KOR. JOUR. MICROBIOL. June, 1987 p.87-93 Vol. 25, No. 2

Properties of an 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 properties of the plasmid pKU41 isolated from *Pseudomonas putida* KU190 have been investigated. pKU41 was defined as an R plasmid having a transmissible ampicillin and tetracycline resistance determinant, and could be classified as a plasmid belonging to IncP-1 group according to incompatibility grouping.
KEY WORDS □ R plasmid, *Pseudomonas putida*

Bacteria belonging to the genus *Pseudomonas* are able to metabolize an enormous range of natural and synthetic organic compounds, and also important agent of degradation of toxic compound that pollute the environment. The unique metabolic pathways starting from peculiar compounds in these *Pseudomonas* have required extensive genetic analysis of these bacteria. It will provides the basis for their future use as biocatalysts in chemical industries.

Studies of R factor in *Pseudomonas* has been reported in *Pseudomonas aeruginosa* as common hospital pathogen. The R factor originated from *P. aeruginosa* specified multiple drug resistance and was transferable to a variety of species (Datta *et al.*, 1971; Grinsted *et al.* 1972; Saunders and Grinsted, 1972; Olsen and Shipley, 1973). Chakrabarty *et al.* (1975) reported the transformation of *Pseudomonas putida* with an R factor DNA (RP1 plasmid). After this, a number of investigators have also reported the transformation of cell of *Pseudomonas* (Nagahari and Sakaguchi, 1978; Mercer and Loutit, 1979) and genetic analysis of chromosomal DNA with Inc P-1 group plasmid (Hedges and Jacob, 1977; Holloway, 1978).

Gene cloning have been taken to develop cloning vectors from some of these R factors. However, most of the plasmid found in *Pseudomonas* species have high molecular weight and it makes their in vitro manipulation difficult. Plasmid pBR322 (Boliver *et al.*, 1977), one of the most widely used plasmid vector, cannot replicate in soil bacteria (Bagdasarian *et al.*, 1979) and although it was used to clone *P. aeruginosa*, some gene of *P. aeruginosa* may not be expressed in *E. coli* background (Holloway *et al.*, 1979). Therefore an appropriate cloning vector in *Pseudomonas* required. Bagdasarian *et al.* (1979) and Wood *et al.* (1981) have reported the construction of a cloning vector for *P. aeruginosa* to overcome this difficulty but they were unable to introduce it into *Pseudomonas* species. Another plasmid vector also has been developed by Ditta *et al.* (1980), which was derived from the broad-host-range plasmid RK2. Recently, host-vector systems consisting of bacterium and its vector plasmid have proven of

immense value for the cloning, amplification and analysis of DNA fragment from a wide variety of organisms. Bagdasarian *et al.* (1981) and Olsen *et al.* (1982) reported the development of host-vector system for gene cloning in *Pseudomonas.*

This paper describes some of properties of the plasmid pKU41 isolated from *P. putida* KU190. And it is one of the basic studies involved in development of a cloning vector for self-cloning of *Pseudomonas putida.*

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in the study are shown in table 1. *P. putida* KU190 is a strain isolated in our laboratory (Kim and Lee, 1984) and pKU41 is a cryptic plasmid isolated from *P. putida* KU190 (Kim *et al.*, 1986).

Media and growth condition

LB medium was used for all tests and growth of bacterial cells and M9 minimal medium (Maniatis *et al.*, 1982) was used for incompatibility test. *P. putida* and *E. coli* were grown with shaking at 30°C and 37°C, respectively.

Determination of antibiotic resistance.

Antibiotic resistance was determinated by the procedure of Norström *et al.* (1968). The cells were grown exponentially in L-broth. The culture was diluted and spread on L-agar plates containing different concentrations of the antibiotic to be tested. Antibiotic resistance is defined as the highest concentration of an antibiotic at which 100% of the cells plated form colonies.

Curing

The method of Rheinwald *et al.* (1973) was used as follows. Cells were inoculated into 1m*l* of L-broth containing mitomycin C in 5μg increment from 5 to 35μg/m*l* and incubated with shaking for 36h. Then, 0.1m*l* of the culture was spread on L-agar and replicated to the appropriate test plates for scoring.

Conjugation

Transfer studies was carried out by broth mating. Overnight cultures in L-broth were diluted and mixed at a donor recipient ratio of 1:10. After incubation for 60 min without shaking, the mixtures were plated directly on L-agar supplemented with the appropricate antibiotics.

Stability test

The method of sakaguchi (1981) was used. After growth in antibiotic-free medium the cells were diluted and plated on L-agar plates containing the appropriate antibiotics. Single colonies from individual plates were spotted on individual antibiotic-containing plates to score for the resistance pattern of individual colonies.

Incompatibility test

To determine the incompatibility group, The test was processed by conjugal mating with

Table 1. *Bacterial strains and plasmids used.*

Designation	Relevant properties (size)	References or Sources
Bacterial strains		
P. putida KU 190	Sal$^+$Cat$^+$	Kim and Lee (1984)
P. putida TN 1032	try$^-$leu$^-$benl$^-$Sm^{r}rec	Nakazawa (1983)
P. putida TN 1307	try$^-$leu$^-$benl$^-$Sm^{r}rec	Nakazawa (1983)
E. coli HB 101	r$^-$m$^-$F$^-$pro$^-$leu$^-$ara$^-$gal$^-$rec	Boyer and Roulland Dussoix (1969)
Plasmids (Inc group)		
RP4 (P-1)	Tra$^+$Ap^{r}Km^{r}Te^r (56kb)	Meyers *et al.* (1976)
RK2 (P-1)	Tra$^+$Ap^{r}Km^{r}Te^r (56kb)	Jobanputra and Datta (1974)
pMG1 (P-2)	Tra$^+$Gm^{r}Sm^{r}Su^{r}Hg^r (470kb)	Hansen and Olsen (1978)
Rms 148 (P-7)	Tra$^+$Sm^r (145kb)	Sagai *et al.* (1976)
pKU41	(41.0kb)	Kim *et al.* (1986)

Sal$^+$; salicylate utilization Cat$^+$; catechol utilization Tra; conjugal transfer

bacteria containing a plasmid of different Inc-group. A broth culture of KU190 was mixed with each of a series of broth cultures carrying known plasmids of different Inc-group. After incubation during the working day, each mixture were plated on L-agar supplemented appropriate drug to select for plasmid transfer.

Isolation of plasmid DNA

For detection of plasmid, the method of Kado and Liu (1981) was used. For obtaining large quantities of plasmids, the method of Tanaka and Weisblum (1975) was used. Cells were grown overnight in 25m*l* of L-broth. The culture was inoculated in 1*l* of L-broth and permitted to grown for 8h with shaking. The cells were harvested by low-speed centrifugation and dissolved in 10m*l* of 50mM Tris-25% (w/v) sucrose (pH 8.0), and 5m*l* of lysozyme solution was added. After the mixture was kept on ice for 5min, 4m*l* of 0.25M EDTA (Na salt, pH 8.0) was added. Also after 5 min, 5m*l* of 5M NaCl and 2m*l* of 10% SDS were added and mixed. The mixture was incubated on ice for 24h, and the lysate was centrifuged. The clear supernatant was carefully decanted. To the supernatant 1/2 vol. of a polyethylene glycol solution (40% (w/v) PEG 6000) was added and mixed by inversion. The mixture was kept at 4°C overnight. The DNA precipitate was pelleted by centrifugation and redissolved in TE buffer (0.05M Tris-0.02M EDTA, pH 8.0). Then 8.0g of CsCl and 40m*l* of an etidium bromide solution (1% (w.v)) were added. The density of the solution was adjusted to 1.60 g/m*l* by addition of a small amount TE buffer. Ultracentrifugation was carried out in an RP 65T rotor at 38,000rpm and for 48h, Under ultraviolet illumination, plasmid DNA was collected, and was extracted three times with isopropyl solution saturated NaCl. The plasmid DNA solution was dialized against three changes of TE buffer.

Transformation

The procedure described by Nagkazawa (1983) was used for tromformation. Cells were grown overnight in L-broth with shaking. A portion (1.5m*l*) of such a culture was inoculated in 20m*l* of 0.5M sucrose in L-broth and grown for 3-4h. The cells were harvested by centrifugation and washed with 10m*l* of 10mM NaCl in 0.5M sucrose solution. The cells were again contrifugated, and resuspended in 10m*l* of 50mM $CaCl_2$ in 0.5M sucrose solution. The cells were kept ice for 20min, harvested by centrifugation and resuspended in 2m*l* of 50mM $CaCl_2$. 0.2m*l* of these cells was then mixed with 0.1m*l* of DNA solution and incubated at 0°C for 60min. The cell DNA mixture was then subjected to a heat pulse at 42°C for 2min, chilled, and finally diluted in 10 volume of fresh L-broth. The cells were allowed to grown at 30°C on a shaker and aliquots were plated on L-agar plates containing the appropriate antibiotics.

Electrophoresis

Electrophoresis of DNA was carried out in agarose gel as described by Casse *et al.* (1979). Sample were run in 0.8% agarose slab gels in a horizontal electrophoresis apparatus filled with electrophoresis buffer (40mM Tris-acetate, 20mM sodium acetate and 1mM Na_2-EDTA). Samples of DNA were mixed with 0.015% bromophenol blue in 60% sucrose, 60mM EDTA. Electophoresis was performed at 120V for 3-4h.

RESULTS AND DISCUSSION

Antibiotic resistance of *P. putida* KU190

Antibiotics used were ampicillin, carbenicillin, chloramphenicol, gentamycin, kanamycin, rifampicin and streptomycin. *P. putida* KU190 was resistant to ampicillin, carbenicillin, chloramphenicol and tetracyclin whereas sensitive to gentamycin, kanamycin, rifampicin and streptomycin (Table 2).

Curing of pKU41

Curing is one of the main criteria for determining character encoded by plasmid. The strain treated with mitomycin C (25ug/m*l*) lost resistant to ampicillin, carbenicillin and tetracyclin (Table 3). Accordingly, resistance to these antibiotics is specified by pKU41, and pKU41 was defined as an R plasmic having the resistance-determining gene.

Transfer ability of pKU41

Conjugal mating was carried out in order to

Table 2. *Resistance of P. putida KU 190 to various antibiotics*

Antibiotics	Concentration of antibiotics (μg/ml)						
	25	50	100	200	400	800	1600
Ap	+	+	+	+	+	+	-
Cb	+	+	+	+	+	+	+
Cm	+	+	+	+	+	+	+
Gm	-	-	-	-	-	-	-
Km	-	-	-	-	-	-	-
Rif	+	-	-	-	-	-	-
Sm	+	-	-	-	-	-	-
Tc	+	+	-	-	-	-	-

Ap, ampicillin; Cb, carbenicillin; Cm, chloramphenicol Gm, gentamycin; Km, kanamycin; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline; +, growth; -, no growth

Table 3. *Effect of mitomycin C (25 μg/ml) on curing*

Selective marker	Concentration of antibiotics (μg/ml)	Frequency of curing (%)[a]
Ap	800	21.4
Cb	1600	29.4
Tc	50	16.0

a; No. of cured colonies/total No. of examined colonies

study the transfer ability of the plasmid pKU41. The transconjugants was obtained from *P. putida* TN1307, TN1032 and *E. coli* HB101 at the frequency between 10^{-6}-10^{-5} (Table 4). Therefore pKU41 can be transferred to *E. coli* as well as strains of *P. putida* (Fig. 1). And it is one of properties of IncP-1 group plasmid.

Table 4. *Transfer frequency of pKU41 by mating.*

Donor	Recipient	Selective marker (conc. of marker)	Transfer frequency[a]
KU 190 (pKU41)	*P. putida* TN 1307	Ap (800 μg/ml) Tc (25 μg/ml) Sm (100 μg/ml)	2.5×10^{-5}
	P. putida TN 1032	Ap (800 μg/ml) Tc (25 μg/ml) Sm (100 μg/ml)	1.1×10^{-5}
	E. coli HB 101	Ap (800 μg/ml) Tc (25 μg/ml) Sm (100 μg/ml)	1.0×10^{-6}

a; No. of trasconjugants/No. of donor cells

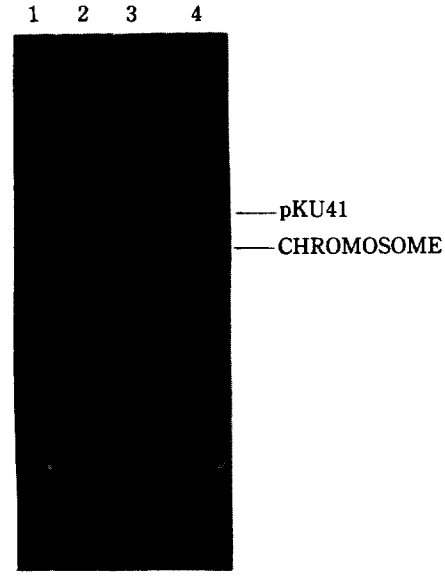

Fig. 1. *Agarose gel electrophoresis of transconjugants.*
lane 1; wild type, KU190 (pKU41)
lane 2; *P. putida* TN1307 (pKU41)
lane 3; *P. putida* TN1032 (pKU41)
lane 4; *E. coli* HB101 (pKU41)

Stability of pKU41

Studies of stability of pKU41 was carried out using antibiotic resistance of transconjugant acquired the plasmid. pKU41 was very stable in both *P. putida* TN1307 (98.0%) and *E. coli* HB101 (97.5%) as in the case of wild strain (99.7%) (Table 5).

Table 5. *Stability of pKU41 in different strains*

Host strains	Selective marker[a]	Stability(%)[b]
P. putida KU190 (pKU 41)	Ap, Tc	99.7
P. putida TN1307 (pKU 41)	Ap, Tc	98.0
E. coli HB101 (pKU 41)	Ap, Tc	97.5

[a]; The concentration of ampicillin (Ap) and tetracycline (Tc) were 600 μg/ml and 20 μg/ml
[b]; Stability is indicated as the percentage of cells resistant to ampicillin and tetracycline after one generation in antibiotic free L-broth.

Table 6. *Incompatibility of pKU41 for different plasmids.*

Donor	Recipient	Plasmid (Inc. group)	Selective marker	Conc. of marker	No. of colonies on master plate
KU190	*E. coli*	RP_4-RK_2	Km	50 μg/ml	N.D
(pKU41)	C 600	(p-1)	Ap	800 μg/ml	
			Tc	25 μg/ml	
	P. aeruginosa	pMG1	Gm	25 μg/ml	$5.2X10^2$
	PAO 303	(p-2)	Ap	800 μg/ml	
			Tc	25 μg/ml	
	P. aeruginosa	Rms 148	Sm	100 μg/ml	5.2X10
	PAO 303	(p-7)	Ap	800 μg/ml	
			Tc	25 μg/ml	

ND; Not detected.

Incompatibility of pKU41

Incompatibility is currently the basis of plasmid classification. Therefore, nearly all plasmids may be assigned to one of a relatively small number of such groups. The incompatibility properties of pKU41 were tested by conjugal mating and the results was scored as the colonies retaining determinants carried by the income and resident plasmid; A high percent figure therefore indicates compatibility, a low figure incompatibility. pKU41 was seem to be compatible with pMG1 as P-2 and Rms 148 as P-7 and incompatible with RP_4 and RK_2 as P-1 (Table 6). This suggests that pKU41 fall therefore into the same incompatibility group as RP_4 and RK_2.

Transformation of *P. putida* and *E. coli* with pKU41

Transformation exhibit a spectrum of host expressing gene of plasmid. Althought the frequency of transformants obtained differs for *P. putida* ($2.8x10^{-5}$) and *E. coli* ($7.8x10^{-7}$), transformant containing pKU41 could be obtained from the two strains (Table 7). And the presence of pKU41 in

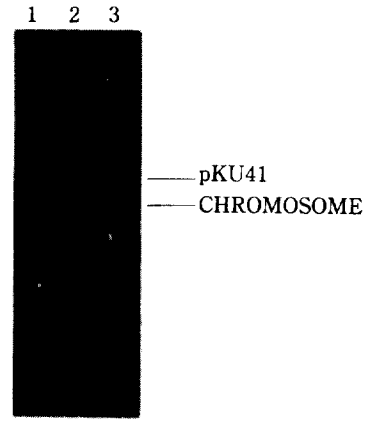

Fig. 2. *Agarose gel electrophoresis of transformants.*
lane 1; wild type. KU190 (pKU41)
transformants
lane 2; *P. putida* TN1307 (pKU41)
lane 3; *E. coli* HB101 (pKU41)

Table 7. *Transformation frequency for antibiotic resistance characters specified by pKU 41.*

Donor plasmid	Recipient	Antibiotic maker	Selective conc.	Transformation frequency
pKU41	*P. putida*	Ap	800 μg/ml	$2.8x10^{-5}$
	TN 1307	Tc	25 μg/ml	
	E. coli	Ap	800 μg/ml	$7.8x10^{-7}$
	HB 101	Tc	25 μg/ml	

Transformation frequency: transformant No./μg DNA/viable cells

both strain led to cellular resistance to ampicillin and tetracyclin (Fig. 2). It suggests that pKU41 can be used to transform both *P. putida* and *E. coli* strain to antibiotic-resistance characters.

As stated in the introduction, R plasmid originated *P. aeruginosa* such RP1 and RP4 are well documented, there is so far no report concerning an R plasmid isolated from *P. putida* as soil saprophytes. However, Grinsted *et al.* (1972) indicated the base composition of an R factor obtained from *P. aeruginosa* (RP1) is more characteristic of terrestrial and aquatic saprophytic *Pseudomonas* than *P aeruginosa.* Thus terrestrial saprophytic *Pseudomonas* may be the natural hosts for R factor.

The occurence of pKU41 as R plasmid in *P. putida* may be extremely useful because pKU41 having a transmissible and ampicillin and teracycline resistance determinant would be of value in an attempt to development for a cloning vector in *Pseudomonas.*

적 요

Pseudomonas putida KU 190에서 분리한 plasmid pKU 41의 특성을 조사하였다. pKU 41은 전달성 및 앰피실린과 테트라싸이클린에 내성을 나타내는 유전자를 지닌 R. plasmid인 것으로 밝혀졌으며, 불화합성에 따르는 분류로는 IncP-1군인 것으로 나타났다.

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(Received Apr. 28, 1987)