

Construction of *Rhizobium*-*E. coli* Shuttle Vector Using Replication and Mobilization Function of Indigenous Multicopy Plasmid from *Rhizobium*

Cho Moo Je¹, Pyung Gyun Shin², Young Ju Choe²,

Kyu Young Kang² and Han Dae Yun²

Department of Biochemistry¹ and Agricultural Chemistry², Gyeongsang National University,
Chinju 660-701, Korea

Rhizobium Multicopy plasmid 의 복제 및 이주 기능을 이용한 *Rhizobium*-*E. coli* Shuttle Vector 구축

조무제¹ · 신평균² · 최영주² · 강규영² · 윤한대²

경상대학교 자연과학대학 생화학과¹, 농과대학 농화학과²

ABSTRACT: The vector, pGUR19, for *Rhizobium* gene manipulation, was constructed by combining the replication and mobilization function of indigenous multicopy plasmid from *Acacia* (*Robinia pseudoacacia* L.) *Rhizobia* sp86 with *E. coli* cloning vehicle, pBR322. The vector could be efficiently mobilized by RP4 *tra* function incorporated into chromosome of *E. coli* named SM10 and efficiently transferred to various gram negative hosts including *Rhizobium* and *Agrobacterium* by transformation. Mobilization frequency of the constructed vector was ranged from 1.2×10^{-2} (*E. coli* HB 101) to 4.6×10^{-4} (*A. tumefaciens* 15955) and transformation frequency was ranged from 5.4×10^{-7} (*E. coli* HB101) to 1.2×10^{-10} (*A. tumefaciens* 15955). The vector, pGUR19, was stably replicated and maintained in a variety of *Rhizobium* and *Agrobacterium*.

KEY WORDS □ *Rhizobium*, Shuttle vector, *Acacia Rhizobia* sp86, pBR322, Broad host range vector

Construction of genetically engineered effective *Rhizobium* has been attempted because of the economic importance, nitrogen fixation, of the genus, but the attempts have not been succeeded for moment mainly due to lack of suitable vectors for *Rhizobium* gene manipulation. The cloning vehicles useful in a variety of *Rhizobiaceas* family require not only broad host range replication origin but also their efficient conjugative transfer function. There are natural plasmids such as *IncP* plasmid RK2 (Ditta *et al.*, 1985; 1980), *IncW* plasmid pSa (Leemans *et al.*, 1982; Tait *et al.*, 1983), and *IncQ* plasmid RSF1010 (Bagdasarian *et al.*, 1981; Bagdasarian & Timmis, 1982; Nagahari &

Sakaguchi, 1987; Priefer *et al.*, 1985; Scherzinger *et al.*, 1984), which are useful in *Rhizobium* as well as a wide variety of gram negative bacteria. But many of them were not completely satisfactory for the purpose of *Rhizobium* gene manipulation, because of molecular weight, versatility of cloning sites and stability of the vectors in *Rhizobium* hosts that we are interested in. In this paper, we have constructed pGUR19 vector which comined the replication and mobilization function of indigenous multicopy plasmid from *Acacia Rhizobia* sp86 and *E. coli* vector pBR322. In addition, the mobilization and transformation characteristics and stability of the constructed vector pGUR19 in different

hosts were examined.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this experiment are listed in Table 1.

Media

E. coli *Rhizobium* and *Agrobacterium* were grown in L-broth (tryptone, 1%; yeast extract, 0.5%; NaCl, 0.5%; and glucose, 0.2%), AMA (mannitol, 1%; yeast extract, 0.1%; MgSO₄·7H₂O, 0.02%; K₂HPO₄, 0.05%; and FeCl₃, 4.88 mg/l) and TY-medium (tryptone, 1%; yeast extract, 0.5%; and CaCl₂ 0.09%), respectively. For the conjugation and transformation of *Rhizobium* and *Agrobacterium*, LY-medium (yeast extract, 0.5%; tryptone, 1%; NaCl, 0.5%; CaCl₂, 0.09%; and glucose, 0.2%) was used. Antibiotics were used at the following concentration unless otherwise indicated: streptomycin (Sm), 150 µg/ml; ampicillin (Ap), 100 µg/ml; tetracycline (Tc), 10 µg/ml.

Transformation

Transformation of *E. coli* was performed by the procedure described by Morrison (1977) and *Rhizobium* and *Agrobacterium* were transformed by the

so called freeze-thaw method described by Selvaraj *et al.* (1981).

Conjugation

Conjugation of the bacteria on the membrane filter was followed by the procedure described by Berry and Atherly (1984). About 10⁹ cells, each of the donor and recipient, were mixed and filtered the suspension onto 0.45 µm Millipore filters. The filters were incubated at 30°C on nonselective agar plates for 3-6 hours before the cells were resuspended and plated on selective medium.

Isolation of plasmids

Plasmids from *E. coli* were isolated from lysozyme and SDS lysed cells and purified by ultracentrifugation with CsCl-ethidium bromide (=1.58 g/cm³). The rapid detection of plasmid pattern in *E. coli* was followed by the alkali lysis method (Maniatis *et al.*, 1982), and the procedure described by Kado and Liu (1981) were adapted for the detection of plasmid in *Rhizobium* and *Agrobacterium*.

Restriction endonuclease digestion and ligation

All the other restriction enzyme digestions were carried out under the condition of the supplier's instruction (New England BioLabs). The ligation was carried out in ligation buffer (50 mM Tris-HCl,

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant genotype or phenotype	Source
<i>E. coli</i>		
SM10	Rec ⁻ derivative of C600 with RP4-2Tc::Mu integrated in the chromosome	19
S17.1	Rec ⁻ derivative of 294 with RP4-Tc::Mu Km::Tn7 in the chromosome	19
HB101	<i>pro Leu Thi lacY Sm^r endA recA hsrR hsrM</i>	5
<i>R. meliloti</i> 102F51	Wild type Nod ⁺ Nif ⁺ Sm ^r	Nitragine, USA
<i>R. fredii</i> USDA193	Wild type Nod ⁺ Nif ⁺ on "Peking cultivar"; Nod ⁺ Nif ⁻ on North American cultivars	H. Keyser, USDA
<i>R. leguminosarum</i> 897	Phe Trp Sm ^r	10
<i>A. tumefaciens</i> 15955	Octopine-type wild type	22
Plasmids		
pBR322	Ar ^r , Tc ^r	4
pRK290	Tc ^r	8
RP4	Ap ^r , Tc ^r , Km ^r	7
pASR186		This experiment

pH 8.0, 10 mM MgCl₂, 10 mM ATP, 40 mM dithiothreitol) using 1 μ g of restriction endonuclease digested plasmid DNA and 2 to 4 units of T₄-DNA ligase. The reaction mixture was incubated at 16°C for 16 hours for ligation (Maniatis *et al.*, 1982).

Estimation of plasmid stability

Stability of the constructed plasmid vector was determined by the procedure described by Prier *et al.* (1985). The single colony harboring the plasmid was inoculated into liquid media in the absence of antibiotics. Immediately after inoculation, bacteria was counted on non-selective medium. After culturing until the end of log phase, the culture was transferred to fresh new non-selective medium to continue the non-selective growth or plated onto non-selective agar plates. In each case, 100 colonies were tested for retention of the plasmid by the plasmid encoded antibiotic resistance marker and agarose gel electrophoresis.

RESULTS AND DISCUSSION

Isolation of pASR186 and pASR286 Plasmid

Several *Rhizobium* strains were screened for the presence of a small multicopy plasmid by the method of Eckhart (1978) or Kado and Liu (1981). In *Acacia Rhizobia* sp86 isolated from the *Acacia (Robinia pseudoacacia L.)* root nodules grown in southern part of Korea, these methods revealed relatively small three plasmids, approximately 15, 9 and 5kb in size, together with two larger extrachromosomal replicons (Fig. 1). The 15 and 5kb plasmids were named as pASR186 and pASR286, respectively and were purified by sucrose density gradient ultracentrifugation for the construction of vector for *Rhizobium* gene manipulation. The *Acacia Rhizobia* sp86 was sensitive to tetracycline, chloramphenicol, kanamycin, sulfonamide and neomycin, but resistant to streptomycin (150 μ g/ml) and ampicillin (25 μ g/ml). The pASR186 was observed to be transmissible by RP4 *tra* function but pASR286 was nontransmissible.

Restriction mapping of pASR 186

In order to construct restriction map, purified pASR186 DNA was digested with *Bam*HI, *Pst*I,

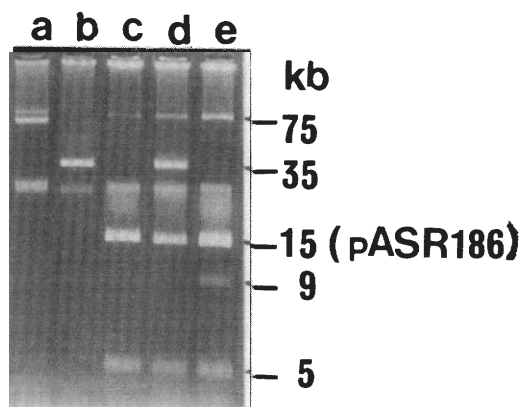


Fig. 1. Indigenous plasmid pattern of the isolated *Acacia Rhizobia* from *Acacia (Robinia Pseudoacacia L.)* root nodules; lane e, *Acacia Rhizobia* SP86.

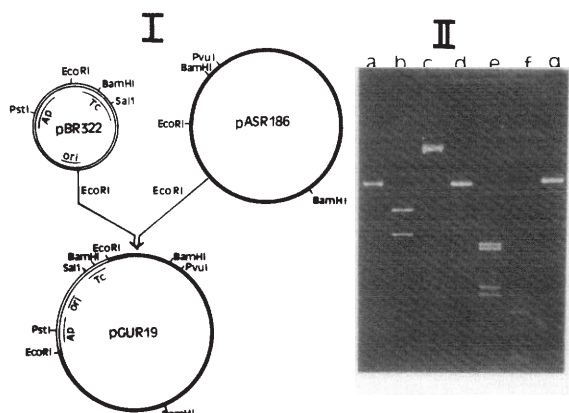


Fig. 2. Strategy for construction of *Rhizobium-E. coli* shuttle vector combining indigenous multicopy plasmid pASR186 from *Acacia Rhizobia* SP86 and *E. coli* plasmid vector pBR322, and physical maps of the pASR186 and the constructed pGUR19 vector(I).

Restriction pattern of pASR186(II): a, uncutted circular form; b, *Bam*HI; c, *Eco*RI; d, *Hind*III; e, *Hinc*II; f, *Pvu*II; g, *Sal*I.

*Eco*RI, *Hind*III, *Hinc*II, *Pvu*II and *Sal*I (Fig. 2-II). With the several double digestion analysis (data not shown), rough restriction map was constructed (Fig. 2-I).

Construction of vector

The strategy for construction of *Rhizobium-E. coli* shuttle vector with the replication and mobilization function from pASR186 and selection marker and cloning site from pBR322 is shown in Fig. 2-I. The pGUR19 vector was constructed by com-

binning *Eco*RI-linearized pBR322 with *Eco*RI-linearized and dephosphorylated pASR196. With the ligation mixture, *E. coli* SM10 was transformed and Ap^r and Tc^r transformants were selected for the pGUR19 vector. The selected transformants were analyzed for the plasmid sizing by agarose gel electrophoresis and correct orientation of the plasmid was analyzed by restriction digestion. The *E. coli* SM10 transformants which contain RP4-specific transfer function in chromosome (Simon *et al.*, 1982) and have correctly sized plasmid constructed from pASR186 and pBR322 were used for conjugation donor to *R. meliloti* 102F51.

R. meliloti 102F51 transconjugants having Ap^r and Tc^r were selected and the stable maintenance and replication of the introduced vector in *R. meliloti* host was analyzed by agarose gel electrophoresis. The constructed vector pGUR19 was observed to be stably maintained in *R. meliloti* 102F51 host without integration onto chromosome or formation of multimeric forms (Fig. 3). Therefore the vector was further characterized for the host range and stability in the different *Rhizobium* and *Agrobacterium* hosts.

Host Range

The mobilization of the pGUR19 vector into *R.*

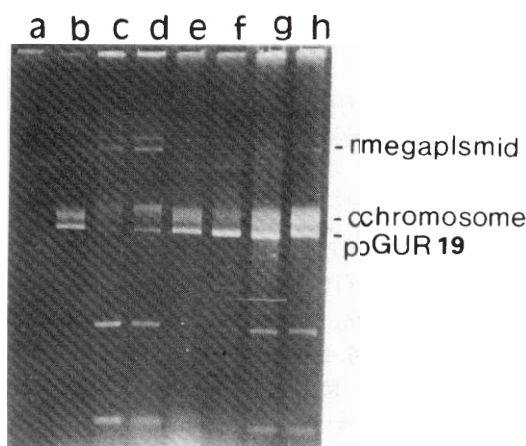


Fig. 3. Agarose gel electrophoretic patterns of different *Rhizobium* and *Agrobacterium* transconjugants containing constructed pGUR19 vector: a, *E. coli* HB101 (pGUR19); b, *A. tumefaciens* 15955 (pGUR19); c and d, *R. fredii* USDA193 (pGUR19); e and f, *R. meliloti* 102F51 (pGUR19); g and h, *R. leguminosarum* 897 (pGUR19).

meliloti 102F51, *R. leguminosarum* 897, *R. fredii* USDA193 and *A. tumefaciens* 15955 were tested with *E. coli* SM10 (pGUR 19) which contained RP4-specific *tra* function in chromosome for the mobilization of the vector. The vector could be mobilized into all the hosts tested with the mobilization frequency of 1.2×10^{-2} in *E. coli* HB 101 recipient and 4.6×10^{-4} in *A. tumefaciens* 15955. These mobilization frequencies of pGUR19 were slightly lower than those of pGUR9 constructed previously (unpublished data) combining replication and mobilization function from RSF1010 with selection marker and cloning sites from pACYC184, but comparable to those of pRK2501 or pRK249 (Ditta *et al.*, 1985) in most hosts tested (Table 2). The vector could also be transferred to a variety of *Rhizobium* and *Agrobacterium* by transformation. Transformation frequencies were ranged from 5.4×10^{-7} in *E. coli* HB101 to 1.2×10^{10} in *A. tumefaciens*. These frequencies were slightly lower than those of pGUR9 and other broad host range vectors such as pSUP104 and pRK290, but usable in some *Rhizobium* hosts (Table 3).

Stability

Stability of the pGUR19 vector was tested for the stable maintenance in *Rhizobium* and *Agrobacterium* hosts without selective pressure of antibi-

Table 2. Host range and mobilization frequencies of pGUR19 vector.

Recipient strain	Mobilization frequency for donor strain ^a
	<i>E. coli</i> SM10 (pGUR19)
<i>E. coli</i> HB101	1.2×10^{-2}
<i>R. meliloti</i> 102F51	1.7×10^{-2}
<i>R. fredii</i> USDA193	2.3×10^{-3}
<i>R. leguminosarum</i> 897	5.6×10^{-3}
<i>A. tumefaciens</i> 15955	4.6×10^{-4}

^a Mobilization frequencies into these recipients were determined with the mobilizing donor strain *E. coli* SM10. All the mating were performed on membrane filter (Bollivar *et al.*, 1977). Transconjugants were selected for the acquisition of one of the plasmid-encoded resistance marker, tetracycline with the concentration of 50 micrograms per milliliter in *E. coli*, 25 micrograms per milliliter in *Rhizobium* and *Agrobacterium* recipients.

Table 3. Transformation frequencies of pGUR19 vector in different hosts.

Host strains	Transformation frequency ^a
<i>E. coli</i> HB101	5.4×10^{-7}
SM10	3.7×10^{-7}
S17.1	6.2×10^{-7}
<i>R. meliloti</i> 102F51	3.2×10^{-8}
<i>R. fredii</i> USDA193	6.4×10^{-9}
<i>R. leguminosarum</i> 897	2.1×10^{-9}
<i>A. tumefaciens</i> 15955	1.2×10^{-10}

^aTransformation frequencies were expressed per viable cell and the transformants were selected with tetracycline resistance with the concentration 15 micrograms per milliliter in *E. coli* and 25 micrograms in *Rhizobium* and *Agrobacterium* host.

otics. The vector was fairly stable in *R. meliloti* 102F51 and *R. fredii* USDA193 but unstable in *R. leguminosarum* 897 and *A. tumefaciens* 15955 (Table 4). Loss of the plasmid vector or formation of

Table 4. Stability of pGUR 19 vector in different hosts.

Host strain	Antibiotics conc. ^a	% Retention of marker at generation		
		20	40	80
<i>R. meliloti</i> 102F51	Tc25	100	100	100
<i>R. leguminosarum</i> 897	Tc25	85	63	51
<i>A. tumefaciens</i> 15955	Tc25	91	74	59
<i>E. coli</i> HB101	Tc50	100	100	100

^aSubscript indicates the concentration of antibiotics in micrograms per milliliter; Tc, tetracycline and stability of the vector plasmid was tested as described in the text.

multimeric form was observed in some transconjugants in the unstable hosts after several generations. The stability of the pGUR19 in *R. meliloti* 102F51 and *R. fredii* USDA193 hosts was observed to be comparable to other broad host range vector with stable maintenance of the plasmid after 60 generations.

적 요

Acacia Rhizobia sp86 으로부터 15 kb 의 multicopy tansmissible plasmid pASR 186 을 분리하고 pASR 186 의 replication 및 mobilization function 과 *E. coli* plasmid vector pBR332 의 selection marker 및 cloning site 를 연결하여 *Rhizobium* 숙주에서 안정하게 복제 및 유지되는 vector 구축을 시도한 결과 목적하는 pGUR 19 을 얻었다. pGUR 19 는 RP 4 tra 기능을 염색체에 도입하여 만든 *E. coli* SM10 의 도움으로 여러 종류의 *Rhizobium* 및 *Agrobacterium* 에 접합에 의하여 전이될 수 있었으며 형질전환에 의한 도입도 가능하였다.

여러 종류의 Rec⁺ *Rhizobium* 및 *Agrobacterium* 숙주에 도입된 pGUR 19 vector 의 안정성을 조사한 결과 *R. leguminosarum* 897 및 *A. tumefaciens* 15955 에서는 비교적 불안정하였으나 *R. meliloti* 102F51 및 *R. fredii* USDA 193 에서는 매우 안정하였다.

ACKNOWLEDGMENT

We thank A. Pühler for kind providing *E. coli* SM10 and H. Keyser for *R. fredii* USDA193 strain.

This research was supported in part by the 1985 Genetic Engineering Research Grant from Ministry of Education.

REFERENCES

1. Bagdasarian, M., R. Lurz, B. Ruckert, F.C.H. Frandlin, M. M. Bagdasarian, J. Frey and K.N. Timmis, 1981. Specific purpose plasmid cloning vectors. II. Broad host range, high copy number RSF1010-derived vectors and host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237-247.
2. Bagdasarian, M. and K.N. Timmis, 1982. Host vector systems for gene cloning in *Pseudomonas*. *Curr. Top. Microbial. Immunol.* **96**, 46-47.
3. Berry, J.O. and A.G. Atherly, 1984. Induced plasmid-genome rearrangement in *Rhizobium japonicum*. *J. Bacteriol.* **157**, 218-224.
4. Bolivar, R., R.L. Rodriguez, P.J. Green, M.C. Betlach, H.C. Heynecker, H.W. Boyer, J.H. Croso and S. Falkow, 1977. Construction and

- characterization of new cloning vehicles. II. A multipurpose gene cloning system. *Gene* **2**, 95-113.
5. Boyer, H.W. and D. Rouland-Dussoix, 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. *J. Mol. Biol.* **41**, 459-472.
6. Ditta, G., T. Schmidhauser, E. Yakobson, Y. Lu, X-W. Liang, D.R. Finlay, D. Guiney and D.R. Helinski, 1985. Plasmids related to the broad host range vector pRK290 useful for gene cloning and for monitoring gene expression. *Plasmid* **13**, 149-153.
7. Ditta, G., S. Stanfeld, D. Corbin and D.R. Helinski. 1980. Broad host range DNA cloning system from gram negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. (USA)*. **77**, 7347-7351.
8. Eckhardt, T. 1978. A rapid method for ⁺ e identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* **1**, 584-588.
9. Johnston, A.W.B. and J.E. Beringer, 1976. Pea root nodules containing more than one *Rhizobium* species. *Nature* **263**, 502-504.
10. Kado, C.L. and S.T. Liu, 1981. Rapid Procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* **145**, 1365-1373.
11. Leemans, J., J. Langenaking, H. De Greve, R. Deblaere, M. van Montagu and J. Schell. 1982. Broad host range cloning vectors derived from the *Inc W* Plasmid pSa. *Gene* **19**, 361-364.
12. Maniatis, R., E.F. Fritsch and J. Sambrook, 1982. Molecular cloning. Cold Spring Harbor Laboratory. Cold Spring Harbor. N.Y.
13. Morrison, P.A., 1977. Transformation in *E. coli*: Cryogenic preservation of competent cells. *J. Bacteriol.* **132**, 349-351.
14. Nagahari, K. and K. Sakaguchi, 1978. RSF1010 plasmid as a potentially useful vector in *Pseudomonas* species. *J. Bacteriol.* **133**, 1527-1529.
15. Priefer, U.B., R. Simon and A. Pühler, 1985. Extension of the host range of *E. coli* vectors by incorporation of RSF1010 replication and mobilization functions. *J. Bacteriol.* **163**, 324-330.
16. Scherzinger, E., M.M. Bagdasarian, P. Scholz, B. Ruckert and M. Bagdasarian, 1984. Replication of the broad host range plasmid RSF1010: Requirement for three plasmid encoded protein. *Proc. Natl. Acad. Sci. (USA)*. **81**, 654-658.
17. Selvaraj, G. and V.N. Iyer, 1981. Genetic transformation of *Rhizobium meliloti* by plasmid DNA. *Gene* **15**, 279-283.
18. Simon, R., U. Priefer and A. Pühler, 1982. A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in gram negative bacteria. *Biotechnology* **1**, 784-791.
19. Tait, R.C., T.J. Close, R.C. Lunquist, M. Hagiya, R.L. Rodriguez and C.I. Kado, 1983. Construction and characterization of a versatile broad host range cloning system for gram negative bacteria. *Biotechnology* **1**, 269-275.
20. Van Vliet, F., B. Silva, M. van Montagu and J. Schell, 1978. Transfer of RP4: Mu plasmids to *Agrobacterium tumefaciens*. *Plasmid* **1**, 446-455.
21. Zaenen, I., N. van Larebeke, H. Teachy, M. van Montagu and J. Shell, 1974. Supercoiled circular DNA in crown gall inducing *Agrobacterium* strains. *J. Mol. Biol.* **96**, 109-127.
- (Received Feb. 24, 1989)