INTEGRATION OF A PLASMID CARRYING THE *attP* SITE INTO THE CHROMOSOME OF *RHIZOBIUM* INSENSITIVE TO THE LYSOGENIC PHAGE INFECTION

TOSHIKI UCHIUMI,* SHIRO HIGASHI, AND MIKIKO ABE

Department of Biology, Faculty of Science, Kagoshima University,
Kagoshima 890, Japan

(Received June 2, 1993)

Fourteen strains belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* were investigated for susceptibility to infection by a lyso-genic phage (phage ϕ U) from *Rhizobium leguminosarum* biovar *trifolii*. All strains tested were resistant to phage ϕ U infection; however, the phage was able to adsorb to *R. leguminosarum* biovar *viciae* strain K5. Plasmid pCI6 carrying the attachment site (*attP*) of phage ϕ U was introduced into all these strains by conjugation. The tetracycline resistance (*Tc*^r) gene on pCI6 was used as a selectable marker. When *R. leguminosarum* biovar *viciae* strain K5 was used as a recipient, *Tc*^r transconjugants appeared at high frequency (4.41×10^{-3}). Southern hybridization with the *attP* fragment of phage ϕ U indicates that pCI6 is integrated into the chromosome of 10 transconjugants. Furthermore, the restriction map of pCI6 integration region on the chromosome of strain K5 was shown to be identical to that of *R. leguminosarum* biovar *trifolii* strain 4S which was the original host strain of phage ϕ U.

Many different wide host range cloning vectors have contributed to the molecular genetic analysis of the *Rhizobium*-legume symbiosis (5, 22, 26, 27). Although interactions among genes need to be investigated, it is difficult to stably maintain multiple plasmids in a *Rhizobium* cell. Especially during nodulation, loss of vectors and their cloned genes are a serious problem for genetic analysis of *Rhizobium*. Incompatibility between recombinant plasmids and the endogenous plasmids in *Rhizobium* is also thought to occur. For accurate analysis of gene expression, stable and efficient expression of the cloned gene(s) will be important.

One effective means to overcome these problems is to integrate the cloned genes into the chromosome of *Rhizobium*. Marker exchange (31) and homologous

* Address reprint requests to: Mr. Toshiki Uchiumi, Department of Biology, Faculty of Science, Kagoshima University, 1-21-35 Korimoto, Kagoshima 890, Japan.

recombination using suicide plasmids (36) were developed and applied to the genetic investigations of *Rhizobium*. The repeat sequence of *Bradyrhizobium japonicum* may be useful for the integration of cloned genes into the chromosome (2). Kunnimalaiyaan et al. (24) reported integration of the cloned *hup* (uptake-hydrogenase) gene into the chromosome of *Rhizobium* strain G36-84. Homologous sequences were created both on the *hup* gene-containing cosmid pHU52 and on the chromosome of *Rhizobium* strain G36-84 by introducing transposon Tn5 into each of them. Homologous recombination between pHU52::Tn5 and chromosome::Tn5 occurred at the Tn5 site when plasmid pPH1JI, which was incompatible with pHU52, was transferred into the *Rhizobium* cell. The integrated *hup* gene was stably maintained and expressed under symbiotic conditions (23). Hermes et al. (12) reported another plasmid vector system which integrated into the chromosome of *Rhizobium meliloti*. They constructed the system with a suicide plasmid vector (pSUP202 or pSUP106) carrying the attachment site (*attP*) of lysogenic phage 16-3. The plasmids carrying *attP* were integrated into the chromosome of *R. meliloti* by site specific recombination between *attP* and *attB* (attachment site of host bacteria). The system is elaborate and suitable for maintaining cloned genes stably in *Rhizobium* cells. However, the application of the system is limited to some *R. meliloti* strains which are susceptible to phage 16-3 infection because this system requires special helper phage infection.

Previously, we cloned the *attP* fragment of lysogenic phage ϕ U, which lysogenizes *Rhizobium leguminosarum* biovar *trifolii* strain 4S, into suicide vector pSUP 202 (39). The recombinant plasmid pCI6 could be transferred from *Escherichia coli* S17-1 to *R. leguminosarum* biovar *trifolii* strain 4S and its lysogenic strain 4S (ϕ U) by conjugation. Plasmid pCI6 could integrate into the *Rhizobium* chromosome at high frequency by site specific recombination between *attP* on pCI6 and *attB* on the bacterial chromosome, the same mechanism as phage ϕ U integration, and could be stably maintained in *Rhizobium* under conditions of selective pressure. After integration, the *attP* fragment on pCI6 was divided into the two fragments of *attL* and *attR* (left and right border), and these two fragments could be detected by Southern hybridization with the labelled *attP* probe (39). No helper phage was required for pCI6 integration into the chromosome of *Rhizobium*.

In this paper, we report integration of plasmid pCI6 into the chromosome of a *Rhizobium* strain which is not susceptible to phage ϕ U infection. The restriction map of the *attB* region for phage ϕ U integration on the *Rhizobium* chromosome is also reported.

MATERIALS AND METHODS

Phage, plasmid and bacterial strains. Lysogenic phage ϕ U lysogenizes wild-type *Rhizobium leguminosarum* biovar *trifolii* strain 4S. Isolation and characterization of phage ϕ U were described previously (38). Plasmid pCI6 was constructed by insertion of the 6.0 kb *Eco*RI fragment (*attP* fragment) of phage ϕ U into the *Eco*RI

site of the suicide vector pSUP202 (39). Plasmid pCI6 can integrate into the chromosome of strain 4S and its lysogenic strain 4S(ϕ U) by site specific recombination. *R. leguminosarum* biovar *trifolii* strain CI101 is one of the pCI6-integrated derivatives of strain 4S (39). Bacterial strains used as conjugation recipients are listed in Table 1. *Escherichia coli* S17-1 harbouring pCI6 was used as a conjugation donor.

Culture conditions. *Rhizobium* and *Bradyrhizobium* strains were maintained on mannitol-yeast agar medium (19). For bacterial mating, plasmid isolation and phage sensitivity tests, they were cultured in TY medium (4). *Agrobacterium* strains were grown in LB medium (21). For screening of *Rhizobium* and *Bradyrhizobium* transconjugants after mating, Sherwood's minimal agar plates (35) were used. MinA agar plates (6) were employed for screening of *Agrobacterium* transconjugants. *E. coli* S17-1 harbouring pCI6 were cultured in LB medium containing ampicillin (Amp, 50 μ g/ml) and tetracycline (Tc, 20 μ g/ml).

Phage sensitivity test. Phage ϕ U lysate was prepared by phage induction of lysogenic strain *R. leguminosarum* biovar *trifolii* 4S(ϕ U) with mitomycin C (38). Phage ϕ U lysate was spotted on a soft agar (0.7% agar) layer containing the test bacterial strain overlaid on a 1.5% agar plate. Plaque formation on the lawn of the test bacterial strain was observed after 24–72 h incubation at 28°C.

Phage induction test. The test bacteria were cultured overnight at 28°C in TY liquid medium. Thirty microliters of the culture was inoculated into 3 ml of fresh TY medium. After 6 h incubation, mitomycin C was added at a final concentration of 0.1 μ g/ml followed by overnight incubation. Cells were removed by centrifugation ($5,000 \times g$, 3 min) and the supernatant was filtrated through a membrane filter (0.22 μ m, HAWPO1300, Millipore, U.S.A.), and then spotted onto soft agar overlays containing *R. leguminosarum* biovar *trifolii* strain 4S to detect phage.

Phage ϕ U adsorption test. The adsorption rate of phage ϕ U to the bacterial strains was determined by free phage titration (3). Bacterial cells (ca. 10^8 colony-forming units) were inoculated into 10 ml of fresh TY- (for *Rhizobium* and *Bradyrhizobium* strains) or LB- (for *Agrobacterium* strains) liquid medium. Phage ϕ U lysate (ca. 10^7 plaque-forming units) was added and the culture was incubated at 30°C with gentle shaking. Samples were withdrawn at regular time intervals and free phage were titrated on *R. leguminosarum* biovar *trifolii* strain 4S as indicator.

Bacterial matings. Donor *E. coli* S17-1 (pCI6) was cultured in LB medium containing Tc and Amp for 24 h. Recipient *Rhizobium* and *Agrobacterium* strains were grown in TY and LB medium for 24 h, respectively. The *Bradyrhizobium* strain was grown in TY medium for 48 h, before use as a recipient. Donor and recipient cells (100 μ l of each) were mixed and washed twice with water. The washed bacteria were suspended in 100 μ l of water, and 50 μ l of the mixture was spotted onto a membrane filter (HAWPO1300, Millipore) on a TY agar plate. After overnight incubation at 28°C, the bacteria grown on the filter were collected and washed twice with water. The bacterial mixture was resuspended with water and plated onto Sherwood's minimal medium (for *Rhizobium* and *Bradyrhizobium*)

or MinA agar medium (for *Agrobacterium*) containing 20 µg/ml of Tc for selection of transconjugants. Frequency of transconjugants appearance was calculated after 4 days incubation at 28°C.

Isolation of DNA and restriction endonuclease digestion. Plasmids were isolated from *E. coli* according to Hattori et al. (10). Total cellular DNA from *Rhizobium* strains were prepared as described previously (7,14). Restriction enzyme digestions of DNA were as described by Sambrook et al. (33).

Agarose gel electrophoresis and Southern hybridization. The conditions for agarose gel electrophoresis were described elsewhere (39). Plasmid pCI6, the cloned *attP* fragment (6.0 kb *EcoRI* fragment) of phage ϕ U, and pSUP202 (36) were labelled by random priming with Klenow fragment in the presence of digoxigenin (8) and used as probes for Southern hybridization. Hybridization conditions and colorimetric detection of hybridized probes were performed according to the manufacturer's instructions (Boehringer Mannheim, Germany).

RESULTS

Susceptibility of Rhizobium, Bradyrhizobium, and Agrobacterium strains to phage ϕ U infection

Phage ϕ U lysate was spotted on top agar containing each of the bacteria listed in Table 1 and plaque formation was observed. The phage titer of the lysate (ca. 10^{10} plaque-forming units/ml) was high enough to form a distinguishable clear zone on a lawn of bacteria which were susceptible (sensitive) to phage ϕ U infection. *R. leguminosarum* biovar *trifolii* strains 4S and 4S(ϕ U) were used as sensitive and resistant controls, respectively. All bacterial strains used as pCI6 recipients were resistant to phage ϕ U infection.

Adsorption of phage ϕ U

Adsorption experiments were performed and typical profiles are shown in Fig. 1. Phage ϕ U efficiently adsorbed to *R. leguminosarum* biovar *trifolii* strain 4S, which is the original host strain of phage ϕ U. Viable phage progeny became apparent after 4 h incubation and increased more than 10-fold above the initial phage titer after 6 h incubation. Phage ϕ U adsorbed to *R. leguminosarum* biovar *viciae* strain K5 with kinetics similar to adsorption to strain 4S within the first 2 h of incubation; however, viable phage progeny were not detected with strain K5 even after 6 h incubation. Phage ϕ U could not adsorb to the other strains used as pCI6 recipients, as exemplified by *Agrobacterium tumefaciens* LBA4404.

Plasmid pCI6 transfer to strains of Rhizobiaceae which are resistant to phage ϕ U

E. coli S17-1 harbouring pCI6 was mated with *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* strains which were resistant to phage ϕ U infection. The tetracycline resistance gene (Tc^r) on pCI6 was used as a selectable marker. Tc^r transconjugants arose only from *R. leguminosarum* biovar *viciae* K5 as a recipient at the high

Table 1. Relevant characteristics of members of the Rhizobiaceae tested for ϕ U susceptibility.

Strain ^a	Susceptibility to phage ϕ U ^b	Frequency of pCI6 integration ^c	Reference or sources
<i>Rhizobium leguminosarum</i>			
biovar <i>trifolii</i> 4S	S	4.40×10^{-3}	(13)
biovar <i>trifolii</i> 4S(ϕ U)	R	2.94×10^{-4}	(38, 39)
biovar <i>trifolii</i> 0403	R	—	(1)
biovar <i>trifolii</i> A6R1	R	—	T.F.A.C. ^d
biovar <i>trifolii</i> A602	R	—	T.F.A.C. ^d
biovar <i>viciae</i> K5	R, A	4.41×10^{-3}	(18)
biovar <i>viciae</i> A400	R	—	T.F.A.C. ^d
biovar <i>phaseoli</i> CNPAF150	R	—	Dr. S. A. Ricardo ^e
biovar <i>phaseoli</i> RM1	R	—	(34)
<i>Rhizobium meliloti</i> 1021	R	—	(29)
<i>Rhizobium meliloti</i> A703	R	—	I.A.M. ^f
<i>Rhizobium fredii</i> USDA193	R	—	(20)
<i>Bradyrhizobium japonicum</i> USDA110	R	—	(25)
<i>Agrobacterium tumefaciens</i> A136	R	—	(37)
<i>Agrobacterium tumefaciens</i> LBA4404	R	—	(15)
<i>Agrobacterium rhizogenes</i> NIAES1724	R	—	(16)

^a All strains except for *R. leguminosarum* biovar *trifolii* strains 4S and 4S(ϕ U) were tested as recipients.

^b S, sensitive; R, resistant; A, adsorptive.

^c The frequency of pCI6 integration in strains 4S and 4S(ϕ U) was reported in Uchiumi et al. (39). —, transconjugant did not appear.

^d *Rhizobium* collection at the Tokachi Federation of Agriculture Cooperative, Tokachi, Japan.

^e Department of Plant Pathology, University of Wisconsin-Madison.

^f Culture collection at the Institute of Applied Microbiology, Tokyo, Japan.

frequency of 4.41×10^{-3} (Table 1). No transconjugant arose from the other strains of Rhizobiaceae. It was expected that pCI6 integrated into the chromosome or autonomously replicated as a plasmid in the transconjugants. Another possibility was that strain K5 was already lysogenized with phage ϕ U. If strain K5 was a lysogenic strain, this strain might be resistant to phage ϕ U infection and pCI6 could integrate into the chromosome of the strain, the same as in the case of strain 4S (ϕ U). However, phage induction test and genomic Southern hybridization using total phage ϕ U DNA as a probe showed that strain K5 did not have a ϕ U prophage in its genome. Ten transconjugants arising from recipient strain K5 were randomly selected and referred to as CIK50 series.

Site of pCI6 integration on chromosome

Total cellular DNAs from transconjugant CIK50 series were analyzed by 0.7% agarose gel electrophoresis, and Southern blot was hybridized with a labelled *attP* probe. Fragmented chromosomal DNA and at least two large plasmids were detected on agarose gel stained with ethidium bromide; however, no DNA corresponding in size to plasmid pCI6 was detected (data not shown). The labelled *attP*

Fig. 1. Phage ϕ U adsorption to different species of Rhizobiaceae.

Bacterial cells and phage ϕ U were mixed and kept at 30°C. Free phage were titrated at the times indicated. N_0 , initial phage titer; N , free phage titer in each sample. ●, *R. leguminosarum* biovar *trifolii* strain 4S; ▲, *R. leguminosarum* biovar *viciae* strain K5; ■, *A. tumefaciens* strain LBA4404.

probe hybridized with fragmented chromosomal DNA and did not hybridize with the two large plasmids (data not shown). These results indicate that pCI6 is integrated into the chromosome of parent strain K5.

Total cellular DNAs prepared from transconjugant CIK50 series and strain CI101 (one of the pCI6-integrated strains derived from *R. leguminosarum* biovar *trifolii* strain 4S), were digested with restriction endonuclease *Eco*RI, separated on 0.7% agarose gel, and hybridized with *attP* and pSUP202 probes. Because the hybridization patterns of CIK50 series were completely identical, the results of strain CIK51 are shown in Fig. 2 representing for transconjugant CIK50 series. Plasmid pCI6 yielded two fragments corresponding to the phage ϕ U *attP* fragment (6.0 kb) and suicide plasmid pSUP202 (ca. 8 kb) by *Eco*RI digestion (Fig. 2, *Eco*RI digests, lane pCI6. See also Fig. 5). Two hybridizing fragments detected in strain CI101 by *attP* probe (Fig. 2, lane CI101) have already been identified as *attL* and *attR* (39). The length of two fragments detected in strain CIK51 by *attP* probe (Fig. 2, lane CIK51) were indistinguishable from the two fragments (*attL* and

Fig. 2. Agarose gel electrophoresis and Southern hybridization of *Eco*RI-digested DNA from *Rhizobium* strains.

Total cellular DNA from strains 4S, CI101, K5 and CIK51, and plasmid pCI6 were digested with *Eco*RI and separated on 0.7% agarose gel. Southern blotted filters were hybridized sequentially with the *attP* fragment of phage ϕ U then with pSUP202. Hybridized fragments are indicated by arrowheads ($\blacktriangle$). *Hind*III-digested λ DNA was used as the size marker (lane M).

attR) observed in strain CI101. When pSUP202 was used as a probe, a DNA fragment corresponding to pSUP202, which was cut out from pCI6-integrated chromosome, was detected (Fig. 2, lanes CI101 and CIK51. See also Fig. 5). These results of the hybridization experiments suggest that pCI6 is integrated intact into the same location on the chromosome in strains CIK51 and CI101.

Construction of the restriction map of the attB region and the chromosome into which pCI6 is integrated

Total cellular DNA from strain CI101 was digested with *Eco*RI, *Hind*III, *Pst*I and *Bam*HI, and Southern blots were hybridized with labelled pCI6 probe. Because the *attP* fragment on pCI6 splits into the two chimeric DNA junction (*attL* and *attR*) containing parts of the *attP* fragment and adjacent chromosomal DNA after integration, two new fragments must be detected in strain CI101 whereas *attP* fragment must be missing in the strain. By comparison to the restriction fragments of pCI6, the hybridizing fragments were identified as internal pCI6 fragments or as *attL* and *attR* (indicated by open and closed arrowheads in Fig. 3. See also Fig. 5).

Fig. 3

Fig. 4

In *EcoRI* digestion, the 6.0 kb *attP* fragment (Fig. 3, *EcoRI*, the lower band in lane pCI6) was missing whereas two fragments of 8.0 kb and 5.6 kb were detected in strain CI101 (Fig. 3, *EcoRI*, lane CI101). The 8.0 kb chimeric fragment (indicated by an arrowhead with asterisk) electrophoretically overlapped with pSUP202 cut out from pCI6-integrated chromosome (see also Fig. 2). The *attP* site locates on the 9 kb *HindIII* fragments of pCI6 (Fig. 3, *HindIII*, the upper band in lane pCI6). This fragment split and generated two fragments of 9 kb and 2.9 kb *HindIII* fragments in strain CI101 (Fig. 3, *HindIII*, lane CI101), although the chimeric 9 kb *HindIII* fragment (indicated by an arrowhead with asterisk) was not electrophoretically distinguishable from the *HindIII* fragment of pCI6. Two chimeric fragments of 5.0 kb and ca. 1 kb were detected in DNA from strain CI101 digested with *PstI* (indicated by open and closed arrowheads in Fig. 3, *PstI*, lane CI101). The 5.0 kb *PstI* fragment (indicated by an open arrowhead in Fig. 3, *PstI*), one of the chimeric fragments, was assigned as the left border (*attL*) even though complete genetic and physical maps of phage ϕ U and ϕ U prophage had not yet been constructed. The *BamHI* site is unique on pCI6 so that only two fragments of 20 kb and 12 kb were detected in DNA from strain CI101 digested with *BamHI*. It was deduced that the *attB* site must be located on an 18 kb *BamHI* chromosomal fragment of strain 4S, because the length of linearized pCI6 was approximately 14 kb while the two fragments total 32 kb.

Double restriction endonuclease digests of total cellular DNA from strains CIK51 and CI101 were hybridized with the *attP* probe (Fig. 4). The hybridization patterns were completely identical in both strains. This result also supports the conclusion that the integration sites (*attB*) for pCI6 on the chromosome of strains K5 and 4S are in the same location. Using *attP* as a probe, the DNA fragments corresponding to *attL* and *attR* (indicated by open and closed arrowheads in Fig. 4) were easily identifiable based on the restriction map of pCI6; however, it could not be decided which of the two fragments corresponds to *attL* in *BamHI-EcoRI*

Fig. 3. Detection of *attL* and *attR* fragments in strain CI101.

Restriction endonuclease-digested DNA of strain CI101 was hybridized with digoxigenin-labelled pCI6. Then, hybridized fragments detected in strain CI101 were compared with the restriction fragments of pCI6. The DNA fragments identified as containing *attL* and *attR* are indicated by closed arrowheads ($\blacktriangleleft$). The 5.0 kb chimeric *PstI* fragment which is indicated by an open arrowhead ($\triangleleft$) was assigned as the left border. The chimeric 8 kb *EcoRI* and 9 kb *HindIII* fragments in strain CI101 ($\blacktriangleleft$ *) electrophoretically overlapped with the *EcoRI* and *HindIII* fragments of pCI6, respectively (see RESULTS in detail).

Fig. 4. Southern hybridization of double restriction endonuclease digests of DNA from strains CIK51 and CI101.

Total cellular DNA from strains CIK51 and CI101 were digested with two different restriction endonucleases and hybridized with the *attP* fragment of phage ϕ U. *HindIII*-digested λ DNA mixed with pHY marker (Takara, Japan) was used as size marker (lane M). The *attL* ($\triangleleft$) and *attR* ($\blacktriangleleft$) were assigned as described in RESULTS. The *attL* and *attR* could not be decided among the *EcoRI-BamHI* double digested DNA fragments.

Fig. 5. Schematic drawing of pCI6 integration into the chromosome of *Rhizobium* by *attP-attB* recombination.

The restriction map of the *attB* region on the *Rhizobium* chromosome was deduced from the restriction maps of pCI6 and pCI6-integrated chromosome. The *BamHI* and *EcoRI* sites whose exact locations could not be determined are shown as dotted lines with question marks. The *BamHI(?)*-*BamHI(?)* fragment and the *EcoRI(?)*-*EcoRI(?)* fragment in the map of the *attB* region are 18 kb and 7.7 kb in length, respectively. The *attP* fragment of phage ϕ U in pCI6 is indicated by an open box. The 1.0 kb region where the *attP* and the *attB* must be located are indicated by closed and hatched boxes, respectively. The restriction sites of *PstI** on pCI6 and on chromosome are described in RESULTS.

double digests. The restriction map of the chromosomes of strains CIK51 and CI101 into which pCI6 is integrated at the *attB* site of parent strains K5 and 4S was prepared from the hybridization data (Fig. 5). The exact location of the flanking chromosomal *EcoRI* and *BamHI* sites could not be determined and are drawn as dotted lines with question marks. Since the *PstI* fragment corresponding to *attR* is 1.0 kb in length, the *attP* and *attB* must be located within approximately 1 kb of the *PstI* sites (labelled *PstI** in Fig. 5) on pCI6 and bacterial chromosome, respectively.

DISCUSSION

Interspecific DNA homologies between members of the Rhizobiaceae has been demonstrated using DNA reassociation methods. Heberlein et al. (11) confirmed the close relationship between the so-called "fast-growing" rhizobia and *Agrobacterium*. Jarvis et al. (17) reported that the average of the relative reassociation value of the DNAs from *R. leguminosarum* biovar *trifolii* and *R. leguminosarum* biovar

viciae was 70% (range, 49 to 94%). Therefore, there is a good possibility that small segments of DNA may be conserved between different members of the Rhizobiaceae. The plasmid vector pCI6, which was constructed by cloning the *attP* fragment of phage ϕ U into the suicide vector pSUP202, could integrate into the chromosome of *R. leguminosarum* biovar *trifolii* strain 4S. If the *attB* site for phage ϕ U was conserved in other bacterial strains belonging to the family Rhizobiaceae, it might be possible to integrate pCI6 into the chromosome of the strain regardless of the susceptibility to phage ϕ U infection. Conjugation experiments were performed using 14 strains of Rhizobiaceae as recipients and *E. coli* strain S17-1 harbouring pCI6 as a donor. The Tc^r transconjugants were recovered from the conjugation performed with *R. leguminosarum* biovar *viciae* strain K5, which was not a susceptible strain to phage ϕ U infection, as a recipient. The frequency of transconjugant appearance was estimated at 4.41×10^{-3} per recipient cell. Southern hybridization with the *attP* fragment of phage ϕ U and pSUP202 against one of the resulting Tc^r transconjugants (strain CIK51) proved that pCI6 integrated intact into the chromosome of strain K5 by *attP-attB* mediated recombination. It has been reported that the integration of the lysogenic phage into the host bacterial chromosome is mediated by integrase coded on the phage genome and IHF (integration host factor) coded on the host genome (9,32). It is expected that integrase gene coded on pCI6 was expressed and functioned efficiently in strain K5 considering the high efficiency of integration of pCI6 into the chromosome. However, it is not known if IHF exists in *R. leguminosarum* biovar *viciae* strain K5 or *R. leguminosarum* biovar *trifolii* strain 4S.

Southern hybridization with *attP* and pSUP202 probes revealed that the pCI6 integration site on the chromosome, i.e. *attB*, was in the same location in *R. leguminosarum* biovar *viciae* strain K5 and *R. leguminosarum* biovar *trifolii* strain 4S. The restriction sites for *Bam*HI, *Eco*RI, *Pst*I, and *Hind*III were identical within a region of at least 18 kb encompassing the *Bam*HI fragment on which the *attB* site located. These data confirm that the *attB* site and its adjacent region were conserved in strain K5 and strain 4S, independent of susceptibility to phage ϕ U infection.

The host range of phage ϕ U seemed to be limited to certain strains of *R. leguminosarum* biovar *trifolii* by a plaque formation test. However, phage ϕ U adsorption experiments revealed that *R. leguminosarum* biovar *viciae* strain K5 had the ability to adsorb phage ϕ U. This indicates that the gene for the specific receptor for phage ϕ U is conserved and must be expressed in strain K5 as well as in strain 4S. The receptor for λ and its related coliphages has been identified as the LamB protein, which is located in the outer membrane of *E. coli* strain K12 and functions as a maltodextrin porter (30,40). The cloned *lamB* gene was transferred into *Rhizobium*, *Agrobacterium* and *Pseudomonas* (28). *R. meliloti* carrying *lamB* could adsorb λ cI857 and was quantitatively transduced with in vitro λ -packaged cosmid. Analogously, phage ϕ U attached to the receptor on the surface of strain K5 will inject its genomic DNA into the bacterial cell. However, phage progeny could not

be detected with strain K5. The gene expression of phage ϕ U is likely to be inhibited by the host restriction system in strain K5 after phage DNA injection.

The plasmid pCI6 integrated into the chromosome not only *R. leguminosarum* biovar *trifolii* strain 4S but *R. leguminosarum* biovar *viciae* strain K5 at high frequency. The Tc^r gene on pCI6 was efficiently expressed and stably maintained after several generations under the condition of selective pressure. It suggested that plasmid pCI6 will serve as a useful vector for interspecific genetic analysis on *Rhizobium*.

We wish to thank Dr. Estelle Hrabak for her critical reading of the manuscript and helpful comments.

REFERENCES

- 1) Abe, M., Sherwood, J. E., Hollingsworth, R. I., and Dazzo, F. B., Stimulation of clover root hair infection by lectin-binding oligosaccharides from the capsular and extracellular polysaccharides of *Rhizobium trifolii*. *J. Bacteriol.*, **160**, 517–520 (1984).
- 2) Acuña, G., Alvarez-Morales, A., Hahn, M., and Hennecke, H., A vector for the site-directed, genomic integration of foreign DNA into soybean root-nodule bacteria. *Plant Mol. Biol.*, **9**, 41–50 (1987).
- 3) Adams, M. H., Bacteriophages, Interscience Publications, London (1959).
- 4) Beringer, J. E., R-factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.*, **84**, 188–198 (1974).
- 5) Beringer, J. E., Johnston, A. W. B., and Kondorosi, A., Genetic maps of *Rhizobium leguminosarum*, *R. meliloti*, *R. phaseoli* and *R. trifolii*. In Genetic Maps, Vol. 4., ed. by O'Brien, S. J., Cold Spring Harbor Laboratory Press, New York (1987), p. 245–251.
- 6) Bevan, M., Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.*, **12**, 8711–8721 (1984).
- 7) Casse, F., Boucher, C., Julliot, J. S., Michel, H., and Dénarié, J., Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. *J. Gen. Microbiol.*, **113**, 229–242 (1979).
- 8) Dooley, S., Radtke, J., and Unteregger, G., Rapid detection of DNA binding factors using protein-blotting and digoxigenin-dUTP marked probes. *Nucleic Acids Res.*, **16**, 11839 (1988).
- 9) Friedman, D. I., Integration host factor: A protein for all reasons. *Cell*, **55**, 545–554 (1988).
- 10) Hattori, M., Hidaka, S., and Sakaki, Y., Sequence analysis of a *Kpn*I family member near the 3' end of human β -globin gene. *Nucleic Acids Res.*, **13**, 7813–7827 (1985).
- 11) Heberlein, G. T., Deley, J., and Tijtgat, R., Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium*, and *Chromobacterium*. *J. Bacteriol.*, **94**, 116–124 (1967).
- 12) Hermesz, E., Olasz, F., Dorgai, L., and Orosz, L., Stable incorporation of genetic material into the chromosome of *Rhizobium meliloti* 41: Construction of an integrative vector system. *Gene*, **119**, 9–15 (1992).
- 13) Higashi, S. and Abe, M., Promotion of infection thread formation by substances from *Rhizobium trifolii*. *J. Appl. Environ. Microbiol.*, **39**, 297–301 (1980).
- 14) Higashi, S., Uchiumi, T., and Abe, M., Elimination of *Rhizobium* infectivity by temperature treatment. *J. Gen. Appl. Microbiol.*, **29**, 281–285 (1983).
- 15) Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J., and Schilperoort, R. A., A binary vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature*, **303**, 179–180 (1983).

- 16) Isogai, A., Fukuchi, N., Hayashi, M., Kamada, H., Harada, H., and Suzuki, A., Mikimopine, an opine in hairy roots of tobacco induced by *Agrobacterium rhizogenes*. *Phytochemistry*, **29**, 3131–3134 (1990).
- 17) Jarvis, B. D. W., Dick, A. G., and Greenwood, R. M., Deoxyribonucleic acid homology among strains of *Rhizobium trifolii* and related species. *Int. J. Syst. Bacteriol.*, **30**, 42–52 (1980).
- 18) Kawaguchi, M., Sekine, M., and Syono, K., Isolation of *Rhizobium leguminosarum* biovar *viciae* variants with indole-3-acetamide hydrolase activity. *Plant Cell Physiol.*, **31**, 449–455 (1990).
- 19) Keele, B. B., Hamilton, P. B., and Elkan, G. H., Glucose catabolism in *Rhizobium japonicum*. *J. Bacteriol.*, **97**, 1184–1191 (1969).
- 20) Keyser, H. H., Bohloul, B. B., Hu, T. S., and Weber, D. F., Fast-growing *Rhizobia* isolated from root nodules of soybean. *Science*, **215**, 1631–1632 (1982).
- 21) Koncz, C., De Greve, H., Andre, D., Deboeck, F., Van Montagu, M., and Schell, J., The opine synthase genes carried by Ti plasmids contain all signals necessary for expression in plants. *EMBO J.*, **2**, 1590–1603 (1983).
- 22) Kondorosi, A. and Johnston, A. W. B., The genetics of *Rhizobium*. In *Biology of the Rhizobiaceae*, ed. by Giles, K. and Atherly, A., Academic Press, New York (1981), p. 191–224.
- 23) Kunnimalaiyaan, M. and Lodha, M. L., Expression of chromosome-integrated hydrogen-uptake genes in *Cicer-Rhizobium*. *J. Plant Biochem. Biotech.*, **1**, 19–21 (1992).
- 24) Kunnimalaiyaan, M., Lodha, M. L., and Sreekumar, K. R., Integration of *hup* cosmid pHU52 into the chromosomal DNA of *Cicer-Rhizobium* using Tn5 as a homologous sequence. *World J. Microbiol.*, **8**, 567–572 (1992).
- 25) Kuykendall, L. D. and Elkan, G. H., *Rhizobium japonicum* derivatives differing in nitrogen-fixing efficiency and carbohydrate utilization. *Appl. Environ. Microbiol.*, **32**, 511–519 (1976).
- 26) Long, S. R., Genetics of *Rhizobium* nodulation. In *Plant Microbe Interactions*, ed. by Kosuge, T. and Nester, E., Macmillan, New York (1984), p. 265–306.
- 27) Long, S. R., *Rhizobium* genetics. *Annu. Rev. Genet.*, **23**, 483–506 (1989).
- 28) Ludwig, R., Gene tandem-mediated selection of coliphage λ -receptive *Agrobacterium*, *Pseudomonas*, and *Rhizobium* strains. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 3334–3338 (1987).
- 29) Meade, H., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M., Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J. Bacteriol.*, **149**, 114–122 (1982).
- 30) Randall-Hazelbauer, L. and Schwartz, M., Isolation of the bacteriophage lambda receptor from *Escherichia coli*. *J. Bacteriol.*, **116**, 1436–1446 (1973).
- 31) Ruvkun, G. B. and Ausubel, F. M., A general method for site directed mutagenesis in prokaryotes. *Nature*, **289**, 85–88 (1981).
- 32) Sadowski, P., Site-specific recombinases: Changing partners and doing the twist. *J. Bacteriol.*, **165**, 341–347 (1986).
- 33) Sambrook, J., Fritsch, E. F., and Maniatis, T., *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989).
- 34) Schwent, R. M., Abe, M., and Higashi, S., Cytological study of the root nodule of *Vigna mungo* (Fabaceae). *J. Ariz.-Nev. Acad. Sci.*, **18**, 22–25 (1983).
- 35) Sherwood, M. T., Improved synthetic medium for the growth of *Rhizobium*. *J. Appl. Bacteriol.*, **33**, 708–713 (1970).
- 36) Simon, R., Preiher, U., and Pühler, A., A broad host range mobilization system for *in vivo* genetic engineering; transposon mutagenesis gram-negative bacteria. *Biotechnology*, **1**, 784–791 (1983).
- 37) Stachel, S. E., An, G., Flores, C., and Nester, E. W., A Tn3 *lacZ* transposon for the random generation of β -galactosidase gene fusions: Application to the analysis of the gene expression in *Agrobacterium*. *EMBO J.*, **4**, 891–898 (1985).
- 38) Uchiumi, T., Ono, Y., Abe, M., and Higashi, S., Phage induction of lysogenic *Rhizobium leguminosarum* biovar *trifolii* in both the free-living and the symbiot form. *J. Gen. Microbiol.*, **135**, 3133–3141 (1989).

- 39) Uchiumi, T., Higashi, S., and Abe, M., A chromosome integrative vector system utilizing DNA fragments of a lysogenic phage of *Rhizobium leguminosarum*. *J. Gen. Microbiol.*, **139**, 2371–2377 (1993).
- 40) Wandersman, C., Schwartz, M., and Ferenci, T., *Escherichia coli* mutants impaired in maltodextrin transport. *J. Bacteriol.*, **140**, 1–13 (1979).