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Characteristics of *Pseudomonas* sp. degrading 2-methyl-4-chlorophenoxyacetic acid

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2-메틸-4—클로로페녹시 아세트산을 분해하는 *Pseudomonas* 균주의 특성

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Abstract: From the soil and river samples, some bacterial strains degrading chlorinated aromatic hydrocarbons were isolated and identified. Of the isolates, seven strains of *Pseudomonas* sp. harbouring plasmids were selected for their prominent degradative ability to 2-methyl -4-chlorophenoxyacetic acid. By agarose gel electrophoresis and curing experiment it was found that the genes for 2-methyl-4-chlorophenoxyacetic acid degradaiton were encoded on the plasmids in these selected strains. Antibiotic resistance and degradative ability for other herbicides of the strains were tested.

Key words: *Pseudomonase* sp., 2-melthyl-4-chlorophenoxyacetic acid

2, 4-dichlorophenoxyacetate (2, 4-D), 2-methyl-4-chlorophenoxyacetate (MCPA), 2, 4-dichlorophenol (DCP), and 3-chlorobenzoate (3CB) are widely used herbicides for the past several decades (Sharpee *et al.*, 1973; Dorn *et al.*, 1974). These chlorinated herbicides have become one of the most serious pollution sources, because they may potentially cause carcinogenic and teratogenic effects on human beings and other beneficial live stocks and they are more persistent and toxic than non -chlorinated aromatic compounds.

Microbial metabolism of MCPA, 2, 4-D, DCP, and 3CB were reported (Gaunt and Evance, 1971; Evance *et al.*, 1971; Hartman *et al.*, 1979), and plasmids were isolated from *Alcaligenes* degrading chlorinated herbicides (Fisher *et al.*, 1978; Don and Pemberton, 1981). Genetic and physical map of pJP4 plasmid, one of them stated above, was generated (Don and Pemberton, 1985), and restriction map of 3CB-degradative plasmid was also generated (Chatterjee and Chakrabarty, 1984).

In the present paper we describe on the characteristics of *Pseudomonas* sp. strains degrading MCPA, and the reationship between the degradative ability of MCPA and the plasmids harboured in the strains.

MATERIALS AND METHODS

Bacterial strains and plasmids used

Bacterial strains and plasmids used in this study are shown in Table 1.

Table 1. *Bacterial strains and plasmids used.*

Strain/plasmid	Relevant characteristics	References
Pseudomonas sp.		
KU49, KU199, KU213, KU366, KU426, KU559, KU563	MCPA$^+$	This work
Escherichia coli		
C600/RP 4	*r*$^-$ *m*$^-$ *thi*$^-$ *leu*$^-$ *thr*$^-$/ Ap^r Tc^r Km^r Tra$^+$	Barth and Grinter (1977)
HB101/pRK290	*r*$^-$ *m*$^-$ *pro*$^-$ *leu*$^-$ *str*^{r}/ Km^r	Yasuda and Taguchi (1983)

Media and growth conditions

Pseudomonas sp. were grown with shaking at 30℃ in L-broth medium (Miller, 1972) or minimal medium (Whiteside and Alexander, 1963). The promonent MCPA-degradative strains were selected by measuring the optical density spectrophotometrically at 600 nm after 24 hr culture in minimal liquid media supplemented with MCPA as a sole source of carbon and energy. For the other herbicides utility test, the strains were inoculated on minimal agar media supplemented with 2, 4-D, 3CB, and DCP as a sole source of carbon, respectively.

Isolation of plasmid DNA

Crude lysates of plasmid DNAs were prepared according to the method of Hansen and Olsen (1978). The following steps show amounts used for a 40-m*l* culture input. Cells were grown to approximately 2×10^8 cells per m*l* in L-broth medium and harvested by centrifugation. The pellet was resuspended in 1.35 m*l* of 25% sucrose-0.05 M Tris (pH 8.0), and 0.1 m*l* of lysozyme (10 mg/m*l* in 0.25 M Tris, pH 8.0) was added. After the mixture was put in an ice-water bath for 5 min, 0.5 m*l* of Na_2EDTA (0.25 M, pH 8.0) was added and the tubes were chilled for 5 min once more in ice water. The addition of 0.5 m*l* sodium dodecyl sulfate (20%, wt/vol, in TE), followed by heat pulse and mixing, produced a clear viscous solution of lysed cells. Immediately 1.25 m*l* of NaCl (5 M) was added and the tubes were chilled in an ice-water bath and refregerated (4℃) for 6hr or overnight as convenient. After centrifugation, to the supernatant 0.313 volume of PEG 6000 (42% in 10 mM sodium phosphate buffer, pH 7.0) was added and the tubes were refrigerated for overnight and centrifuged. The pellet was suspended in TES (20 mM Tris-HCl-5 mM EDTA-100 mM NaCl, pH 8.0) buffer.

Response to antibiotics

Ampicillin, tetracyclin, kanamycin, chloramphenicol, streptomycin, and gentamycin were used. Strains were grown overnight in L-broth to exponential phase. A portion of such a culture was plated on L agar media containing various antibiotics, respectively. The cells were grown at 30℃ for 48 hr.

Curing test

Cells were grown at 30℃ in L-broth supplemented with various concentration of mitomycin C for 24-72 hr. The cultures were then diluted and plated on L agar media. Well-seperated colonies were then replicated to minimal media supplemented with corresponding carbon source.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed in a horizontal gel of 0.6% agarose in TAE buffer composed of 0.4 M Tris, 0.2 M sodium acetate, 0.01 M EDTA at 8.0. Gels were run at 100 V for 3 hr. Gels were stained in solution of ethidium bromide (1μg/m*l*) for 10 min, rinsed and photographed under UV illumination.

RESULTS AND DISSCUSSION

Selection and characterization of the MCPA degrading bacteria

From the soil and river samples collected mainly in the Gyeong-gi Province, 744 strains of bacteria degrading chlorinated aromatic hydrocarbons were isolated and identified. Of the isolates, seven strains of *Pseudomonas* sp. harbouring plasmids were selected for their prominent degradative ability of MCPA.

Table 2. *Patterns of utilization of other herbicides by the MCPA-degradative strains.*

Strain	MCPA	2,4-D	3CB	DCP
KU49	+	+	+	+
KU199	+	+	−	−
KU213	+	+	−	−
KU366	+	+	+	+
KU426	+	+	+	+
KU559	+	+	+	+
KU563	+	+	+	+

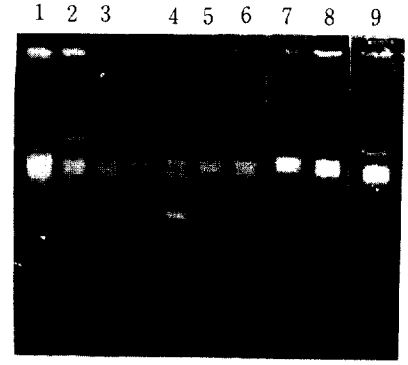

Fig.1. *Agarose gel electrophoresis of plasmids isolated from MCPA-degradative Pseudomonas.*

1. *Pseudomonas* sp. KU366
2. *Escherchia coli* (RP4)
3. *Pseudomonas* sp. KU49
4. *Escherichia coli* (pRK290)
5. *Pseudomonas* sp. KU199
6. *Pseudomonas* sp. KU213
7. *Pseudomonas* sp. KU559
8. *Pseudomonas* sp. KU426
9. *Pseudomonas* sp. KU563

Patterns of utilization of other herbicides and resistance to various antibiotics of the selected isolates were shown in Table 2 and Table 3, respectively. Five strains of *Psudomonas* sp. could utilize 2,4-D, 3CB and DCP as well as MCPA as a sole source of carbon and energy, while the other two strains couldn't utilitize 3 CB and DCP. All MCPA degrading strains tested were sensitive to gentamycin, while resistance to various antibiotics of the strains were varied according to the strains.

Detection of plasmid DNA

All selected strains of *Pseudomonas* sp. degrading MCPA (KU49, 199, 212, 365, 426, 559, and 563) contained plasmids. As shown in Fig.1, one strain of *Pseudomonas* sp., KU366 contained three plasmids (pKU12, 13, 14) and two strains, KU49 (pKU1, 2), KU213 (pKU4, 11), had two plasmids each, while the other 4 strains, KU199(pKU3), KU426(pKU 15), KU559(pKU16), and KU563(pKU17), had one plasmid.

Curing of cells harbouring plasmids

To clarify the relationships between biodegradability of MCPA and their plasmids, curing tests were accomplished with mitomycin C

Table 3. *Resistance of MCPA-degradative strains of various antibiotics.*

Antibiotics	Ap			Tc			Km			Sm			Cm			Gm		
Conc. (μg/ml) Strain	100	200	400	12.5	25	50	12.5	25	50	25	50	100	50	100	200	25	50	100
KU49	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	−	−	−
KU199	−	−	−	+	+	−	+	+	+	+	+	+	+	+	+	−	−	−
KU213	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−
KU366	+	+	+	+	+	+	−	−	−	+	+	+	+	+	−	−	−	−
KU426	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	−	−	−
KU559	+	+	+	+	+	+	−	−	−	+	−	−	+	+	+	−	−	−
KU563	+	+	+	+	+	+	−	−	−	+	−	−	+	+	+	−	−	−

Abbr. Ap; ampicillin Tc; tetracyclin Km; kanamycin
Sm; streptomycin Cm; chloramphenicol Gm; gentamycin

Table 4. *Curing of MCPA-degradative strains by mitomycin C.*

Strains	Mitomycin C (μg/ml)	Number of colonies tested	Used marker for curing	Frequency of curing (%)
Pseudomonas sp.				
KU49	12.5	800	MCPA	16.5
KU213	5.0	345	MCPA	10.1
KU366	40.0	400	MCPA	18.0
KU426	40.0	400	MCPA	11.0
KU559	20.0	400	MCPA	12.0
KU563	25.0	400	MCPA	9.3

* KU199 was hard to be cured by treatment with mitomycin C.

(MC). As can be seen in table 4, sublethal concentration of KU213 was 5μg MC/m*l* and KU199 could not grow with 0.1 ug Mc/m*l*. As the result on curing, of the 7 strains harbouring plasmids, 6 strains showed the resistance to mitomycin C and lost the degradative ability for chlorinated hydrocarbons. Agarose gel electrophoresis of cured strains were shown in Fig.2. It was found by frequency of curing and agarose gel electrophoresis of cured strains that the loss of biodegrability was not due to the mutation of genes, but to loss of plasmids encoding degradative genes. As shown in Fig.2, the cured strains of KU49, KU213, and KU366 didn't have any plasmid at all, and therefore it was not clear that which of the plasmids harboured in these strains encoded the degradative genes.

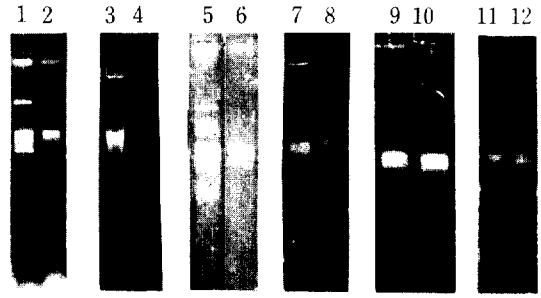

Fig.2. *Agarose gel electrophoresis of the plasmid-harbouring and their cured strains.*

1. *Pseudomonas* sp. KU49
2. cured strain of *P.* sp. KU49
3. *Pseudomonas* sp. KU213
4. cured strain of *P.* sp. KU213
5. *Pseudomonas* sp. KU366
6. cured strain of *P.* sp. KU366
7. *Pseudomonas* sp. KU426
8. cured strain of *P.* sp. KU426
9. *Pseudomonas* sp. KU559
10. cured strain of *P.* sp. KU559
11. *Pseudomonas* sp. KU563
12. cured strain of *P.* sp. KU563

적 요

염소계 방향족 탄화수소를 분해하는 다수의 세균을 토양과 하천 표품으로부터 분리 하여 동정하였다. 이들중 2-메틸-4-클로로페녹시 아세트산을 강력하게 분해하고, 또한 플라스미드를 갖고있는 7 군주의 *Pseudomonas* sp.를 선별하였다. 2-메틸-4-클로로페녹시 아세트산의 분해 유전자가 플라스미드에 기인함을 전기영동과 큐어링 실험에 의해서 확인하였다. 항생물질에 대한 내성과 다른 세초세에 대한 분해능도 아울러 조사하였다.

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