomal linkage of beta

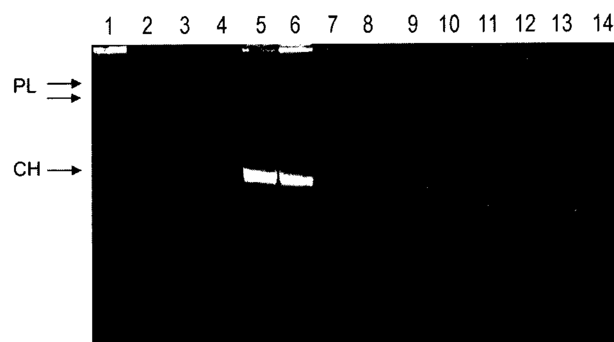

Fig. 5. Plasmid profiles of the isolates.
Lanes: 1, DB1; 2, DB2; 3, DB3; 4, DB4; 5, DB5; 6, DB6; 7, DB7; 8, DB8; 9, DB9a; 10, DB9b; 11, DB10a; 12, DB10b; 13, DB11a; 14, DB11b. PL, plasmid band; CH, chromosomal band.

oxidation systems to 2,4-D degradative plasmid, leading to its complete mineralization.

PCR Amplification of the *tfd* Genes

To investigate whether the 2,4-DB-degrading isolates have any *tfd*-like genes of the 2,4-D degradative plasmid pJP4, PCR amplification was performed for every isolate using specific primers selected from the internal sequences of the *tfdA*, *B*, and *C* genes. Among the 14 isolates, strain DB2 produced an amplified DNA band of the expected size only from the *tfdA* gene, and strain DB9a produced an expected DNA band only from the *tfdC* gene (data not shown). The other isolates did not give any DNA bands with any of the primers of the *tfdA*, *B*, and *C* genes. It has been reported that about 25% of the 47 2,4-D-degrading bacteria isolated under 2,4-D selection had all of the *tfdA*, *B*, *C*, and *D* genes [19]. All of the isolates, which were enriched under 2,4-DB selection, were able to degrade 2,4-D as well as 2,4-DB, but the occurrence frequency of the *tfd* genes in these strains was very low. The results suggest that the isolates have degradative genes dissimilar from those of the previously described 2,4-D-degrading strains, and that different groups of microorganisms are enriched depending on the applied selection, though the enriched strains share a common property of degrading the same compound.

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