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

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Rhizobium Muliticopy plasmid의 복제 및 이주 기능을 이용한 *Rhizobium-E. coli* Shuttle Vector 구축

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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 *Inc*P plasmid RK2 (Ditta *et al.*, 1985; 1980), *Inc*W plasmid pSa (Leemans *et al.*, 1982; Tait *et al.*, 1983), and *Inc*Q 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 bacteial 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%; $MgSO_4.7H_2O$, 0.02%; K_2HPO_4, 0.05%; and $FeCl_3$, 4.88 mg/*l*) and TY-medium (tryptone, 1%; yeast extract, 0.5%; and $CaCl_2$ 0.09%), respectively. For the conjugation and transformation of *Rhizobium* and *Agrobacterium*, LY-medium (yeast extract, 0.5%; tryptone, 1%; NaCl, 0.5%; $CaCl_2$, 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 10^9 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/cm^3). 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
E. coli		
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	*pro Leu Thi lac*Y Sm^r *end*A *rec*A *hsr*R *hsr*M	5
R. meliloti 102F51	Wild type Nod$^+$ Nif$^+$ Sm^r	Nitragine, USA
R. fredii USDA193	Wild type Nod$^+$ Nif$^+$ on "Peking cultivar"; Nod$^+$ Nif$^-$ on North American cultivars	H. Keyser, USDA
R. leguminosarum 897	Phe Trp Sm^r	10
A. tumefaciens 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 $MgCl_2$, 10 mM ATP, 40 mM dithiothreitol) using 1 μg of restriction endonuclease digested plasmid DNA and 2 to 4 units of T_4-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 Priefer *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 μg/ml) and ampicillin (25 μg/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, *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).

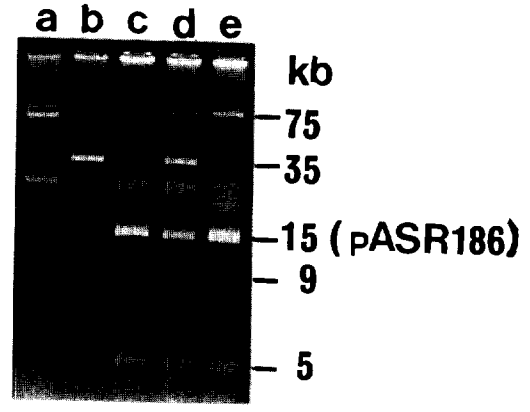

Fig. 1. *Indigenous plasmid pattern of the isolated Acacia Rhizobia from Acacia (Robinia Pseudoacacia L) root nodules; lane e, Acacia Rhizobia SP86.*

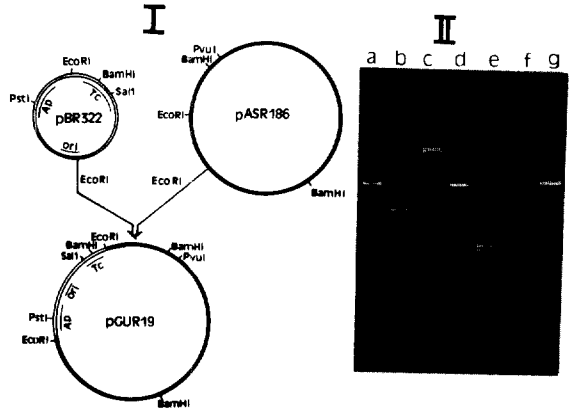

Fig. 2. *Stratagy for construction of Rhizobium-E. coli shuttle vector combining indigenous multycopy plasmid pASR186 from Acacia Rhizobia SP86 and E. coli plasmid vector pBR322, and physical maps of the pASR186 and the constructed pGUR19 vector(I).*
Restriction pattern of pASR186(II): a, uncutted circular form; b, *Bam*HI; c, *Eco*RI; d, *Hind*III; e, *Hinc*II; f, *Pvu*II; g, *Sal*I.

Construction of vector

The stratagy 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-

bining *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. 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 recepient and 4.6×10^{-4} in *A. tumefacienns* 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).

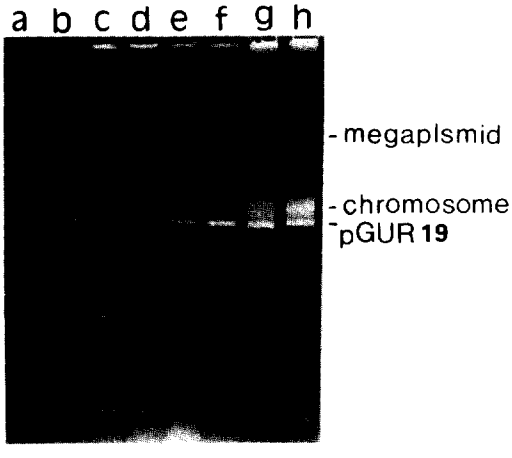

Fig. 3. *Agarose gel electrophoretic patterns of different Rhizobium and Agrobacterium transconjugants containing constructed pGUR19 vector: a, E. coli HB101 (pGUR19); b, A. tumefaciens 15955 (pGUR19); c and d, R. fredii USDA193 (pGUR19); e and f, R. meliloti 102F51 (PGUR19); g and h, R. leguminosarum 897 (pGUR19).*

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]
	E. coli SM10 (pGUR19)
E. coli HB101	1.2×10^{-2}
R. meliloti 102F51	1.7×10^{-2}
R. fredii USDA193	2.3×10^{-3}
R. leguminosarum 897	5.6×10^{-3}
A. tumefaciens 15955	4.6×10^{-4}

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

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

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

[a]Transformation frequencies were expressed per viable cell and the transformants were selected with tetracycline resistance with the concentration 15 micrograms per mililiter in *E. coli* and 25 micrograms in *Rhizobium* and *Agrobacterium* host.

Table 4. *Stability of pGUR19 vector in different hosts.*

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

[a]Subscript indicates the concentration of antibiotics in micrograms per mililiter: Tc, tetracycline and stability of the vector plasmid was tested as described in the text.

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 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 sp86으로부터 15kb의 multicopy tansimissible plasmid pASR186을 분리하고 pASR186의 replication 및 mobilization function과 *E. coli* plasmid vector pBR332의 selection marker 및 cloning site를 연결하여 *Rhizobium* 숙주에서 안정하게 복제 및 유지되는 vector 구축을 시도한 결과 목적하는 pGUR19을 얻었다. pGUR19는 RP4 *tra* 기능을 염색체에 도입하여 만든 *E. coli* SM10의 도움으로 여러 종류의 *Rhizobium* 및 *Agrobacterium*에 접합에 의하여 전이될 수 있었으며 형질전환에 의한 도입도 가능하였다.

여러 종류의 Rec$^+$ *Rhizobium* 및 *Agrobacterium* 숙주에 도입된 pGUR19 vector의 안정성을 조사한 결과 *R. leguminosarum* 897 및 *A. tumefaciens* 15955에서는 비교적 불안정하였으나 *R. meliloti* 102F51 및 *R. fredii* USDA193에서는 매우 안정하였다.

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