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Characterization of R plasmid pKU41 from *Pseudomonas putida* KU190

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Pseudomonas putida KU 190에서 분리한 R plasmid pKU 41의 특성

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ABSTRACT: The location of R-determinants, Ap^r and Tc^r, and replication origin in pKU41 determined using the construction of miniplasmid by the BamHI and the HindIII restriction fragment from pKU41 and the cloning of the restriction fragments from pKU41 into pSY343. The gene encoding resistance to ampicillin (Ap) as well as replication origin in pKU41 were located on the region overlapping BamHI B fragment and HindIII A fragment. The gene encoding resistance to tetracycline (Tc) was located on the region of the HindIII C fragment, which was cleaved by BamHI as well.
KEY WORDS □ R-determinant replication origin.

Within the *Pseudomonas* species there are many strains of great important for medical, industrial and environmental research. Particulary, the bacterium *pseudomonas putida* has diverse metabolic pathway and novel regulatory mechanism (Stanier *et al.*, 1966). For that reason, detailed information about the genetic organization of the genus *pseudomonas* has been required and it was desirable to find an apporpriate cloning vector in *Pseudomonas* with the recent development of molecular cloning.

For genetic analysis of *Pseudomonas aeruginosa*, Inc P-1 group R-plasmids have interest as cloning vector carring of chromosomal gene because of their extraordinary broad host range among gram-negative organisms. (Chakrabarty, 1975; Jacob and Grinter, 1975; Holloway, 1978; Meyer *et al.*, 1977; Meyer, 1979). But the size of such R-prime plasmid (<40 megadalton) make their *in vitro* manipulation difficult. pBR 322, the well-characterized vector, is also used to clone *P. aeruginosa* genes into *Escherichia coli*. However, some genes of *P. aeruginosa* may not be expressed in an *E. coli* background.

As an alternative proposal, Bagdasarian *et al.* (1979) described the construction of serveral hybrid plasmids derived from plasmid RSF1010 (Nagahari and Sakaguchi, 1978) which can be used as cloning vector in *Pseudomonas spp.* They also described the construction of a potentially more versatile vectors composed of plasmids pBR322 and RSF1010 but were unable to introduce it into *Pseudomonas* (Bagdasarian *et al.*, 1981). Recently, hybrid plasmid derived from the exiting vector plasmids has been constructed to overcome this difficult with the development of recombinant DNA techniques (Wood *et al.*, 1981). Particulary, host vector system for self cloning in

Pseudomonas has been developed, since naturally occurring plasmids have been able to modified (Olsen *et al.*, 1982; Meyer and Shapiro, 1980).

Plasmid pKU41 is an R plasmid originating from *P. putida* KU190 and can be classified as a plasmid belonging to Inc P-1 group (Joo and Lee, 1987). And the restriction map of this plasmid was constructed previously and it has been found that the restriction endonuclease BamHI and Hind III each cleave pKU41 at only three sites (Kim *et al.*, 1986). In this paper, we describe the location of R-determinants and replication origin in pKU41 and this study have been performed in order to develope as the vector for cloning of various useful genes in *Pseudomonas putida.*

MATERIALS AND METHODS

Bacterial strains and plasmids

These are shown in table 1. *P. putida* TN 1307 (rec^-) was used as recipient for transformation.

Media, antibiotics and growth condition

For all experiments, L-broth was used for liquid culture and with 1.5% agar for solid medium. Antibiotics were used at a concentration of 600 μg/m*l* ampicillin (Ap), 25 μg/m*l* tetracyclin (Tc) 100 μg/m*l* streptomycin (Sm) and 50 μg/m*l* kanamycin (Km). Incubation temperature for *P. putida* was 30°C.

Purification of plasmid DNA, transformation and agarose gel electrophoresis

The procedure used for these was done as previously described (Joo and Lee, 1987).

Restriction endonuclease digestion and ligation

These were performed with modification as described by Maniatis *et al* (1982). Purified DNA was digested with restriction endonuclease in the buffer containing 10 mM Tris-HCl (pH 7.5)-50 mM NaCl-10 mM $MgCl_2$-1 mM dithiothreitol. Incubation was at 37°C for at least 1h. After restriction enzyme digestion, restriction fragment analysis was done horizontal agarose gel (w/v, 0.6%) electrophoresis. Ligation was carried out by using the T_4 ligase at 40°C for 20 h in a ligation buffer containing 66 mM Tris-HCl-50 mM $MgCl_2$, 50 mM dithiothreitol, and 10 mM ATP. The ligation mixture was used directly to transform competent cell of *P. putida* TN 1307.

Table 1. *Bacterial strains and plasmids used.*

Bacterial strains and plasmids	Relevant characteristics(size)	References or sources
Bacterial strains		
Pseudomonas putida		
KU190	Sal^+ Cat^+	Kim and Lee (1984)
TN1307	try^- leu^- benl Sm^r rec	Nakazawa(1983)
Plasmids		
pKU41	Tra^+ Ap^r Tc^r (41kb)	Kim *et al.*(1986)
pSY343	Tra^- Km^r(9.5kb)	Yasuda *et al.* (1983)
pMB2	Ap^r(17.5kb)	This work
pMB12	Ap^r(35.6kb)	This work
pMB23	Ap^r(22.9kb)	This work
pMH1	Ap^r(19.1kb)	This work
pMH12	Ap^r(34.1kb)	This work
pMH31	Ap^r Tc^r(26.0kb)	This work
pVB2	Ap^r Km^r(27.0kb)	This work
pVH1	Ap^r Km^r(28.6kb)	This work
pVH3	Tc^r Km^r(16.4kb)	This work

Sal^+; salicylate utilization Cat^+; catechol utilization rec; recombinant defective strain Tra; conjugal transfer

Recovery of DNA segments from agarose gels

Specific restriction fragments were recovered after electrophoretic saparation by the following procedure (Chen and Thomas, 1980). The position of the required fragment was identified by visualization on a transilluminator and a small block of agarose containing DNA band was cut out. The gel slice was weighed and mixed with 3 vol. of 8 M $NaClO_4$ and dissolved in a few minutes at room temperature. A 6 mm GF/C disk was placed on sheets of GF/A paper which serve as a blotter and washed with 100 μ*l* 6 M $NaClO_4$ in 10 mM Tris (pH 7.5) and 1 mM EDTA. The sample was delivered from a plastic micropipet tip to the center of the GF/C disk and allowed to pass through to the GF/A sheet. The DNA was retained on the GF/C

disk while the dissolved agarose was washed through with buffered 6 M $NaClO_4$. The residual $NaClO_4$ was removed by 95% ethanol. The GF/C disk was allowed to air dry for 3 min., then transfered to a 0.5 m*l* Eppendorf centrifuge tube and bathed in 1 mM Tris (pH 7.5), 0.1 mM EDTA. The tube was capped and incubated at 37°C for 30 min. A small hole was then pierced in the bottom from the outside. This tube was then nested in a 1.5 m*l* Eppendorf tube and centrifuged for 10 S to transfer the extract from the inner to the outer tube.

Construction of miniplasmid

Based on the restriction map of pKU41, a series of miniplasmids of pKU41 were constructed by digesting pKU41 DNA with restriction enzyme and ligating fragment of the digested DNA. The constructed miniplasmid was transformed into *P. putida* TN1307 (rec^-).

Cloning procedure

Cloning procedure was as described by Timmis and Frey (1984). The vector DNA (pSY343) was digested with the selected restriction enzyme. The digested DNA was extracted with phenol and ether, and precipitated with isopropanol, then suspended in LBR (10 mM Tris HCl-10 mM $MgCl_2$, pH 7.4). The suspension and restriction fragments of pKU41 to be cloned were mixed and ligated in ligation buffer with T_4 ligase. The reaction was carried out at 14°C for overnight, after which the mixture was used for transformation. Each of the antibiotic resistances conferred by pKU41 and the vector-associated antibiotic resistance maker (Km) were used for selecting transformants.

RESULTS AND DISCUSSION

Location of the Ap^r and Tc^r determinants on the restriction map of pKU41

To confirm location of R-determinants, Ap^r and Tc^r, of pKU41, we carried out construction of miniplasmid by self-ligating the BamHI and Hind III restriction fragments from pKU41 (Fig. 1,2) and cloning the restriction fragments into pSY343 (Fig. 3,4).

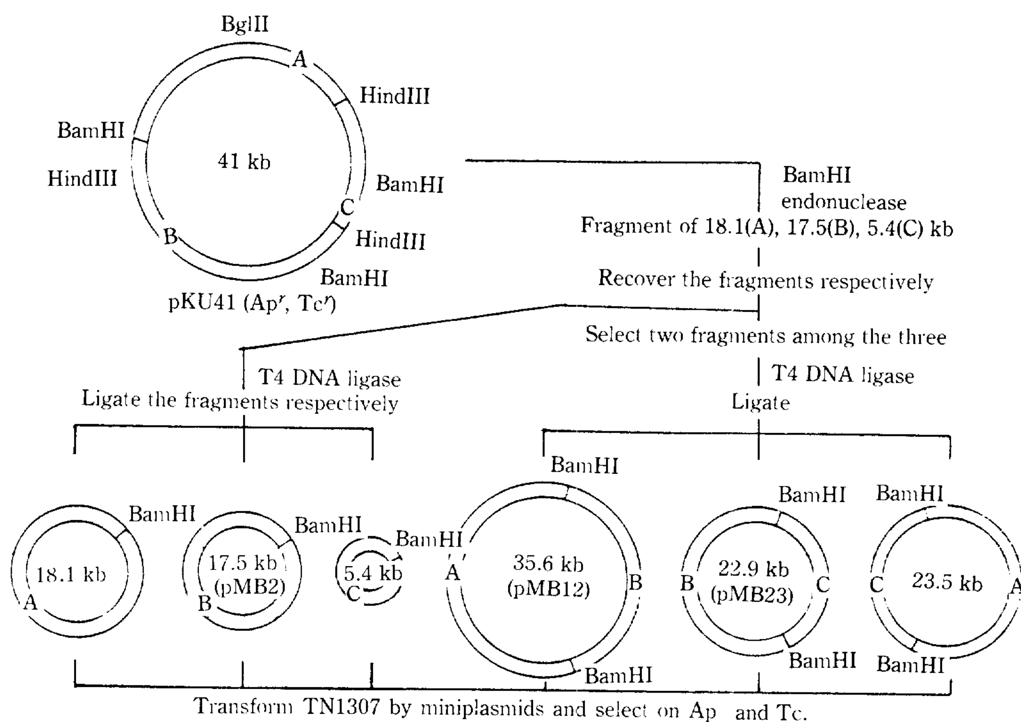

Fig. 1. *Construction of miniplasmids from the BamHI restriction fragments of pKU41.*

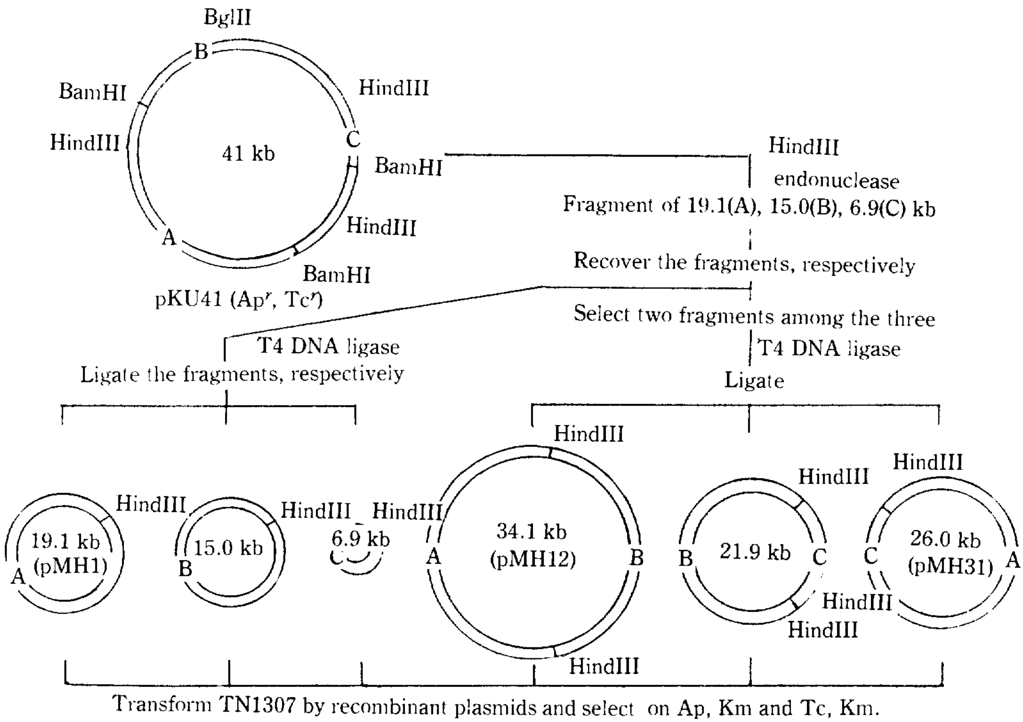

Fig. 2. *Construction of miniplasmids from the HindIII restriction fragments of pKU41.*

Ori
9.5 kb HindIII
Km
BamHI
pSY343 (Km^r)
BamHI
endonuclease
1 Fragment
BglII
A
BamHI
HindIII
41 kb
HindIII
BamHI
C
HindIII
B
BamHI
pKU41 (Ap^r, Tc^r)
BamHI
endonuclease
Fragment of 18.1(A), 17.5(B), 5.4(C) kb
Recover the fragments, respectively
T4 DNA ligase
Ligate
27.6 kb
27.0 kb (pVB2)
14.9 kb
Transform TN1307 by miniplasmids
and select on Ap and Tc.

Fig. 3. *Construction of recombinant plasmids from pSY343 and the BamHI restriction fragments of pKU41.*

Ori
9.5 kb HindIII
Km
BamHI
pSY343 (Km^r)
HindIII
endonuclease
1 Fragment
BglII
B
BamHI
HindIII
41 kb
HindIII
C
BamHI
HindIII
A
BamHI
pKU41 (Ap^r, Tc^r)
HindIII
endonuclease
Fragment of 19.1(A), 15.0(B), 6.9(C) kb
Recover the fragments, respectively
T4 DNA ligase
Ligate
28.6 kb (pVH1)
24.5 kb
16.4 kb (pVH3)
Transform TN1307 by recombinant plasmids
and select on Ap, Km and Tc, Km.

Fig. 4. *Construction of recombinant plasmids from pSY343 and the HindIII restriction fragments of pKU41.*

As the results on transformation with the constructed miniplasmid, Ap^r transformants were obtained when transformation was carried out with miniplasmid included the BamHI B fragment or the Hind III A fragment (Table 2,3) and Tc^r transformants were obtained when transformation was carried out with miniplasmid composed of the Hind III C and Hind III A fragment (Table 2,3). As the reqults on the cloning experiment, Ap^r transformants were obtained when the BamHI B fragment or the Hind III A fragment was introduced into the restriction site of pSY343 (Table, 4,5) and Tc^r transformants were obtained when the Hind III C fragment was introduced. These results indicate that Ap^r determinant is located on the BamHI B fragment or the Hind III A fragment and Tc^r determinant is located on the Hind III C fragment. And in above results, no Tc^r transformants were obtained when the fragments digested with BamHI were cloned. It indicate that the regions of gene encoding Tc resistance has a site of cleavage by BamHI. According to evidence reported by Jacob *et al.* (1976), cloning vector has a restriction site for insertional inactivation when foreign DNA was cloned into the vector. Therefore, the region of gene encoding Tc resistance in pKU41 is cleaved by Bam HI and insertion of foreign DNA into the cleaved site can be monitored by inactivation of Tc resistance. Accordingly, this site may be regarded as proper site for insertional

Table 2. *Transformation efficiency by the self-ligated BamHI restriction fragments of pKU41.*

DNA fragment	Size of fragment(kb)	Selective marker	Transformation efficiency[a]
A	18.1	Ap	N.D
		Tc	N.D
B	17.5	Ap	1.2×10^{-5}
		Tc	N.D
C	5.4	Ap	N.D
		Tc	N.D
A + B[b]	35.6	Ap	1.0×10^{-5}
		Tc	N.D
B + C[b]	22.9	Ap	1.0×10^{-6}
		Tc	N.D
C + A[b]	23.5	Ap	N.D
		Tc	N.D

a; Number of transformant/Number of recipient cell.
b; Two BamHI fragments ligated with T4 DNA ligase.
N.D; Not detected.

Table 3. *Transformation efficiency by the self-ligated HindIII restriction fragment of pKU41.*

DNA fragment	Size of fragment(kb)	Selective marker	Transformation efficiency[a]
A	19.1	Ap	2.4×10^{-4}
		Tc	N.D
B	15.0	Ap	N.D
		Tc	N.D
C	6.9	Ap	N.D
		Tc	N.D
A + B[b]	34.1	Ap	2.0×10^{-6}
		Tc	N.D
B + C[b]	21.9	Ap	N.D
		Tc	N.D
C + A[b]	26.0	Ap	2.0×10^{-6}
		Tc	2.0×10^{-4}

a; Number of transformant/Number of recipient cell.
b; Two HindIII fragments ligated with T4 DNA ligase.
N.D; Not detected.

Table 4. *Transformation efficiency by recombinant plasmid inserted the BamHI restriction fragment of pKU41.*

Vector	Cloned fragment (size kb)	recombinant plasmid size (kb)	Selective markers	Transformation efficiency*
pSY343	A (18.1)	27.6	Ap, Km	N.D
			Tc, Km	N.D
pSY343	B (17.5)	27.0	Ap, Km	4.0×10^{-3}
			Tc, Km	N.D
pSY343	C (5.4)	14.9	Ap, Km	N.D
			Tc, Km	N.D

*; Number of transformant/Number of recipient cell.
N.D; Not detected.

Table 5. *Transformation efficiency by recombinant plasmid inserted the HindIII restriction fragments of pKU41.*

Vector	Cloned fragment (size kb)	recombinant plasmid size (kb)	Selective markers	Transformation efficiency*
pSY343	A (18.1)	28.6	Ap, Km	5.0×10^{-4}
			Tc, Km	N.D
pSY343	B (15.0)	24.5	Ap, Km	N.D
			Tc, Km	N.D
pSY343	C (6.9)	16.4	Ap, Km	N.D
			Tc, Km	2.1×10^{-4}

*; Number of transformant/Number of recipient cell.
N.D; Not detected.

inactivation.

Replication origin (OriV)

To determine the region required for replication functions, we constructed *in vitro* miniplasmid by self-ligating the restriction fragments from pKU41. In the results on transformation with the constructed miniplasmid, transformants were able to obtain when transformation by only the BamHI B fragment or the Hind III A fragment was carried out (Table 2,3) and Tc^r transformants were able to obtain only when the Hind C fragment was linked the Hind III A fragment (Table 3). According to report described by Tait *et al.* (1982), a self-replicating fragment has the region essential for replication involved the replication origin (OriV). Therefore, that the BamHI B fragment or the Hind III A fragment appears origin-containing fragment and the fragments has the region encoded information required for replication of the plasmid.

From all the results described above, we could prepared a partial genetic map of plasmid pKU41 on the restriction map of the plasmid (Fig. 5). That is, Ap^r determinant as well as replication origin (OriV) were located within the 15 kb region overlapping the BamHI B fragment and the Hind III A fragment. Tc^r determinant was located in the 6.9 kb region of Hind III C fragment, which was cleaved by BamHI as well.

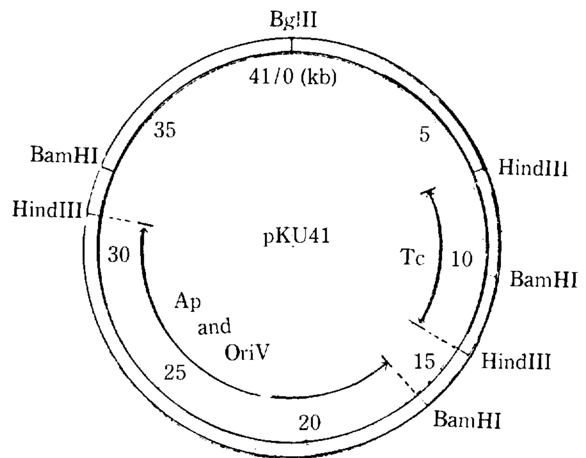

Fig. 5. *Genetic map of pKU41.*

This paper describe the location of its R-determinants and replication origin to develop pKU41, as an ideal vector for self-cloning in *Pseudomonas putida.* Therefore, mini-replicator plasmid derived from pKU41 may be as more functional vectors for gene cloning of *Pseudomonas* by detailed studies on the genetic organization in the future.

적 요

플라스미드 pKU41의 앰피실린과 테트라싸이클린 항생제내성 유전자와 복제개시점 위치를, pKU41의 BamHI과 HindIII 제한 절편에 의한 miniplasmid 제조와 함께 그 제한 효소절편을 pSY343에 클로닝 함으로써 결정하였다. pKU41의 복제개시점과 앰피실린에 대한 내성유전자는 BamHI B 절편과 HindIII a 절편이 겹쳐지는 부위에 위치하였고, 테트라싸이클린에 대한 내성 유전자는 HindIII c 절편상에 위치하였으며, 이는 또한 BamHI에 의해 절단되는 부위이기도 하였다.

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