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Genetic and Phenotypic Diversity of (*R/S*)-Mecoprop [2-(2-Methyl-4-Chlorophenoxy)Propionic Acid]-Degrading Bacteria Isolated from Soils

Jong-Sung Lim, Mee-Kum Jung, Mi-Soon Kim, Jae-Hyung Ahn, and Jong-Ok Ka*

School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, South Korea

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Twelve mecoprop-degrading bacteria were isolated from soil samples, and their genetic and phenotypic characteristics were investigated. Analysis of 16S rDNA sequences indicated that the isolates were related to members of the genus *Sphingomonas*. Ten different chromosomal DNA patterns were obtained by polymerase-chain-reaction (PCR) amplification of repetitive extragenic palindromic (REP) sequences from the 12 isolates. The isolates were found to be able to utilize the chiral herbicide mecoprop as a sole source of carbon and energy. While seven of the isolates were able to degrade both (*R*)- and (*S*)-mecoprop, four isolates exhibited enantioselective degradation of the (*S*)-type and one isolate could degrade only the (*R*)-enantiomer. All of the isolates were observed to possess plasmid DNAs. When certain plasmids were removed from isolates MP11, MP15, and MP23, those strains could no longer degrade mecoprop. This compelling result suggests that plasmid DNAs, in this case, conferred the ability to degrade the herbicide. The isolates MP13, MP15, and MP24 were identified as the same strain; however, they exhibited different plasmid profiles. This indicates that these isolates acquired different mecoprop-degradative plasmids in different soils through natural gene transfer.

Key words: mecoprop-degrading bacteria, biodegradation, mecoprop, bacterial diversity

Phenoxyalkanoic acid herbicides have enormous agricultural utility, and thus many studies have focused on their persistence and fate in natural environments. (Torstensson *et al.*, 1975; Smith and Hayden, 1981; Smith and Aubin, 1991). 2-(4-chloro-2-methylphenoxy) propionic acid (mecoprop, MCPP) is a chlorinated phenoxyalkanoic herbicide, and is used to control broad-leaved weeds in cereals and grassland. While phenoxyacetic acids, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA), are achiral, phenoxypropionic acids, including mecoprop and 2-(2,4-chlorophenoxy)propionic acid (dichlorprop, 2,4-DP), are chiral due to an asymmetrically substituted carbon atom. Most commercial formulations of mecoprop are racemic mixtures, containing both the *R* and *S* enantiomers of mecoprop, although only the *R* enantiomer has been reported to be herbicidally active (Aberg, 1956).

Among the phenoxyalkanoic acids, 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 the chiral phenoxyalkanoic acid herbicide mecoprop (Horvath *et al.*, 1990; Tett *et al.*, 1994). The main reason for this dearth of information is that, unlike the 2,4-D-degrading organisms, mecoprop-degrading microorganisms are fairly rare and thus not easily isolated in pure cultures on laboratory media. Mecoprop is 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 and continuous cultivation to obtain degradation-positive cultures of the herbicide, 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 complete mineralization of the herbicides in nature can only occur through the syntrophism of a consortium of microorganisms (Tett *et al.*, 1994).

In this work, we studied the occurrence of mecoprop-degrading microorganisms in two different types of natural soils, i.e., agricultural soils with no history of mecoprop treatment, and golf course soils, which had been routinely treated with the herbicide for several years. We were able to isolate twelve mecoprop-degrading bacteria from golf course soil samples, but none from agricultural soils. We investigated species diversity by 16S rDNA sequence analysis and REP-PCR patterns of chromosomes, and described physiological and genetic properties of the isolates on mecoprop biodegradation.

* To whom correspondence should be addressed.
(Tel) 82-2-880-4673; (Fax) 82-2-871-2095
(E-mail) kagil@plaza.snu.ac.kr

Materials and Methods

Media and culture conditions

All isolates were cultivated on mineral medium (Park and Ka, 2003) containing mecoprop at a concentration of 300 μg/ml. Peptone-tryptone-yeast extract-glucose (PTYG) medium containing (per liter) 0.25 g of peptone (Difco, USA), 0.25 g tryptone (Difco, USA), 0.5 g of yeast extract (Difco, USA), 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 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-methyl-4-chlorophenoxy)propionic acid (MCPP, mecoprop), 2-(2,4-dichlorophenoxy) propionic acid (2,4-DP, dichlorprop), 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB), and 4-(2-methyl-4-chlorophenoxy)butyric acid (MCPB) were obtained from Aldrich Chemical Co., Milwaukee, USA, and analytical grade 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA), and (*RS*)-enantiomers of mecoprop were purchased from Sigma Chemical Co. (USA).

Analysis of phenoxyalkanoic acids

HPLC analyses were performed on a Shimadzu LC-10 series high-performance liquid chromatography (Shimadzu Co., Tokyo, Japan). A Nucleodex-α-PM column (200×4 mm) with permethylated α-cyclodextrin (Macherey-Nagel, Düren, Germany) was used to separate mecoprop enantiomers, and a UV detector was set at 230 nm. The eluent system consisted of 70% methanol and 30% NaH_2PO_4 (50 mM, pH 3.0) at a flow rate of 0.7 ml/min. Retention times were typically 8.5 min for (*R*)-mecoprop, and 11.5 min for (*S*)-mecoprop. UV spectra were recorded with a UVIKON 860 spectrophotometer (Kontron Ins., Milan, Italy) at wavelengths from 340 to 220 nm.

Isolation of mecoprop-degraders

Soil samples were taken from South Korean golf courses and agricultural fields in various locations. Golf courses selected in this study had been treated with racemic mecoprop for several years, while the upland fields had undergone normal agricultural practices growing dry crops, such as legumes and barley for more than 10 years, and had no history of mecoprop treatment. Samples from the top 15 cm of soil were taken, sifted through a 2-mm-pore sieve, and kept at 4 °C prior to use. 100 g of each soil sample was transferred to each 250-ml sterile beaker, treated with racemic mecoprop dissolved in a 0.1 M NaH_2PO_4 buffer (pH 7.0) to a concentration of 300 ppm, and thoroughly mixed. Four weeks after mecoprop 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. After homogenization, 0.1 ml samples of appropriate 10-fold dilutions were inoculated into test tubes containing 3 ml of mecoprop medium (300 μg/ml). The tubes were incubated at 30°C for 4 weeks and the amount of mecoprop degradation was ascertained by spectrophotometry and HPLC. The culture of the terminal positive tube (showing substantial cell growth and less than 50% of the mecoprop 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 mecoprop degradation in fresh mecoprop medium, before strain purification.

16S rDNA sequence analysis

Total genomic DNA was extracted 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 an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit according to manufacturers instructions (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).

Nucleotide sequence accession numbers

The partial 16S rDNA sequences of the 12 isolates have been deposited in the GenBank under the following accession numbers: MP1, AY521007; MP9, AY521008; MP11, AY521009; MP12, AY521010; MP13, AY521011; MP15, AY521012; MP18, AY521013; MP19, AY521014; MP20, AY521015; MP23, AY521016; MP24, AY521017; MP27, AY521018.

Colony REP-PCR

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 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 1.5% agarose gels.

Degradative phenotype analysis

Each strain was grown on PTYG medium. Cells were 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. Ali-

quots of suspended cells were inoculated into culture tubes, each of which contained mineral medium supplemented with a 300 μg/ml concentration of one of the structural analogs. After 4 weeks of incubation, the cultures were centrifuged to remove cellular material, and UV spectra were measured from 340 to 220 nm to determine the degree of phenoxyacetate degradation.

Axenic culture experiment

After growth in 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 racemic mecoprop medium (300 μg/ml) 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 mecoprop degradation.

Plasmid detection and conjugation

In order to detect plasmid DNA, cells were lysed using a modified form (Ka and Tiedje, 1994) of the previously published procedure (Kado and Liu, 1981). Plasmid curing was performed by the sodium dodecyl sulfate method (Crosa *et al.*, 1994). To analyze transferability of the mecoprop degradative phenotype in the isolates, matings were performed on membrane filters, as described by Willetts (Willetts, 1988), using *Alcaligenes eutrophus* JMP228 and *Pseudomonas cepacia* DBO1 as recipients. Transconjugants were selected on mecoprop mineral media containing either rifampicin (100 μg/ml) for *Alcaligenes eutrophus* JMP228, or kanamycin (75 μg/ml) for *Pseudomonas cepacia* DBO1, 1.5% Noble agar (Difco, USA), and 300 μg/ml of mecoprop.

PCR amplification of the* tfd *genes

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

Results and Discussion

Isolation of mecoprop-degrading bacteria

Both agricultural and golf course soil samples were subjected to a simple enrichment procedure to isolate mecoprop-degrading microorganisms. However, none of the 67 agricultural soil samples showed any detectable mecoprop degradation during 4 weeks of incubation. Thus, no mecoprop-degrading organisms were recognized in any of the agricultural samples. By way of contrast, 12 of the 23 golf course soils, which had been treated with mecoprop for several years, exhibited positive degradation of the herbicide. Twelve mecoprop-degrading bacteria were isolated and purified from the positive tubes of 12 different soils (Table 1).

It usually takes a long time to enrich microbial cultures which are able to degrade mecoprop or dichlorprop, 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). This difficulty suggested that microorganisms which possess 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, as we observed no positive degradation of mecoprop in any of the 67 different agricultural soil samples. In contrast, about 52% of the golf course soil samples exhibited positive degradation of the herbicide, and twelve strains able to independently mineralize (*R*) and/or (*S*)-type mecoprops were readily isolated from these soils with a simple enrichment procedure. The results suggest that mecoprop-degrading microorganisms are ubiquitous in soils which

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

Isolate	GenBank accession no.	Soil sites	Nearest relative	Similarity(%)[a]
MP 1	AY521007	Seongnam, Gyeonggi-do	*Sphingomonas wittichii*	95
MP 9	AY521008	Seongnam, Gyeonggi-do	*Sphingomonas agrestis*	99
MP11	AY521009	Yongin, Gyeonggi-do	*Sphingomonas suberfaciens*	97
MP12	AY521010	Incheon, Gyeonggi-do	*Sphingomonas* sp. VM506	98
MP13	AY521011	Incheon, Gyeonggi-do	*Sphingomonas agrestis*	99
MP15	AY521012	Yangsan, Gyeongnam	*Sphingomonas agrestis*	99
MP18	AY521013	Jinhae, Gyeongnam	*Sphingomonas* sp. VM506	98
MP19	AY521014	Gyeongsan, Gyeongbuk	*Sphingomonas* sp. JS1	98
MP20	AY521015	Gyeongsan, Gyeongbuk	*Sphingomonas* sp. JS1	97
MP23	AY521016	Jeju-si, Jeju-do	*Sphingomonas agrestis*	98
MP24	AY521017	Namjeju-gun, Jeju-do	*Sphingomonas agrestis*	99
MP27	AY521018	Gyeongsan, Gyeongbuk	*Sphingomonas* sp. JS1	97

[a]Based upon approximately 400 nucleotide positions at the 5 end of the 16S rRNA gene

are routinely treated with mecoprop, but are, at best, very rare in environments with no history of mecoprop treatment.

16S rDNA sequence and REP-PCR analyses

16S rDNA sequence analysis confirmed that all isolates were related to members of the genus *Sphingomonas* (Table 1). The isolates were all Gram-negative, and belonged to the alpha subgroup of Proteobacteria. Although the mecoprop-degrading strains were isolated from different soils, some isolates were most closely related to the same species, notably, *S. agrestis* and *Sphingomonas* sp. JS1. REP-PCR experiments were performed in order to study genomic relatedness among the isolates that appeared most closely related. It was revealed that the 12 isolates produced 10 different DNA fingerprint patterns (Fig. 1). This suggests that, although the isolates all belonged to the genus *Sphingomonas*, they possessed radically different genomic fingerprints. Strains MP13, MP15, and MP24, however, which were classified as *S. agrestis*, exhibited identical genomic DNA fingerprints. This indicated that the three strains are either siblings, or at least very closely related. Since all of the strains were isolated from different soil samples, their detection frequencies reflect their ubiquity in the soils examined.

Enantioselective degradation of (RS)-mecoprop in axenic cultures

To understand the preferential degradation patterns of the enantiomers of racemic mixtures by the mecoprop degraders, each strain was grown on PTYG, and inoculated into racemic mecoprop medium. The growth and mecoprop degradation curves of representative strains are shown in Fig. 2. Isolates were classified into five groups, according to their growth characteristics on racemic mecoprop. Group I contained strain MP11, which degraded both enantiomers almost simultaneously and grew rapidly after an initial lag period of about 1 day (Fig. 2A). Group II included strains MP13, MP19, MP20, and MP23, which also could degrade both enantiomers, but degraded (*S*)-mecoprop preferentially (Fig. 2B). Group III contained strains MP12 and MP15, which both degraded mecoprop in an enantioselective manner, but exhibited a long lag phase of about 4 days before the strains began to grow on (*R*)-mecoprop (Fig. 2C). Group IV consisted of strains MP1, MP9, MP18, and MP24, which could degrade (*S*)-mecoprop, but were unable to degrade (*R*)-mecoprop (Fig. 2D). Group V contained strain MP27, which could not degrade (*S*)-mecoprop, but degraded (*R*)-mecoprop after

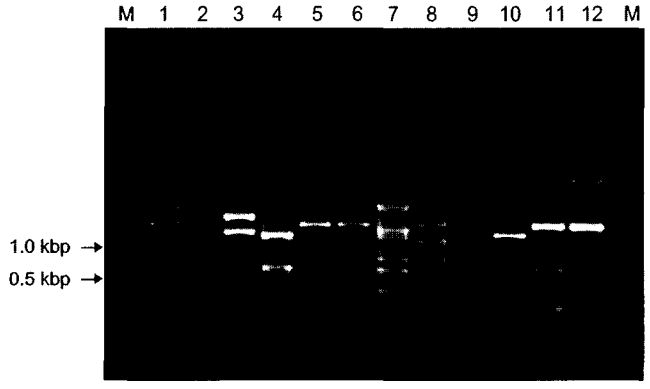

Fig. 1. REP-PCR band patterns of the isolates. Lanes : 1, MP1; 2, MP9; 3, MP11; 4, MP12; 5, MP13; 6, MP15; 7, MP18; 8, MP19; 9, MP20; 10, MP23; 11, MP24; 12, MP27; M, DNA size marker.

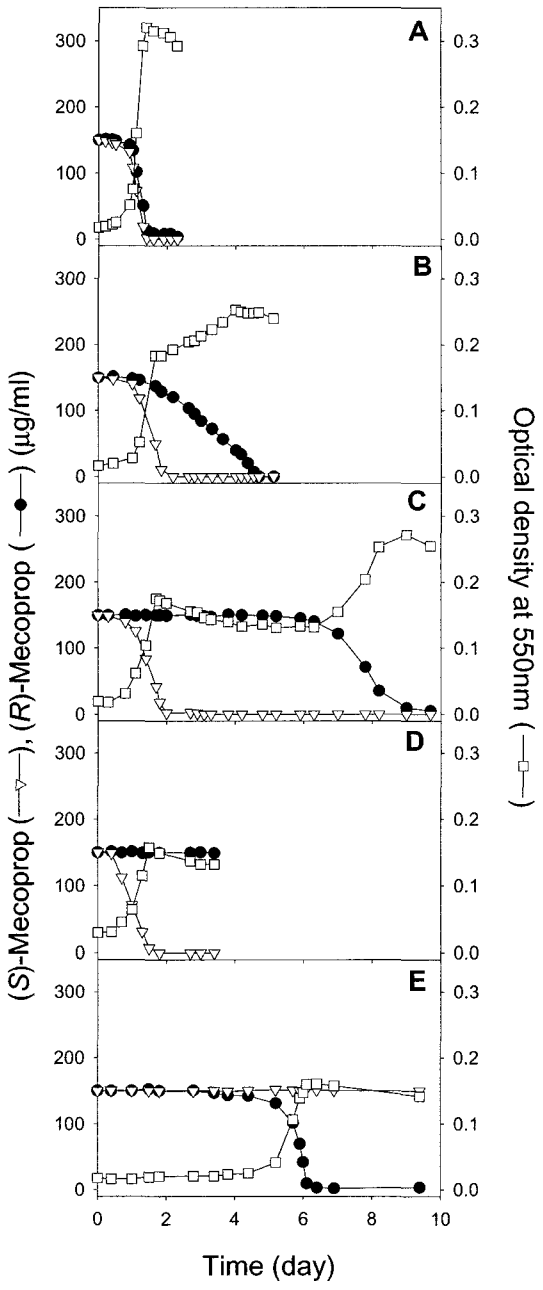

Fig. 2. Growth patterns of mecoprop-degrading bacteria on racemic mecoprop. Disappearance of mecoprop enantiomers and growth by strain MP11 (A), strain MP19 (B), strain MP15 (C), strain MP1 (D), and strain MP27 (E). Each point is the mean for two replicate liquid cultures.

Table 2. Substrate utilization patterns by the mecoprop-degrading isolates.

Isolate[a]	Substrate[b]					
	Mecoprop	Dichlorprop	2,4-D	MCPA	2,4-DB	MCPB
MP 1	+(S)	+(S)	+	++	-	-
MP 9	+(S)	+(S)	+	++	-	-
MP11	++	++	+	++	-	-
MP12	++	++	+	++	-	-
MP13	++	++	+	++	-	-
MP15	++	++	+	++	-	-
MP18	+(S)	+(S)	+	++	-	-
MP19	++	++	+	++	-	-
MP20	++	++	-	-	-	-
MP23	++	++	+	++	-	-
MP24	+(S)	+(S)	+	++	-	-
MP27	+(R)	+(R)	+	++	-	-

[a]The isolates were grown on PTYG 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 >0.23); +, 40 to 60% reduction in peak height and moderate growth (optical density at 550 nm >0.14); –, <10% reduction in peak height and very scant growth (optical density at 550 nm <0.01); +(S), utilization of only (*S*)-type enantiomer; +(R), utilization of only (*R*)-type enantiomer. mecoprop, 2-(2-methyl-4-chlorophenoxy)propionic acid; dichlorprop, 2-(2,4-dichlorophenoxy)propionic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MCPA, 2-methyl-4-chlorophenoxyacetic acid; 2,4-DB, 4-(2,4-dichlorophenoxy)butyric acid; MCPB, 4-(2-methyl-4-chlorophenoxy)butyric acid.

an initial lag period of about 5 days (Fig. 2E). The final culture absorbances of groups I, II, and III, which degraded both enantiomers, were about twice as high as those of groups IV and V, which degraded only one enantiomer. Observed growth characteristics on racemic mecoprop indicate that (*RS*)-mecoprop degradation patterns in the isolates are much more diverse than those of the enantioselective mecoprop-degraders, *Alcaligenes denitrificans* and *Sphingomonas herbicidovorans* MH (Tett *et al.*, 1994; Zipper *et al.*, 1996). *A. denitrificans* could degrade only the (*S*) enantiomer of mecoprop, like our group IV strains, and *S. herbicidovorans* MH showed a sequential degradation pattern, similar to our group II strains. The strains belonging to groups I, III, and V of this study exhibited new growth characteristics on mecoprop, indicating that racemic mecoprop biodegradation mechanisms are very diverse among mecoprop-degrading microbial populations in nature. The diverse enantiomer-specific degradation patterns of the isolates support the previous observation that separate enzyme systems are required for the degradation of each mecoprop enantiomer (Zipper *et al.*, 1996).

Degradative diversity analysis

Isolates were grown on PTYG medium, and examined for their ability to degrade other mecoprop-related compounds. All mecoprop-degrading isolates, except strain MP20, were also able to degrade related herbicides, such as (*RS*)-dichlorprop, 2,4-D, and MCPA (Table 2). When group IV strains, which could not degrade (*R*)-type mecoprop, were inoculated into minimal medium containing pure (*R*)-type dichlorprop as the sole carbon source, they were unable to degrade and grow on the (*R*)-type dichlorprop. In contrast, strain MP27 of group V, which degraded only (*R*)-mecoprop, was able to degrade only (*R*)-dichlorprop. The other isolates, which could degrade (*RS*)-mecoprop, also degraded (*RS*)-dichlorprop. These results suggested that degradation enzyme systems for mecoprop enantiomers in the isolates are closely related to those for dichlorprop enantiomers. All isolates degraded 2,4-D to completion, but growth yields were lower than with (*RS*)-mecoprop. It is of note that most of the isolates could utilize (*S*)-mecoprop as a carbon and energy source, while relatively limited populations degraded the (*R*)-mecoprop. These results are in agreement with the observation that the herbicidally active (*R*)-mecoprop was more slowly degraded than the inactive (*S*)-type enantiomer in environmental samples (Heron and Christensen, 1992; Müller and Buser, 1997).

Plasmid detection and its relationship to mecoprop degradation phenotype

When the isolates were subjected to Kado's plasmid detection procedure (Kado and Liu, 1981), all isolates exhibited one to five plasmid DNA bands (Fig. 3). These plasmids were stably maintained in cells cultivated for two months,

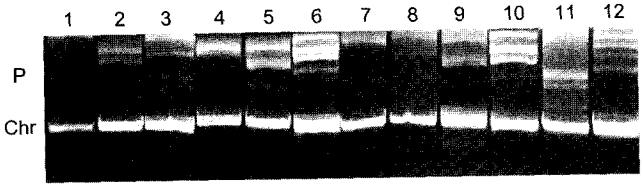

Fig. 3. Plasmid profiles of the isolates. Lanes : 1, MP1; 2, MP9; 3, MP11; 4, MP12; 5, MP13; 6, MP15; 7, MP18; 8, MP19; 9, MP20; 10, MP23; 11, MP24; 12, MP27. P, plasmid band; Chr, chromosomal band.

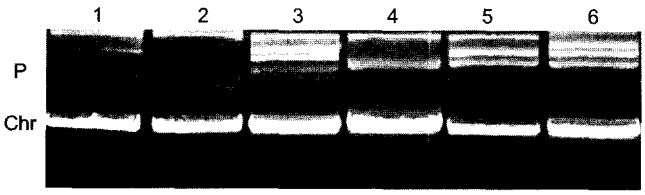

Fig. 4. Plasmid profiles of the representative isolates and their cured strains. Lanes : 1, MP11; 2, cured strain of MP11; 3, MP15; 4, cured strain of MP15; 5, MP23; 6, cured strain of MP23. P, plasmid band; Chr, chromosomal band.

with repeated transfers into Luria-Bertani medium. To investigate the relationship between mecoprop degradation phenotype and these plasmids, some representative isolates were subjected to sodium dodecyl sulfate plasmid curing procedures (Fig. 4). The cured strain of MP11 lost the ability to degrade (*RS*)-mecoprop when it lost the second plasmid from the top (Fig. 4, lane 2). Initially, strain MP15 was able to degrade both (*RS*)-mecoprop and (*RS*)-dichlorprop. However, when the second to fifth plasmids from the top were lost, producing smaller plasmids (Fig. 4, lane 4), the cured strain of MP15 could no longer degrade any of the enantiomers. In the case of strain MP23, part of the first plasmid was eliminated through the curing procedure, producing a smaller plasmid (Fig. 4, lane 6), and the resultant cured strain could not degrade (*RS*)-mecoprop, (*RS*)-dichlorprop, or 2,4-D. These results suggested that plasmids contained the essential mecoprop-degradation genes. When the plasmid-containing isolates were mated with two antibiotic-resistant recipients, *Pseudomonas cepacia* DBO1 and *Alcaligenes eutrophus* JMP228, the mecoprop degradation phenotype was not transferred to either of the two recipients at a detectable frequency ($<10^{-9}$).

Interestingly, strains MP13, MP15, and MP24 exhibited different plasmid profiles, although they were identified as the same strain, *Sphingomonas agrestis,* based on their colony morphologies, genomic DNA fingerprints, and 16S rDNA sequences. These three isolates showed different growth characteristics on racemic mecoprop, each strain belonging to three different groups (Fig. 2). The substrate utilization pattern of strain MP24 was also different from those of strains MP13 and MP15 (Table 2). Since the three isolates identified as the same strain had the same genomic DNA fingerprint, their differences in mecoprop degradation properties were clearly due to their different plasmid profiles. Most of the previous mecoprop-degrading microorganisms were isolated through long periods of subcultivation from multi-membered mixed cultures, suggesting that the mecoprop-degradative pathway in a pure culture resulted from the exchange of genetic information between several organisms in the mixed culture (Horvath *et al.*, 1990; Tett *et al.*, 1994). Genes specifying pesticide-biodegradative ability are often contained on plasmids, which can facilitate the transfer of genetic information among microbial populations (Pemberton *et al.*, 1979). Our results indicated that the three isolates acquired different catabolic plasmid DNAs from their neighboring microbial communities, while they were living in different soils under mecoprop treatment. The different plasmid profiles leading to distinct catabolic activities in the same isolates demonstrate how microbial populations adapt and evolve in natural environments amended with herbicides. In our laboratory, we are currently investigating how each plasmid of the three strains is involved in the degradation of (*RS*)-mecoprop, and related phenoxy herbicides.

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 mecoprop, 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).

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