The Journal of Microbiology, June 1997, p.117-122

Vol. 35, No. 2

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

Min-Jae Chung, Se-Young Shin, Yong-Keun Park[1], Kyung-Hee Min[1] and Jong-Ok Ka*

Department of Agricultural Biology, Seoul National University, Suwon 441-744, Korea
[1]Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea

(Received March 29, 1997 / Accepted April 25, 1997)

Several dominant 4-CPA-degrading bacteria were isolated from agricultural soils. Most of the isolates were identified as *Burkholderia* species by fatty acid methyl ester (FAME) analysis, but they were distinct in chromosomal patterns obtained by PCR amplification of repetitive extragenic palindromic (REP) sequences. These strains were generally restricted in their substrate utilization capabilities. The 4-CPA degradative enzymes were inducible by 4-CPA and some isolates appeared to mineralize 4-CPA via formation of 4-chlorophenol and 4-chlorocatechol as intermediates during its biodegradation pathway. Plasmid DNAs were not detected from most of the isolates and their 4-CPA degradation phenotype was not transferred to other bacteria, suggesting that the 4-CPA genes were on the chromosomal DNA. The 4-CPA degradation patterns in axenic cultures and natural soils varied depending on the strains and soils. The inoculation of 4-CPA degraders much improved the removal of 4-CPA from the 4-CPA treated soils.

Key words: biodegradation, 4-chlorophenoxyacetic acid, *Burkholderia* sp.

Large amounts of man-made chlorinated organic chemicals have been used as pesticides in agriculture. Among them, chlorinated phenoxyalkanoates are of the most extensively used pesticides. Many studies have reported on the isolation and characterization of phenoxyacid pesticide-degrading microorganisms, and their degradation enzymes and genes have been extensively described (5, 7, 13-14, 19). These pesticides are also known to be degraded via formation of chlorinated phenols and catechols during their biodegradation pathways (5, 9). Some phenoxyacid pesticides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA), are readily mineralized by a number of microorganisms present in soil and stimulate substantial growth of the corresponding microbial population (3, 8, 15, 20). 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-chlorophenoxyacetic acid (4-CPA) is one of the chlorinated phenoxy alkanoic pesticides and is closely related to 2,4-D in its structure and action mechanism. 4-CPA belongs to the class of hormone herbicides and possesses plant growth regulating activity. It is used for improving fruit setting on tomato, for inhibiting sprout formation in mung beans, and for fruit thinning in peaches. This compound reaches the soil mainly during the application process and by transfer from plants to the soil by rainwater. The 4-CPA applied to leaves is also readily absorbed and translocated to all parts of the treated plant and exuded from the root to the surrounding rhizosphere soil.

If a pesticide is to be of wide application in agricultural practice, its degradation and persistence should be analyzed to protect the growth of the succeeding crop and the environment. Despite continual agricultural use of 4-CPA, however, little information is available about its fate in the soil, the distribution of 4-CPA degrading microorganisms, their species diversity and physiology, and the biodegradation pathway.

In this study, we isolated several dominant 4-CPA-degrading bacteria from agricultural soils and investigated their diversity and physiological and genetic properties. In addition, soil microcosms were used to study 4-CPA degradation patterns in natural soils with and without the inoculation of 4-CPA degrading bacteria.

* To whom correspondence should be addressed

Materials and Methods

Media and culture conditions

All isolates were maintained on MMO mineral medium (18) containing 4-CPA at a concentration of 300 ppm (μg/ml). Peptone-tryptone-yeast extract-glucose (PTYG) medium containing (per liter) 0.25 g of peptone (Difco), 0.25 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 0.5 g of glucose, 0.03 g of magnesium sulfate, and 0.003 g of calcium chloride was used for strain purification and colony production for repetitive extragenic palindromic PCR (REP-PCR).

Chemicals

4-chlorophenoxyacetic acid (4-CPA), 2-chlorophenoxyacetic acid (2-CPA), 3-chlorobenzoic acid (3-CB), and salicylate were obtained from Aldrich Chemical Co. Analytical grade 2,4-dichlorophenoxyacetic acid (2,4-D), phenoxyacetic acid (PA), and 2-methyl-4-chlorophenoxyacetic acid (MCPA) were obtained from Sigma Chemical Co.. 4-nitrophenoxyacetic acid (4-NPAA) was obtained from Timothy Sassanella of Michigan State University.

Isolation of bacterial strains

Agricultural soil samples were taken from diverse countrywide sites. 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 a sterilized 0.85% saline solution by shaking the preparation on a rotary shaker (200 rpm). Samples (0.1 ml) of appropriate 10-fold dilutions were inoculated into most-probable-number tubes containing 3 ml of 4-CPA mineral medium (MMO mineral medium containing 300 ppm of 4-CPA). The tubes were incubated at 30°C for 3 weeks and degradation of 4-CPA was analyzed by spectrophotometry. The culture in the tube containing the highest dilution that exhibited 4-CPA 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 4-CPA degradation in fresh 4-CPA mineral medium before strain purification.

FAME analysis

The isolates were cultured on tryptic soy agar medium at 28°C for 48 to 72 h. Cells were harvested from the plates by scraping with a sterile glass loop and used for fatty acid methyl ester (FAME) analysis. Saponification, methylation, and extraction were performed by using the procedure described in the MIDI manual (Microbial Identification, Inc.) (17).

Colony REP-PCR

The colony REP-PCR was performed using BOXA1R as a primer as described previously (4, 20). 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.

Degradation phenotype analysis

Each strain was cultured in 4-CPA mineral medium to produce cells induced to metabolize 4-CPA and in PTYG medium to produce cells not induced for 4-CPA metabolism. Cultures were grown at 30°C and aerated by shaking at 200 rpm in an incubator shaker. Cells in the late log phase 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. Aliquots of suspended cells were inoculated into culture tubes, each of which contained MMO mineral medium supplemented with one of the structural analogs at a concentration of 300 ppm. After incubation, cultures were centrifuged to remove cellular material, and UV absorption was measured to determine the degradation of phenoxyacetates.

Axenic culture experiment

After growth in 4-CPA mineral medium and PTYG medium as described above, cells were harvested, washed, and prepared in a sodium phosphate buffer. Aliquots of suspended cells were inoculated into duplicate flasks containing 200 ml of 4-CPA (300 ppm) mineral medium. All cultures were incubated at 30°C and were aerated by shaking at 200 rpm on a rotary shaker.

Analysis of 4-CPA degradation pathway

Strains were streaked onto 4-CPA minimal plates to produce cells induced to metabolize 4-CPA and onto PTYG plates to produce cells not induced to metabolize 4-CPA. To assay the 2,4-D esterase activity, 4-NPAA solution (20 mM) was sprayed onto each plates and then further incubated at 30°C during observations for color changes. To analyze 2,4-D esterase activity in liquid cultures, each strain was inoculated into 4-CPA mineral medium and into PTYG medium. When cells reached the late log phase, the 4-NPAA solution was added into each culture tube

Table 1. Identification of 4-CPA-degrading isolates by FAME analysis

Isolate	Soil site of isolation[a]	Identity as determined by FAME analysis
CPA1	Taean, Chungchongnam-Do	*Burkholderia cepacia*
CPA2	Poun, Chungchongbuk-Do	*Burkholderia gladioli*
CPA3	Wonju, Kangwon-Do	Unidentifiable[b]
CPA4	Kosong, Kyungsangnam-Do	*Burkholderia cepacia*
CPA5	Sunchun, Chollanam-Do	*Burkholderia cepacia*
CPA6	Pukcheju, Cheju-Do	*Burkholderia cepacia*

[a] All soils were from agricultural fields.
[b] The isolate could not be identified due to its poor growth on laboratory medium.

at a final concentration of 20 mM and then further incubated for color changes. For the resting cell experiment, bacterial strains were cultured, harvested, and prepared in sodium phosphate buffer as described above, and then inoculated into MMO mineral medium supplemented with 4-chlorophenol or 4-chlorocatechol at a concentration of 50 ppm. The disappearance of each intermediate was monitored by spectrophotometry.

Degradation of 4-CPA in soil

The isolate CPA5 (Table 1) was grown at 30°C in PTYG medium, harvested, washed, and prepared in sodium phosphate buffer. The agricultural soil samples were sifted, adjusted to a water content of ca. 30% (wt/wt), and inoculated with strain CPA5 at a density of 1.0×10^6 cells/g soil. The soil was thoroughly mixed, and 300 g was transferred to each beaker. Two other replicates were not inoculated with the strain CPA5. Inoculated and uninoculated soils were treated with 4-CPA dissolved in 0.1 M NaH_2PO_4 buffer (pH 7.0) to a concentration of 300 ppm and thoroughly mixed. The disappearance of 4-CPA from soil was monitored by high-performance liquid chromatography (12), and the soils were respiked with 4-CPA (300 ppm) after it was removed until a total of 5 cycles of degradation had been completed.

Results and Discussion

Degradation of 4-CPA

4-CPA was completely degraded by the strains isolated from the agricultural soils (Fig. 1). The isolated bacteria utilized it as their sole carbon and energy source, producing a significant biomass in 4-CPA mineral medium.

FAME and REP-PCR analysis

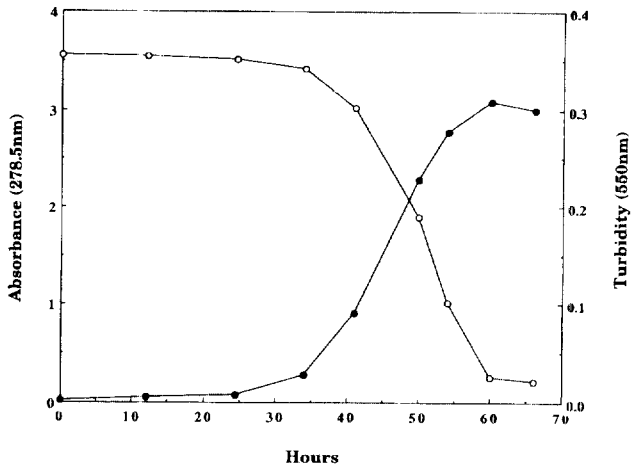

Fig. 1. Disappearance of UV absorption spectrum (- O -) and growth of bacteria during biodegradation of 4-CPA by strain CPA6(- ● -).

The isolates that could be reasonably identified by the FAME results are shown in Table 1. All of the isolates were *Burkholderia* species, except strain CPA3, which could not be identified due to its poor growth on tryptic soy agar medium.

A colony REP-PCR experiment was performed to study the genomic relatedness of the isolates (Fig. 2). REP-PCR analysis of the isolates revealed that these bacteria produced 6 different DNA fingerprint patterns, suggesting that none of them were identical to each other. The strains CPA4 and CPA6, which were identified by FAME analysis as *Burkholderia cepacia*, shared two common bands, while the other strains appeared to have no common bands.

Growth of 4-CPA-degrading bacteria in axenic cultures

To understand axenic growth patterns of the 4-CPA degraders, each strain was inoculated into 4-CPA mineral medium under both induced and uninduced conditions (Fig. 3). Under uninduced conditions, strains CPA3 and CPA6 exhibited short lag periods (ca. 25 h) and thereafter began to grow exponentially. Strains CPA4 and CPA5 exhibited relatively longer lag periods (ca. 50 h) and strain CPA1 showed the longest lag period (ca. 80 h). Under induced conditions, by contrast, most of the isolates did not exhibit any significant lag periods, suggesting that 4-CPA degradative enzymes were inducible by the presence of the substrate 4-CPA. The inducibility of 4-CPA degradative enzymes was not observed in strain CPA2. This strain exhibited similar growth patterns under both induced and uninduced conditions (Fig. 3), suggesting that its 4-CPA degradative enzymes were

Fig. 2. REP-PCR patterns of isolates. Lanes : 1, strain CPA6; 2, strain CPA2; 3, strain CPA5; 4, strain CPA3; 5, strain CPA1; 6, strain CPA4; 7, *Hin*dIII digested lambda DNA standard.

constitutively produced.

Degradative diversity analysis

The isolates were grown on 4-CPA mineral medium and PTYG medium and then examined for their ability to degrade other compounds related to 4-CPA. The isolates were generally restricted in their substrate utilization abilities (data not shown), although some of the isolates such as CPA 3, CPA4, and CPA6 could degrade 3-CB and salicylate in addition to 4-CPA. It is of interest to note that none of these bacteria could utilize 2,4-D as a carbon source, while many of the isolates selected for 2,4-D degradation were able to mineralize 4-CPA (11).

Analysis of 4-CPA degradation pathway

To investigate the degradation pathway of 4-CPA, strains were grown on either 4-CPA mineral plates to produce cells induced for 4-CPA metabolism or PTYG plates to produce uninduced cells. 2,4-D esterase activities were then assayed by using the 4-NPAA test procedure (Table 2). The 2,4-D esterase

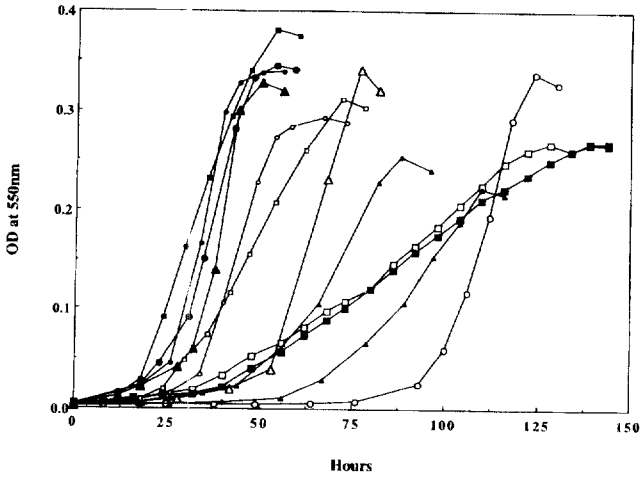

Fig. 3. Growth patterns of 4-CPA-degrading bacteria in axenic culture. Symbols; ● and ○, strain CPA1; ■ and □, strain CPA2; ▪ and ▫, strain CPA3; ▲ and △, strain CPA4; ▲ and △, strain CPA5; ● and ○, strain CPA6. The bacteria were either adapted (solid symbols) or not adapted (open symbols) for 4-CPA metabolism. Each point is the mean for two replicate liquid cultures. OD, optical density.

is known to be encoded by the *tfd*A gene in most of the 2,4-D degraders and is specific for the first step in the major 2,4-D degradation pathway, converting 2,4-D to 2,4-dichlorophenol (16). 4-NPAA has an acetic acid group linked to the nitroaromatic ring via an ether bond. When the ether bond is enzymatically cleaved by the 2,4-D esterase activity, the resulting colony produces a yellow color due to the product, 4-chlorophenol. All of the isolates, except strain CPA2, rapidly turned yellow within sev-

Table 2. Activities of 2,4-D esterase in 4-CPA-degrading isolates[a]

Strain	Growth condition	2,4-D esterase activity	
		plate assay	liquid culture assay
CPA1	A	+	+
	U		
CPA2	A	+	+
	U	+	+
CPA3	A	+	+
	U		
CPA4	A	+	+
	U		
CPA5	A	+	+
	U		
CPA6	A	+	+
	U		

[a] The bacteria were grown in 4-CPA mineral medium (A) or in PTYG medium (U). Then 4-NPAA was applied and the cells were incubated at 30°C for development of intensive yellow color. +, Development of intensive yellow color within 1 hr; -, no development of yellow color in 18 hrs.

eral minutes on 4-CPA mineral plates, but no color change was observed on PTYG plates. Similar results were also observed from the liquid culture experiment, indicating that the isolated 4-CPA-degrading bacteria had 2,4-D esterase activities only when induced by 4-CPA. Interestingly, the strain CPA2 exhibited yellow color under both induced and uninduced conditions, further supporting that the enzymes were constitutively produced in this strain.

Among the isolates, the resting cells of strain CPA4 and CPA6 grown on 4-CPA also immediately metabolized 4-chlorophenol and 4-chlorocatechol without a lag phase, while the cells not induced for 4-CPA metabolism could not degrade these intermediates within 7 hr's incubation. These results strongly suggested that the two isolates mineralized 4-CPA via formation of 4-chlorophenol and 4-chlorocatechol during its degradation pathway, which could represent a different metabolic pathway from the proposed one on a pseudomonad bacterium that could not attack 4-chlorophenol (6).

Transferability of 4-CPA phenotype

To investigate whether 4-CPA degradative genes

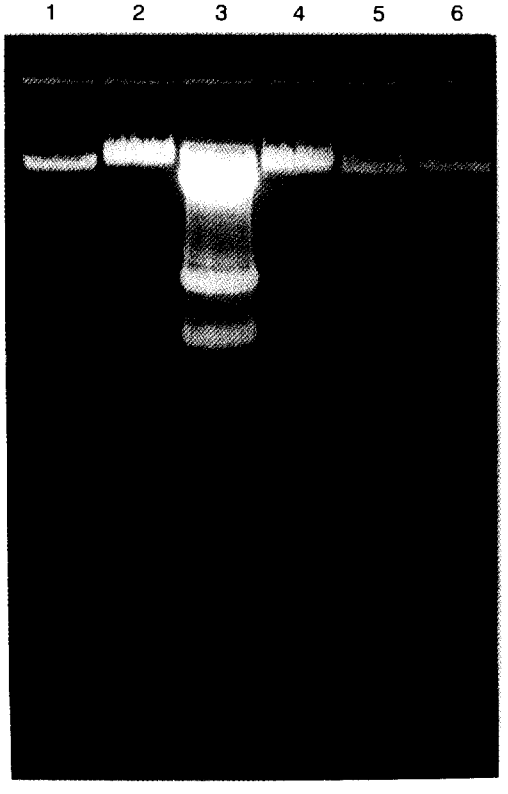

Fig. 4. Agarose gel electrophoresis for detection of plasmids in 4-CPA-degrading bacteria. Lanes: 1, strain CPA1; 2, strain CPA6; 3, strain CPA2; 4, stran CPA3; 5, strain CPA5; 6, strain CPA4. Chr, chromosomal and linear DNA.

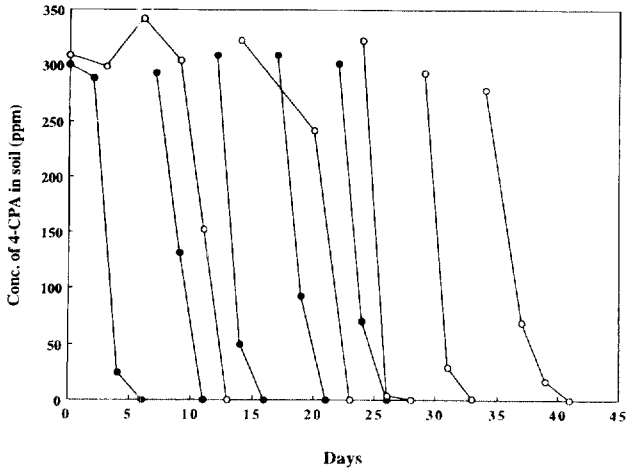

Fig. 5. Degradation of 4-CPA in Chungchongbuk-Do soil during 5 repeated additions in microcosms inoculated with strain CPA5(●–) and with only indigenous microbial population (–○).

are transmissible to other bacteria, strains were mated with antibiotics-resistant recipients such as *Pseudomonas cepacia* and *Alcaligenes* sp. (10). It was observed that the 4-CPA$^+$ phenotype was not transferred at a detectable frequency ($<10^{-9}$) from all of the isolates, suggesting that the 4-CPA genes were contained on chromosomal DNA in these strains. When the isolates were subjected to Kado's plasmid detection procedure (11), only strain CPA2 exhibited plasmid DNA bands (Fig. 4).

Degradation of 4-CPA in soil

The patterns of degradation of 4-CPA in natural soils were analyzed with and without 4-CPA de-

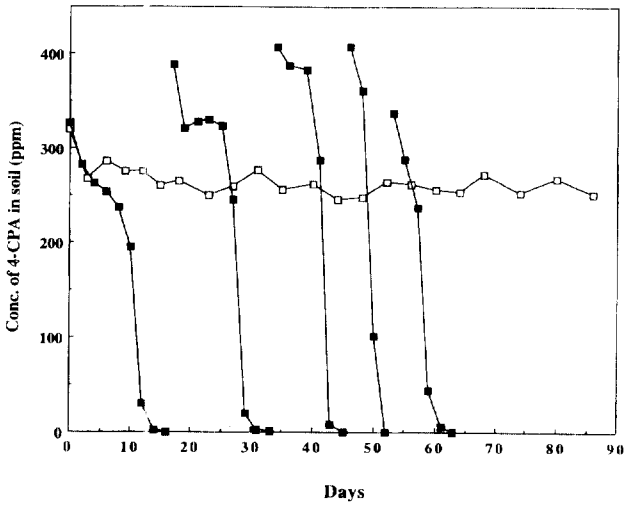

Fig. 6. Degradation of 4-CPA in Kyungki-Do soil during 5 repeated additions in microcosms inoculated with strain CPA5 (–■–) and with only indigenous microbial population (–□).

graders. 4-CPA was observed to be quickly degraded after short lag periods in soils inoculated with or containing 4-CPA degraders (Fig. 5). It took about 1 week for each addition of 300 ppm of 4-CPA to be degraded when the soil bacterial community was adapted for degradation of 4-CPA. In a field soil sample from Suwon, Kyungki-Do, which was not inoculated with 4-CPA degraders, however, the treated 4-CPA was not degraded during the experiment (Fig. 6). The results suggested that the 4-CPA used in agricultural soils containing no 4-CPA degraders could persist in the soils for quite a long time, possibly leading to environmental pollution problems. The 4-CPA-degrading strains isolated in this study thus could help in removing persistent 4-CPA through in situ bioremediation.

Acknowledgements

This study was supported by Korean Ministry of Education through Research Fund (1995) and in part by the Korea Science and Engineering Foundation (KOSEF) through the Research Center for Molecular Microbiology at Seoul National University.

References

1. **Alexander, M.** 1965. Biodegradation: problems of molecular recalcitrance and microbial fallibility. *Adv. Appl. Microbiol.* **7**, 35-80.
2. **Alexander, M.** 1969. Microbial degradation and biological effects of pesticides in soil. *Soil Biol. Rev. Res. Nat. Resour. Res.* (UNESCO) **9**, 209-240.
3. **Biederbeck, V.O., C.A. Campbell, and A.E. Smith.** 1987. Effects of long-term 2,4-D field applications on soil biochemical processes. *J. Environ. Qual.* **16**, 257-262.
4. **de Bruijn, F.J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of Rhizobium meliloti isolates and other soil bacteria. *Appl. Environ. Microbiol.* **58**, 2180-2187.
5. **Don, R.H., A.J. Weightman, H.-J. Knackmuss, and K. N. Timmis.** 1985. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). *J. Bacteriol.* **161**, 85-90.
6. **Evans, W.C., S.W. Smith, P. Moss, and H.N. Fernley.** 1971. Bacterial metabolism of 4-chlorophenoxyacetate. *Biochem. J.* **122**, 509-517.
7. **Fisher, P.R., J. Appleton, and J.M. Pemberton.** 1978. Isolation and charterization of the pesticide-degrading plasmid pJP1 from Alcaligenes paradoxus. *J. Bacteriol.* **135**, 798-804.
8. **Foster, R.K., and R.B. Mckercher.** 1973. Laboratory incubation studies of chlorophenoxyacetic acids in chernozemic soils. *Soil Biol. Biochem.* **5**, 333-337.
9. **Gaunt, J.K. and W.C. Evans.** 1971. Metabolism of 4-chloro-2-methyl-phenoxyacetate by a soil pseudomonad. Preliminary evidence for the metabolic pathway. *Biochem. J.* **122**, 519-526.
10. **Hong, S.M., Y.J. Ahn, Y.K. Park, K.H. Min, C.K. Kim, and J.O. Ka.** 1995. Effects of genetically different 2,4-D-degradative plasmids on degradation phenotype and competitiveness of soil microorganisms. *J. Microbiol.* **33(3)**, 208-214.
11. **Ka, J.O., W.E. Holben, and J.M. Tiedje.** 1994. Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-treated field soils. *Appl. Environ. Microbiol.* **60**, 1106-1115.
12. **Ka, J.O., W.E. Holben, and J.M. Tiedje.** 1994. Use of gene probes to aid in recovery and identification of functionally dominant 2,4-dichlorophenoxyacetic acid-degrading populations in soil. Appl. Environ. Microbiol. 60, 1116-1120.
13. **Kilbane, J.J., D.K. Chatterjee, J.S. Karns, S.T. Kellogg, and A. M. Chakrabarty.** 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of Pseudomonas cepacia. *Appl. Environ. Microbiol.* **44**, 72-78.
14. **Lappin, H.M., M.P. Greaves, and J.H. Slater.** 1985. Degradation of the herbicide mecoprop 2-(2-methyl 4-chlorophenoxy)-propionic acid by a synergistic community. *Appl. Environ. Microbiol.* **49**, 429-433.
15. **Loos, M.A., I.F. Schlosser, and W.R. Mapham.** 1979. Phenoxy herbicide degradation in soils: quantitative studies of 2,4-D and MCPA-degrading microbial populations. *Soil Biol. Biochem.* **1**, 377-385.
16. **Sassanella, T.M.** (Michigan State Univers4ity) Personal communication.
17. **Sasser, M.** 1990. Technical note 102: tracking a strain using the Microbial Identification System. MIS Inc., North Newark, Del.
18. **Stanier, R.Y., N.J. Palleroni, and M. Doudoroff,** 1966. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.* **43**, 159-271.
19. **Streber, W.R., K.N. Timmis, and M.H. Zenk.** 1987. Analysis, cloning, and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene *tfd*A of *Alcaligenes eutrophus* JMP134. *J. Bacteriol.* **169**, 2950-2955.
20. **Torstensson, N.T. L., J. Stark, and B. Goransson.** 1975. The effect of repeated applications of 2,4-D and MCPA on their breakdown in soil. *Weed Res.* **15**, 159-164.
21. **Versalovic, J., M. Schneider, F.J. de Bruijn, and J. R. Lupski.** 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods *Mol. Cell. Biol.* **5**, 25-40.