KOR. JOUR. MICROBIOL. December, 1986 p. 394-399 Vol. 24, No. 4

Characteristics of MCPA Plasmid isolated from *Pseudomonas* sp.*

Lee, Y.N., D.S. Choi, S.H. Eun, and Y.D. Park
Department of Biology, College of Science, Korea University, Seoul, Korea

Pseudomonas 에서 분리한 MCPA 플라스미드의 특성

이영록 · 최대성 · 은성호 · 박영두
고려대학교 이과대학 생물학과

Abstract: From the lysates of the 7 selected strains of *Pseudomonas* utilizing 2-methyl -4-chlorophenoxyactate as a sole source of carbon and energy, several MCPA plasmids, which encodes genes for the degradation of 2-methyl-4-chlorophenoxyacetate, were isolated, and measured their molecular weight as well as genetic characters such as resistance to antibiotics and degradative ability of other chlorinated herbicides. Transmissibility of the MCPA plasmids, pKU1, pKU15, and pKU17 was tested by conjugation or transformation and the restriction pattern of pKU15 for Pvu II, Hind III, EcoR I, Xho I, Bgl II, and Ava II was analyzed.
Key words: MCPA plasmid, *Pseudomonas* sp.

In our previous paper (Eun *et al.*, 1986), we reported on the occurence of MCPA plasmid in *Pseudomonas* strains, which encoded the genes for the degradation of 2-methyl -4-chlorophenoxyacetic acid. In the present study, we describe on the other characteristics of the MCPA plasmids such as molecular weight, restriction patterns, other genes encoded, and transmissibility.

MATERIALS AND METHODS

Bacterial strains and plasmids

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

Media and culture conditions

E. coli and *P. aeruginosa* were grown at 37℃, and *P. putida* and *P.* sp. were grown at 30℃ in L-broth or minimal media (Whiteside and Alexander, 1963).

Isolation of plasmid DNA

Crude lysates of plasmid DNAs were prepared according to the method of Hansen and Olsen (1978). For obtaining large quantities of plasmid DNA, the modified method of Tanaka and Weisblum (1975) was employed. Cells were grown overnight and were diluted 10-fold into fresh L-broth medium. The culture was permitted to grow for 5 hr with shaking. The cells from 1 *l* of culture were suspended in 25 m*l* of 50 mM Tris-HCl-25% sucrose (pH 8.0), and 5 m*l* of lysozyme was added. After the mixture was incubated on ice for 5 min, 12.4 m*l* of 5 M NaCl and 5 m*l* of 10% SDS were added, and mixed rapidely. The mixture was incubated on ice for 2-4 hr, and the lysate was centrifuged. To the supernant the same volume of 20% polyethy-

* This work was supported by a grant from the Korea Green Cross Research Institute

Table 1. *Used bacterial strains and their characteristics.*

Strain/plasmid	Relevant characteristics	References
Pseudomonas aeruginosa		
PAO303 / Rms148	*arg*$^-$/Sm^r Tra$^+$	Sagai *et al.* (1975)
KU141	*sal*$^+$ *amp*^{r} *gen*^{r}	Kim and Lee (1984)
Pseudomonas putida		
TN1032	*trp*$^-$ *leu*$^-$ *ben1*$^-$ *Str*^{r}	Nakazawa and Yokota (1977)
TN1126	*met*$^-$ *trp*$^-$	Nakazawa and Yokota (1977)
Pseudomonas sp.		
KU606	3CB$^+$, 2, 4-D$^+$	Eun *et al.* (1986)
Alcaligenes eutrophus		
JMP228	*Str*^{r}	Don and Pemberton (1981)
Escherichia coli		
C600/RP4	*r*$^-$ *m*$^-$ *thi*$^-$ *leu*$^-$ *thr*$^-$/Ap^r Tc^r Km^r Tra$^+$	Barth and Grinter (1977)
HB101/pRK290	*r*$^-$ *m*$^-$ *pro*$^-$ *leu*$^-$ *Str*^{r} *recA*/Tc^r	Corbin *et al.* (1982)
HB101/pSY343	*r*$^-$ *m*$^-$ *pro*$^-$ *leu*$^-$ *Str*^{r} *recA*/Km^r	Yasuda and Taguchi (1983)

lene glycol, and the mixture was incubated on ice for 2-24 hr. After centrifugation, the precipitate was dissolved in TES (20 mM Tris -HCl-5 mM EDTA-100 mM NaCl, pH 8) buffer. Solid CsCl (0.996 g/m*l*) and ethidium bromide (0.6 mg/m*l*) were added, and the final density was adjusted to 1.610±0.005. The samples was spun at 38,000 rpm for 40 hr in a RP65T rotor. Under ultraviolation, plasmid band was collected, and was extracted three times with isoamyl alcohol and dialized against one fifth of TES buffer.

Bacterial conjugation

Transfer frequencies were determined by mating on membrane filters as described by De Graaf *et al.* (1973). Crosses were made as follows. Exponentially growing donors were mixed with an equal volume of recipient cultures in the late exponential phase (about 2×10^9 colony forming units per m*l*). The mixture was filtered on a Toyo membrane filter (0.2μm pore size, 25-mm diameter, Gelman Science, INC). Membranes were placed on the surface of a freshly prepared Penassay broth agar plate and incubated at 30℃ for 5 hr. Bacteria were then suspended in 1 m*l* of saline, and 0.2 m*l* sample of suitable dilutions were spread on the selective medium. Conjugation frequencies were determined after the titration of donor cells on LB agar plate.

Transformation procedure

The transformation procedure is carried out as described by Nakazawa (1983). The cells of *P. putida* TN1126 or *P.* sp. KU606 were grown overnight in L-broth at 27℃ on a shaker. A portion (1.5 m*l*) of such a culture was reinoculated in 20 m*l* of fresh L-broth and grown at 27℃ for 2 hr. The cells were chilled, harvested by centrifugation at 4℃ and washed once with 10 m*l* of 10 mM NaCl -0.5 M sucrose. The cells were kept on ice for 20 min. The cells were again centrifuged and resuspended in 2 m*l* of 50 mM $CaCl_2$. An aliquot (0.2 m*l*) of these cells was then mixed 100 μl of pKU1 or pKU15 DNA solution and 3μl of lysozyme (0.1 mg/m*l*). The cell-DNA mixture was incubated at 0℃ for 60 min and then subjected to a heat pulse at 42℃ for 2 min, chilled and finally diluted in 2.7 m*l* of L-broth. The cells were allowed to grow at 27℃ for 8 hr on a

shaker and aliquots were plated on selection agar medium.

Digestion of plasmid DNA with restriction enzymes

Restriction endonuclease cleavages were carried out in different buffer system. Low buffer (10 mM Tris, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.5) was used for Bgl II, medium buffer (50 mM NaCl, 10 mM Tris -HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.5) for Ava II, Pvu II, and Hind III, and high buffer (100 mM NaCl, 50 mM Tris -HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.5) for EcoRI and Xho I. In most of the cases reaction were carried out at 37℃ for 1 to 3 hr, depending on the concentration of plasmid DNA (0.5 to 3 μg) and the concentration of endonucleasas (6-10 units).

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as previously (Kim *et al.*, 1986). A horizontal gel of 0.7-0.9% agarose in TAE buffer (0.4 M Tris, 0.2 M sodium acetate, 0.01 M EDTA, pH 8.0) was run at 100 V for 3 hr or 50-60 V overnight. Gels were stained in a solution of ethidium bromide (1 μg/ml) for 20 min, rinsed and photographed under UV illumination.

RESULTS AND DISCUSSION

Determination of molecular weight

Molecular weight of the isolated plasmids which encode the MCPA-degradative genes were determined by use of covalently closed circular forms of Rms148, RP4, and pSY343 as standard size markers (Fig.1). The smallest plasmid was pKU14 isolated from *Pseudomonas* sp. KU366 and the largest plasmid was pKU4 isolated from *Pseudomonas* sp. KU213. The molecular weight of pKU4 was estimated at larger than 100 Md because it was larger than that of the largest plasmid of the standard size marker, Rms148. The molecular weights of the plasmids isolated for seven *Pseudomonas* sp. were shwon in Table 2.

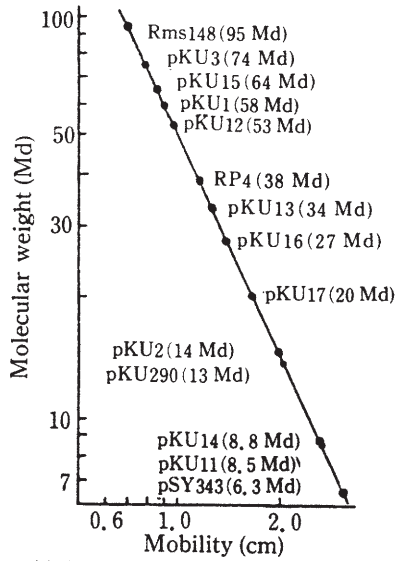

Fig.1. *Molecular weights of MCPA-degradative plasmids.*

1. pKU16
2. pKU15
3. pKU12, 13, 14
4. RP4
5. pKU1, 2
6. pRK290
7. Rms148
8. pKU4, 11
9. pSY343
10. pKU17

Properties of plasmids

The biodegradability for chlorinated aromatics of the strains was shown to be encoded on plasmids by previous curing test. In this study to investigate the additional plasmid -encoded properties, plasmids-harboring strains and cured strains were tested for their ability to degrade other chlorinated aromatics and the resist to various antibiotics and heavy metals. Ampicilin, tetracyclin, chloramphenicol, kanamycin, streptomycin, and gentamycin were used as antibiotics, and $BaCl_2$, $CoNO_3$, $HgCl_2$, and $CaCl_2$ were used as heavy metals. As the results of this test, pKU15, pKU16, and pKU17 are shown to encode the ability to degrade 4 kinds of chlorinated herbicides, and pKU16 conferred the resistance to antibiotics, tetracyclin and ampicillin, and heavy metal, $HgCl_2$, too. Plasmids of KU49, KU213 and KU366 also encoded the ability to degrade two or four kinds of the chlorinated herbicides, and conferred to resist kanamycin in KU49. However which of the plasmids encoded the biodegradability and

Table 2. *Characteristics of the plasmids isolated from Pseudomonas strains degrading MCPA.*

Strain	Plasmid	Phenotype	Molecular weight (Md)
P. sp. KU49	pKU1, 2	MCPA$^+$, 2, 4-D$^+$, 3CB$^+$, DCP$^+$, Km$^+$	58.0, 14.0
P. sp. KU199	pKU3	ND	74.0
P. sp. KU213	pKU4, 11	MCPA$^+$, 2, 4-D$^+$	>100, 8.5
P. sp. KU366	pKU12, 13, 14	MCPA$^+$, 2, 4-D$^+$, 3CB$^+$, DCP$^+$	53.0, 34.0, 8.8
P. sp. KU426	pKU15	MCPA$^+$, 2, 4-D$^+$, 3CB$^+$, DCP$^+$	64.0
P. sp. KU559	pKU16	MCPA$^+$, 2, 4-D$^+$, 3CB$^+$, DCP$^+$, Tc^r, Ap^r, Hg^r	27.0
P. sp. KU563	pKU17	MCPA$^+$, 2, 4-D$^+$, 3CB$^+$, DCP$^+$	20.0

ND; Not determined

resistance to antibiotics in these strains was not confirmed in this study.

Restriction patterns of pKU15

To construct the restriction map and to investigate more detailed genetic properties of the plasmids, among the characterized plasmids, we first selected a plasmid, pKU15. The plasmid DNA was digested with six restriction endonucleases, Pvu II, Hind III, EcoRI, Xho I, Bgl II, and Ava II. The molecular weights

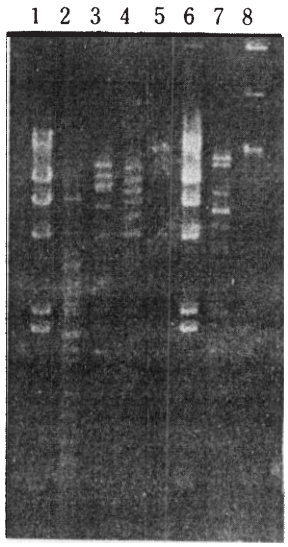

Fig.2. *Agarose gel electrophoresis of pKU15 digested with various restriction enzymes.*

1) λ-Hind III
2) Ava II
3) EcoRI
4) Bgl II
5) Xho I
6) λ-Hind III
7) Hind III
8) Pvu II

Table 3. *The size of the restriction fragment of plasmid pKU15*.*

Fragment	Pvu II	Hind III	EcoRI	Bgl II	Ava II	Xho I
A	52.5	10.0	8.4	8.6	6.0	9.8(2)
B	11.0	8.8	7.6	6.4	5.1	8.6(2)
C		8.0	6.2(2)	5.2(2)	4.8(2)	7.5(2)
D		5.8	5.4	4.6	4.6	4.8
E		5.2	5.1	3.8(2)	4.0	
F		4.6	4.4	3.4(2)	3.4	
G		4.1(2)**	3.8	2.8	2.8(2)	
H		3.8	3.1	2.4	2.4	
I		3.6	2.6	2.0	2.1	
J		1.9	2.4	1.1(2)	1.8(2)	
K		1.7	2.1		1.7	
L		1.3	1.9		1.4	
M		1.0	1.6			
N			1.5			
O			1.2			
Total (Md)	63.5	63.9	63.5	<64.0	<64.0	<64.0

* Fragments smaller than 1.0 Md were not determined.

** The number in parenthesis indicates a probable stoichiometry of 2.

of the DNA fragments were estimated by calibration of the mobility of λ-phage DNA fragments digested with Hind III. The restriction patterns by agarose gel electrophoresis are shown in Fig.2. Pvu II gave two fragments, Xho I more than seven fragments, Bgl II more than fourteen fragments and Ava II more than fifteen fragments. The size of fragments are shwon in Table 3.

Table 4. *Transfer of the ability of MCPA degradation by conjugation.*

Donor	Recipient	Selected marker	Frequency of* transfer
P. sp. KU49	*P. aeruginosa* KU141	MCPA, Gm	1.5×10^{-7}
	P. putida TN1032	MCPA, Sm	1.1×10^{-7}
	A. eutrophus JMP228	MCPA, Sm	3.5×10^{-8}
P. sp. KU563	*P. aeruginosa* KU141	MCPA, Gm	4.0×10^{-8}
P. sp. KU426	*P. aeruginosa* KU141	MCPA, Gm	4.0×10^{-8}

*Frequency of transfer : $\frac{\text{Number of conjugants}}{\text{Number of recipients}}$

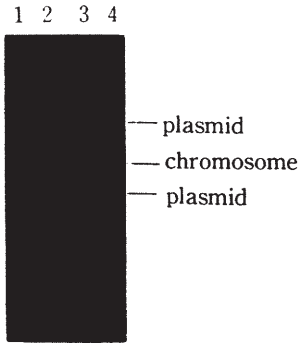

Fig.3. *Agrose gel electrophoresis of conjugants.*
1. *P. aeruginosa* KU141 (recipient)
2. *P. aeruginosa* KU751 (conjugant of KU141 with KU49)
3. *P. aeruginosa* KU751 (conjugant of KU141 with KU49)
4. *P.* sp. KU49 (donor)

Transfer of plasmids by conjugation

To clarify the transmissibility of the plasmids, bacterial conjugations were carried out. Three *Pseudomonas* spp. harbouring MCPA plasmid were used as donors, and *P. aeruginosa* KU141 and *P. putida* TN1032 as recipients. As shown in Table 4, KU49, KU426, and KU563 could transfer the degadative ability for chlorinated aromatics to recipients by frequencies from 3.5×10^{-8} to 1.5×10^{-7}. To clarify which of the plasmids of KU49 encode the degradative ability, plasmid of KU751, conjugant of KU141 with KU49, was isolated and agarose gel electrophoresis of plasmids was shown in Fig.3. As shown in Fig.3, two plasmids of KU49 were cotransferred to KU141, and this suggeted that self-transmissible plasmid could help a nonconjugative but mobilizable plasmid to be tranferred (Freifelder, 1983). Therefore it was not ascertained which of pKU1 and pKU2 was degradative plasmid.

Transformation of *Pseudomonas*

In order to transfer the ability of chlorinated herbicides degradation and to confirm the properties of pKU1, *Pseudomonas* strains were transformed by the plasmid of pKU1 and pKU15. Selection media were two or three kinds of minimal media supplemented with 2, 4-D, 3CB, MCPA, and DCP, respectively. When *P. putida* TN1126 was transformed by pKU1, transformants could be obtained on 2, 4-D, 3CB and MCPA minimal plates (Table 5). By the result of this experiment, pKU1 plasmid of *P.* sp KU49 was proved to encode the ability of chlorinated aromatics degradation.

Table 5. *Transformation of Pseudomonas cells for chlorinated aromatic hydrocarbon degrading characters specified by pKU1 and pKU15 plasmid DNA.*

Plasmid DNA	Recipient	Selected Marker	Transformation* Frequency
pKU1	*P. putida* TN1126	2, 4-D	4.5×10^{-8}
		MCPA	7.8×10^{-8}
		3CB	6.6×10^{-8}
pKU15	*P.* sp. KU606	MCPA	1.3×10^{-8}
		DCP	3.8×10^{-8}

*Transformation frequency : $\frac{\text{Number of transformants}}{\text{Number of recipients}}$

적 요

본 연구실에서 분리 보존하고 있는 MCPA (2-methyl-4-chlorophenoxy acteic acid)를 분해하는 *Pseudomonas* 7 균주들로부터 각각 MCPA 플라스미드를 분리하여 그들의 분자량을 측정함과 동시에 항생제 내성 및 다른 제초제를 분해하는 유전자의 존재여부등 그 특성을 조사하였다.

또한 MCPA 플라스미드 pKU1, pKU15 및 pKU17등의 접합 또는 형질전환등에 의한 종간 및 속간 전달능을 조사하고 pKU15의 Pvu II, Hind III, EcoRI, Xho I, Bgl II 및 Ava II등 제한효소에 대한 소화패턴등을 해석하였다.

REFERENCES

1. Barth, P.T. and N.J. Grinter, 1977. Map of plasmid RP4 derived by insertion of transposon C. *J. Mol. Biol.*, **113**, 455-474.
2. Corbin, D., G. Ditta, and D.R. Helinski, 1982. Clustering of nitrogen fixation (*nif*) genes in *Rhizobium meliloti*. *J. Bacteriol.* **149**, 221-228.
3. De Graaf, J., P.C. Kreuning, and P. Van De Putte, 1973. Host controlled restriction and modification of bacteriophage Mu and Mu-promoted chromosome mobilization in *Citrobacter freundi*. *Mol. Gen. Genet.*, **123**, 283-288.
4. Don, R.H. and J.M. Pemberton, 1981. Properties of six pesticide degrading plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *J. Bacteriol.*, **145**, 681-686.
5. Eun, S.H., Y.D. Park, and Y.N. Lee, 1986. Characteristics of *Pseudomonas* sp. degrading 2-methyl-4-chlorophenoxyacetic acid. Kor. *J. Microbiol.*, **24**, 389-393.
6. Freifelder, D., 1983. Mechanism for rearrangement and exchange of genetic material: I. Plasmid. In Molecular Biology. Science Books International, Boston.
7. Hansen J.B. and R.H. Olsen, 1978. Isolation of large plasmids and characterization of P2 incompatibility group plasmids pMG1 and pMG5. *J. Bacteriol.*, **135**, 227-238.
8. Kim, J.K. and Y.N. Lee, 1984. Isolation and identification of *Pseudomonas* utilizing hydrocarbon. *Kor. Jour. Microbiol.*, **22**, 29-34.
9. Kim, H.K., S.K. Koh, and Y.N. Lee, 1986. Restriction map of a cryptic plasmid from *Pseudomonas putida*. *Kor. Jour. Microbiol.*, **24**, 7-11.
10. Nakazawa, T. and T. Yokota, 1977. Isolation of a mutant TOL plasmid with increased activity and transmissibility from *Pseudomonas putida* (*arvilla*) mt-2. *J. Bacteriol.*, **129**, 39-46.
11. Nakazawa, t., 1983. *Pseudomonas;* In Practical techniques of genetic recombination. vol 4, 73-84 (in Japanese).
12. Sagai, H., V. Krcmery, K. Hasuda, S. Iyobe, H. Knothe, and S. Mitsuhashi, 1975. R factor-mediated resistance to amino-glycoside antibiotics in *Pseudomonas aeruginosa*. *Japan. J. Microbiol.*, **19**, 427.
13. Tanaka, T. and B. Weisblum, 1975. Construction of a colicin E1-R factor composite plasmid *in vitro* : Means for amplification of deoxyribonucleic acid. *J. Bacteriol.*, **121**, 345-362.
14. Whiteside, J.S. and M. Alexander, 1963. Measurement of microbiological effects of herbicide. *Weeds*, 8, 204-213.
15. Yasuda. S. and T. Taguchi, 1983. Overproduction of *Escherichia coli* replication proteins by the use of runaway-replication plasmid. *J. Bacteriol.*, **154**, 1153-1161.

(Received Nov. 9, 1986)