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Transfer of RP4::Mu cts and RP4::mini-Mu from *E. coli* to *Pseudomonas* sp.

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RP4 :: Mu cts 및 RP4 :: mini-Mu의 *Pseudomonas* sp.로의 전달

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ABSTRACT: Chromosomal gene transferable hybrid plasmids, RP4::Mu cts and RP4::mini-Mu, were transferred by conjugation from *E. coli* to *Pseudomonas* strains. In order to use for recipient cells of RP4::Mu cts and RP4::mini-Mu, plasmid-free *Pseudomonas* strains were characterized for their antibiotic resistance, aromatic hydrocarbon utility and degradation patterns of chlorinated herbicide. Transfer frequencies of RP4::mini-Mu exhibited about 10^{-2} to 10^{-4}, while those of RP4::Mu cts exhibited very low value of 10^{-7} in recipients tested except *Pseudomonas aeruginosa* KU557. Existance of hybrid plasmids in *Pseudomonas* transconjugants were identified by their antibiotic resistance and agarose gel electrophoresis. In case of RP4::Mu cts transconjugants it was also confirmed by demonstrating that they were capable of releasing phage and forming plaques at 43°C. Plaque forming unit of the transconjugants was about 10^5. It was shown by the stability test that RP4::Mu cts and RP4::mini-Mu in *Pseudomonas* were relatively stable.

KEY WORDS□RP4::Mu cts, RP4::mini-Mu, *Pseudomonas* sp.

Soil and water microbial populations, particularly members of the genus *Pseudomonas,* are capable of degrading various xenobiotics such as aromatic compounds and chlorinated herbicides as a source of carbon and energy or as a form of cometabolism(Gunsalus and Marshall, 1971; Horvath, 1971). Therefore these *Pseudomonas* strains have attracted attention to their potential uses in the production of industrial chemicals from cheap sources of carbon and in the degradation or detoxification of chemical wastes that contribute to environmental pollution because of the wide use of agricultural pesticides or herbicides and release of industrial wastes(Lehrbach *et al.,* 1984).

In *Pseudomonas* strains, these degradative concerning genes are mainly placed in plasmid (Chakrabarty, 1976) but some chromosome borne (Wheelis, 1975). In order to understand the biochemical and enzymatic pathways of degradative genes and to increase the versatile degradative abilities respectively, we can use degradative plasmids in natural (Knackmuss, 1981) or by cloning of degradative genes on to plasmid or bacteriophage vectors(Franklin *et al.,* 1981).

The use of chromosome transferable R plasmid leads to an increase in available genetic information on a wide variety of non-enteric gram-negative bacteria-*Pseudomonas, Agrobacterium, Rhizobium* (Williams, 1981). The first R plasmid of *E. coli* shown to promote transfer of chromosome has broad host range. In most cases, however, chromosome transfer occurs at low frequencies and from a limited number of sites.

While thermoinducible mutator phage Mu is able to integrate randomly in nonspecific sites of host chromosomes and mediates chromosomal rearrangements of bacterial genes into a plasmid during the lytic cycles(Van Gijsegem *et al.*, 1982). But unfortunately the host range of Mu is restricted to certain strains of the family *Enterobacteriaceae* (Howe and Bade, 1975). Therefore, the hybrid plasmid RP4::Mu was constructed and transferred by conjugation into *Klebsiella pneumoniae, Pseudomonas solanacearum,* and *Rhizobium meliloti* (Denarie *et al.*, 1976; Boucher *et al.*, 1977). RP4::Mu cts 62 has been shown to mediate heterospecific transfer of genes from *K. pneumoniae* to *E. coli* including thr, leu, and trp genes and the genes specifying the enzymes aryl sulfatase and tyramine oxidase(Murooka *et al.*, 1981).

However, the use of intact Mu to make R primes has some drawbacks. Firstly, it is necessary to integrate Mu into the chromosome of the donor strain. Secondly, it is necessary to use a recipient strain which is lysogenic for Mu(to avoid zygotic induction) and resistant to Mu (to avoid reinfection and killing of the recipient by the phages released upon induction of the donor). These problems can be overcome partially by replacing Mu with a mini-Mu derivatives, such as Mu 3A, which is deleted for all of the Mu lethal functions but retains the transposition-related properties (Faelen *et al.*, 1978).

The RP4::mini-Mu(pULB113; RP4::Mu3A) created by Van Gijsegem and Toussaint(1982) can promote transfer of the *E. coli* chromosome, can form R-prime plasmids, and can promote transfer of the Mu-lysogenic *Enterobacteriaceae* chromosome to *E. coli* in heterospecific matings. The *bla* gene of *Enterobacter cloacae* was transferred to *E. coli* by use of pULB113 (B. Wiedemann, 1984). Thus, these plasmids could be a powerful tool for genetic mapping and *in vivo* gene cloning. In contrast with RP4::Mu, an RP4 carrying a mini-Mu is transmitted efficiently even in heterospecific matings.

In this study, we have selected the *Pseudomonas* strains which have a hydrocarbon utilizable and chlorinated herbicide degradative genes in their chromosome. Then, as a step towards understanding the genetic organization and *in vivo* gene cloning of the Chromosome-borne degradative genes, RP4::Mu cts and RP4::mini-Mu in *E. coli* were transferred to these *Pseudomonas* strains by conjugation and were tested for their usefulness as tools in *in vivo* genetic engineering of *Pseudomonas.*

MATERIALS AND METHODS

Bacterial strains and phage

The bacterial strains and phage used in this study are described in Table 1.

Media

Table 1. *Bacterial strains and their characteristics*

Strains	Characteristics or genotype	Ref. or sources
E. coli		
C600(RP4)	r⁻m⁻thr⁻leu⁻thi⁻/ Ap^{r}Tc^{r}Km^{r}Tra$^+$, IncP4(RP4)	Depicker *et al.* (1977)
JC5466(pULB11)	Trp⁻his⁻recA56/Ap^{r}Tc^{r}Km^r Tra$^+$(RP4::Mu cts62)	J. Denarie(1976)
MXR(pULB113)	FΔ(lac-pro) thi⁻galE recA/ Tra$^+$Ap^{r}Km^{r}Tc^r(RP4::mini-Mu)	F. Van Gijsegem (1982)
Pseudomonas sp.		
KU349	wild type	Oh *et al.*(1987)
KU373	wild type	Oh *et al.*(1987)
Pseudomonas aeruginosa		
KU557	wild type	Oh *et al.*(1987)

L-Broth was used for routine cultivation of bacteria as well as diluent for phages. Penassay broth agar plate medium was used for bacterial matings. The supplemented LB media were as follows: LBKT, LB medium containing kanamycin (25μg/m*l*) and tetracycline (25μg/m*l*); LBCM, LB medium containing 5mM $CaCl_2$ and 10mM $MgSO_4$(Agar medium contained 200mM $MgSO_4$.). M9(Maniatis *et al.*, 1982), Mineral salt medium A(Whiteside and Alexander, 1963) and Mineral salt medium B(Chatterjee *et al.*, 1981) were used as a minimal medium.

Antibiotic resistance test

For the determination of resistance to various antibiotics, bacterial suspensions were plated on LB agar containing different concentrations of various antibiotics respectively and incubated for 48 hrs at 30°C.

Chlorinated herbicide utility test

The selected *Pseudomonas* strains were inoculated into Mineral salt agar medium A supplemented with 2.4-D (2.4-dichlorophenoxy acetate), MCPA(2-methyl-4-chlorophenoxy acetate), and DCP(2,4-dichlorophenol) as a sole carbon source and Mineral salt agar medium B supplemented with 3-CB(3-chlorobenzoate) as a sole carbon source. These were incubated at 30°C for three to seven days.

Hydrocarbon utility test

The selected *Pseudomonas* strains were inoculated into M9 minimal medium containing each aromatic hydrocarbon instead of glucose and incubated for one week at 30°C as previously(Kim and Lee, 1984). Used carbon sources were salicylate, toluene, xylene, benzene, cyclohexane, octane, naphthalene, camphor and catechol.

Bacterial mating

Bacterial matings were performed on membrane filters by the method of De Graff *et al.* (1973).

Plasmid isolation

The modification of the method of Kado and Liu(1981) was used to isolate plasmids from transconjugants.

Agarose gel electrophoresis

For electrophoresis, horizontal mini-gel chamber(Maruzen Oil Biochemical Co.) was used. 40 μl DNA samples including 10 μl of SEB(60% w/v sucrose-60mM EDTA-0.015% bromophenol blue) were subjected to electrophoresis in 0.6% agarose mini-gel dissolved in E buffer. Electrophoresis was carried out at 50V, for 2 hrs. The gel was then stained in a solution of etidium bromide(10 μg/m*l*) in water for 15 min and observed by an transilluminator (TM 36, UV Product Co.)

Production of phage Mu by thermoinduction

The method of Murooka *et al.* (1981) was used for preparation of Mu cts lysates from the purified lysogens.

A. Preparation of phage lysates

Overnight cultures of lysogens of *E. coli* and *Pseudomonas* sp., grown in LBKT medium at 30°C, were diluted with LBCM medium and grown to a density of about 10^8 bacteria per m*l*. Then the cultures were incubated at 43°C for 1 hr with vigorous shaking(160 rpm) and at 37°C until lysis was observed. And chloroform was added. The mixture was shaken on a vortex mixer, kept at room temperature for 15 min, then centrifuged (10 min, 7000 rpm). Obtained supernatants, phages, were stored over a few drops of chloroform at 4°C.

B. Plaque formation by phage

Phage titers were determined as follows. Indicator *E. coli* C600 was grown in LBCM medium to a density of 10^9 bacteria per m*l*. Appropriate dilutions of the lysates were mixed with the culture of *E. coli* C600 and incubated at 30°C for 30 min to allow adsorption.

After top agar was added, the mixture was poured into LBCM agar plates and incubated overnight at 43°C.

Estimation of stability of RP4::Mu cts and RP4::mini-Mu in *Pseudomonas* sp.

A modification of the method of Galas and Chandler (1982) was employed.

Overnight cultures of the *Pseudomonas* lysogens, grown in LBKT at 30°C, were diluted by a factor of 10^6 and incubated to saturation. The subculturing was repeated five times to obtain a culture grown for 100 or more generations. The final culture was plated on LB agar media.

In case of RP4::Mu cts, isolated colonies were replicated onto LBKT agar plates and onto two LB agar plates without antibiotics. Then LBKT agar plate and one LB agar plate were incubated at 30°C, while another LB agar plate was incubated at 43°C. In case of RP4::mini-Mu, isolated colonies were replicated onto LBKT agar plate and incubated at 30°C.

RESULTS AND DISCUSSIONS

Antibiotic resistance of *Pseudomonas*

In order to use for recipient cells of RP4::Mu cts or RP4::mini-Mu, plasmid-free *Pseudomonas* strains were selected and tested for their antibiotic resistance.

As shown in Table 2, *E. coli* JC5466, RP4::Mu cts donor, had strong resistance for kanamycin (200 μg/ml), tetracycline(200 μg/ml), rifampicin (50 μg/ml). And *E. coli* MXR, RP4::mini-Mu donor, had also strong resistance for kanamycin (200 μg/ml), tetracycline(200 μg/ml).

On the other hand, All *Pseudomonas* strains that selected as recipients of RP4::Mu cts and RP4::mini-Mu were sensitive to kanamycin and tetracycline, but had strong chloramphenicol resistance(400 μg/ml). Besides *Pseudomonas* sp. KU373 had a little resistance for gentamycine (20 μg/ml) and *Pseudomonas aeruginosa* KU557 had a little resistance for streptomycin (50 μg/ml). Therefore, selected *Pseudomonas* strains have a suitable antibiotic resistance marker as recipients of RP4::Mu cts or RP4::mini-Mu.

Carbon source utility

The selected *Pseudomonas* strains utilized catechol as a sole carbon source, but benzene, camphor, cyclohexane, naphthalene, octane, salicylate, toluene, and xylene were not utilized by these strains. Also, as shown in Table 3, *Pseudomonas* strains exhibit an versatile degradative abilities for 2.4-D, MCPA and 3-CB, but they were not capable of degrading DCP due to its toxicity. It was identified that *Pseudomonas* strains had no plasmid band in them by electrophoresis after

Table 2. *Resistance of the strains for various antibiotics*

Antibiotics (μg/ml)	Tc		Km		Cm		Sm		Gm		Rif	
Strains	100	200	100	200	200	400	20	50	20	50	20	50
E. coli C600	+	+	+	+	−	−	−	−	−	−	−	−
JC5466	+	+	+	+	−	−	−	−	−	−	+	+
MXR	+	+	+	+	−	−	−	−	−	−	−	−
Pseudomonas sp. KU349	−	−	−	−	+	+	−	−	−	−	−	−
KU373	−	−	−	−	+	+	−	−	+	−	−	−
Pseudomonas aeruginosa KU557	−	−	−	−	+	+	+	+	−	−	−	−

Abbr.; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol; Sm, streptomycin; Gm, gentamycin; Rif, rifampicin

Table 3. *Degradation patterns of chlorinated herbicides by pseudomonas*

Herbicides (μg/ml)	2,4-D		MCPA		3-CB		DCP	
Strains	500	1000	500	1000	500	1000	250	500
Pseudomonas sp. KU349	−	−	+	+	+	+	−	−
KU373	−	−	+	+	+	+	−	−
Pseudomonas aeruginosa KU557	+	−	+	+	−	−	−	−

Abbr.: 2,4-D, 2,4-dichlorophenoxy acetate; MCPA, 2-methyl-4-chlorophenoxy acetate; 3-CB, 3-chlorobenzoate DCP, 2,4-dichlorophenol

Table 4. *Transfer frequencies of RP4::Mu cts and RP4::mini-Mu from E. coli to Pseudomonas strains*

Recipient strains	selected marker	Transfer frequency RP4::Mu cts	RP4::mini-Mu
Pseudomonas sp.			
KU349	Km, Tc, Cm	1.2×10^{-7}	6.3×10^{-3}
KU373	Km, Tc, Cm	3.3×10^{-7}	1.0×10^{-4}
Pseudomonas aeruginosa KU557	Km, Tc, Cm	3.7×10^{-2}	4.0×10^{-2}

plasmid isolation. By this result, it is considered that these degradative concerning genes are chromosomal borne.

Introduction of RP4::Mu cts and RP4::mini-Mu into *Pseudomonas*

RP4::Mu cts and RP4::mini-Mu in *E. coli* were transferred into *Pseudomonas* by conjugation on membrane filters respectively. Transfer frequencies of these plasmids were expressed as the number of transconjugants per the number of initial donors.

As indicated in Table 4, RP4::Mu cts and RP4::mini-Mu were transferred to the *Pseudomonas* recipients except *Pseudomonas aeruginosa* KU557 at a frequency of 10^{-7} and 10^{-4} to 10^{-3} respectively. The transfer frequency was increased approximately 10^3-fold when RP4:: mini-Mu were transferred. These results suggest that the conjugative transfer of the mini-Mu genome, which is deleted for all of the Mu lethal functions but retains the transposition related properties, into *Pseudomonas* does not result in detectable zygotic induction or restriction. Whereas the conjugative transfer of the intact Mu cts results in strong zygotic induction which destroys the plasmid or kills the recipients(Lejeune *et al.*, 1983). On the other hand, the two R plasmids were transferred at about the same frequency of 10^{-2} when the recipient was *Pseudomonas aeruginosa* KU557, showing that the zygotic induction or restriction does not significantly occur. But the reason for this phenomenon is not sure.

Identification of plasmids in transconjugants

In order to verify that the *Pseudomonas* transconjugants have a RP4::Mu cts or RP4:: mini-Mu, we checked for their antibiotic resistance markers, and purified plasmid DNAs were analyzed by agarose gel electrophoresis. All the antibiotic resistance markers carried by RP4 were expressed in the *Pseudomonas* sp. and levels of resistance were comparable to those found in the *E. coli* donor. Besides, as shown in Fig. 1 and Fig. 2, the wild type *Pseudomonas* strains had no plasmid band. But all *Pseudomonas* transconjugants had a plasmid band at the same site in the *E. coli* donor.

In case of RP4::Mu cts lysogens, the presence of phage Mu was confirmed by demonstrating

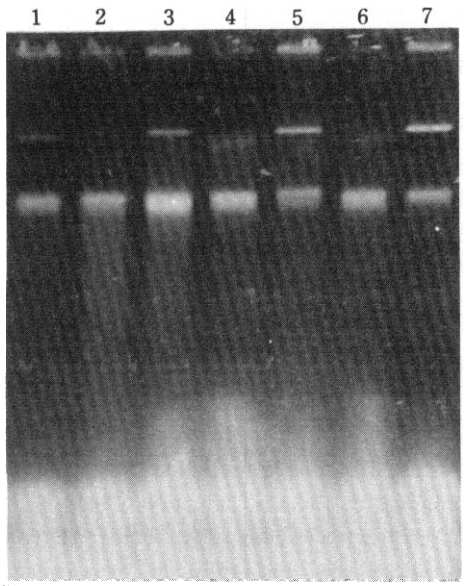

Fig. 1. *Agarose gel electrophoresis of RP4::Mu cts isolated from transconjugants.*

lane 1. *E. coli* JC5466(Donor)
2. *Pseudomonas* sp. KU349(Recipient)
3. *Pseudomonas* sp. KU349A(Transconjugant)
4. *Pseudomonas* sp. KU373(Recipient)
5. *Pseudomonas* sp. KU373A(Transconjugant)
6. *Pseudomonas aeruginosa* KU557(Recipient)
7. *Pseudomonas aeruginosa* KU557A(Transconjugant)

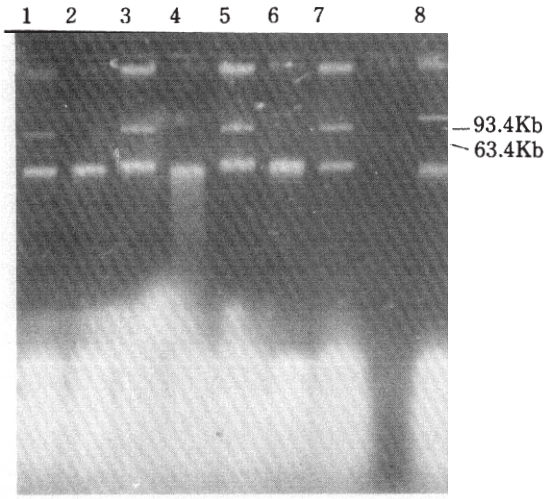

Fig. 2. *Agarose gel electrophoresis of RP4::mini-Mu isolated from transconjugants.*

lane 1. *E. coli* MXR(Donor)
2. *Pseudomonas* sp. KU349(Recipient)
3. *Pseudomonas* sp. KU349A(Transconjugant)
4. *Pseudomonas* sp. KU373(Recipient)
5. *Pseudomonas* sp. KU373A(Transconjugant)
6. *Pseudomonas aeruginosa* KU557(Recipient)
7. *Pseudomonas aeruginosa* KU557A(Transconjugant)
8. *E. coli* JC5466(RP4::Mu cts)

that the kanamycin, tetracycline resistant clones were capable of releasing phage and forming plaques on LBCM agar plates at 43°C. This is due to that cells lysogenic for Mu cts are temperature inducible owing to a mutation in the repressor gene C(Boram and Abelson, 1971). Lysis was ascertained by decrease in turbidity and by formation of cell-flocks at the bottom of the flasks. A restriction and modification deficient *E. coli* C600 was used as an indicator for the assay of Mu phage.

Table 5 shows phage yield from various lysogens obtained by thermal induction. Phage yield from *E. coli* JC5466 was about 10^{10} P.F.U. (Plaque Forming Unit) and those from the *Pseudomonas* lysogens were about 10^5.

These results showed that Mu expressed its gene in *Pseudomonas* transconjugants suggesting that RP4::Mu cts in *Pseudomonas* sp. can mediate transfer of chromosomal genes by formation of prime plasmid.

Stability of RP4::Mu cts and RP4::mini-Mu in *Pseudomonas* sp.

Table 5. *Mu phage yield by thermal induction from E. coli and Pseudomonas sp. carrying RP4::Mu cts*

Bacterial strains	Plaque forming unit
E. coli JC5466	2.3×10^{10}
Pseudomonas sp. KU349	5.5×10^5
KU373	8.7×10^5
Pseudomonas aeruginosa KU557	7.3×10^5

Stability was tested by presence of antibiotic resistance markers of RP4 plasmid after several frequent subcultures in non-selective LB media.

Table 6 shows the different patterns of stability that 80 to 87 percent of *Pseudomonas* sp. KU349 and *Pseudomonas aeruginosa* KU557 have maintained antibiotic resistance after serial transfers. *Pseudomonas* sp. KU373 had a low stability(less than 10%). However, when lysogens of *Pseudomonas* sp. KU373 stocked in LB agar for 3 to 4 weeks at 15°C were tested, the stabilities were significantly higher (80 to 90%) than those of subculturing(data not shown).

The loss of transferred plasmid markers might be interpreted as excision by reciprocal recombination between two flanking Mu cts or mini-Mu sequences. Lejeune (1983) reported that *rec* A influenced on the stability of the transposed marker on the plasmid. The *Pseudomonas* sp. with antibiotic resistance markers, having RP4::Mu cts, grew well at 30 °C but very poorly at 43 °C, whereas antibiotic sensitive strains well at 43 °C. From these results, we can conclude that the most transconjugants possessing RP4 plasmid have also Mu phage.

In this study we presented evidence that RP4::

Table 6. *Stability of RP4::Mu cts and RP4::mini-Mu in Pseudomonas sp.*

Bacterial strains	RP4::Mu cts		RP4::mini-Mu
	Tc, Km	Mu cts	Tc, Km
Pseudomonas sp. KU349	87%	87%	86%
KU373	8%	8%	7%
Pseudomonas aeruginosa KU557	80%	80%	81%

Mu cts and RP4::mini-Mu were able to be transferred efficiently into *Pseudomonas* sp. and retained stably in the new hosts. Therefore RP4:: Mu cts and especially RP4::mini-Mu could be a powerful tool for genetic study of *Pseudomonas* strains.

적 요

염색체 유전자를 전달시키기 위한 기초작업으로 RP4 : : Mu cts와 RP4 : : mini-Mu 잡종 플라스미드를 접합에 의하여 *E. coli*로 부터 *Pseudomonas*로 전달시켰다.

RP4 : : Mu cts와 RP4 : : mini-Mu의 수용세포는 플라스미드를 가지지 아니하는 *Pseudomonas* 균주들의 항생제 내성, 탄화수소 자화능 등의 유전적 지표를 조사하여 사용하였다.

RP4 : : mini-Mu는 10^{-2}-10^{-4}의 빈도로 전달되었으며 RP4 : : Mu cts는 *Pseudomonas aeruginosa* KU 557로는 10^{-2}의 빈도로, 그 이외의 수용세포로는 10^{-7}의 빈도로 전달되었다.

접합체에 전달된 플라스미드의 존재는 암피실린, 카나마이신, 테트라싸이클린에 대한 내성과 전기영동에 의해 확인하였으며 특히 RP4 : : Mu cts는 43℃에서의 플라크 형성으로도 확인하였다. 접합체들로 부터 생성된 Mu 파아지는 약 10^{5}의 P. F. U.를 나타냈으며 전달된 RP4 : : Mu cts와 RP4 : : mini-Mu는 접합체들에서 비교적 안정한 것으로 밝혀졌다.

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