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A genetic map of the R-factor pKU10 isolated from *Pseudomonas putida*

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*Pseudomonas putida*에서 분리한 R-factor pKU 10의 유전자 지도

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ABSTRACT: A genetic map of the IncP-1 group plasmid pKU10 has been prepared through the construction of recombinant plasmids containing various fragments of pKU10. Phenotypic analysis of these derivatives has identified the location of genes encoding resistance to ampicillin, tetracyclin, and chloramphenicol. The region involved in conferring resistance to ampicillin was located around two *Pst*I sites that are 1.0Kb apart. The tetracyclin resistance gene was mapped on the region of *Hind*III E fragment and a part of *Hind*III D fragment, and the determinant for chloramphenicol resistance gene was localized on *Hind*III D fragment.

KEY WORDS□vector in *Pseudomonas*, genetic map

The most versatile vectors in *E. coli* have limitation to be used as a cloning vector in *Pseudomonas* (Bagdasarian *et al.*, 1979). Accordingly, it is necessary to develop intrinsic cloning vectors in *Pseudomonas*. In our laboratory, the naturally occuring IncP-1 group plasmid, pKU10, was originally isolated from a *Pseudomonas putida*. The plasmid pKU10 is nonconjugative, but can be mobilized by conjugative SAL plasmid pKU7 (Kim *et al.*, 1987). pKU10 confers resistance to the antibiotics ampicillin, tetracyclin, and chloramphenicol, has a size of 9.4Kb, and has unique cleavage sites for *Eco*RI, *Xho*I, *Sal*I, *Bgl*II, and *Sma*I. It is stably expressed in *Pseudomonas* and *E. coli* cells (Lim and Lee, 1987).

In order to clarify the regions of pKU10 involved in conferring resistance to antibiotics, we have inserted foreign DNA into pKU10 DNA and have cloned various regions of pKU10 DNA into the plasmid pUC19 (Yanisch-Perron *et al.*, 1985). Analysis of the phenotypic properties of these pKU10 derivatives has allowed the construction of a genetic map of the plasmid indicating the position of genes involved in antibiotics resistances of pKU10.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this work are described in Table 1.

Media and growth conditions

The L broth (Miller, 1972) was used as a complete medium and PAS medium (Chatterjee *et al.*, 1981), as a minimal medium. Incubations were carried out at 30°C for *P. putida* and at 37°C for *E. coli*. Antibiotics were used at the following concentrations unless otherwise indicated: ampicillin, 600 μg/ml; tetracyclin, 12.5 μg/ml; chloramphenicol, 800 μg/ml.

Table 1. *Used bacterial strains and plasmids*

Strain/Plasmid	Relevant property	Reference or sources
Pseudomonas putida		
KU816(pKU10)	/Ap^r Tc^r Cm^r	Kim *et al.* (1987)
KU218RL	*rif ade rec Sal*$^+$	Lee and Lee (1988)
TN1126	*met trp*	Nakazawa and Yokota (1977)
Escherichia coli		
DH1	*F*$^-$ *recA EndA gyrA thi hsdR supE relA*	Yanisch-Perron *et al.* (1985)
JM83(pUC19)	/Ap^r	Yanisch-Perron *et al.* (1985)
HB101(pSY343)	/Km^r	Yasuda and Takagi (1983)
C600(pBR322)	/Ap^r Tc^r	Bolivar *et al.* (1977)
(pDC100)	/3CB$^+$ Ap^r Sm^r	Frantz *et al.* (1987)

Isolation of plasmid DNA

Crude lysates of plasmid DNAs were prepared by the alkaline lysis procedure (Maniatis *et al.*, 1982). To obtain large quantities of plasmid DNA, the method of Hansen and Olsen (1978) was employed. Plasmid DNA was purified by ultracentrifugation(RP 65T rotor) at 36000 rpm for 48hr at 18°C to equilibrium in CsCl-EtBr gradients. Under ultraviolet illumination, plasmid band was collected and extracted three times with water-saturated n-butanol, and plasmid solution was dialized against several changes of TE buffer for 48hr.

Transformation

The transformation procedure of *Pseudomonas* was essentially same as described by Molholt and Doskočil (1978). Competent cells of *E. coli* were prepared and transformed as described by Perbal (1984). Viable cell count assays on cell and DNA mixtures were performed by serially diluting 10-fold in L-broth and plating 100 μl on L agar followed by incubation for 48hr.

Restriction enzyme analysis and agarose gel electrophoresis

Restriction enzymes were purchased from Bethesda Research Laboratories and used according to the instructions of the manufacturer. Crude plasmid solution and DNA fragments were analyzed by 0.7% and 1% agarose gel electrophoresis, respectively, in TAE buffer composed of 0.4M tris, 0.2M sodium acetate, and 0.01M EDTA at pH 8.0 (Kado and Liu, 1979). Gels were run at 100V for 2-3hr and were stained in a solution of ethidium bromide(1 $\mu g/ml$) for 20 min, rinsed and photographed under UV illumination.

Recovery of DNA fragment from agarose gel

After agarose gel electrophoresis the gel was immersed in water containing ethidium bromide (1 $\mu g/ml$) for 30min at room temperature. Using a sharp scalpel, a slice of agarose containing desired DNA was cut and placed into a dialysis bag which was filled with 0.5X TAE buffer. The bag was immersed in a shallow layer of 0.5X TAE in a electrophoresis tank and applied electric field at constant 100V for 2hr. After checking the elution of DNA from agarose gel using UV light, polarity of the current was reversed for 2min and all the buffer surrounding the gel slice was recovered (McDonell *et al.*, 1977).

Ligation of DNA fragments

Plasmid DNA fragments to be ligated were extracted once each with phenol/chloroform(1:1) and chloroform/isoamylalcohol(24:1), precipitated using 0.1 volume of Na acetate(3M, pH 7.0) and 2 volumes of ethanol and cooling to -70°C. The precipitates were collected by centrifugation and resuspended in 20mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 10mM dithiothreitol and 0.6mM ATP, to which was added T_4 DNA ligase (BRL). The mixture was incubated overnight at 4°C.

RESULTS AND DISCUSSION

Insertion of foreign DNA into pKU10

To examine whether the resistance determinants for antibiotics exist on the region containing two *Pst*I sites, we constructed a hybrid plasmid as shown in Fig. 1. pKU10 DNA was doubly digested with *Eco*RI and *Pst*I, and 7.7Kb fragment was electroeluted. Partial double digestion of pDC100 (Frantz *et al.*, 1987) with *Eco*RI and *Pst*I followed by electroelution of 7.7Kb *Eco*RI-*Pst*I segment with genes for degradation of 3-chlorobenzoate(3CB). The 7.7Kb fragment of pKU10 and 7.7Kb fragment of pDC100 were ligated and used to transform *P. putida* TN1126, followed by selection for degradation of 3CB. The recombinant plasmid was designated pKUH3. This plasmid was phenotypically Ap^s Tc^r. It suggested that the region involved in conferring resistance to ampicillin was located around the two *Pst*I sites that are 1.0Kb apart. The hybrid plasmid pKUH3 should have a molecular size of 15.4Kb theoretically, however, the plasmid extracted from transformants has a molecular size of about 150Kb (Fig. 2).

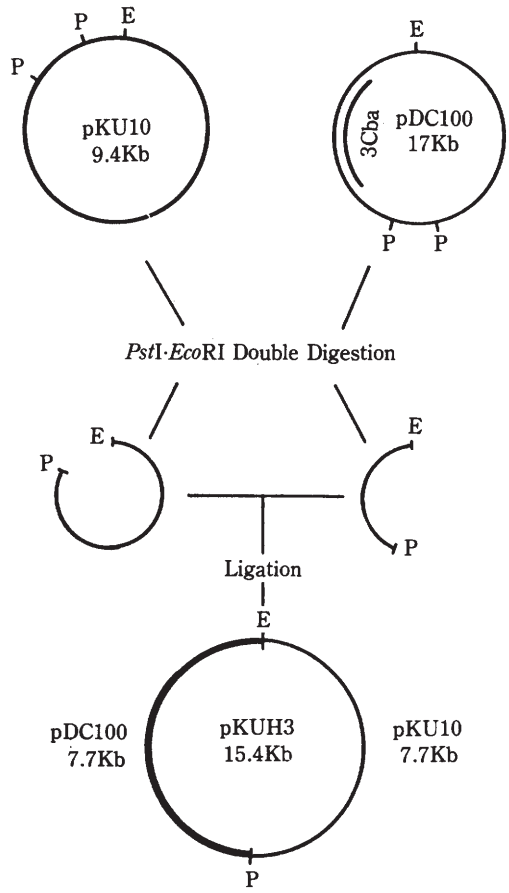

Fig. 1. *Construction of hybrid plasmid pKUH3.*

To investigate the reason for this difference of molecular size, we have transformed *P. putida* TN1126 and *P. putida* KU218RL, $recA^-$ strain with pDC100. When TN1126 was used as a recipient, a molecular size of plasmid extracted from the transformant was about 180Kb(Fig. 3). However the molecular size of plasmid isolated from the transformed KU218RL was 17Kb, same as that of pDC100 in *E. coli* (Fig. 3). Based on this result, it is supposed that pDC100 forms multimer in TN1126, $recA^+$ strain. Therefore, hybrid plasmid pKUH3 in TN1126 may exist also as a multimer.

Construction of recombinant plasmids containing pUC19 and pKU10 DNA

In order to determine the regions of pKU10 involved in resistance to tetracyclin and chloramphenicol, we have cloned various regions of pKU10 in the plasmid pUC19 (Yanisch-Perron *et*

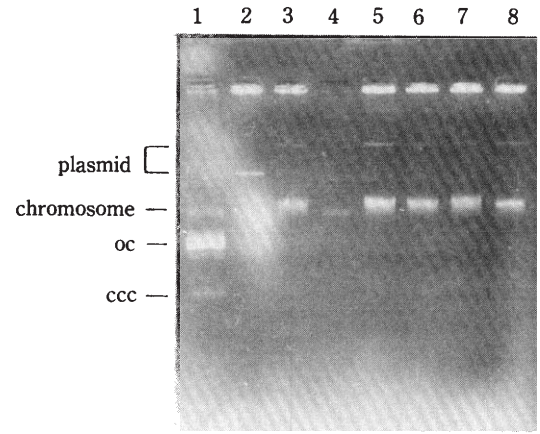

Fig. 2. *Agarose gel electrophoresis of DNA from various P. putida strains;*
1. *P. putida* KU816(pKU10)
2. *P. putida* KU220(RP4; 56Kb)
3. *P. putida* TN1126(transformant)
4. *P. putida* TN1126(recipient)
5-8. *P. putida* TN1126(transformant)

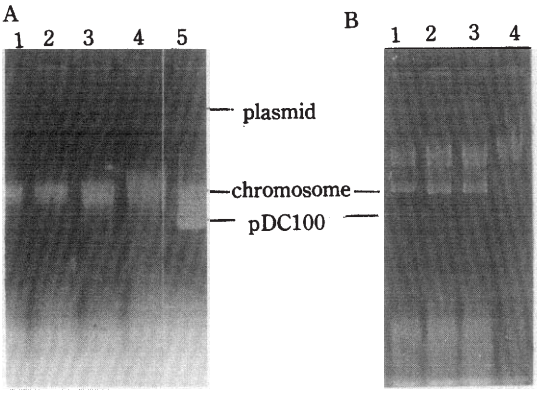

Fig. 3. *Agarose gel electrophoresis of DNA from;*
A) 1. *P. putida* TN1126(recipient)
2 -4. *P. putida* TN1126(transformants)
5. *E. coli*(donor: pDC100)
B) 1. *E. coli*(donor: pDC100)
2 -3. *P. putida* KU218RL(transformants)
4. *P. putida* KU218RL(recipient)

al., 1985). The DNA of pKU10 and of a vector plasmid pUC19 were cleaved by the appropriate restriction enzymes, and ligated. The ligated DNAs were used to transform *E. coli* DH1 cells.

The hybrid plasmid carrying the *Eco*RI-*Hind*III fragment with coordinates 0 to 2.85Kb of pKU10 and hybrid plasmid harbouring *Pst*I-*Xho*I fragment 8.7Kb to 1.6Kb of pKU10 were constructed as schemes of Fig. 4 and Fig. 5, respectively. The resulting hybrid plasmids pKUH4(Fig. 4) and pKUH5(Fig. 5) did not confer resistance to tetracyclin and chloramphenicol.

By ligation of *Hind*III fragments of pKU10 to *Hind*III-cleaved pUC19, five recombinant plasmids containing pUC19 and pKU10 DNA were obtained(Fig.6) and characterized with respect to their antibiotic resistance phenotype. The hybrid

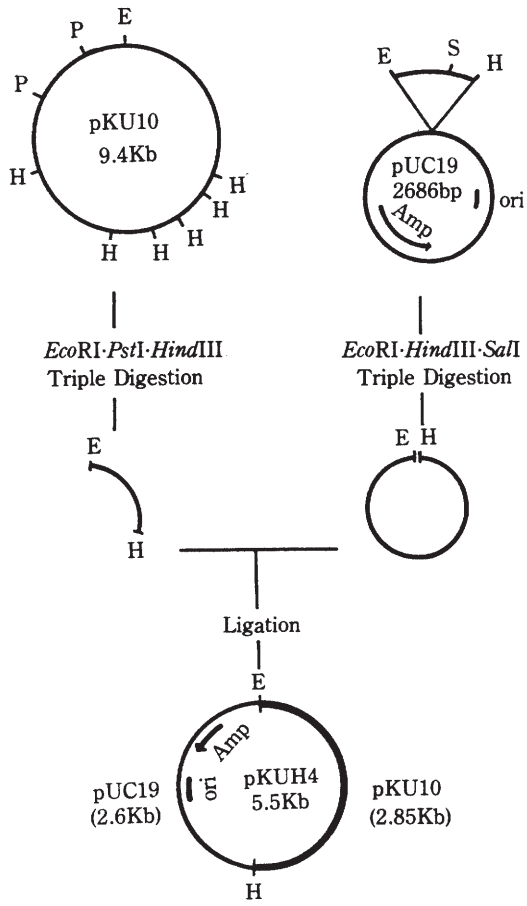

Fig. 4. *Construction of hybrid plasmid pKUH4.*

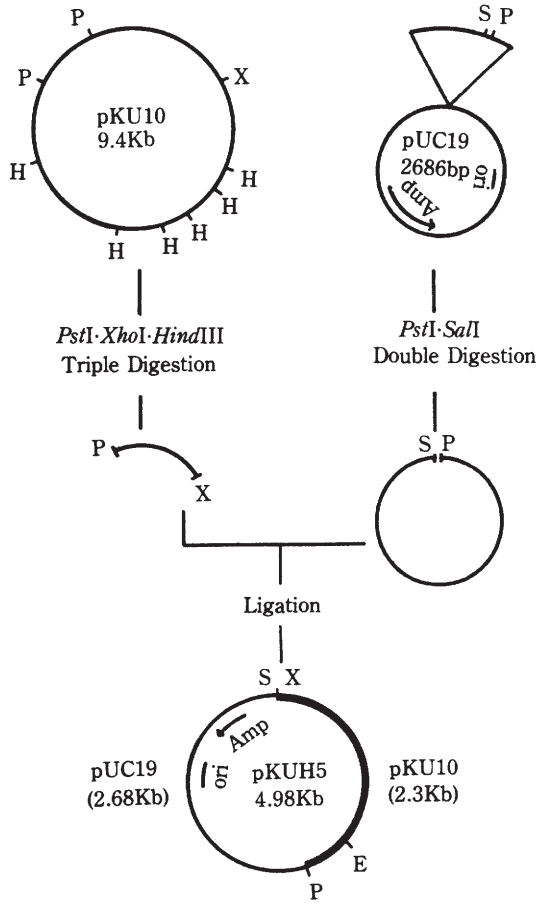

Fig. 5. *Construction of hybrid plasmid pKUH5.*

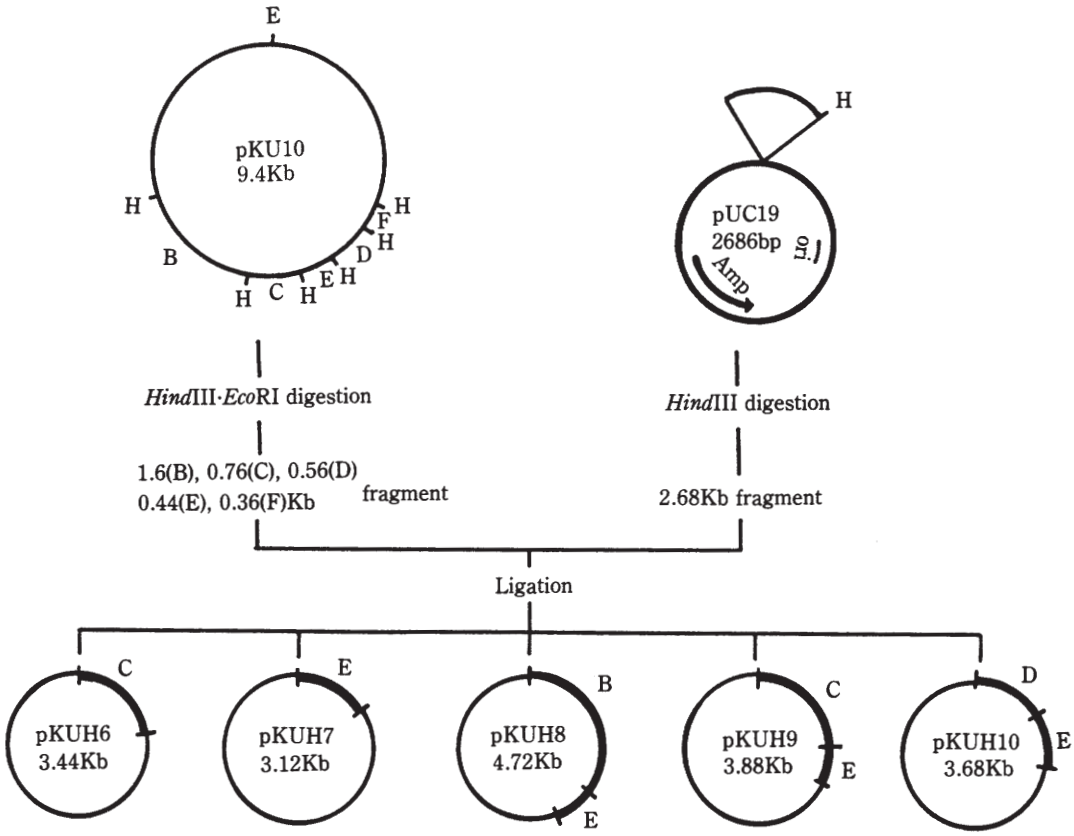

Fig. 6. *Construction of hybrid plasmids pKUH6, pKUH7, pKUH8, pKUH9, and pKUH10.*

plasmid pKUH6 containing only *Hind*III C fragment of pKU10 conferred resistance to Ap, which may originate from pUC19. The hybrid plasmids, pKUH7, pKUH8, and pKUH9 all contained *Hind*III E fragment were resistant to Ap and Tc, whereas pKUH10 carrying *Hind*III E and D fragment was resistant to Ap, Tc, and Cm. The clone containing pKUH10 grew rapidly on the Tc-containing medium, whereas the clones harbouring pKUH7, pKUH8, and pKUH9 grew slowly. The pKU10 *Hind*III D fragment is believed to harbor gene(s) necessary for efficient expression of the structural genes borne on pKU10 *Hind*III E fragment.

Therefore, the *Hind*III D fragment of pKU10 was found to carry gene for resistance to Cm, and the gene for resistance to Tc existed on the region of *Hind*III E and a part of D fragment (Fig. 7).

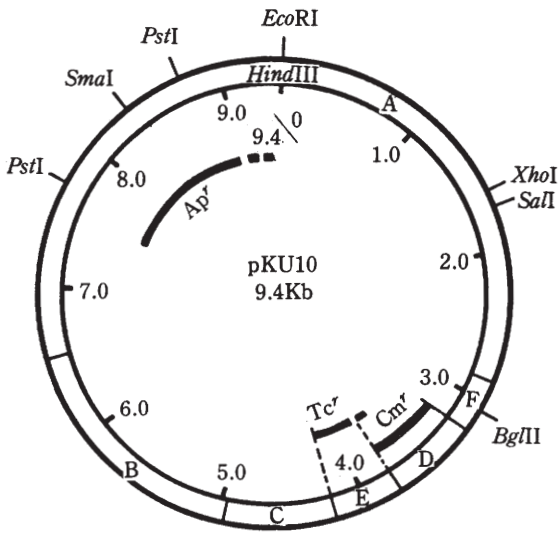

Fig. 7. *Genetic map of pKU10.*

적 요

IncP-1군에 속하는 플라스미드 pKU10의 유전자지도는 pKU10 DNA에 외부 DNA를 삽입시키거나, pKU10의 여러 절편들을 벡터 pUC19에 클로닝시키므로써 작성하였다. 조립된 여러 하이브리드 플라스미드들의 표현형을 분석하여 암피실린, 테트라사이클린, 클로람페니콜등에 대한 내성 유전자의 위치를 밝혀내었다. 암피실린 내성 유전자는 1.0Kb 떨어진 *Pst* I 절단부위의 양쪽에 걸쳐 존재하며, 테트라사이클린 내성 유전자는 *Hind* III E 절편 및 D 절편의 일부에 까지 존재하며, 클로람페니콜 내성 유전자는 *Hind* III D 절편에 존재하는 것을 알 수 있었다.

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