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Genetic and Phenotypic Diversity of Dichlorprop-Degrading Bacteria Isolated from Soils

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Nine dichlorprop-degrading bacteria and three pairs of bacteria showing syntrophic metabolism of the herbicide were isolated from soils, and their genetic and phenotypic characteristics were investigated. Analysis of 16S rDNA sequences indicated that the isolates were related to members of the genera, *Sphingomonas*, *Herbaspirillum*, and *Bradyrhizobium*. Twelve different chromosomal DNA patterns were obtained by polymerase-chain-reaction (PCR) amplification of repetitive extragenic palindromic (REP) sequences from the 15 isolates. The isolates were able to utilize the herbicide dichlorprop as a sole source of carbon and energy and their dichlorprop degradative pathways were induced by the presence of dichlorprop. Most of the isolates and syntrophic pairs were able to degrade both (*R*)- and (*S*)-dichlorprop, but strain DP522 exhibited enantioselective degradation of (*S*)-dichlorprop. The isolates degraded 2,4-dichlorophenoxyacetic acid, 2-methyl-4-chlorophenoxyacetic acid, and mecoprop, in addition to dichlorprop. Oxygen uptake experiments indicated that most of the isolates degraded dichlorprop through 2,4-dichlorophenol.

Key words: bacteria, biodegradation, dichlorprop, diversity

Chlorinated phenoxyalkanoates are widely used, and thus many studies have focused on their persistence and fate in the natural environment (Torstensson *et al.*, 1975; Smith and Hayden, 1981; Smith and Aubin, 1991). Some phenoxyacid herbicides, such as 2,4-dichlorophenoxy acetic 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 (Foster and Mckercher, 1973; Loos *et al.*, 1979; Biederbeck *et al.*, 1987). 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 (Alexander, 1965). 2-(2,4-dichlorophenoxy) propionic acid (dichlorprop, 2,4-DP) is one of the chlorinated phenoxy alkanoic herbicides and is used to control broad-leaved weeds in cereals and grassland. While the phenoxyacetic acids such as 2,4-D and MCPA are achiral, the phenoxypropionic acids including dichlorprop and 2-(2-methyl-4-chlorophenoxy) propionic acid (mecoprop, MCPP) are chiral because they possess an asymmetrically substituted C-atom. Most commercial formulations of dichlorprop are racemic mixtures, containing both the (*R*) and the (*S*) enantiomers of dichlorprop, though only the (*R*) enantiomer has been reported to be herbicidally active (Aberg, 1956).

Among the phenoxyalkanoic aicds, 2,4-D has been most extensively studied regarding its environmental fate, metabolizing microorganisms, degradation pathways, and degradative genes and enzymes (Loos *et al.*, 1967; Don *et al.*, 1985; Pieper *et al.*, 1988; Ka *et al.*, 1994; Chung and Ka, 1998). In contrast, relatively little information is available on the microorganisms able to degrade chiral phenoxyalkanoic acid herbicides such as dichlorprop and mecoprop (Horvath *et al.*, 1990; Tett *et al.*, 1994). The main limitation is that, unlike the 2,4-D-degrading organisms, the dichlorprop- and mecoprop-degrading microorganisms are not ubiquitous in the environment and thus not easily isolated in pure cultures on laboratory media. These chiral herbicides are considered more recalcitrant than 2,4-D due to the propionic acid side chain linked to the oxygen atom of the phenoxyacid moiety (Alexander and Aleem, 1961). It requires long continuous cultivation to obtain degradation-positive cultures of the herbicides and the prolonged enrichment often results in multi-membered mixed cultures (Lappin *et al.*, 1985; Tett *et al.*, 1994). The difficulty of obtaining pure cultures implies that the complete mineralization of the herbicides can only occur through the syntrophism of a consortium of microorganisms in nature (Tett *et al.*, 1994).

In this work, we studied the occurrence of dichlorprop-degrading microorganisms in two different types of natural

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soils, i.e., agricultural soils with no history of dichlorprop amendments and golf course soils routinely treated with the herbicide for several years. Through a simple enrichment procedure, we were able to isolate nine dichlorprop-degrading bacteria and three pairs of syntrophic bacteria co-mineralizing the herbicide from golf course soils, but no microorganisms were purified from the agricultural soils. We investigated their species diversity by 16S rDNA sequence analysis and REP-PCR patterns of chromosomes, and described their physiological and genetic properties related to dichlorprop biodegradation.

Materials and Methods

Media and culture conditions

PTYG medium contained (in grams per liter) peptone (0.25), tryptone (0.25), yeast extract (0.5), glucose (0.5), magnesium sulfate (0.03), calcium chloride (0.003), and agar (for plates only, 15). The dichlorprop mineral medium used for enrichment, isolation, and maintenance of isolates contained (in grams per liter) dichlorprop (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/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 dichlorprop 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., Korea).

Chemicals

2-(2,4-dichlorophenoxy)propionic acid (2,4-DP, dichlorprop), 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB), 4-(2-methyl-4-chlorophenoxy)butyric acid (MCPB), and 2-(2-methyl-4-chlorophenoxy)propionic acid (MCPP, mecoprop) were obtained from Aldrich Chemical Co., Milwaukee, USA, and analytical grade 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenol (2,4-DCP), 3,5-dichlorocatechol, 2-methyl-4-chlorophenoxyacetic acid (MCPA), and (*R*)-enantiomer of dichlorprop were purchased from Sigma Chemical Co., St. Louis, USA.

Isolation of dichlorprop-degraders

Soil samples were taken from golf courses and agricultural fields of various locations in South Korea. The golf courses selected in this study have been treated with racemic dichlorprop for several years, while the upland fields, which have been under normal agricultural practices growing dry crops, such as legumes and barley, for more than 10 years, have no history of dichlorprop amendments. 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 100 g of each soil sample was transferred to each 250 ml sterile beaker, and then treated with racemic dichlorprop dissolved in 0.1 M NaH_2PO_4 buffer (pH 7.0) to a concentration of 300 ppm and thoroughly mixed. Four weeks after the dichlorprop application, a 10 g soil sample from each beaker was homogenized with 95 ml of a 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 test tubes containing 3 ml of dichlorprop medium (300 ppm). The tubes were incubated at 30°C for 4 weeks and degradation of dichlorprop was analyzed by spectrophotometry and reverse-phase HPLC on μBondapack C18 column (3.9 by 300 mm; Waters, Milford, MA, USA) and a UV detector set at 282 nm; methanol 0.1% phosphoric acid (60 : 40) was used as the eluant. The culture of the terminal positive tube showing substantial cell growth and less than 20% of the dichlorprop 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 dichlorprop degradation in fresh dichlorprop medium before strain purification.

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 (Lane, 1991). The amplified 16S rRNA genes were sequenced using a ABI Prism BigDye Terminator Cycle Sequencing Ready Kit according to the manufacturers instruction (Perkin-Elmer) with the sequencing primers 27f and 519r (Hugenholtz *et al.*, 1998). Approximately 400 unambiguous nucleotide positions were used for comparison to the data in GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). Sequences from nearest relatives were identified from the Ribosome Database Project (RDP) using the SIMILARITY-RANK program of the RDP (Maidak *et al.*, 2000)

Colony REP-PCR

The colony REP-PCR was performed using BOXA1R as a primer as described previously (de Bruijn, 1992; Versalovic *et al.*, 1994). 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 on dichlorprop medium (300 ppm) or on PTYG medium. Cells were then harvested by cen-

trifugation 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 at 282 nm to determine the degradation of phenoxyacetates.

Axenic culture experiment

After growth in dichlorprop 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 dichlorprop 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 dichlorprop.

Plasmid detection and conjugation

For detection of plasmid DNA, cells were lysed using a modified form (Ka and Tiedje, 1994) of the procedure previously published (Kado and Liu, 1981). Curing of plasmid was performed by the sodium dodecyl sulfate method (Crosa *et al.*, 1994). To analyze the transferability of the dichlorprop degradative phenotype of the isolates, matings were performed on membrane filters as described by Willetts (Willetts, 1988), using *Alcaligenes eutrophus* JMP228 and *Pseudomonas cepacia* DBO1 as the recipients. Transconjugants were selected on dichlorprop mineral medium containing appropriate antibiotics, 1.5% Noble agar, and 300 μg/ml of dichlorprop.

Respirometric experiments

Each strain was grown in dichlorprop medium or glucose medium. Cells were then harvested at 4°C, washed twice, and resuspended in mineral medium. The resuspended cells were kept on 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 according to the manufacturers manual (Yellow Springs Instrument Co., Inc, Ohio). 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 replicates 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.*, using bovine serum albumin as a standard (Lowry *et al.*, 1951) and activities were expressed as μl O_2/h/mg protein.

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 (Don *et al.*, 1985). The PCR cycles and primers for the *tfd* genes were previously reported (Berthelet *et al.*, 1996; Vallaeys *et al.*, 1996; Kamagata *et al.*, 1997). The amplification of the *tfdA, B,* and *C* genes with the corresponding primers was expected to produce a 362 bp, 205 bp, and 361 bp DNA fragment, respectively.

Results

Isolation of dichlorprop-degrading bacteria

Both agricultural soils and golf course soils were subjected to the simple enrichment procedure to isolate dichlorprop-degrading microorganisms. However, none of the 75 agricultural soil samples showed any detectable degradation of dichlorprop during the incubation time. Thus, no organisms able to degrade dichlorprop were purified from any of the agricultural soils. In contrast, 12 of the 17 golf course soils, which had been treated with dichlorprop for several years, exhibited positive degradation of the herbicide. Nine dichlorprop-degrading bacteria were isolated and purified from the positive tubes of 9 different soils (Table 1). Except for strain DP522, which degraded only about 50% of the racemic herbicide, dichlorprop in mineral medium was completely degraded by the isolates, and no dead-end products accumulated during biodegradation of the herbicide when analyzed with high-performance liquid chromatography. Since dichlorprop is highly water-soluble and not volatile, its disappearance is due to microbial degradation. Figure 1 shows typical growth and degradation curves by strain DP535 on dichlorprop medium. However, some enriched cultures failed to produce pure strains able to degrade dichlorprop. When the different colony types from each of these cultures were combined on dichlorprop

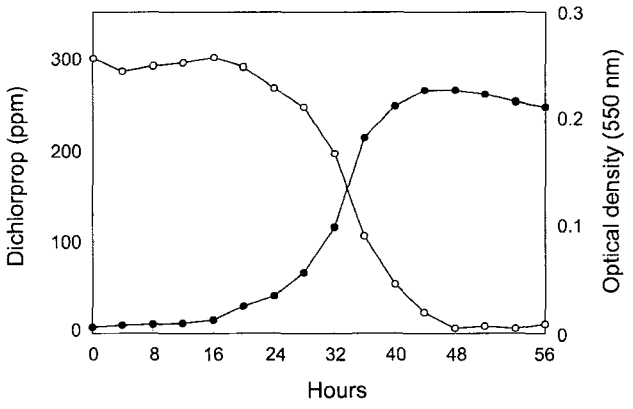

Fig. 1. Disappearance of dichlorprop (○) and growth of strain DP535 (●) during its growth in mineral medium. Each point is the mean for two replicate liquid cultures.

Table 1. Nearest relatives of the dichlorprop-degrading isolates based upon 16S rDNA sequence analysis

Isolate[a]	GenBank accession no.	Soil sites	Nearest relative	Similarity(%)[b]
DP522	AY227691	Koyang, Kyongggido	*Sphingomonas agrestis*	98
DP523	AY227692	Koyang, Kyongggido	*Sphingomonas asaccharolytica*	98
DP524	AY227693	Koyang, Kyongggido	*Sphingomonas* sp. CD	99
DP535	AY227694	Kimpo, Kyongggido	*Sphingomonas asaccharolytica*	98
DP542	AY227695	Koyang, Kyongggido	*Sphingomonas agrestis*	98
DP611	AY227696	Chungju, Chungbuk	*Bradyrhizobium* sp.	99
DP622	AY227697	Gwangju, Kyongggido	*Sphingomonas asaccharolytica*	98
DP661	AY227698	Gapyeong, Kyongggido	*Sphingomonas asaccharolytica*	98
DP682	AY227699	Yongin, Kyongggido	*Sphingomonas agrestis*	99
DP51a	AY227700	Yongin, Kyongggido	*Sphingomonas* sp. SRS2	99
DP51b	AY227701	Yongin, Kyongggido	*Herbaspirillum seropedicae*	98
DP52a	AY227702	Koyang, Kyongggido	*Sphingomonas* sp. SRS2	99
DP52b	AY227703	Koyang, Kyongggido	*Herbaspirillum frisingense*	100
DP67a	AY227704	Yongin, Kyongggido	Uncultured eubacterium WR1130	98
DP67b	AY227705	Yongin, Kyongggido	*Herbaspirillum seropedicae*	98

[a]Syntrophic bacteria were denoted by a and b.
[b]Based upon approximately 400 nucleotide positions at the 5' end of the 16S rRNA gene

medium, some mixed cultures were able to grow and mineralize the herbicide. From these mixed cultures, three pairs of presumably syntrophic bacteria (denoted by a and b) capable of degrading dichlorprop were isolated from three different soils (Table 1).

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 *Sphingomonas*, *Bradyrhizobium*, and *Herbaspirillum* (Table 1). All of the isolates were Gram-negative and belong to the alpha and beta subgroups of Proteobacteria. Although the dichlorprop-degrading strains were isolated from different soil locations, some isolates were most closely related to the same species, such as *Sphingomonas asaccharolytica* and *Sphingomonas agrestis*. A REP-PCR experiment was performed to study the genomic relatedness among the isolates closely related according to the 16S rDNA sequence analysis (Fig. 2). It was revealed that the 15 isolates produced 12 different DNA fingerprint patterns. An identical REP-PCR pattern was observed for strains DP523, DP535, DP622, and DP661, which were classified as *Sphingomonas asaccharolytica*. Identical patterns indicate that the strains are very closely related or siblings. Since all of the strains were isolated from different soil samples, their detection frequencies reflect their ubiquitousness in the soils examined. Interestingly, the three pairs of syntrophic bacteria produced six different DNA fingerprint patterns (Fig. 2), suggesting that each isolate is a distinct strain.

Growth of dichlorprop-degrading bacteria in axenic cultures

To understand axenic growth patterns of the dichlorprop degraders, the strains were inoculated into racemic dichlo-

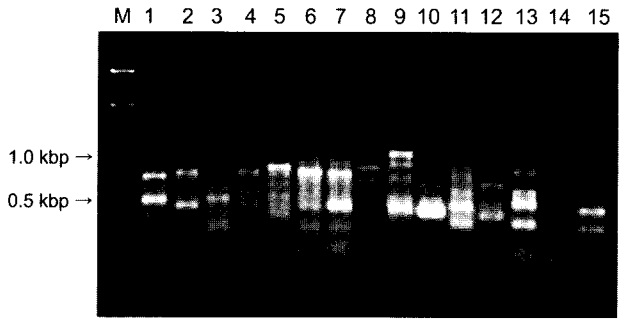

Fig. 2. REP-PCR band patterns of the isolates. Lanes: 1, DP522; 2, DP523; 3, DP524; 4, DP535; 5, DP542; 6, DP622; 7, DP661; 8, DP682; 9, DP611; 10, DP51a; 11, DP51b; 12, DP52a; 13, DP52b; 14, DP67a; 15, DP67b; M, DNA size marker.

rprop medium after growth on the herbicide (inducing) or on PTYG (non-inducing). The growth curves of some representative strains are shown in Figure 3. Among the isolates capable of degrading dichlorprop alone, strains DP523, DP535, DP542, DP622, and DP661 grew quickly on dichlorprop medium, showing short lag period (ca. 16 h). Strains DP522 and DP682 exhibited relatively a longer lag periods (ca. 30 h), and strain DP524 and DP611 showed the longest lag periods (ca. 60−110 h) under uninduced conditions. In contrast, when adapted to dichlorprop metabolism, the isolates degraded the herbicide much more quickly, suggesting that the dichlorprop degradative pathways are inducible by the presence of the herbicide. The inducibility of dichlorprop degradative pathways of the isolates was confirmed in respirometry experiments conducted with adapted or not adapted cells. Respiratory activities on dichlorprop ranged from 42.4 (syntrophic pair DP67ab) to 227.7 (strain DP523) μl O_2/h/mg protein for the isolates grown on dichlorprop, but the cells cultivated on glucose oxidized the herbicide

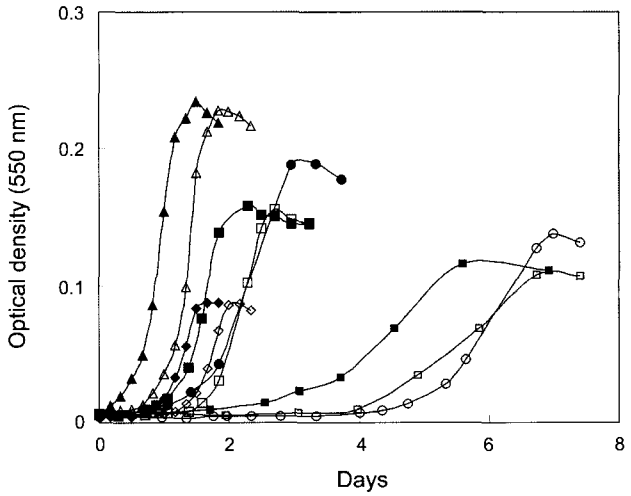

Fig. 3. Growth of dichlorprop-degrading bacteria on racemic dichlorprop. Symbols: ○ and ●, DP611; ◇ and ◆, DP522; □ and ■, DP682; △ and ▲, DP535; □ and ■, DP67ab. The inoculated bacteria were either adapted (solid symbol) or not adapted (open symbol) to dichlorprop metabolism. Each point is the mean for two replicate liquid cultures. OD, optical density.

much more slowly. For the syntrophic bacteria, groups DP51ab, DP52ab, and DP67ab showed a growth pattern similar to each other, exhibiting ca. 96 h of lag period.

Degradative diversity analysis

The isolates were grown on dichlorprop or PTYG medium, and then examined for their ability to degrade other compounds related to dichlorprop. All of the pure isolates capable of degrading dichlorprop independently were also able to mineralize 2,4-D, MCPP, and MCPA, whether they were adapted to dichlorprop metabolism or not (Table 2). When each strain was inoculated into minimal medium containing pure (*R*)-type dichlorprop as the sole carbon source, only strain DP522 of the isolates was not able to degrade and grow on it. In fact, this strain exhibited lower culture absorbance than the other pure isolates. About 50% of the initial racemic dichlorprop was not degraded during the axenic culture experiment. Thus, this strain has enantioselective degradation property on chiral dichlorprop, degrading only (*S*)-type dichlorprop. Interestingly, this strain was also able to degrade MCPB only under dichlorprop-adapted conditions, suggesting

Table 2. Patterns of utilization of substrates by the isolates

Isolate	Pregrowth condition[a]	Substrate[b]					
		2,4-DP	2,4-D	MCPP	MCPA	2,4-DB	MCPB
DP522	A	+	++	+	++	-	++
	U	+	++	+	++	-	-
DP523	A	++	++	++	++	-	-
	U	++	++	++	++	-	-
DP524	A	++	++	++	++	-	-
	U	++	++	++	++	-	-
DP535	A	++	++	++	++	-	-
	U	++	++	++	++	-	-
DP542	A	++	++	++	++	-	-
	U	++	++	++	++	-	-
DP611	A	++	++	++	++	-	-
	U	++	++	++	++	-	-
DP622	A	++	++	++	++	-	-
	U	++	++	++	++	-	-
DP661	A	++	++	++	++	-	-
	U	++	++	++	++	-	-
DP682	A	++	++	++	++	-	-
	U	++	++	++	++	-	-
DP51ab	A	++	++	++	-	++	-
	U	++	++	++	-	++	-
DP52ab	A	++	++	++	++	-	-
	U	++	++	++	++	-	-
DP67ab	A	++	++	-	++	-	-
	U	++	++	-	++	-	-

[a]The isolates were grown on 2,4-DP (A) or PTYG (U) before the test of substrate utilization.

[b]Test for the utilization of substrates. ++, >95% reduction in peak height as determined by UV scanning and substantial growth (optical density at 550 nm > 0.12); +, 40 to 60% reduction in peak height and moderate growth (optical density at 550 nm > 0.08); −, < 15% reduction in peak height and very scant growth (optical density at 550 nm <0.01). 2,4-DP, 2-(2,4-dichlorophenoxy)propionic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MCPP, 2-(2-methyl-4-chlorophenoxy)propionic acid; MCPA, 2-methyl-4-chlorophenoxyacetic acid; 2,4-DB, 4-(2,4-dichlorophenoxy)butyric acid; MCPB, 4-(2-methyl-4-chlorophenoxy)butyric acid.

Table 3. Respiratory activities of whole cells of representative dichlorprop-degrading bacteria grown on dichlorprop

Substrate[a]	% Respiratory activity of bacterial cells[b]								
	DP522	DP523	DP524	DP542	DP682	DP611	DP51ab	DP52ab	DP67ab
dichlorprop	100 (115.0)	100 (227.7)	100 (102.4)	100 (145.3)	100 (116.3)	100 (109.8)	100 (88.7)	100 (155.9)	100 (42.4)
2,4-D	65	48	44	17	60	19	21	31	44
2,4-DCP	86	54	36	48	86	43	75	16	62
3,5-DCC	62	56	132	62	138	65	58	15	174
Glucose	15	2	12	10	6	4	5	12	6

[a]2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol.
[b]Respiratory activities in [μl O_2 h^{-1} (mg protein)$^{-1}$] are given in parentheses. The values are the mean of two replicate assays of two independent cultures.

that this compound is probably metabolized because of its structural similarity to dichlorprop or its metabolites. None of the isolates could degrade 2,4-DB.

Each of the two-member communities was also able to degrade 2,4-D as well as dichlorprop syntrophically, while no single organism alone could degrade any of the compounds (Table 2). Interestingly, the DP51ab pair was capable of synergistically mineralizing 2,4-DB, which was not degraded by any of the pure isolates. The group DP52ab showed a similar utilization pattern to the pure isolates capable of independently degrading dichlorprop, while group DP67ab was not able to degrade MCPP.

Respirometric experiments

To investigate the diversity of dichlorprop degradation pathways among the isolates, each strain and the syntrophic pairs were grown on dichlorprop medium or on glucose medium. They were then analyzed for oxygen uptake with various substrates, including the assumed intermediates of dichlorprop, such as 2,4-D, 2,4-DCP and 3,5-DCC (Table 3). Dichlorprop-grown cells of strain DP682 rapidly oxidized dichlorprop, 2,4-D, 2,4-DCP, and 3,5-DCC. This result indicates that this strain degrades dichlorprop through the formation of 2,4-DCP and 3,5-DCC, which has been reported as a degradation pathway for dichlorprop in a *Flavobacterium* sp. strain MH (Horvath *et al.*, 1990). Other isolates, such as DP522, DP523, and DP67ab, exhibited similar oxygen uptake patterns to strain DP682. Strains DP542, DP611, and DP51ab also showed high oxygen consumption with 2,4-DCP and 3,5-DCC, but their oxygen uptake rates with 2,4-D were much lower compared to those of the DP682 type. At present, it is not clear whether dichlorprop is degraded via 2,4-D formed by decarboxylation of the herbicide in DP682 types. On the other hand, syntrophic group DP52ab adapted to dichlorprop metabolism had much lower oxygen consumption with 2,4-DCP and 3,5-DCC, suggesting that this syntrophic pair utilizes dichlorprop using a dissimilar degradation pathway from the DP682 types. Most of the cells grown on dichlorprop showed low oxygen consumption with glucose.

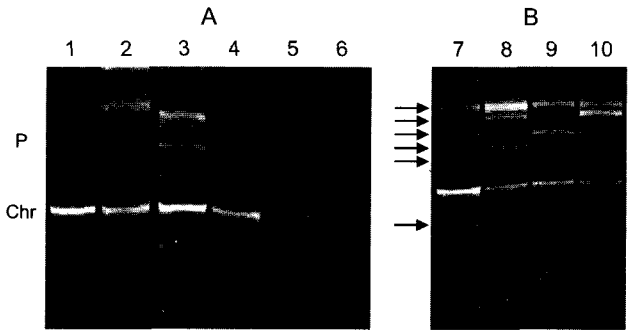

Fig. 4. Plasmid profiles of the isolates. Lanes: 1, DP522; 2, DP524; 3, DP542; 4, DP682; 5, DP51a; 6, DP67a; 7, DP542; 8, DP542-1; 9, DP542-2; 10, DP542-3. P, plasmid band; Chr, chromosomal band. The arrows indicate six plasmid bands.

Plasmid detection and transferability of dichlorprop degradation phenotype

When the isolates were subjected to Kado's plasmid detection procedure, six strains among the 15 isolates exhibited one to six plasmid DNA bands (Fig. 4A). These plasmids were stably maintained in cells cultivated for two months with repeated transfers in Luria-Bertani medium and were not easily cured with sodium dodecyl sulfate, except strain DP542. Initially, strain DP542 was able to degrade both (*S*)-and (*R*)-enantiomers of dichlorprop and MCPP. However, when the second and the sixth plasmids from the top were lost (Fig. 4B, lane 9), the cured strain DP542-2 could not degrade (*R*)-type enantiomers any more. Since cured strains (DP542-1 and DP542-3) without the third or the fourth plasmid (Fig. 4B, lanes 8 and 10) were still able to degrade the herbicides completely, the second and the sixth plasmids appeared to have crucial roles in degradation of (*R*)-type dichlorprop and MCPP in strain DP542. When the isolates containing plasmid were mated with two antibiotic-resistant recipients, *Pseudomonas cepacia* DBO1 and *Alcaligenes eutrophus* JMP228, the dichlorprop degradation phenotype was not transferred to any of the two recipients at a detectable frequency ($<10^{-9}$) from any of the isolates.

PCR amplification of the tfd genes

Since all of the pure isolates and the syntrophic pairs were able to degrade 2,4-D as well as dichlorprop, we investigated whether the isolates have any *tfd*-like genes of the 2,4-D degradative plasmid pJP4. When PCR amplification was performed for every isolate using specific primers selected from the internal sequences of the *tfdA*, *B*, and *C* genes, none of the isolates exhibited any PCR DNA bands (data not shown). The results indicate that the genes involved in 2,4-D degradation in these isolates do not have any significant homology with the well-known *tfd* genes of the 2,4-D degradative plasmid pJP4 (Don *et al.*, 1985).

Discussion

We isolated dominant dichlorprop-degrading bacteria from golf course soils and analyzed their diversity and properties on dichlorprop degradation. Unlike 2,4-D and MCPA, which are easily degraded by soil microorganisms, dichlorprop and MCPP have a propionic acid side chain linked to the chiral carbon atom, which decreases the susceptibility of the herbicide to microbial attack (Alexander and Aleem, 1961). It usually takes a long time to enrich microbial cultures able to degrade dichlorprop or MCPP, and many of the researches have had to use soil-column techniques and chemostat cultures to enrich them (Lappin *et al.*, 1985; Horvath *et al.*, 1990). The inability to easily isolate pure cultures capable of degrading dichlorprop or MCPP suggested that the soil microorganisms possessing the complete degradative pathway required to mineralize the herbicides are very rare in natural environments (Tett *et al.*, 1994). This was true for the agricultural soils tested in this work, showing no positive degradation of dichlorprop in all of the 75 different agricultural soil samples. In contrast, about 70% of the golf course soil samples exhibited positive degradation of the herbicide, and thus nine strains able to independently mineralize dichlorprop and three pairs of syntrophic degraders were readily isolated from these soils with a simple enrichment procedure. The results suggest that the dichlorprop-degrading microorganisms are ubiquitous in the soils routinely treated with dichlorprop, while they are very rare in the environments with no history of dichlorprop amendments.

Most of the previous dichlorprop- and MCPP-degrading microorganisms were isolated through long periods of subcultivation from multi-membered mixed cultures, suggesting that the degradative pathway of the herbicide in a pure culture resulted from the combination of the genetic information of several organisms in the mixed culture (Horvath *et al.*, 1990; Tett *et al.*, 1994). Genes specifying biodegradation of the pesticides are often contained on plasmids, which can facilitate the transfer of genetic information among microbial populations (Pemberton *et al.*, 1979). Among our isolates independently capable of degrading dichlorprop, most strains possessed one to six plasmids and only two species, *S. asaccharolytica* and *Bradyrhizobium* sp., did not show any plasmid DNA (Fig. 4), suggesting that the plasmids could have crucial roles in development of the dichlorprop degradative pathway. It is of note that strain DP542 harbored six different plasmids. This strain was initially able to mineralize 2,4-D, MCPA, and both (*R*)-and (*S*)-enantiomers of dichlorprop and MCPP. However, the cured strain without the second and the sixth plasmids could not degrade the (*R*)-enantiomers of the herbicides any more. The results suggest that the degradation of each enantiomer is catalyzed by each specific enzyme and the genetic information required for enantioselective degradation of the herbicides is on the separate plasmid in strain DP542.

The herbicidally active (*R*)-type enantiomers of dichlorprop and MCPP were found to be more slowly degraded than the inactive (*S*)-type enantiomers in environmental samples (Heron and Christensen, 1992; Müller and Buser, 1997). However, it is not yet known which enantiomer is perferentially utilized as a carbon and energy source by pure cultures. In the case of MCPP, both enantiomers were used as growth substrates by *S. herbicidovorans*, while *Alcaligenes denitrificans* degraded only the (*R*)-type enantiomer (Tett *et al.*, 1994; Zipper *et al.*, 1996). In our study, all of the isolates were able to utilize both enantiomers as growth substrates, except strain DP522 which could not mineralize the (*R*)-enantiomer. The results indicate that the microorganisms capable of degrading both enantiomers of the herbicides are dominant in natural environments, while the microbial populations degrading only one type of enantiomer could be rare.

All of our isolates and syntrophic pairs could degrade 2,4-D, in addition to dichlorprop, but none of them gave any PCR-amplified DNA bands with any of the primers of the *tfdA*, *B*, and *C* genes. It has been reported that about 25% of the forty-seven 2,4-D-degrading bacteria isolated under 2,4-D selection had all of the *tfdA, B*, *C*, and *D* genes (Ka *et al.*, 1994). Our results suggest that the isolates, which were isolated under dichlorprop selection, utilize 2,4-D using dissimilar degradative genes from those of the previously described 2,4-D-degrading strains, and thus that different groups of microorganisms are enriched depending on the applied selection, even though the enriched strains share a common property of degrading the same compound.

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