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Restriction map of a cryptic plasmid from *Pseudomonas putida**

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*Pseudomonas putida*로 부터 분리한 cryptic플라스미드의 制限酵素地圖

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We screened lysates of the laboratory strains of pseudomonads utilizing hydrocarbon by agarose gel electrophoresis and cesium chloride-ethidium bromide equilibrium centrifugation, to find an intrinsic plasmid as a vector and to examine the relationship between the plasmid and hydrocarbon degradation. Only one strain from the examined strains, *Pseudomonas putida* KU190, contained a plasmid. We named the plasmid pKU41. The molecular size of pKU41 was determined as 41kb, using covalently closed circular forms of RP4 and pSY343 as standard size markers. The restriction sites of pKU41 for *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III and *Sal*I were 3, 1, 3, 6, and more than 13, respectively. With double or triple digestion, restriction map of pKU41 was constructed for *Bam*HI, *Bgl*II and *Hind*III. For elucidation on the biological function of the plasmid, test was conducted on the ability of hydrocarbon utilization of the host strain but no apparent relationship was observed.

Bacteria belonging to the genus *Pseudomonas* have a wide variety of habitats, in soil, fresh water and the marine environment, and they can be use a variety of organic compounds including synthetic chemicals as a sole carbon source. Moreover, several plasmids which confer the ability to degrade octane, camphor, salicylate, xylene, or naphthalene have been isolated from *P. putida* strains (Chakrabarty, 1976). TOL plasmids have wide host range including *E. coli* (Benson and Shapiro, 1978). However, *tol* genes are weakly expressed in *E. coli* (Nakazawa *et al.*, 1978). This might be due to the lack of suitable information or a different information requirement for the expression of *tol* genes in *E. coli* cells, possibly relating to their membrane barrier to substrates or the accumulation of toxic intermediates. Inouye *et al.* (1981) suggested that *Pseudomonas* promoter sites are recognized poorly by the RNA polymerases in other gram-negative bacteria, including *E. coli*, and that on the contrary, the expression of the *E. coli* gene in *Pseudomonas* is very efficient.

Chakrabarty *et al.* (1975) reported the transformation of *P. putida* with RP1 plasmid DNA which has a mass of 38Md. Nagahari (1980) constructed previously a hybrid plasmid consisting of RP4 and a fragment containing *E. coli* tryptophan operon using *Eco*RI restriction endonuclease and T4 ligase. However, because of its high molecular weight, it is difficult to handle. Failure to construct a mini RP4 plasmid with various restriction

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endonucleases suggested a scattering of the genes that are essential to the replication and maintenance of RP4 plasmid (Barth and Grinter, 1977). Recently, RSF1010, a derivative from streptomycin and sulfonamide resistance factor R6, is used for a cloning vector in *Pseudomonas* cells. However, an intrinsic plasmid of *Pseudomonas* sp. would be preferable as a vector for biosafety.

In the present study, we have delineated tentative physical map of an intrinsic plasmid harbored by *P. putida*, and examined the relationship between the plasmid and hydrocarbon degradation.

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 in this experiment.*

Bacterial strains and plasmids	Genetic Marker (Molecular size)	Reference
Strains		
P. putida KU 185	SAL, CAT	Kim and Lee (1984)
KU 188	SAL, CAT	Kim and Lee (1984)
KU 189	SAL, CAT	Kim and Lee (1984)
KU 190	SAL, CAT	Kim and Lee (1984)
KU 191	SAL, CAT	Kim and Lee (1984)
KU 212	SAL, CAT	Kim and Lee (1984)
KU 218	SAL, CAT	Kim and Lee (1984)
KU 220	SAL, CAT	Kim and Lee (1984)
P. aeruginosa		
KU 94	CAT	Kim and Lee (1984)
KU 141	SAL, CAT	Kim and Lee (1984)
Plasmids		
pSY 343	Km^r (9.5Kb)	Yasuda et al. (1983)
RP 4	Ap^r, Km^r, Tc^r (56.4 Kb)	Barth and Grinter (1977)
pKU 41	(41 Kb)	This experiment

SAL : salicylate, CAT : catechol, Km : kanamycin, Ap : ampicillin, Tc : tetracycline

Media and growth conditions

P. aeruginosa and *P. putida* were grown with shaking at 37°C and 30°C, respectively, in L-broth medium (Miller, 1972) or M9 medium (Maniatis *et al.*, 1982).

Curing test

Cells were grown at 30°C in L-broth supplemented with various concentration of curing agents for 24-72hr. The culture was then diluted and plated on L-agar. Colonies were then replica-plated to another L-agar plate, and also M9 agar medium supplemented with salicylate or catechol instead of glucose.

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 5hr with shaking. The cells from 1l of culture were suspended in 25ml of 50mM Tris-HCl-25% sucrose (pH8), and 5ml of lysozyme was added. After the mixture was incubated on ice for 5 min, 12.4ml of 5M NaCl and 5ml of 10% SDS were added, and mixed rapidly. The mixture was incubated on ice for 2-4hr, and the lysate was centrifuged. To the supernatant the same volume of 20% polyethylene glycol, and the mixture was incubated on ice for 2-24hr. After centrifugation, the precipitate was dissolved in TES (20mM Tris-HCl-5mMEDTA-100mM NaCl, pH8) buffer. Solid CsCl (0.996g/ml) and ethidium bromide (0.6mg/ml) were added, and the final density was adjusted to 1.610 + 0.005. The sample was spun at 38,000rpm for 40hr in a RP65T rotor. Under ultraviolet illumination, plasmid band was collected, and was extracted three times with isoamyl alcohol and dialized against one fifth of TES buffer.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed in a vertical slab gel of 0.7-0.9% agarose in TAE buffer composed of 0.4M Tris, 0.2M sodium acetate, 0.01M EDTA at pH8.0. Gels were run at 100V for

3hr or 50-60V overnight. Gels were stained in a solution of ethidium bromide (1ug/ml) for 20min, rinsed and photographed under UV illumination.

Digestion of plasmid DNA with restriction endonucleases

Restriction endonuclease cleavages were carried out in different buffer systems. Low buffer (10mM Tris-HCl, 10mM $MgCl_2$, 1mM dithiothreitol, pH7.5) was used for *Bgl*II, medium buffer (50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM dithiothreitol, pH7.5) for *Bam*HI and *Hind*III, and high buffer (100mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$, 1mM dithiothreitol, pH7.5) for *Eco*RI, *Sal*I and *Xho*I. In most of the cases reactions were carried out at 37°C for 1 to 3 hr, depending on the concentration of plasmid DNA (0.5 to 3μg) and the concentration of endonucleases (6-10 units).

RESULTS AND DISCUSSION

Detection of plasmid DNA and determination of molecular size

The isolated strains including *P. putida* and *P. aeruginosa* except *P. putida* KU190 were already investigated their hydrocarbon utilization, and they were cured to some hydrocarbons (Chung and Lee, 1984). Among them, *P. putida* contained a plasmid (Fig. 1). We named the plasmid pKU41. We could not detect any plasmid in cured strains. It was found that strain KU191 had lost the ability

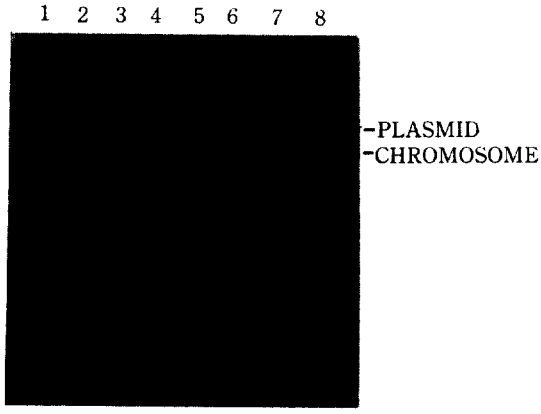

Fig. 1. *Plasmid from P. putida strains.*
1. KU220, 2. KU218, 3. KU212, 4. KU191, 5. KU190, 6. KU189, 7. KU188, 9. KU185

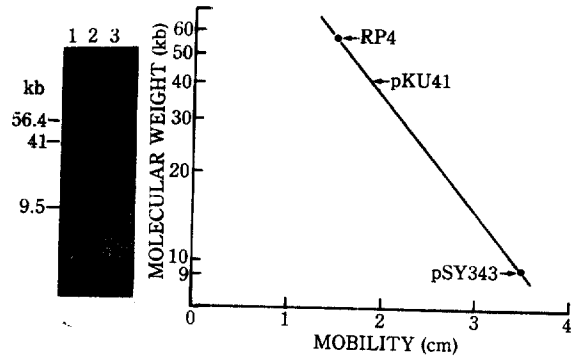

Fig. 2. *Molecular size of pKU41.*
1. pSY343, 2. pKU41, 3. RP4

of salicylate utilization during laboratory preservation of more than 1 year. However, our present method for detection of pseudomonad plasmid may not have been the best.

The plasmid isolated from *P. putida* KU190 harboring only a single plasmid was subsequently purified by CsCl-EtBr density gradient centrifugation. The molecular size was estimated at 41Kb using covalently closed circular forms of RP4 and pSY343 as standard size markers (Fig. 2).

Table 2. *The sizes of the restriction fragments of plasmid pKU41*.*

Restriction enzyme / Fragment	*Bam*HI	*Bgl*II	*Eco*RI	*Hind*III	*Sal*I
A	18.1	41	9.8	19.1	14.6
B	17.5		9.2	15.0	4.0
C	5.4		7.8	6.9	3.6
D			5.8		2.6
E			5.0		2.5
F			3.4		2.1
G					2.05(2)**
H					2.0
I					1.95
J					1.8(2)
K					1.65
Total(Kb)	41	41	41	41	41>

*Fragments smaller than 1.5 kb were not determined.

**The numbers in parentheses indicate a probable stoichiometry of 2.

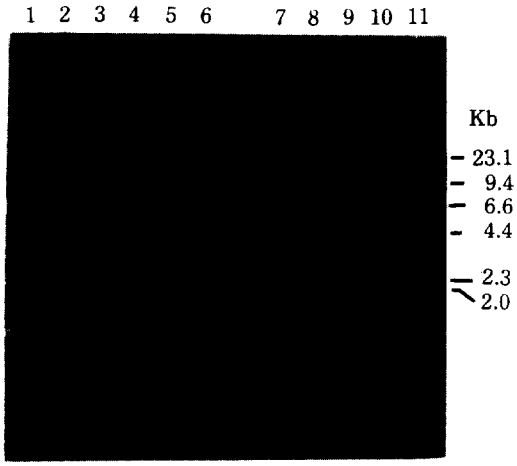

Fig. 3. *Agarose gel electrophoresis of pKU41 – digested with various restriction enzymes.*
1. *Bam*HI + *Hind*III, 2. *Eco*RI + *Hind*III, 3. *Bam*HI + *Bgl*II, 4. λ-*Hind*III, 5, *Xho*I, 6. *Sal*I, 7. *Hind*III, 8. *Eco*RI, 9. *Bgl*II, 10. *Bam*HI, 11. λ-*Hind* III

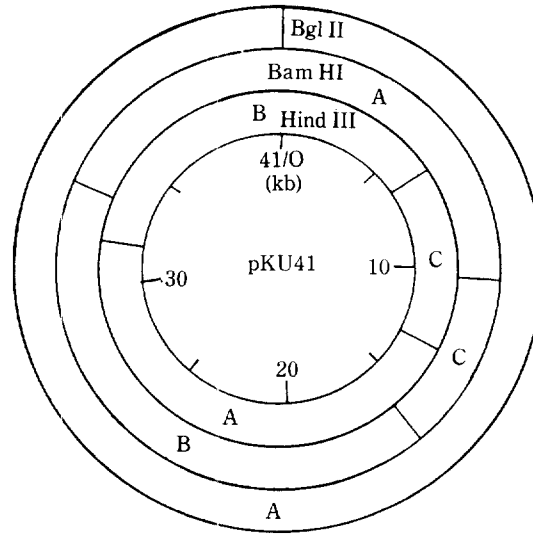

Fig. 4. *Restriction enzyme map of pKU 41.*

Restriction patterns and restriction map of pKU41

The purified plasmid DNA was digested with six restriction endonucleases, *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Sal*I and *Xho*I, then subjected to agarose gel electrophoresis. The molecular sizes of DNA fragments were estimated after calibration of the mobility of λ phage DNA fragments digested with *Hind*III. The restriction patterns on agarose gel are shown in Fig. 3. *Bam*HI gave three fragments, *Bgl*II a single fragment, *Eco*RI six fragments. *Hind*III three fragments, and *Sal*I more than thirteen fragments, whereas the plasmid was not digested with *Xho*I. The sizes of fragments generated are shown in Table 2. Because the sum of the molecular sizes of fragments by each enzyme approximated the molecular size of the plasmid determined by CCC form, most fragments were probably detected, although some smaller fragments might escaped our observations.

Electrophoretic pattern of some double digestion of pKU41 is shown in Fig. 3, and triple digestion was also carried out. The restriction map was constructed according to these information (Fig. 4).

Biological function of pKU41

In order to elucidate the biological function of this plasmid test was conducted on the ability of hydrocarbon utilization of the host strain. *P. putida* could grow on salicylate and catechol as sole carbon source. The ability of hydrocarbon utilization could not be cured by treatment of the strain with acridine orange, mitomycin C or ethidium bromide. Thus, it is not clear whether a relationship exists between hydrocarbon utilization and this plasmid.

Although no biological function of pKU41 is known so far, it may be useful as a vector in pseudomonad, if a genetic marker such as antibiotic resistance is added to this plasmid and a smaller-sized plasmid is constructed by *in vitro* manipulation.

적 요

*Pseudomonas*의 분해계 플라스미드와 이들에 유용한 벡터를 개발하기 위해서 본 연구실에서 분리, 보존하고 있는 *Pseudomonas* 중에서 *P. putida* KU 190으로부터 하나의 플라스미드를 분리하여 그 특성을 해석코저 하였다. size marker로 RP4와 pSY 343를 사용하여 플라스미드의 크기를 결정한 바 41 Kb로 나타났으며, 이 플라스미드를

pKU 41라 명명하였다.

제한효소 *Bgl*II, *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I등으로 이 플라스미드를 소화하여 이들의 제한효소 pattern을 조사한 결과, *Bgl*II가 1, *Bam*HI이 3, *Hin*dIII는 3, *Eco*RI은 6 그리고 *Sal*I은 13개 이상의 절단부위를 가지고 있었다. 이 제한효소의 절단부위, 토막들의 크기로부터 *Bgl*II, *Bam*HI, *Hin*dIII에 대한 지도를 작성하였다.

플라스미드의 생물학적 기능을 조사하기 위하여 탄화수소 자화능에 대한 큐어링 실험을 하였으나 이로부터 뚜렷한 관련성 여부를 관찰할 수 없었다.

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. Benson, S. and Shapiro, 1978. Tol is a broad-host-range plasmid. *J. Bacteriol.* **135,** 278-280.
3. Chakrabarty, A.M., J.R. Mylroie, D.A. Friello and J.G. Vacca, 1975. Transformation of *Pseudomonas putida* and *Escherichia coli* with plasmid linked drug-resistance factor DNA. *Proc. Natl. Acad. Sci. USA* **72,** 3647-3651.
4. Chakrabarty, A.M., 1976. Plasmids in *Pseudomonas. Ann. Rev. Genet.* **10,** 7-30.
5. Chung, I.H. and Y.N. Lee, 1984. On the relationship between the utilizing capacities of hydrocarbons and degradative plasmids in crude oil-degrading bacteria. *The Sciences and Technologies, Korea University,* **25,** 261-266.
6. Hansen, J.B. and R.H. Olsen, 1978. Isolation of large plasmids and characterization of P2 incompatability group plasmids pMG1 and pMG5. *J. Bacteriol.* **135,** 227-238.
7. Inouye, S., A. Nakazawa and T. Nakazawa, 1981. Molecular cloning of TOL genes and *xyl*B and *xyl*E in *Escherichia coli. J. Bacteriol.* **145,** 1137-1143.
8. Kim, J.K. and Y.N. Lee, 1984. Isolation and Identification of *Pseudomonas* utilizing hydrocarbon. *Kor. Jour. Microbiol.* **22,** 29-34.
9. Maniatis, T., E.F. Fritsch and J. Sambrook, 1982. Molecular cloning (A laboratory manual). Cold Spring Harbor Laboratory.
10. Miller, J.H., 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory.
11. Nagahari, K., 1980. Development of molecular cloning systems in *Pseudomonas.* In "Molecular breeding and genetics of applied microorganisms," Sakaguchi, K. and M. Okanishi ed., Kodansha Academy Press, pp. 124-127.
12. Nakazawa, T., E. Hayashi, T. Yokoda, Y. Ebina and A. Nakazawa, 1978. Isolation of TOL and RP4 recombinants by integrative suppression. *J. Bacteriol.* **134,** 270-277.
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,** 354-362.
14. Yasuda, S. and T. Taguchi, 1983. Overproduction of *Escherichia coli* replication proteins by the use of runaway-replication plasmid. *J. Bacteriol.* **154,** 1153-1161.

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