Isolation and Characterization of 2-Methyl-4-Chlorophenoxyacetic Acid-Degrading Bacteria from Agricultural Soils

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Seven numerically dominant 2-methyl-4-chlorophenoxyacetic acid (MCPA)-degrading bacteria were isolated from agricultural soils. The isolates utilized the herbicide MCPA as a sole carbon source, producing significant biomass in MCPA mineral medium. They exhibited diverse herbicide degradation capabilities, but most of them grew very slowly in mineral medium containing herbicide. The chromosomal DNA patterns of the isolates obtained by polymerase chain reaction amplification of repetitive extragenic palindromic sequences were distinct from each other. One isolate, SH3, which was identified as *Sphingomonas* species by fatty acid methyl ester analysis, was able to degrade 5 different phenoxyacetic acid herbicides within 4 days. This strain contains two plasmids, and the smaller one has a crucial role in herbicide degradation. MCPA treated into agricultural soils without indigenous MCPA-degraders persisted for a long time, but the application of the isolate SH3 resulted in rapid decline of MCPA concentration in the soil.

Key words : *biodegradation, 2-methyl-4-dichlorophenoxyacetic acid, bacteria.*

Large amounts of man-made chlorinated organic chemicals have been used in agriculture as herbicides and pesticides. Among them, 2-methyl-4-chlorophenoxyacetic acid (MCPA) is a selective hormone-type herbicide widely 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, MCPA is known to be easily degraded in agricultural soils within 1 to 4 months.[1]

There have been sporadic reports on microbial degradation of MCPA in soil and isolation of MCPA-utilizing microorganisms.[2-4] The persistence of MCPA in soil is longer than that of 2,4-dichlorophenoxyacetic acid and 4-chloro phenoxyacetic acid,[5] and the degradation rate of MCPA in soil is enhanced by amendment with fertilizers and organic materials.[6] Repeated amendments with phenoxy herbicides, such as MCPA or 2,4-D, accelerate the degrada-tion rate of the herbicide in soil and proliferate the herbicide-degrading microorganisms.[7] Microorganisms that have been reported to be capable of degrading MCPA belong to several genera, including *Arthrobacter*, *Pseudomonas*, and *Alcali-genes*.[8-11] It is also reported that MCPA is metabolized mainly via formation of 3-chloro-5-methylcatechol using ortho- or meta-cleavage pathway.[9]

However, the previous studies on MCPA-degrading isolates have mainly focused on degradative enzyme systems and pathways, thus giving little information on the phenotypic and genetic diversity of MCPA-degrading microorganisms. Furthermore, since many of the previously described MCPA-degrading microrganisms were initially isolated for their ability to metabolize other phenoxy herbicides, e.g. 2,4-D or mecoprop, few studies have been available on physiological and ecological properties of MCPA-degrading microorganisms isolated from MCPA selection.

In this study, we isolated seven numerically dominant MCPA-degrading bacteria from ninety three agricultural soils amended with MCPA, and investigated their diversity, physiological and genetic properties. In addition, soil microcosms were used to study MCPA degradation patterns in natural field soils with and without the inoculated MCPA-degrading isolates.

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Abbreviations: 4-CPA, 4-chlorophenoxyacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DB, 2,4-dichlorophenoxybutyric acid; 2,4-DP, 2-(2,4-dichlorophenoxy)propionic acid; FAME, fatty acid methyl ester; HPLC, high performance liquid chromatography; MCPA, 2-methyl-4-chlorophenoxyacetic acid; MCPB, 4-(2-methyl-4-chlorophenoxy)butyric acid; MCPP, 2-(2-methyl-4-chlorophenoxy)propionic acid; MPN, most pobable number; PTYG, peptone-tryptone-yeast extract-glucose; REP-PCR, repetitive extragenic palindromic sequence-polymerase chain reaction; SMB, standard mineral base.

Materials and Methods

Media and culture conditions. All isolates were maintained on SMB mineral medium[12] containing MCPA at a concentration of 300 μg/ml. PTYG medium containing peptone 0.25 g, tryptone 0.25 g, yeast extract 0.5 g, glucose 0.5 g, magnesium sulfate 0.03 g, and calcium chloride 0.03 g in 1 liter of sterile distilled water was used for strain purification and colony production for the REP-PCR.

Chemicals. Analytical grade MCPA and 2,4-D were obtained from Sigma Chemical Co., and 4-CPA, 2,4-DP, MCPP, 2,4-DB, and MCPB were obtained from Aldrich Chemical Co.

Isolation of bacterial strains. Agricultural field soil samples were taken from diverse countrywide sites in Korea. Samples from the top 15 cm of field soil were taken and kept at 4°C prior to use. Each soil was treated with MCPA at a concentration of 300 μg/g and incubated at room temperature for one month. A 10 g of soil sample was homogenized in 95 ml of a sterilized saline solution (0.85%) by shaking on a rotary shaker (200 rpm). Samples (0.1 ml) of appro-priate 10-fold dilutions were inoculated into MPN tubes containing 3 ml of MCPA mineral medium (SMB mineral medium containing 300 μg/ml of MCPA). The tubes were incubated at 30°C for three weeks and degradation of MCPA was analyzed by spectrophotometry. The culture of the ter-minal positive tube showing MCPA 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 MCPA degradation in fresh MCPA mineral medium before strain purification.

FAME analysis. The isolates were cultured on tryptic soy agar at 28°C for 48 to 72 h, then the cells were harvested from the plates by scraping with a sterile glass loop and used for FAME analysis. Saponification, methylation, and extraction were performed according to the procedure previously described in the MIDI manual (Microbial Identification, Inc.).[13]

Colony REP-PCR. The colony REP-PCR was performed using BOXA1R as a primer as described previously.[14,15] Each isolate was grown on the PTYG agar for 24 to 48 h, then a small amount of the cells were 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 MCPA mineral medium or PTYG medium, 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 the suspended cells were inoculated into duplicate flasks containing 200 ml of MCPA (300 μg/ml) mineral medium at a final density of OD_{550}=0.005 for the axenic growth experiment or 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 the cultures were regularly removed to determine cell growth and degradation of MCPA.

Degradative phenotype analysis. Each strain was cultured in PTYG medium. Cells were then harvested, washed, and prepared in sodium phosphate buffer as described above. Aliquots of the 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 μg/ml. After three week incubation, the cultures were centrifuged to remove the cellular material, and the UV absorption was measured to determine the degradation of chloroorganic compounds.

Table 1. MCPA-degrading bacteria and their isolation soil sites.

Isolates	Soil sites[a]
SH1	Chunam, Chungchongnam-Do
SH2	Yangsan, Kyungsangnam-Do
SH3	Chunan, Chungchongnam-Do
SH4	Namcheju, Cheju-Do
SH5	Yagok, Chungchongbuk-Do
SH6	Yangpyoung, Kyungki-Do
SH7	Chungwon, Chungchongbuk-Do

[a]All soils were from agriculture fields.

Plasmid detection and conjugation. For detection of plasmid DNA, cells were lysed using a modified form[16] of the procedure of Kado and Liu.[17] To analyze the transferability of the MCPA degradative phenotype of the isolates, matings were performed on membrane filters as described by Willetts.[18] Transconjugants were selected on 2,4-D mineral medium containing 300 μg/ml MCPA, appropriate antibiotics, and 1.5% Noble agar.

Degradation of MCPA in soil. Two soil samples with or without the indigenous MCPA-degrading microorganisms were obtained from agricultural fields. The soil was stored at field moisture levels at 4°C until used. The isolate SH3 showing the highest degradation rate of MCPA among the isolates (Table 1) was chosen as the inoculum strain. The strain SH3 was grown at 30°C in PTYG medium, harvested, washed, and prepared in sodium phosphate buffer. Each of the soil samples was inoculated with the strain SH3 at a density of 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 strain SH3. Inoculated and uninoculated soils were treated with MCPA dissolved in 0.1 M NaH_2PO_4 buffer (pH 7.0) to a concentration of 300 μg/g and thoroughly mixed. The disappearance of MCPA from soil was monitored by HPLC,[19] and the soils were respiked with MCPA (300 μg/g) after it was removed until a total of 3 cycles of degradation had been completed.

Results and Discussion

Distribution of MCPA-degrading microorganisms. The numbers of MCPA-degrading microorganisms in diverse agricultural soils were estimated with MPN procedure. The MCPA-degrading populations generally ranged from 0 to 3.4×10^2 cells/g soil in ninety three different agricultural soils, and 86% of the soil samples apparently did not show any positive degradation of MCPA during the incubation time. The population values for MCPA-degraders in the tested soils are relatively lower than the previous result,

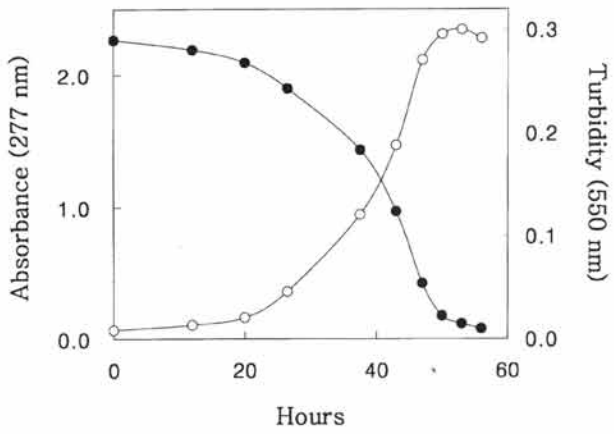

Fig. 1. Disappearance of MCPA (●—●) and growth of bacterial strain SH3 (○—○) during biodegradation of MCPA. Each point is the mean for two replicate liquid cultures.

0.34 to 1377 cells/g,[20] and the observation that only 14% of the tested soils showed positive degradation of MCPA suggests that the MCPA-degrading microrganisms are not widely distributed in our agricultural soils.

Degradation of MCPA. MCPA in mineral medium was completely degraded by the isolates, and no intermediate products were detected by HPLC analysis. Fig. 1 shows typical growth and degradation curves of strain SH3 on MCPA medium. The isolates were able to utilize MCPA as a sole carbon and energy source, as indicated by significant biomass increases in MCPA mineral medium.

FAME and REP-PCR analysis. FAME method was used to analyze the phylogenetic properties of the isolates, but only the SH3 strain of the isolates was reasonably identified as a *Sphingomonas* species. The other strains could not be identified due to their poor growth on laboratory media.

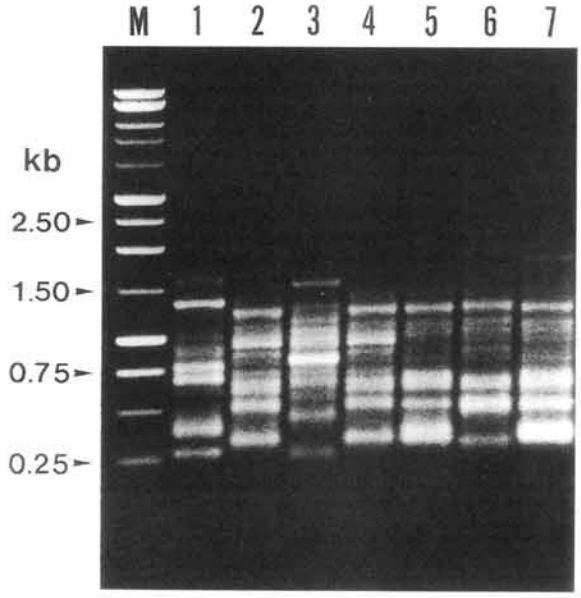

Fig. 2. REP-PCR patterns of strains. Lanes: 1, SH1; 2, SH2; 3, SH3; 4, SH4; 5, SH5; 6, SH6; 7, SH7; and M, 1 kb ladder DNA size marker.

A colony REP-PCR experiment was performed to study the genomic relatedness of the isolates (Fig. 2). The analysis of REP-PCR band patterns with densitometer revealed that the isolates produced 6 different DNA fingerprint patterns. Strains SH2 and SH4 isolated from different locations exhibited the same band pattern, suggesting that they were very closely related in their chromosome structure. Strains SH5 and SH7 also showed a similar band pattern.

Growth properties of the isolates. To understand axenic growth patterns of the MCPA degraders, the isolates were inoculated into MCPA mineral medium under induced and uninduced conditions. One of the most unique features of the isolates was that most of them are slow-growing bacteria compared to previously reported MCPA degraders.[11] Fig. 3 shows typical growth patterns of strains SH1 and SH3 on MCPA medium. Under the uninduced condition, strain SH3 exhibited short lag period (ca. 1 day) and thereafter began to grow exponentially. Strain SH1 exhibited relatively longer lag period (ca. 3 days). On the other hand, the rest of the isolates took about 30 days to completely mineralize 300 μg/ml of MCPA. These slow-growing isolates had long lag periods (10 to 20 days) and their doubling times during the exponential growth phase were about 20 times longer than that of strain SH3. The previously described microorganisms able to degrade phenoxy herbicides grow well in herbicide medium, and it usually takes several days to completely mineralize the herbicides.[11] Recently, Kamagata *et al.* reported on the isolation of slow-growing, oligotrophic bacteria, but their isolates still completely mineralize the herbicide within 1 week.[21] Our strains are not oligotrophic, since they grow in mineral medium containing more than 1000 μg/ml of MCPA.

Under induced condition, our isolates grew more quickly (Fig. 3), suggesting that their MCPA degradative enzymes were inducible by the presence of MCPA. The inducibility of MCPA degradative enzymes of the isolates was confirmed

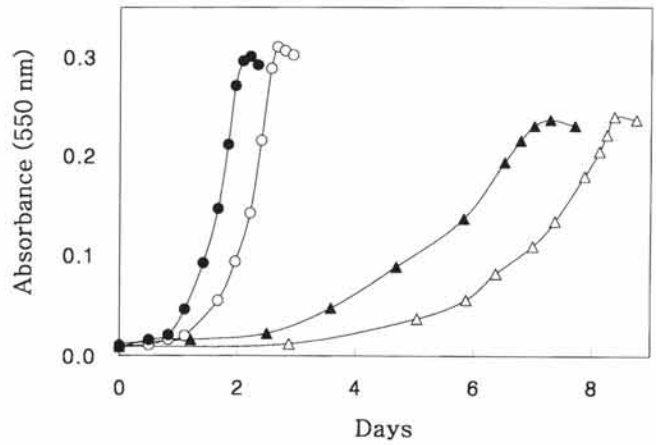

Fig. 3. Growth patterns of MCPA-degrading bacteria in axenic culture. Symbols: ●—● and ○—○, strain SH3; ▲—▲ and △—△, strain SH1. The bacteria were either adapted (solid symbols) or not adapted (open symbols) to MCPA metabolism. Each point is the mean for two replicate flask cultures.

Table 2. Ability of MCPA-degrading strains to degrade other phenoxy acid herbicides.

Herbicide	Strains[a]						
	SH1	SH2	SH3	SH4	SH5	SH6	SH7
MCPA	+	+	+	+	+	+	+
2,4-D	+	+	+	+	+	+	+
MCPB	-	+	-	+	+	-	-
2,4-DB	+	+	-	+	+	-	+
4CPA	+	+	+	+	-	-	+
MCPP	-	-	+	-	-	-	-
2,4-DP	-	-	+	-	-	-	-

[a]The bacterial strains were grown on PTYG and then tested for herbicide utilization capabilities. +, >90% reduction in peak height from UV scanning and substantial growth (OD_{550}>0.25); -, <15% reduction in peak and very scant growth (OD_{550}<0.01).

in resting cell experiments conducted with cells adapted or not adapted to MCPA metabolism. The cells adapted to MCPA metabolism mineralized MCPA quickly without any lag period, while the unadapted cells could not degrade it during the incubation period.

Degradative diversity analysis. The isolates were grown on PTYG medium, then examined for their ability to degrade other compounds related to MCPA (Table 2). The isolates exhibited diverse patterns in their herbicide utilization abilities. Strains SH1, SH2, SH4, and SH7 were able to utilize MCPA, 2,4-D, 2,4-DB and 4-CPA as the sole carbon sources, as indicated by complete disappearance of absorbance peak and by substantial cell growth. Strain SH6 was restricted in its herbicide degradation spectrum. Of the isolates, strain SH3 shows the most interesting degradation capability, since it rapidly degraded five different herbicides, including MCPP and 2,4-DP which have a three-carbon side chain.

Plasmid profile of the isolates. When the isolates were subjected to Kado's plasmid detection procedure, only strain SH3 exhibited two plasmid bands and the other strains contained no plasmids (Fig. 4). To investigate whether the MCPA degradative genes were transmissible to other bacteria, the isolates were mated with antibiotics-resistant recipients, *Pseudomonas cepacia* and *Alcaligenes* sp. In all of the isolates, the MCPA degradation phenotype was not transferred at a detectable frequency ($<10^{-9}$) into the two recipients, suggesting that their MCPA degradation genes were located in the chromosomal DNA or on the untransmissible plasmid. Through a curing process using sodium dodecyl sulfate, a cured strain was obtained from strain SH3. This cured strain did not have the smaller one of the two plasmids and concomitantly lost the ability to degrade MCPP and 2,4-DP. Moreover, its MCPA and 2,4-D degradation rates were significantly decreased, taking 20 to 30 days to com-pletely mineralize 300 μg/ml of MCPA. This result suggests that the smaller plasmid of strain SH3 has very important genes for metabolism of herbicides. In future studies, more attention needs to be placed on

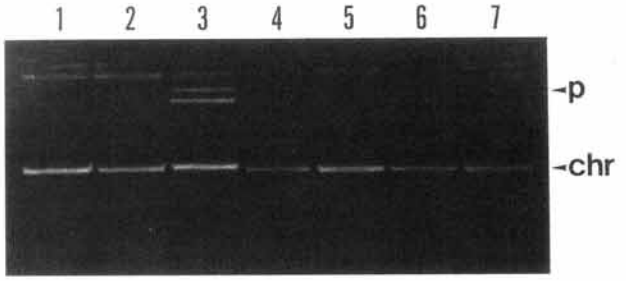

Fig. 4. Plasmid profiles of MCPA-degraders. Lanes: 1, SH1; 2, SH2; 3, SH3; 4, SH4; 5, SH5; 6, SH6; and 7, SH7.

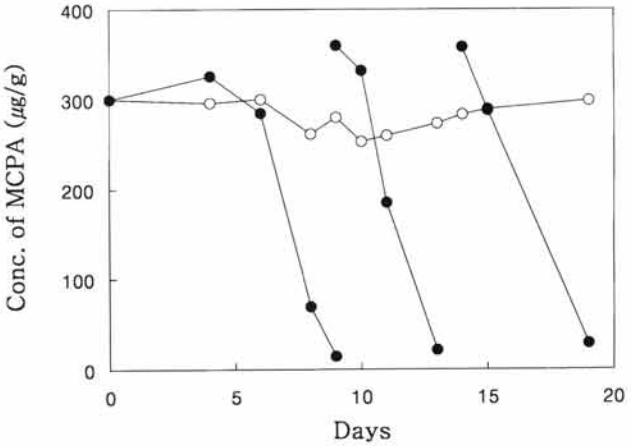

Fig. 5. Degradation of MCPA in soil microcosms inoculated with strain SH3 (●—●) or with only indigenous microbial population (○—○). Each point is the mean for two replicate microcosms.

understanding the role of this new degradative plasmid in herbicide metabolism.

Degradation of MCPA in soil. The patterns of degradation of MCPA in natural field soils were analyzed with and without MCPA degraders. MCPA was observed to be quickly degraded after short lag periods in field soils with indigenous MCPA degraders. The first dose of MCPA (300 μg/g) in this soil was degraded within two weeks, and it took only about one week for the second and third doses of MCPA to be completely degraded, respectively. However, MCPA was persistent for quite a long time in natural field soils without any indigenous MCPA-degrading microorganisms (Fig. 5). The application of one of the isolates into this natural field soil at a density of 10^6 cells/g soil resulted in quick degradation of MCPA, taking about 1 week for the complete removal of 300 μg/g of MCPA in adapted soils. The result suggested that MCPA used in agricultural practices could persist in the soils without MCPA degraders and that the MCPA-degrading strains isolated in this study thus could help in removing the persistent MCPA residues through in situ bioremediation.

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