Characteristics of the R plasmid pKU10 isolated from *Pseudomonas putida*

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*Pseudomonas putida*에서 분리한 플라스미드 pKU 10의 특성

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ABSTRACT: The characteristics of the plasmid pKU10 isolated from *Pseudomonas putida* KU816 were investigated and its restriction map was constructed. The pKU10 plasmid was a small R plasmid carrying genes for resistance to ampicillin, tetracyclin, and chloramphenicol, and cured by treatment with mitomycin C. The molecular size of pKU10 was estimated to be 9.4Kb. *Pseudomonas* strains and *E. coli* cells could be transformed for antibiotic resistance characters specified by pKU10 plasmid DNA. By incompatibility test with other plasmids, pKU10 is grouped into IncP-1. *Eco*RI, *Xho*I, *Sal*I, *Bgl*II, and *Sma*I cleaved pKU10 once, while *Pst*I cleaved at two sites, and *Hind*III cleaved at six sites. The restriction map was constructed by partial and complete digestion of the purified plasmid DNA with single, double, or triple restriction enzymes. Thus, pKU10 is expected to be used for a cloning vector in *Pseudomonas* cells.

KEY WORDS□R plasmid; a cloning vector in *Pseudomonas*

Soil microbial populations, particularly members of the genus *Pseudomonas,* have a nutritional versatility and are capable of the degradation of a range of complex, synthetic and naturally occuring aromatic and aliphatic compounds. For genetic analysis and manipulation of such bacteria, it is desirable to find out or construct appropriate cloning vectors. However, most of the plasmids found in *Pseudomonas* have masses more than 20Md (Shapiro, 1977). For example RP4 plasmid has a wide host range among gram-negative bacteria, but because of its high molecular weight, it is difficult to handle. Failure to construct a mini RP4 plasmid with various restriction endonucleases suggested a scattering of the genes that are essential to the replication and maintenance of RP4 plasmid (Barth and Grinter, 1977). The most versatile vectors currently available in *E. coli,* such as pBR322, pUC19, and pACYC184, cannot also replicate in soil bacteria because they are narrow host range plasmids (Bagdasarian *et al.,* 1979). Recently RSF1010 (Nagahari and Sakaguchi, 1978) found in *E. coli* and their derivatives (Priefer *et al.,* 1985) are used as cloning vector in *Pseudomonas.* However, they contain few restriction endonucleases cleavage sites. Accordingly, it is necessary to develop intrinsic cloning vectors in *Pseudomonas.* In our laboratory, an R plasmid with a prospect to be a useful cloning vector was isolated from *Pseudomonas putida* (Kim *et al.,* 1987).

In this study, in order to evaluate the potential usefulness of new plasmid pKU10 as a vector in *Pseudomonas* species, characteristics of pKU10 were investigated and physical map using restric-

* This work was supported by a grant from The Korea Science and Engineering Foundation.

tion endonucleases was generated.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this work are described in Table 1. *P. putida* KU 816 having pKU10 is a cured strain of KU806 harbouring both pKU7 and pKU10 as described previously (Kim *et al.*, 1987).

Media and growth conditions

The L broth (Miller, 1972) was used as a complete medium and was solidified with 1.5% agar. Incubations were carried out at 30°C for *P. putida* and at 37°C for *P. aeruginosa* and *E. coli*. Antibiotics were used at the following concentrations unless otherwise indicated: ampicillin, 600μg/ml; tetracyclin, 12.5μg/ml; chloramphenicol, 800 μg/ml.

Response to antibiotics

The antibiotics used were ampicillin, tetracyclin, kanamycin, chloramphenicol, streptomycin, and gentamycin. Strains were grown overnight in L broth to exponential phase. A portion of such a culture was plated on L agar media containing various antibiotics. The cells were grown at 30°C for 48hr.

Curing test

Curing test was carried out according to the procedure of Datta. *et al.* (1979) (except using mitomycin C as a curing agent).

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 36000rpm 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

Table 1. *Used bacterial strains and their characteristics*

Strain/plasmid	Relevant characteristics	Source or Reference
Pseudomonas putida		
KU816 (pKU10)	/pKU10 (Ap^r Tc^r Cm^r)	Kim *et al.* (1987)
TN1307	*trp leu benl str recA*	Nakazawa and Yokota (1977)
TN1126	*met trp*	Nakazawa and Yokota (1977)
TN1126 (RSF1010)	/RSF1010 (Sm^r)	Transformation of TN1126 with RSF1010
TN1126 (pKT230)	/pKT230 (Km^r Sm^r)	Transformation of TN1126 with pKT230
Pseudomonas aeruginosa		
PA0303 (pMGl)	*arg* /pMGl (Sm^r)	Hansen and Olsen (1978)
PA0303 (Rms148)	*arg* /Rms148 (Sm^r)	Jacoby (1977)
PA08 (R18)	*met ilv str* /R18 (Km^r)	Issac and Holloway (1968)
PA02003	*arg str cml recA*	Wood *et al.* (1981)
Escherichia coli		
C600	*r m thr leu thi*	Uozumi, T
K060	wild type (C strain)	Uozumi, T.
HB101	*r m pro leu str recA*	Boyer and Rouland-Dussiox (1969)
C600 (RP4)	/RP4 (Ap^r Tc^r Km^r)	Barth and Grinter (1977)
JM83 (pUC19)	/pUC19 (Ap^r lacZ$^-$)	Transformation of JM83 with pUC19
20SO (pTS1045)	/pTS1045 (Sm^r Su^r Ap^r *xylE*)	Nakazawa (1983)

(1984). Viable cell count assays on cell and DNA mixtures were performed by serially diluting 10-fold in L-broth and plating 100μl on L agar followed by incubation for 48hr.

Determination of plasmid incompatibility

Plasmid incompatibility was determined by transformation with various pairs of plasmids. A strain containing a particular plasmid was transformed with pKU10 DNA, and the cells were plated on medium that selects for the incoming and resident plasmid. By this test, plasmids are judged incompatible if transformed colonies do not occur.

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 (1μg/ml) for 20min, rinsed and photographed under UV illumination.

RESULTS AND DISCUSSION

Examination of antibiotic markers

In order to examine the genetic markers of *P. putida* KU816 harbouring plasmid pKU10, resistance to various antibiotics was examined. KU816 showed resistance to chloramphenicol, ampicillin, and tetracyclin, but showed little resistance to gentamycin, streptomycin and kanamycin.

Curing of antibiotic resistance

To determine whether the genes for antibiotic resistance are present on plasmid or not, curing and transformation experiments were carried out. From the result of curing test by treatment with minimum inhibitory concentration (25μg/ml) of mitomycin C (Table 2), loss of resistance to ampicillin, tetracyclin as well as plasmid pKU10 was observed. Because the *P. putida* strains are inherently resistant to chloramphenicol (Meyer *et al.*, 1982), it was not ascertained by curing test whether the pKU10 encoded the genes for chloramphenicol-resistance or not.

Table 2. *Effect of mitomycin C(25μg/ml) on curing the resistance to Ap, Tc, and Cm.**

Selective marker	Total no. examined	Concentration of antibiotic(μg/ml)	Frequency of curing (%)
Ap	770	800	17.6
Tc	770	25	24.5
Cm	770	1600	0

* Abbr. Ap; ampicillin, Tc; tetracyclin, Cm; chloramphenicol

Transformation of *Pseudomonas* and *E. coli* with pKU10

To determine the genetic markers of pKU10 and to investigate whether the genes specified by pKU10, which was isolated from *P. putida*, were expressed in other *Pseudomonas* species and genus or not, transformation experiment was accomplished. The restriction systems of *P. putida* and *P. aeruginosa* are highly effective barrier to the uptake of heterologous DNA by bacterial cells (Holloway, 1965; Bagdasarian *et al.*, 1979). Therefore mutants defective in restriction (TN1126; Nakazawa, 1983) and recombination (TN1307, PAO 2003) were used as recipients. *E. coli* strains used were also mutants defective in restriction (HB101, C600) and *E. coli* C strain (K060). The ability of

Table 3. *Transformation of Pseudomonas and E. coli strains by pKU10 plasmid DNA*

Recipient	Selection	Efficiency*
P. putida TN1307	Sm, Ap	3.6×10^{-5}
	Sm, Tc	1.0×10^{-6}
TN1126	Ap	2.6×10^{-6}
	Tc	2.4×10^{-6}
P. aeruginosa PA02003	Ap	8.0×10^{-5}
E. coli C600	Ap	2.4×10^{-5}
	Tc	5.0×10^{-6}
	Cm	1.3×10^{-5}
E. coli K060	Ap	1.0×10^{-5}
E. coli HB101	Ap	4.0×10^{-5}
	Tc	5.0×10^{-6}
	Cm	1.6×10^{-5}

* Efficiency; $\frac{\text{Number of transformants}}{\text{Number of recipients}}$

Table 4. *Determination of incompatibility group by transformation*

DNA	Recipient	Plasmid(Inc group)	Selection	Efficiency*
pKU10	*E. coli* C600	RP4 (IncP-1)	Km, Cm	0
	P. aeruginosa PA08	R18 (IncP-1)	Km, Cm	0
	P. aeruginosa PA0303	pMG1 (IncP-2)	Sm, Ap, Tc, Cm	1.8×10^{-6}
	P. putida TN1126	RSF1010 (IncP-4)	Sm, Ap, Tc	6.0×10^{-7}
	P. aeruginosa PA0303	Rms148 (IncP-7)	Sm, Ap, Tc	2.0×10^{-5}

* Efficiency; $\frac{\text{Number of transformants}}{\text{Number of recipients}}$

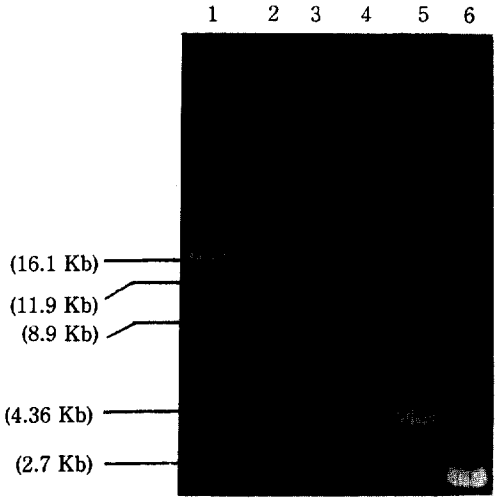
Pseudomonas and *E. coli* cells to be transformed to antibiotic-resistance characters by pKU10 DNA is shown in Table 3.

Pseudomonas and *E. coli* strains were transformed with pKU10 DNA at efficiency of 10^{-6}–10^{-5}. It was observed that *Pseudomonas* cells transformed with pKU10 DNA had resistance to ampicillin and tetracyclin, and transformed *E. coli* cells were resistant to chloramphenicol as well as ampicillin and tetracyclin. Thereby, it is considered that the clarified genetic markers of pKU10 are ampicillin, tetracyclin, and chloramphenicol-resistance. It is also proved that pKU10 can replicate in *P. aeruginosa* and *E. coli* strains as well as in *P. putida*. pKU10 can be mobilized by SAL plasmid pKU7 as a helper plasmid (Kim *et al.*, 1987), whereas pKU10 was not transferred to other strains by conjugation (data not shown).

Examination of incompatibility group

Various kinds of drug resistance plasmids were classified according to their compatibility and their transmissibility to the bacteria of other genera.

Plasmid incompatibility was determined by transformation of bacterial strains harbouring plasmid belonged to several different incompatibility group with pKU10 DNA. The host strains harbouring RP4 and R18 of IncP-1, pMG1 of IncP-2, RSF1010 of IncP-4, and Rms148 of IncP-7 group, respectively, were transformed with pKU10 DNA. Plasmid pKU10 was shown to be incompatible with the IncP-1 plasmid RP4 and R18 (Table 4). Thereby it was confirmed that pKU10 belonged to IncP-1 group.



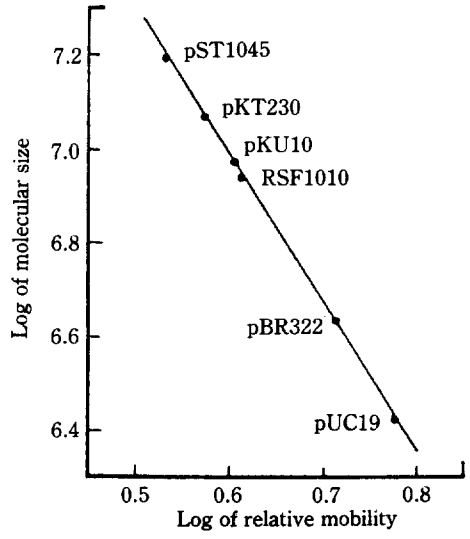


Fig. 1. *Molecular size of pKU10*

1. pTS1045 (16.1Kb) 2. pKT230 (11.9Kb) 3. pKU10 (9.4Kb)
4. RSF1010 (8.9Kb) 5. pBR322 (4.36Kb) 6. pUC19 (2.7Kb)

Determination of molecular size

Fig. 1 shows the standard curve that we constructed from agarose gel electrophoresis data for 5 standard plasmids. pTS1045 (16.1Kb), pKT230 (11.9Kb), RSF1010 (8.9Kb), pBR322 (4.36Kb), and pUC19 (2.7Kb) were used as standard size markers. The molecular size of pKU10 was determined to be 9.4Kb. It is a relatively small plasmid found in genus *Pseudomonas* (Shapiro, 1977).

Restriction map of pKU10

The purified pKU10 DNA was cleaved with several different restriction endonucleases. Out of several restriction enzymes used, *Eco*RI, *Xho*I, *Sal*I, *Bgl*II *Sma*I, *Pst*I, and *Hind*III proved to be the most useful. The restriction patterns on agarose gel are shown in Fig. 2. *Eco*RI, *Xho*I, *Sal*I, *Bgl*II and *Sma*I cleaved pKU10 once, while *Pst*I cleaved at two sites, and *Hind*III cleaved at six

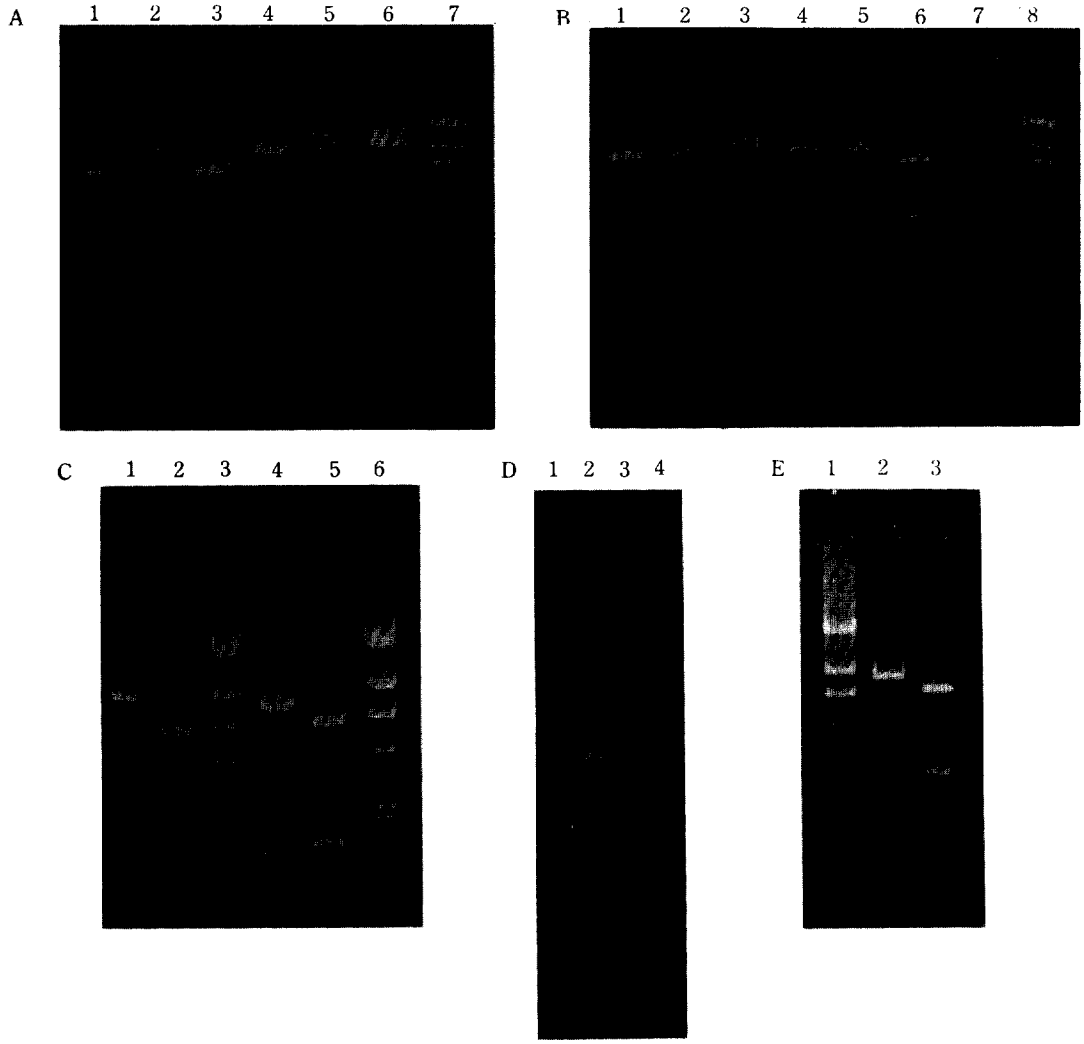


Fig. 2. *Restriction digests of the R plasmid pKU10.*
(A) lane 1, pKU10; lane 2, *Eco*RI; lane 3, *Hind* III; lane 4, *Pst*I; lane 5, *Sal*I; lane 6, *Xho*I; lane 7, λ-*Hind*III, (B) lane 1, *Eco*RI + *Sal*I; lane 2, *Eco* RI + *Xho*I; lane 3, *Sal*I + *Xho*I; lane 4, *Eco* RI + *Sal*I + *Xho*I; lane 5, *Pst*I + *Eco*RI; lane 6, *Pst*I + *Xho*I; lane 7, *Pst*I + *Eco*RI + *Xho*I; lane 8, λ-*Hind*III, (c) lane 1, *Bgl*II; lane 2, *Bgl*II + *Eco* RI; lane 3, λ-*Hind*III; lane 4, *Bgl* II + XhoI; lane 5, *Bgl*II + *Eco*RI + *Xho*I; lane 6, λ-*Hind*III, (D) lane 1, *Hind*III + *Eco*RI; lane 2, *Hind*III; lane 3, *Hind*III + *Pst*I; lane 4, λ-*Hind*III, (E) lane 1, λ-*Hind*III; lane 2, *Sma*I + *Eco*RI; lane 3, *Sma*I + *Xho*I.

Table 5. *Molecular sizes of fragments generated from pKU10 plasmid DNA by various restriction endonucleases used separately or in combinations of two or three.*

Restriction endonuclease	Sizes of the fragments(Kb)*							Total
*Bgl*II	9.4							9.4
*Bgl*II + *Eco*RI	6.3	3.1						9.4
*Bgl*II + *Xho*I	7.9	1.5						9.4
*Bgl*II + *Eco*RI + *Xho*I	6.3	1.6	1.5					9.4
*Eco*RI	9.4							9.4
*Sal*I	9.4							9.4
*Xho*I	9.4							9.4
*Eco*RI + *Sal*I	7.7	1.7						9.4
*Eco*RI + *Xho*I	7.8	1.6						9.4
*Eco*RI + *Sal*I + *Xho*I	7.7	1.6						9.3***
*Hind*III	5.7	1.6	0.74	0.56	0.44	0.36		9.4
*HInd*III + *Eco*RI	2.85(2)**	1.6	0.74	0.56	0.44	0.36		9.4
*Hind*III + *Xho*I	4.45	1.6	1.25	0.74	0.56	0.44	0.36	9.4
*Hind*III + *Pst*I	3.7	1.6	1.0(2)	0.74	0.56	0.44	0.36	9.4
*Hind*III + *Eco*RI + *Xho*I	2.85	1.6(2)	1.25	0.74	0.56	0.44	0.36	9.4
*Pst*I	8.4	1.0						9.4
*Pst*I + *Eco*RI	7.7	1.0	0.7					9.4
*Pst*I + *Xho*I	6.1	2.3	1.0					9.4
*Pst*I + *Eco*RI + *Xho*I	6.1	1.6	1.0	0.7				9.4
*Sma*I	9.4							9.4
*Sma*I + *Eco*RI	8.3	1.1						9.4
*Sma*I + *Xho*I	6.7	2.7						9.4

* The molecular size standards used were *Hind*III fragments of phage λDNA (Daniels *et al.*, 1980)
** The number in parentheses indicate a probable stoichiometry of 2.
*** Fragment smaller than 0.3Kb were not determined.

sites. But the plasmid is not digested with *Bam*HI. The sizes of fragments generated are shown in Table 5. The locations of the cleavage fragments for each of the restriction endonucleases on the circular pKU10 map were then found by carrying out the appropriate series of double or triple digestion, and comparing the fragments with those obtained using each enzyme separately (Fig. 3). The cleavage sites for *Hind*III were particularly constructed by partial digestion.

Usefulness as a cloning vector

pKU10 plasmid has the following merits as a cloning vector; a) small size, b) selection markers for hybrid molecules during transformation, and c) unique cloning sites for many of the commonly used restriction endonucleases. Thus, pKU 10 is expected to be used as a cloning vector in *Pseudomonas* cells.

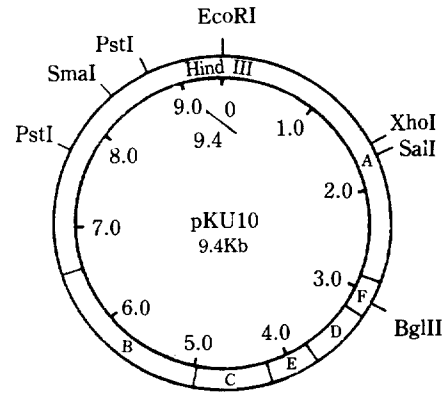


Fig. 3. *Restriction map of pKU10.*
The coordinates are given from *Eco*RI site in clockwise direction: *Eco*RI 0/9.4Kb; *Xho*I 1.6Kb; *Sal*I 1.7Kb; *Bgl*II 3.1Kb; *Sma*I 8.3Kb; *Pst*I 7.7 and 8.7Kb; *Hind*III 2.85, 3.21, 3.77, 4.21, 4.95, and 6.55Kb.

적 요

Pseudomonas putida KU816에서 분리한 플라스미드 pKU10의 여러가지 특성을 조사하고 그 제한효소 지도를 작성하였다. pKU10은 암피실린, 테트라사이클린, 클로람페니콜에 대한 내성 유전자를 갖는 작은 R factor로서 마이토마이신 C에 의하여 큐어링 된다. 플라스미드의 크기는 9.4 Kb로 측정되었다. pKU10은 *Pseudomonas*와 *E. coli*를 숙주로 하였을 때 안정하게 형질 발현이 된다. 또한 pKU10의 불화합성군은 IncP-1으로 조사되었다. *Eco*RI, *Xho*I, *Sal*I, *Bgl*II, *Sma*I은 pKU10 DNA를 한 부위에서 자르고, *Pst*I은 두 부위, *Hind*III는 여섯 부위에서 자른다. 제한 효소 지도는 제한 효소를 이중, 삼중으로 완전 소화시키거나, 부분 소화시켜서 얻었다. pKU10은 *Pseudomonas*속에서 유용한 클로닝 벡터로 이용될 것으로 기대된다.

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(Received Oct. 25, 1987)