KOR. JOUR. MICROBIOL. September, 1986 p. 211 220 Vol 24 No 3

Intergeneric Transfer of Nitrogen Fixation Genes from *Rhizobium leguminosarum* by RP4 :: Mu cts

Huh, Youn-Ju and Yung-Nok Lee
Department of Biology, Korea University

RP4:: Mu cts에 의한 *Rhizobium leguminosarum* 질소고정 유전자의 속간전달에 관한 연구

허연주 · 이영록
고려대학교 생물학과

Abstract: Nitrogen fixation (*nif*) genes of *Rhizobium leguminosarum* were transferred to *nif*⁻ *Klebsiella pneumoniae* and *E. coil* by conjugation after partial heat induction of RP4 :: Mu cts in *Rhizobium* R^+ transconjugant, and the hybrid plasmids in the transconjugant strains were isolated and characterized. In order to transfer the *nif* genes from *Rhizobium*, the hybrid plasmid RP4 :: Mu cts was transferred by conjugation from *E. coil* to the symbiotic nitrogen fixer, *R. leguminosarum*. After stabillity test, the RP4 :: Mu cts in Rhizobium R^+ transconjugant was subjected to partial heat induction by culturing it statically at 38℃ for 16 hours, and then conjugated with the *nif* defective mutant strains of *K. pneumoniae* or *nif* mutant strains of *E. coli* having whole *nif* gene plasmid. Recombinant strains of *K. pneumoniae*, which could grow in a N-free medium and exhibit the nitrogenase activity were selected. However, in the case of *E. coli*, they could grow well in a NA medium containing antibiotices, but hardly grow in a N-free medium. The hybrid plasmids in these transconjugal strains were isolated by gel electrophoresis and compared their molecular sizes.
Key words: Nitrogen fixation gene, *R. leguminosarum*, *K. pseudomiae*.

The temperate bacteriophage Mu inserts its DNA at many different loci in the genome of its host bacteria E. coli K12(Taylor, 1963 ; Bukhari and Zipser, 1972) and can mediate transfer of chromosome markers into other bacteria during its lytic cycle(Faelen et al., 1976). However, its host range is restricted to certain strains of the family Enterobacteriaceae (De Graff *et al.*, 1973). Dénarié et al. (1976) constructed the hybrid plasmid, RP4 :: Mu cts, in order to extend the host range of this phage. RP4 :: Mu cts, which is temperature sensitive at 42℃, and confers resistances to ampicillin, kanamycin and tetracycline, has broad host range and may integrate at many different loci in the host chromosome.

In general, nitrogen fixing bacteria will be divided into two broad categories: free living bacteria and symbiotic bacteria. The free living nitrogen fixer which has been the object of most molecular genetic studies is *Klebsiella pneumoniae*, in which 17 different genes have been identified(Puhler and Klipp, 1981). The symbiotic species studied most extensively belong to the genus *Rhizobium* (Beringer,

1980).

Recently, considerable interest has been devoted to study whether *nif* genes from *K. pneumoniae* are homologous to DNA from other nitrogen fixation species such as *Rhizobium*(Hennecke, 1981). Ruvkun and Ausubel (1980) used cloned *K. pneumoniae nif* DNAs as hybridization probes to identify particular DNA restriction fragments from other organisms that contain sequences homologous to *K. pneumoniae nif* genes. Nuti et al.(1979) demonstrated homology between *K. pneumoniae* nitrogenase genes and DNA from *R. leguminosarum* and *R. meliloti*, and Mazur et al.(1980) discovered that DNA from three species of *cyanobacteria* was also homologous to *K. pneumoniae* nitrogenase genes. On the other hand, Dixon and Postagate (1972) transfered nitrogen fixation genes from *K. pneumoniae* to *E. coli* by using F-like R factor and reported the expression of *nif* genes in *E. coli*. Using R factor, *nif* genes were also transferred from *R. trifolii* to *K. aerogenes* (Dunican and tierney, 1974). But we expected that RP4 :: Mu cts could transfer various genes effectively, so RP4 :: Mu cts would be a more proper vector in the intergeneric transfer of *nif* genes than RP4. Previously, Murooka(1981) reported arysulfatase gene transfer between *E. coli* and *K. aerogenes*, and Takizawa (1983) showed the intergeneric transfer of pullulanase gene from *Klebsiella aerogenes* to *E. coli* by using RP4 :: Mu cts.

In this study, nitrogen fixation genes of *Rhizobium leguminosarum* were transferred to *nif*⁻ *Klebsiella pneumoniae* by conjugation after partial induction of RP4 :: Mu cts in *Rhizobium* R⁺ transconjugant, and the hybrid plasmids in the transconjugal strains were isolated and characterized.

MATERIALS AND METHODS

Bacterial strains and phages

The bacterial strains used in this study are listed in table 1. *E. coli* and *K. pneumoniae* strains (MacNeil et al., 1978 a,b; Merrick et al., 1980) were obtained from professor Uosumi, Tokyo university, and *Rhizobium* strains were isolated in our laboratory (Lee et al., 1985). In experiments of *nif* gene transfer, *Rhizobium leguminosarum* K12C and *R.*

Table 1. *Used bacterial strains and their characteristcs.*

Strains	Characteristics of genotype	References or Sources
E. coli		
E. coli	Trp⁻, his⁻, rec A56, Ap Tc Km(RP4::Mu cts)	J. Dénarié (1976)
UNF1323	His⁺ *nif* L2176 Rif^S	Merrick *et al.* (1980)
UNF1323-1	His⁺ *nif* L2176 Rif^r	Mutagenesis of UNF UNF1323
UNF1323-1A	His⁺ *nif* L2176 Rif^r Tc⁺, Km⁺	UNF1323-1 mated with
UNF1323-1B	His⁺ *nif* L2176 Rif^r Tc⁺, Km⁺	*R. leguminosarum* K12-C
UNF1483	His⁺ *nif* A2732::Tn7 Rif^S	Merrick *et al.* (1980)
UNF1483-1	His⁺ *nif* A2732::Tn7 Rif^r	Mutagenesis of UNF1483
UNF1483-1A	His⁺ *nif* A2732::Tn7 Rif^r Tc⁺, Km⁺	UNF1483-1 mated with *R. leguminosarum* K12C
UNF1483-1B	His⁺ *nif* A2732::Tn7 Rif^r Tc⁺, Km⁺	
Rhizobium		
Rhizobium K10	Cm Sm Gm	Lee *et al.* (1985)
Rhizobium K10A	Ap Tc Km Cm	K10 mated with *E. coli* JC5466
R. leguminorum K12	Cm	Lee *et al.* (1985)
R. legiuminosarum K12C	Ap Tc Km Cm	K12 mated with *E. coli* JC5466
R. japonicum K15	Cm	Lee *et al.* (1985)
R. japonicum K15A	Ap Tc Km Cm	K15 mated with *E. coli*
R. japonicum K15B	Ap Tc Km Cm	JC5466
R. trifolii K20	Cm	Lee *et al.* (1985)
R. trifolii K20A	Ap Tc Km Cm	K20 mated with *E. coli*
R. trifolii K20B	Ap Tc Km Cm	JC5466

Table 2. *Countinued*

Strains	Characteristics of genotype	References or Sources
K. pneumoniae		
UNF910	*nif* K2812::MudAplac rec A56 Rif^S	Merrick *et al.* (1980)
UNF910-1	*nif* K2812::MudAplac rec A56 Rif^r	Mutagenesis of UNF910
UNF910-1A, B, D, E, H, I, K	*nif* K2812::MudAplac rec A56 Rif^r (RP4 ::Mu cts) *nif*$^+$	UNF910-1 mated with *R. leguminosarum* K12C
UNF725	*nif* H2783::MudAplac rec A56 Rif^S	Merrick *et al.* (1980)
UNF725-1	*nif* H2783::MudAplac rec A56 Rif^r	Mutagenesis of UNF725
UNF725-1A, B, C, G, H, I,	*nif* H2783::MudAplac rec A56 Rif^r (RP4::Mu cts)	UNF725-1 mated with *R. leguminosarum* K12C
CK263	*nif* A$^-$2263 Rif^S	Uosumi
CK263-1	*nif* A$^-$2263 Rif^r	Mutagenesis of CK263
CK263-1A, B, C, D, E, F, G, H	*nif*$^-$2263 Rif^r (RP4 ::Mu cts) *nif*$^+$	CK263-1 mated with *R. leguminosarum* K12C

trifolii K20A carring RP4 :: Mu cts were used as donors. They contained *nif* gene and were resistant to Ap (100mg/m*l*), Tc (100mg/m*l*), Km (25mg/m*l*) and Cm (200 mg/m*l*). As recipients, *K. pneumoniae* UNF910-1, UNF725-1, CK236-1, *E. coli* UNF1323-1 and UNF1483-1 were used. They were the derivatives of UNF910, UNF725, CK263, UNF1323 and UNF1483, and resistant to Rifampicin (500mg/m*l*) but sensitive to Tc. and Km. *K. pneumoniae* UNF910, UNF725 and CK263 are *nif* K, H, A gene defective strains, and *E. coli* UNF1323 and UNF1483 are *nif* A, L gene defective mutant strains of *E. coli* having whole *nif* gene plasmid (pRD1).

Media

The rich media used were Penassay broth, consisting of 1.5g beef extract, 1.5g yeast extract, 3.5g NaCl, 1.32g KH_2PO_4, and 3.68 g K_2HPO_4, and LB medium, consisting of 10 g trypton, 5g yeast extract, 5g NaCl and 1g glucose per liter the supplemented LB media used were as follows: LBAKT, LB plus ampiciilin (100mg/m*l*), kanamycin (25mg/m*l*) and tetracycline (100mg/m*l*), and LBCM, LB plus 5mM $CaCl_2 \cdot 2H_2O$ and 200 mM $MgSO_4$. The minimal media used for *Klebsiella* was nitrogen-free(N-free) medium, consisting of 13.9g Na_2HPO_4, 1.7g KH_2PO_4, 2.0g NaCl, 0.2g $MgSO_4$, 0.008g $FeCl_3 \cdot 7H_2O$, 0.001g $Na_2Mo_4 \cdot 2H_2O$, 0.001g Thiamine, 6.0 g glucose and 13g of purified agar (Difco) per liter. For growth of *Rhizobium* strains, YMA, consisting of 10g mannitol, 0.5g K_2SO_4, 0.2g $MgSO_4 \cdot 7H_2O$, 3.0g $CaCO_3$ and 0.2g yeast extract per liter, was used.

Transfer of *nif* gene by thermal induction and conjugation

The modification method of Murooka *et al.* (1981) was used for *nif* gene transfer. *R. leguminosarum* K12C (RP4 :: Mu cts) as a donor was grown in LBAKT at 30℃ overnight and diluted 1 : 100 with a fresh LBCM, and incubated at 38℃ for 16 hours without shaking to form Hfr or RP4 :: Mu cts prime plasmid. *Nif*$^-$ *K. pneumoniae* and *E. coli* strains as recipients were grown in LBCM for 20hrs. 10m*l* of donor cells and 1 m*l* of recipient cells were mated on membrane filters. *Nif*$^+$ transconjugants were selected by spreading on N-free agar plate. *Nif*$^-$ *K. pneumoniae* and *E. coli* can't grow on N -free media. Donor cells were counter selected by adding of antibiotics.

Assay of nitrogenase activity

Nitrogen fixation was assessed for bacterial suspensions grown in N-free medium including glucose (for *E. coli*) or N-free medium including saccharose (for *K. pneumoniae*), under anaerobic conditions, using the C_2H_2 reduction assay by the modification method of Dunican and Tierney (1974).

For this purpose, a 0.0001m*l* aliquot of a mid-logarithmic phase LB culture was added to 5m*l* of N-free medium in a vial (39m*l*). The vials were then capped with serum stoppers, evacuated, filled with N_2, incubated for 2 days at 30℃, and injected with acetylene

and argon gas. ethylene production was measured after 4hrs, at 30℃, by injecting a 1 m*l* gas sample in a Varian Model 3700 gas chromatography fitted with a porapak-R column at 80℃ column temperature, with N_2 as carrier gas at a flow rate of 30cc/min.

On the other hand, in the case of symbiotic nitgogen fixer *R. leguminosarum*, ethylene production was detected as described by Wacek and Brill (1976). Peas were placed in a 20m*l* serum bottle which had been filled with sterile vermiculite and 16m*l* of plant nutrient solution. Then 1m*l* of *R. leguminosarum* suspension was dispensed into each of the sterilized bottles containing the vermiculite. After 14 days incubation, the plant was cut at the base of the stem. A serum stopper was placed on the bottle and 0.4m*l* of 1 atm. acetylene was injected into the bottle. The bottles were incubated at 25℃ for 1 to 3 hours after which 0.5m*l* gas samples were injected into the gas chromatograph.

Plasmid isolation

The method of Kado and Liu (1981) was used. Cells were grown in 3m*l* of L-broth overnight at 30℃ to an optical density at 600nm of 0.8 and pelleted by centrifugation (6.000rpm 7 min., 4℃). The cell pellet was thoroughly suspended in 1m*l* of E buffer(consisting of 0.04M tris., and 0.002M disodium EDTA, adjusting pH7.9 with glacial acetic acid). The cells were lysed by adding 2m*l* of lysing solution, pH12.6 (0.05M tris base and 3% S.D.S.), which was mixed by brief agitation. The solution was heated at 50℃ to 65℃ for 20 min in water bath, and 2 volumes of phenol-chloroform solution (1 : 1, vol/vol) were added. The solution was emulsified by shaking briefly, and the emulsion was broken by centrifugation (8000 rpm, 15 min., 4℃). Portion of the supernatant sample was pipetted into the walls of the gel.

Agarose gel electrophoresis

For electrophoresis, horizontal gel chambers were used. 45m*l* DNA sample including 10m*l* of tracking solution was subjected to electrophoresis in 0.7% agarose gel dissolved in E buffer. A dye solution consists of k0.25% bromocresol blue and glycerol 0.5M tris-acetate, pH7.9. Electrophoresis was carried out at 120V, 37mA, for 3hrs. The gel was then placed in a solution of ethidium bromide in water (50mg/m*l*), stained for 15 min. And photographed on a UV-transilluminator.

RESULTS AND DISCUSSION

Analysis of *Rhizobium* R^+ transconjugants

In order to transfer the *nif* genes from *Rhizobium*, we have previously transferred the hybrid plasmid RP4 :: Mu cts from *E. coli* to the symbiotic nitrogen fixer, *R. leguminosarum* by conjugation. As a result, RP4 :: Mu cts was able to express in it's new host *Rhizobium* and most of the transconjugants conserved drug resistances and phage Mu cts stably. *Rhzobium* R^+ transconjugants were taken up into liquid medium containing

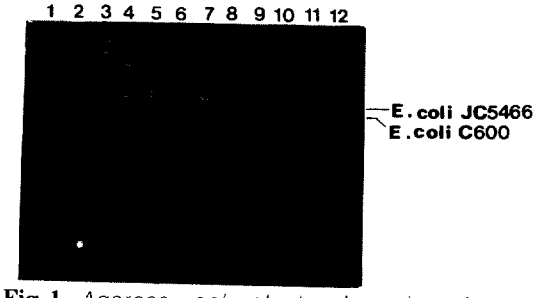

Fig. 1. *Agarose gel electrophoresis of the strains.*

1. *R. leguminosarum* K12C(RP4 :: Mu cts)
2. *R. leguminosarum* K12(Wild type)
3. *Rhizobium* K10A(RP4 :: Mu cts)
4. *Rhizobium* K10(Wild type)
5. *R. trifolii* K20B(RP4 :: Mu cts)
6. *R. trifolii* K20A(RP4 :: Mu cts)
7. *R. trifolli* K20(Wild type)
8. *R. japonicum* K15B(RP4 :: Mu cts)
9. *R. japonicum* K15A(RP4 :: Mu cts)
10. *R. japonicum* 15(Wild type)
11. *E. coli* C600(RP4)
12. *E. coli* JC5466(RP4 :: Mu cts)

Rhizobium transconjugants were taken up into liquid medium containing the four antibiotics. They were grown and processed further to obtain cleared lysates for agarose gel electrophoresis.

the four antibiotics (Tc, Km, Ap, Cm). They were grown and processed further to obtain cleared lysates for agarose gel electrophoresis. As showed in Fig.1, the hybrid plasmids RP4 :: Mu cts of *Rhizobium* R^+ transconjugants were identified on agarose gel and these transconjugants contained plasmids in accordance with our expectation. It was concluded that the recipient and donor plasmids were identical, based on their identical relative mobilities. In addition, transconjugants had two kinds of plasmids. It seems likely that the larger plasmid is a multimer form by rec protein or a RP4 :: Mu cts prime being caused by site specific integrase.

Transfer of the nitrogenase gene from *R. leguminosarum* by RP4 :: Mu cts

Partial heat induction of Mu cts results in formation of Mu-bacterial circular DNA which can be integrated at random into the bacetrial chromosome or into a plasmid. This can lead to transfer of the bacterial sequences carried by this circular DNA to any location in either the chromosome or the plasmid. After a donor *R. leguminosarum* K12C carring RP4 :: Mu cts and recipients *nif*$^-$ *K. pneumoniae* were mated, they were suspended in saline and spreaded on N-free agar plate.

Donor and recipient strains were not able to grow in N-free plate plus antibiotics owing to defect of the *nif* gene. *Nif*$^+$ transconjugants capable of growing on N-free media under the anaerobic condition, for 7 days at 30℃, were selected (Fig.2). As shown in the table 2, *nif* gene transfer frequencies by RP4 :: Mu cts were about 10^{-9}. Those isolates were also grown in N-free media to analyze for acetylene-reducing activity. They reduced acetylene (table 3) and, therefore, contained the *nif* genes. However, as showed in Fig. 3, *nif*$^-$ recipient could not reduce acetylene at all, while donor *R. leguminosarum* K12C could redece the acetylene only in pea nodule. These results suggest that *K. pneumoniae nif* genes K,H,A are homologous to those from *R. leguminosarum*.

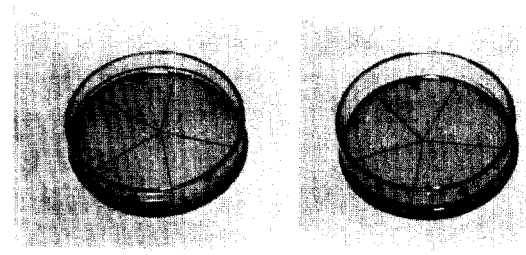

Fig. 2. *Growth of K. pneumoniae strains on N-free agar medium for 7 days at 30℃.*

Lift:
a. *K. pneumoniae* M5al(Wild type)
b. *K. pneumoniae* CK263-1(recipient)
c. *K. pneumoniae* CK263-1B(transconjugant)
d. *R. leguminosarum* K12C(donor)
e. *E. coli* JC5466(RP4 :: Mu cts)
Right:
a. *K. pneumoniae* M5al(Wild type)
b. *K. pneumoniae* UNF910-1(recipient)
c. *K. pneumoniae* UNF910-1H(transconjugant)
d. *K. pneumoniae* UNF725-1B(transconjugant)
e. *K. pneumoniae* UNF725-1(recipient)

In order to select *nif*$^-$ transconjugants, the mixture on membrane filter was placed on nutrient agar plus ampicillin, tetracycline, kanamycine and rifampicin at first and then *nif*$^+$ *K. pneumoniae* were selected on N-free media under the anaerobic condition for 7 days at 30℃.

On the other hand, as table 4 showed,

Table 2. *Nif gene transfer frequencies by RP4 :: Mu cts from R. leguminosarum to K. pneumoniae.*

Donor Strain	Recipient Strain	Selected marker	Transfer frequency
R. leguminosarum K12C (RP4::Mu cts, *Nif*$^+$)	*K. pneumoniae* UNF910-1 (*Nif* K$^-$)	Tc Km Rif *Nif*$^+$	3.4×10^{-9}
	K. pneumoniae UNF725-1 (*Nif* H$^-$)	Tc Km Rif *Nif*$^+$	6.6×10^{-9}
	K. pneumoniae CK263-1 (*Nif* A$^-$)	Tc Km wif *Nif*$^+$	3.2×10^{-9}

R. leguminosarum K12C(RP4::Mu cts) was grown as a donor in LBATK at 30℃ overnight and diluted 1:100 with a fresh LBCM and incubated at 38℃ for 16hours without shaking to form Hfr cells or RP4::Mu cts prime plasmid. *K. pneumoniae* strains as a recipient were grown in LBCM for 20h. 10m*l* of donor cells (10^8 cell per m*l*) and 1m*l* of recipient cells (10^9 cells/m*l*) were mated on membrane filters.

Table 3. *Nitrogenase activities of K. pneumoniae transconjugants.*

Bacterial strains	n mole C_2H_4/tube. hr.
Klebsiella pneumoniae	
M5a1	39
UNF 910-1	0
UNF910-1E	11.7
UNF910-1H	22.1
UNF910-1I	7.15
UNF 725-1	0
UNF725-1B	31.85
UNF725-1C	26
CK 263-1	0
CK 263-1B	33.15
CK 263-1C	49. 4
CK 263-1D	13.65
CK 263-1F	15.6

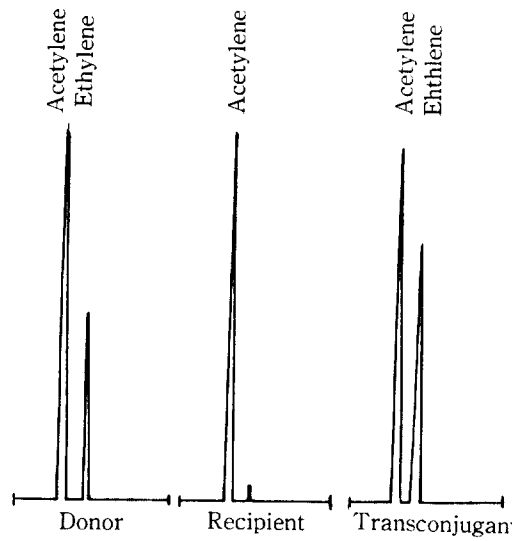

Fig. 3. *Nitrogenase activity of K. pneumoniae transconjugants compared to that of R. leguminosarum and the recipient K. pneumoniae UNF 910-1. K. pneumoniae UNF 725-1. K. pneumoniae CK 263-1.*

transfer frequencies of RP4 : : Mu cts in cross between *R. trifolii* K20A and *nif* mutant strains of *E. coli* having whole *nif* genes plasmid (pRD1) on nutrient agar plate including antibiotics were 10^{-7} to 10^{-8}. However, when they were replicated on N-free media under the anaerobic condition they hardly grew Therefore, they were analyzed for plasmid content by agarose gel electrophoresis. As presented in Fig.4, *E. coli* UNF1323 and *E. coli* UNF1483 had lost their own plasmid pRD1 and instead had only the RP4 :: Mu cts of donor strains (in Fig.4, lane 2 and lane 5). By this result, recipient strains originally contained P1 incompatibility group plasmid pRD1 and donor strains also had hybrid plasmid of P1 incompatibility group plasmid RP4, so when the RP4 : : Mu cts of *Rhizobium* transferred into *E. coli* resident plasmid pRD1 disappeared and thus *nif* gene of *Rhizobium* could not express in *E. coli* having no pRD1.

Identification of intergeneric *nif* gene transfer

Fig.5, 6, 7 showed that intact recipient strains, *K. pneumoniae* UNF910-1, UNF725-1 and CK263-1 had no plasmids but *K. pneumoniae* transconjugants had two dif-

Table 4. *Transfer frequencies of RP 4 : : Mu cts in cross between Rhizobium trifolii K 204 and nif mutant strains of E. coli having whole nif gene plasmid*

Donor Strain	Recipient Strains	Selected Marker	Transfer frequency
Rhizobium trifolii K20A	*E. coli* UNF1483-1(*Nif* A$^-$)	Tc Km Rif	3.4×10^{-7}
(RP4::Mu cts, *Nif*$^+$)	*E. coli* UNF1323-1(*Nif* L$^-$)	Tc Km Rif	6.6×10^{-8}
	E. coli UNF1323-2(*Nif* L$^-$)	Tc Km Rif	3.9×10^{-8}

Rhizobium trifolli K20A as a donor was partially induced by thermal treatment and conjugated with recipient *E. coli*. At first, mixture of donor and recipient was placed on nutrient agar plate plus tetracycline, kanamycin, rifampicin or tetracycline, kanamycin, gentamycin. And then they were replicated on N-free agar media.

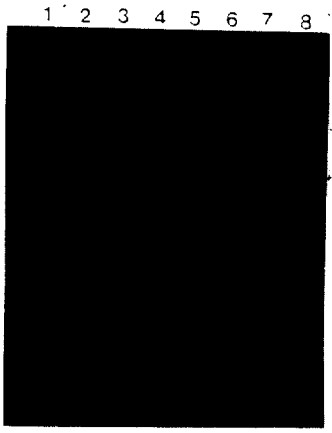

Fig. 4. *Agarose gel electrophoresis of E. coli transconjugants on nutrient agar plate.*

Lane
1. *R. trifolii* K20A(donor)
2. *E. coli* UNF1483-1(recipient)
3. *E. coli* UNF1483-1A(transconjugant)
4. *E. coli* UNF1483-1B(transconjugant)
5. *E. coli* UNF1323-1(recipient)
6. *E. coli* UNF1323-1A(transconjugant)
7. *E. coli* UNF1323-1B(transconjugant)
8. *E. coli* JC5466(RP4 : : Mu cts)

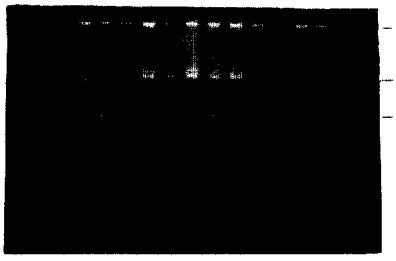

Fig. 5. *Agarose gel electrophoresis of K. pneumoniae CK263-nif⁺ transconjugants*

1. *E. coli* C600(RP4)
2. *E. coli* JC5466(RP4 : : Mu cts)
3. *K. pneumoniae* CK263-1 *nif* K⁻, Rif⁺
4. *K. pneumoniae* CK263-1C(RP4 : : Mu cts *nif*⁺)
5. *K. pneumoniae* CK263-1F(RP4 : : Mu cts *nif*⁺)
6. *K. pneumoniae* CK263-1A(RP4 : : Mu cts *nif*⁺)
7. *K. pneumoniae* CK263-1B(RP4 : : Mu cts *nif*⁺)
8. *K. pneumoniae* CK263-1J(RP4 : : Mu cts *nif*⁺)
9. *K. pneumoniae* CK263-1I(RP4 : : Mu cts *nif*⁺)
10. *K. pneumoniae* CK263-1E(RP4 : : Mu cts *nif*⁺)
11. *K. pneumoniae* CK263-1D(RP4 : : Mu cts *nif*⁺)
12. *E. coli* JC5466 (RP4 : : Mu cts)

ferent sized plasmids like *Rhizobium R*⁺ transconjugants. By Fig.8a. it was suggested that the upper plasmid bands were the OC form or the multimer of RP4 : : Mu cts and the lower plasmids were larger than intact

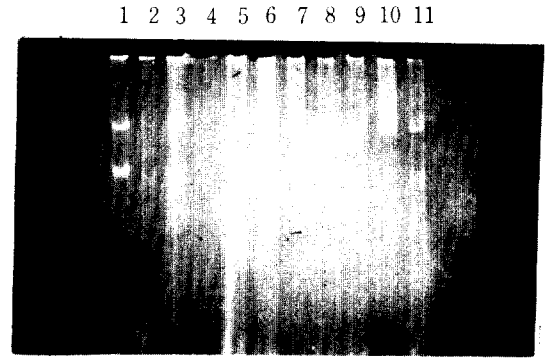

Fig. 6. *Agarose gel electrophoresis of K. pneumoniae UNF 910-1 nif⁺ transconjugants.*

1. *E. coli* C600(RP4)
2. *E. coli* JC5466 (RP4 : : Mu cts)
3. *R. leguminosarum* K12C
4. *K. pneumoniae* UNF910-1(*nif* K⁻, Rif⁺)
5. *K. pneumoniae* UNF910-1H(RP4 : : Mu cts *nif*⁺)
6. *K. pneumoniae* UNF910-1K(RP4 : : Mu cts *nif*⁺)
7. *K. pneumoniae* UNF910-1E(RP4 : : Mu cts *nif*⁺)
8. *K. pneumoniae* UNF910-1I(RP4 : : Mu cts *nif*⁻)
9. *K. pneumoniae* UNF910-1J(RP4 : : Mu cts *nif*⁺)
10. *K. pneumoniae* UNF910-1F(RP4 : : Mu cts *nif*⁺)
11. *K. pneumoniae* UNF910-1L(RP4 : : Mu cts *nif*⁺)

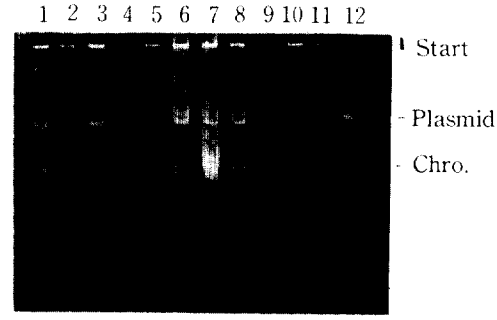

Fig. 7. *Agarose gel electrophoresis of K. pneumoniae UNF 725-1, nif⁺ transconjugants*

1. *E. coli* C600(RP4)
2. *E. coli* JC5466(RP4 : : Mu cts)
3. *R. leguminosarum* K12C
4. *K. pneumoniae* UNF725-1(*nif* H⁻, Rif⁺)
5. *K. pneumoniae* UNF725-1H(RP4 : : Mu cts *nif*⁺)
6. *K. pneumoniae* UNF725-1I(RP4 : : Mu cts *nif*⁺)
7. *K. pneumoniae* UNF725-1A(RP4 : : Mu cts *nif*⁺)
8. *K. pneumoniae* UNF725-1B(RP4 : : Mu cts *nif*⁺)
9. *K. pneumoniae* UNF725-1C(RP4 : : Mu cts *nif*⁺)
10. *K. pneumoniae* UNF725-1D(RP4 : : Mu cts *nif*⁺)
11. *K. pneumoniae* UNF725-1J(RP4 : : Mu cts *nif*⁺)
12. *K. pneumoniae* UNF725-1K(RP4 : : Mu cts *nif*⁺)

vector plasmid RP4 : : Mu cts. Thus, it was probable that transconjugants contain RP4 : : Mu-prime having some chromosomal DNA of donor strain. As Fig.8b indicated, the

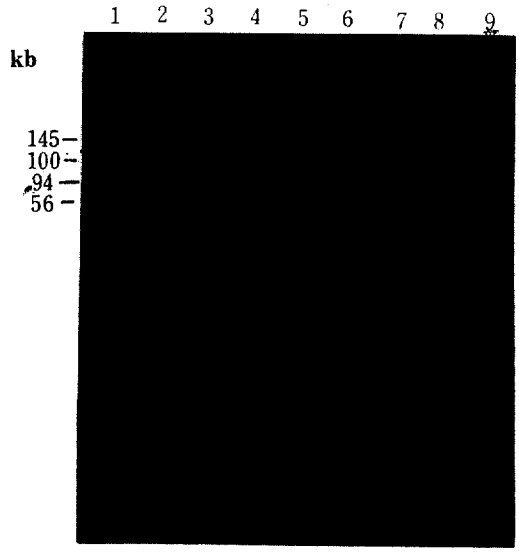

Fig.8a. *Molecular weight of nif⁺ K. pneumoniae transconjugants.*

1. *E. coli* JC5466(RP4 : : Mu cts)
2. *P. aeruginosa* PAO303(Rms 148)
3. *E. coli* C600(RP4)
4. *K.P* UNF910-1E(RP4 : : Mu cts, *nif⁺*)
5. *K.P* UNF725-1B(RP4 : : Mu cts, *nif⁺*)
6. *K.P* UNF725-1C(RP4 : : Mu cts, *nif⁺*)
7. *K.P* CK263-1B(RP4 : : Mu cts, *nif⁺*)
8. *K.P* CK263-1C(RP4 : : Mu cts, *nif⁺*)
9. *E. coli* C600(RP4)

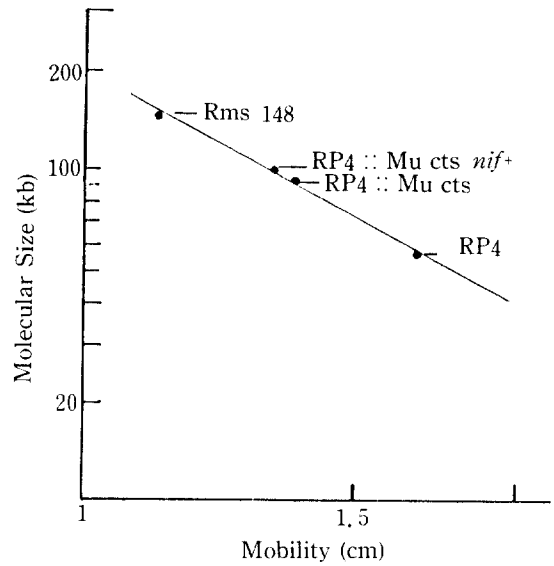

Fig.8b. *The plasmid isolated from K. pneumoniae transconjugants harboring RP4 : : Mu cts containing nif genes.* The molecular size of the plasmid from *K. pneumoniae* transconjugants were estimated at 94 to 100 kb using CCC forms of Rms 148, RP4 and RP4 : : Mu cts as a standard size markers.

molecular size of the plasmids from *K. pneumoniae* transconjugants was estimated at 94 to 100 kb using CCC forms of Rms148, RP4 and RP4 : : Mu cts as standard size markers.

In this paper, *in vivo* intergeneric transfer of the *nif* genes with RP4 : : Mu cts has been described. Therefore, it was confirmed that the *nif* K,H,A DNA of a symbiotic nitrogen fixation bacterium, *Rhizobium leguminosarum* was able to complement those of a free-living bacterium, *K. pneumoniae*. Recently most of the genetic engineering has been accomplished by the technuque of *in virto* recombinant DNA. However, *in vivo* method using RP4 : : Mu cts has advantages in simple procedures and economic standard points and is applicable to a wide variety of nitrogen fixation bacteria such as *Rhizobium, Klebsiella* and *Agrobcteric*, since RP4 : : Mu cts can be introduced, and Mu phage is expressed in these bacteria that are important in agricultural microbiology.

적 요

RP4 : : Mu cts를 부분 열유발시킨 후, ***Rhizobium legumino sarum***의 질소고정 능력을 질소고정 능력이 결손된 ***Klebsiella pneumoniae***와 ***E. coli***에 전달시키고저 하였다.

먼저 ***E. coli***의 RP4 : : Mu cts가 접합에 의해 ***R. leguminosarum***으로 전달되었다. ***Rhizobium leguminosarum***으로 옮겨진 RP4 : : Mu cts의 부분열 유발과 접합에 의해 ***Klebsiella***로 전달된 *Nif* 유전자의 전달빈도는 10^{-9}이었다. 유전자를 가진 접합체 콜로니들은 우선 항생제를 포함한 영양배지에서 선별한 후 혐기성 상태하의 N-결핍배지에서 복제하였는데 이들은 아세틸렌을 환원할 수 있었다. ***K. pneumoniae*** 접합체의 Agarosegel 전기영동에 의하면 두 개의 크기가 다른 플라스미드가 관찰되었다. ***K. pneumoniae*** 접합체의 플라스미드의 크기는 원래의 벡터 플라스미드

인 RP4 :: Mu cts보다 조금 더 큰 것으로 나타났는데 그 크기는 94-100 kb 정도였다.

한편, *R. trifolii* K20A와 전실소고성 유전자를 가지는 플라스미드를 가지고 있지만 일부 유전자의 돌연변이로 질소고정능이 결여된 *E. coli*와 접합시켰을 때, 항생제를 함유한 영양배지상에서의 RP4 :: Mu cts의 전달빈도는 10^{-7} - 10^{-8}이었다. 그러나 이들을 협기상태하의 N-결핍배지에 옮겼을 때에는 잘 자라지 아니하였다. 이 접합체의 Agarose gel 전기영동 실험결과로 수용세포가 원래 가지고 있었던 pRD1플라스미드는 없어지고 공여세포의 RP4 :: Mu cts-prime 플라스미드만 남아있었음을 확인할수 있었다.

REFERENCES

1. Beringer, J.E., 1980. The development of *Rhizobium* genetics. J. of Gen. Microbiology. **116,** 1-7.
2. Bukhari, A.I. and D. Zipser, 1972. Random insertion of Mu-1 DNA with a single gene. Nature (London) *New Biol.* 236-243.
3. Dénarié, J., C. Rosenberg, B. Bergeron, C. Boucher, M. Michel and M. Barate de Bertalmio, 1976. RP4 :: Mu plasmids: obtention, transfer by conjugation into *Klebsiella pneumoniae, Pseudomonas solanacearum,* and *Rhizobium meliloti* and evaluation of potentials as a tool for *in vivo* genetic engineering of gram-negative bacteria. In DNA insertions. A.I. Bukhari, J. Shapiro, and S. Adhya (eds.), New York: Cold Spring Harbor Laboratory.
4. Dixon, R.A. and J.R. Postgate, 1972. Transfer of nitrogen fixation genes from *Klebsiella pneumoniae* to *E. coli.* Nature. **237,** 102-103.
5. Dunican, L.K. and A.B. Tierney, 1974. Genetic transfer of nitrogen fixation from *Rhizobium trifoli* to *Klebsiella aerogenes.* Biochem. and Biophysic. Resear., Comm. **57,** 62-72.
6. Faelen, M. and S. Toussaint, 1976. Bacteriophage Mu-1 : tool to transpose and localize bacterial genes. J. of Mol. Biol. **104,** 525-539.
7. Graaf, J. De, P.C. Kreuning and P. Van De Putte, 1973. Host controlled restriction and modification of bacteriophage Mu-promoted chromosome mobilization in *Citrobacter freundi. Mol. Gen. Genet.* **123,** 283-288.
8. Hennecke, H. and J.R. Mielenz, 1981. Molecular cloning of *Rhizobium japonicum* DNA in *E. coli* and identification of nitrogen fixation (*nif*) genes. In Genetic Engineering of Symbiotic Nitrogen Fixation. pp.41-53. J.M. Lyons, R.C. Valentine, D.A. Phillips, D.W. Rains and R.C. Huffaker(eds.), Plenum Press. New York and London.
9. Kado, C.I. and S.T. Liu, 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* **145,** 1365-1373.
10. Lee, I.Y., Y.J. Huh and Y.N. Lee, 1985. Transfer of RP4 :: Mu cts from *E. coli* to *R. leguminosarum.* The Korean *J. Microbiol.* **23,** 107-114.
11. MacNeil, T., W.J. Brill and M.M. Howe, 1978a. Bacteriophage Mu-induced deletions in a plasmid containing the *nif* genes of *Klebsiella pneumoniae. J. Bact.* **134,** 821-829.
12. MacNeil, T., D. MacNeil, G.P. Roberts, M. Supiano and W.J. Brill, 1978b. Fine-structure mapping and complementation analysis of *nif genes in Klebsiella pneumoniae. J. Bact.* **136,** 253-266.
13. Mazur, B., D., and R. Haselkorn, 1980. Identification of blue-green algae nitrogen fixation genes by using heterologous DNA hybridization probes. *Proc. Natl. Acad. Sci. USA.* **77,** 186-190.
14. Merrick, M., Filser, R. Dixon, C. Elmerich, L. Sibold and J. Houmard, 1980. The use of translocatable genetic ele-

ments to construct a fine-structure map of the *Klebsiella pneumoniae* nitrogen fixation (*nif*) gene cluster. *J. Gen. Microbiol.* **117,** 509-520.

15. Murooka, Y., N. Takizawa and T. Harada, 1981. Introduction of bacteriophage Mu into bacteria of various genera and intergeneric gene transfer by RP4 : : Mu. *J. Bact.* **145,** 358-368.
16. Nuti, M.P., A.A. Lepidi, R.K. prakash, R.A. Schilperoot, and F.C. Cannon, 1979. Evidence for nitrogen fixation genes on indigenous *Rhizobium* plasmids. *Nature.* (London). **282,** 533-535.
17. Pühler, A. and W. Klipp, 1981. Fine -structure analysis of the gene region of N_2-fixation (*nif*) of *pneumoniae.* In Biology of Inorganic Nitrogen and Sulfur. pp. 267-286. H. Boethe and A. Trebst(eds.), Berline: Springer-Verlag.
18. Ruvkun, G.B. and F.M. Ausubel, 1980. Interspecies homology of nitrogenase genes. *Proc. Natl. Acad. Sci. USA.* **77,** 191-195.
19. Takizawa, N., 1983. Intergeneric transfer of pullulanase gene between *Klebsiella aerogenes* and *E. coli* by RP4 : : Mu. Unpublished.
20. Taylor, A.I., 1963. Bacteriophage induced mutation in *E. coli. Proc. Natl. Acad. Sci. USA.* **50,** 1043-1051.
21. Wacek, T.J. and W.J. Brill, 1976. Simple, rapid assay for screening nitrogen -fixing ability in soybean. Crop Science. **16,** 517-522.

(Received Apr. 16, 1986)