A chromosome integrative vector system utilizing DNA fragments of a lysogenic phage of *Rhizobium leguminosarum*

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The attachment site (*attP*) of phage ϕ U, a lysogenic phage of *Rhizobium leguminosarum* biovar *trifolii*, was identified on a 6.0 kb *Eco*RI fragment of the phage DNA. Plasmid pCI6 was constructed by cloning this *Eco*RI fragment into the *Eco*RI site of suicide plasmid vector pSUP202. *Escherichia coli* S17-1 harbouring plasmid pCI6 was mated with wild-type *R. leguminosarum* biovar *trifolii* strain 4S and its lysogenic strain 4S(ϕ U) using tetracycline resistance (Tc^r) as a selection marker. The Tc^r *R. leguminosarum* biovar *trifolii* transconjugants appeared at high frequency (10^{-3} – 10^{-4} per recipient cell in both matings). Southern hybridization with the *attP* fragment and pSUP202 as probes indicated that plasmid pCI6 integrated into the chromosome of all these transconjugants in the same manner as phage ϕ U.

Introduction

The lysogenic phages of *Rhizobium* have contributed to the analysis of *Rhizobium* genes (Kowalski, 1967; Buchanan-Wollaston, 1979; Sik *et al.*, 1980; Finan *et al.*, 1984; Martin & Long, 1984). Phage 16-3, a lysogenic phage of *Rhizobium meliloti*, has been investigated in detail (Dorgai *et al.*, 1981, 1986) and Hermes *et al.* (1992) constructed cloning vectors using the attachment site (*attP*) of phage 16-3. These vectors integrated into the chromosome of *R. meliloti* by site specific recombination between the *attP* site on the vectors and the *attB* site on the host bacterial chromosome with simultaneous infection of specific helper phage. The integrated vectors were stably maintained in *R. meliloti*. This integrative vector system will play an important role in genetic studies of the *Rhizobium*–legume symbiosis, because loss of vectors and cloned genes during nodulation has been a problem (Long, 1989).

The integrative site-specific recombination of lysogenic bacteriophage is well understood in coliphage λ . Phage λ integrates its genome into the host bacterial chromosome by recombination between specific phage (*attP*) and bacterial (*attB*) sites. As a result of integration, two new DNA junctions, *attL* and *attR*, are generated on the left and right borders of the prophage genome. If a lysogenic phage integrates into the chromosome of the host bacteria in the same manner as phage λ , three fragments of *attP*, *attL* and *attR* can be detected by Southern

hybridization. Waldman *et al.* (1986) distinguished *attL* and *attR* fragments among the restriction fragments prepared from the lysogenic *Haemophilus influenzae* strain, L-10. When total DNA of lysogenic strain L-10 digested with restriction endonuclease was hybridized with DNA of lysogenic phage HP1c1, *attL* and *attR* fragments were detected as new bands which did not exist in restriction fragments of vegetative phage DNA. The *attP* fragment was detected as a very faint band, presumably because the copy number of the vegetative phage DNA was very low in the lysogen. Duchrow & Giffhorn (1987) also reported the detection of *attL*, *attR* and *attP* fragments of a lysogenic strain of *Rhodobacter sphaeroides* and its lysogenic phage ϕ Rsg1 in the same way.

Lysogenic phage ϕ U has been shown to lysogenize *Rhizobium leguminosarum* biovar *trifolii* strain 4S (Uchiumi *et al.*, 1989. Phage ϕ U was designated phage U-mole in the report.). It was suggested that phage ϕ U integrates its genomic DNA into the host chromosome during lysogeny, and that the *attP* site may locate on a 6.0 kb *Eco*RI fragment of phage ϕ U DNA (Uchiumi *et al.*, 1989). The DNA fragments corresponding to *attL* and *attR* were thought to be the 7.4 kb and 5.0 kb *Eco*RI fragments also found in the lysogen. We hypothesized that if a plasmid carrying the *attP* fragment could be constructed and introduced into the host *Rhizobium* cell, the recombinant plasmid might integrate into the host chromosome by *attP/attB*-mediated site-specific recombination. Such a construct would be useful for future studies on *Rhizobium* genetics. We report here the

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construction of a suicide plasmid vector carrying the *attP* fragment of phage ϕ U DNA and high frequency integration of this vector into the chromosome of *R. leguminosarum* biovar *trifolii*.

Methods

Phage, plasmids and bacterial strains. The relevant characteristics of the phage, plasmids and bacterial strains used in this study are shown in Table 1. One of the symbiotically effective lysogenic strains, *R. leguminosarum* biovar *trifolii* 4S(ϕ U), was used in this study.

Media and antibiotics. *E. coli* strains were maintained and cultured in LB medium. *Rhizobium* strains were maintained on mannitol–yeast agar medium (Keele *et al.*, 1969). TY medium (Beringer, 1974) was used for DNA preparation, phage ϕ U induction and mating. For selection of transconjugants from *E. coli* and *Rhizobium* mating mixtures, Sherwood's minimal agar plates (Sherwood, 1970) were employed. Antibiotics were used at the following concentrations: tetracycline (Tc) 20 μ g ml⁻¹; chloramphenicol (Cm) 30 μ g ml⁻¹; ampicillin (Ap) 50 μ g ml⁻¹; and mitomycin C for phage induction, 0.1 μ g ml⁻¹.

Isolation of DNA and restriction endonuclease digestion. Plasmid isolation from *E. coli* was done on a small scale according to Birnboim & Doly (1979), for routine analysis. For cloning vector and template for hybridization probes, the plasmid was purified according to Hattori *et al.* (1985). Isolation of phage ϕ U DNA was performed as described by Yamamoto *et al.* (1970) and Dallmann *et al.* (1979). Total cellular DNA from *Rhizobium* was prepared by the method of Casse *et al.* (1979) as modified by Higashi *et al.* (1983). Isolated DNAs were digested with appropriate restriction endonucleases according to standard methods.

Table 1. Bacterial strains, phage and plasmids

	Relevant characteristics*	Reference or source
<i>Rhizobium</i> strains		
4S	Wild-type	Higashi & Abe (1980)
4S(ϕ U)	Lysogen of strain 4S	Uchiumi <i>et al.</i> (1989)
CI101	Transconjugant, Tc ^r	This work
CI301	Transconjugant, Tc ^r	This work
<i>E. coli</i> strains		
S17-1	<i>tra</i> gene on chromosome	Simon <i>et al.</i> (1982)
S17-1(pCI6)	S17-1 harbouring pCI6	This work
JM109	Cloning host for pUC18	Yanisch-Perron <i>et al.</i> (1985)
LE392	Cloning host	Sambrook <i>et al.</i> (1989)
VCS257	Cloning host	Stratagene
Phage ϕ U	Lysogenic phage of <i>Rhizobium</i>	Uchiumi <i>et al.</i> (1989)
Plasmids		
pSUP202	<i>mob</i> ⁺ Tc ^r Ap ^r Cm ^r	Simon <i>et al.</i> (1982)
pCI6	pSUP202 carrying <i>attP</i> of phage ϕ U	This work
pUC18	Ap ^r <i>lacZ'</i>	Yanisch-Perron <i>et al.</i> (1985)

* *mob*⁺, mobilizable by *tra* gene; Tc^r, tetracycline resistant; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant.

Agarose gel electrophoresis. Plasmids from *E. coli*, total cellular DNAs from *Rhizobium* and restriction fragments of DNAs were separated on 0.7% agarose gels. Electrophoresis was performed in TBE-buffer (89 mM-Tris base, 2 mM-EDTA, 89 mM-boric acid, pH 8.4) at 50 V for 14 h for *Rhizobium* plasmids and at 100 V for 90 min for *E. coli* plasmids and restriction fragments. Agarose gels were stained with ethidium bromide and observed in UV light (302 nm).

Isolation of DNA fragments from agarose gel. After agarose gel electrophoresis, the stained DNA band of interest was excised, transferred to a small centrifuge tube with a membrane filter (Ultrafree-C3, Millipore), and isolated under the conditions recommended by the manufacturer. Isolated DNA was directly labelled for use as a hybridization probe or cloned into the appropriate cloning sites on plasmid vector pUC18.

Southern hybridization. After agarose gel electrophoresis, DNAs in the gel were denatured with 200 mM-hydrochloric acid followed by 1 M-sodium hydroxide. DNAs were then transferred to a Nytran NY13N filter (Shleicher and Schüll) by vacuum blotting. The blotted filters were irradiated with UV light (302 nm) for 4 min to bind DNAs strongly to the filters. Plasmid DNA or isolated DNA from the agarose gel were labelled with digoxigenin and used as probes. Hybridization and colorimetric detection were performed according to the manufacturer's instructions (Boehringer Mannheim).

Construction of plasmid pCI6. The 6.0 kb *Eco*RI fragment of phage ϕ U DNA, which was identified as an *attP* fragment in this work, was cloned into an *Eco*RI site of pUC18 and maintained in *E. coli* LE392. Purified pUC18 carrying the *attP* fragment and mobilizable (*mob*⁺) suicide plasmid pSUP202 were digested with *Eco*RI, ligated with T4 DNA ligase, and introduced into *E. coli* VCS257 by electroporation (Gene Pulser, Bio-Rad). Because the *Eco*RI site locates within the Cm^r gene on pSUP202, Ap^r Tc^r Cm^s colonies were selected as transformants and their plasmids were confirmed. Plasmid pSUP202 carrying the *attP* fragment of phage ϕ U was referred to as plasmid pCI6.

Bacterial matings for transfer of plasmid pCI6 into *Rhizobium* cells. In advance of mating, plasmid pCI6 was introduced to *E. coli* S17-1. Donor *E. coli* S17-1 harbouring pCI6 and recipient *Rhizobium* were grown in LB medium and TY medium for 24 h, respectively. A 100 μ l sample (ca. 5×10^7 cells) of each culture was centrifuged, and washed twice with sterile distilled water, then 50 μ l of the mixture was spotted onto sterile filters (HAWP01300, Millipore) on TY agar plates. After overnight incubation at 30 °C, the cells on the filter were washed twice with sterile distilled water and resuspended in 1 ml of sterile distilled water. The conjugated mixture was plated onto minimal medium containing Tc (20 μ g ml⁻¹) for selection of *Rhizobium* transconjugants; 4 d after plating, colonies were counted to estimate the frequency of transconjugant appearance.

Phage sensitivity test and phage induction test. Phage sensitivity and induction with mitomycin C of phage ϕ U in *Rhizobium* strains were determined as described previously (Uchiumi *et al.*, 1989).

Nodulation test. Nodulation and nitrogen fixation ability of *Rhizobium* strains were confirmed on white clover (*Trifolium repens* L. cultivar Ladino) as described by Higashi *et al.* (1983).

Results

Identification of the *attP* fragment of phage ϕ U DNA

If phage ϕ U integrates into the chromosome of *Rhizobium* at the site of the 6.0 kb *Eco*RI fragment of phage ϕ U DNA, then *attL* and *attR* should be detectable among DNA fragments of lysogenic strain 4S(ϕ U) by Southern hybridization using the *Eco*RI 6.0 kb fragment

Fig. 1. Southern hybridization for identification of the *attP* fragment of phage ϕ U DNA. *Eco*RI- and *Bam*HI-digested DNA from *R. leguminosarum* biovar *trifolii* strain 4S(ϕ U) were hybridized with a digoxigenin-labelled 6.0 kb *Eco*RI putative *attP* fragment of phage ϕ U DNA. Lanes 1, phage ϕ U DNA digested with *Eco*RI and *Bam*HI, respectively; lanes 2, strain 4S(ϕ U) DNA digested with *Eco*RI and *Bam*HI, respectively; lanes 1', phage ϕ U DNA hybridized with putative phage ϕ U *attP* fragment; lanes 2', strain 4S(ϕ U) DNA hybridized with putative phage ϕ U *attP* fragment. For explanation of bands indicated by large and small arrowheads, see text.

Fig. 2. Restriction map of the *attP* region of phage ϕ U DNA. This map was prepared by summarizing the results of Southern hybridization against restriction fragments of phage ϕ U and the lysogenic *Rhizobium* strain 4S(ϕ U) probed with the *attP* fragment of phage ϕ U. The open box is a 2.3 kb *Eco*RI-*Pst*I fragment which contains the *attP* site. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I.

as a probe. Total cellular DNA was prepared from *R. leguminosarum* biovar *trifolii* lysogenic strain 4S(ϕ U), and digested with *Eco*RI and *Bam*HI. Southern hybridization of fragments of phage ϕ U and lysogenic strain 4S(ϕ U) DNAs probed with the digoxigenin labelled putative *attP* fragment (6.0 kb *Eco*RI fragment) of phage ϕ U DNA are shown in Fig. 1. Three hybridized bands were detected in fragmented DNA of strain 4S(ϕ U),

whereas only one band was detected in phage ϕ U DNA. This indicates that the 6.0 kb *Eco*RI fragment of phage ϕ U DNA is split in the lysogen. Two fragments which did not exist in phage ϕ U DNA (indicated by large arrowheads in lanes 2') were judged to carry *attL* and *attR* in lysogenic strain 4S(ϕ U). The other fragment (indicated by small arrowheads in lanes 2') was interpreted as the *attP* fragment itself, derived from vegetative phage ϕ U DNA in strain 4S(ϕ U) cells, because corresponding fragments could be detected by agarose gel electrophoresis of phage ϕ U DNA (lanes 1). The *attP* fragment was detected at almost equal intensity to *attL* and *attR* (lanes 2'), contrary to the results reported by Waldman *et al.* (1986) and Duchrow & Giffhorn (1987). The DNA isolation procedure employed in this work is suitable for isolation of large plasmid DNA in *Rhizobium*, and should also favour autonomous replicating phage DNA in the lysogenic strain. When Southern hybridization was performed using other *Eco*RI fragments of phage ϕ U DNA as probes, they hybridized with only one fragment, corresponding to themselves, in lysogenic strain 4S(ϕ U) (data not shown). These hybridization data support the designation of the 6.0 kb *Eco*RI fragment as the *attP* fragment of phage ϕ U DNA.

Restriction map of the *attP* fragment

Phage ϕ U DNA prepared from phage particles was single- and double-digested with restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III and *Pst*I, then hybridized with the 6.0 kb *Eco*RI *attP* fragment for restriction mapping. Fig. 2 shows the restriction map of an 11.7 kb *Pst*I-*Hind*III fragment on which the 6.0 kb *Eco*RI *attP* fragment is located. When Southern hybridization was performed against *Eco*RI and *Bam*HI digests of lysogen 4S(ϕ U) DNA using a 2.3 kb *Eco*RI-*Pst*I fragment (indicated as an open box in Fig. 2) as a probe, two fragments of *attL* and *attR* were detected (data not shown). This indicates that the integration might occur within the 2.3 kb *Eco*RI-*Pst*I fragment.

Transfer of plasmid pCI6 into *Rhizobium*

The *attP* fragment of the 6.0 kb *Eco*RI fragment of phage ϕ U DNA was cloned into the *Eco*RI site of suicide plasmid pSUP202 and referred to as pCI6. *E. coli* S17-1 transformed with pCI6 was used as a conjugation donor. Plasmid pCI6 encodes tetracycline resistance (*Tc*^r) and ampicillin resistance (*Ap*^r) as selection markers, and carries a *mob* gene (*mob*⁺) derived from plasmid RP4. *E. coli* S17-1 is able to transfer *mob*⁺ plasmids such as pCI6 and pSUP202 to other bacterial genera including *Rhizobium* by *trans* function of the *tra* (transfer) gene on its chromosome (Simon *et al.*, 1983).

R. leguminosarum biovar *trifolii* strain 4S and its

Fig. 3. Agarose gel electrophoresis (a) and Southern hybridization (b) of total cellular DNAs from *Rhizobium* strains and plasmid pCI6. Digoxigenin-labelled pCI6 was used as a hybridization probe. Lanes 1, strain 4S; lanes 2, strain 4S(ϕ U); lanes 3, strain CI101; lanes 4, strain CI301; lanes 5, plasmid pCI6 from *E. coli* VCS257 harbouring pCI6. chr, Fragmented chromosome DNA.

lysogenic derivative strain 4S(ϕ U) were mated with *E. coli* S17-1 harbouring pCI6, respectively. *Rhizobium* transconjugants were selected on minimal agar plates containing tetracycline. Ap^r was not used as a selection marker because strains 4S and 4S(ϕ U) are intrinsically resistant to ampicillin. The frequency of appearance of Tc^r transconjugants was estimated (average of three independent mating experiments). Tc^r transconjugants appeared at a high frequency of 4.4×10^{-3} for strain 4S as a recipient and at 2.9×10^{-4} for strain 4S(ϕ U) as a recipient, respectively. No transconjugants were obtained by matings with *E. coli* S17-1 harbouring pSUP202 as a donor. This indicates that pSUP202 could not replicate in *Rhizobium*. Because pCI6 also could not replicate in *Rhizobium*, pCI6 was expected to integrate into the chromosome of *Rhizobium* by site-specific recombination between the *attP* and *attB* sites.

Characteristics of CI strains

Phage ϕ U productivity, sensitivity to phage ϕ U, and symbiotic phenotypes were investigated in ten transconjugants of the CI100 series (recipient, strain 4S) and ten transconjugants of CI300 series [recipient, strain 4S(ϕ U)], respectively. All transconjugants maintained nodulation and nitrogen fixation ability on white clover.

Transconjugants of the CI100 series retained the characteristics of the parent strain 4S, which had no phage ϕ U and was sensitive to phage ϕ U infection. Transconjugants of the CI300 series lost phage ϕ U productivity and were sensitive to phage ϕ U, whereas the parent strain 4S(ϕ U) had phage ϕ U and was resistant to phage ϕ U infection. This indicates that the CI300 series strains have apparently lost the integrated phage ϕ U present in the parent strain 4S(ϕ U). Transfer of pCI6 into the lysogenic strain 4S(ϕ U) may exclude prophage from the chromosome by the integration and excision mechanism.

Representative transconjugants, strains CI101 and CI301 were used in subsequent experiments.

Confirmation of chromosomal integration of pCI6 in *Rhizobium*

To distinguish pCI6 among various possible locations, total cellular DNAs prepared from transconjugants and respective parent strains were analysed by agarose gel electrophoresis and Southern hybridization with the pCI6 probe (Fig. 3). Plasmid pCI6 (lanes 5) was prepared from *E. coli* VCS257 harbouring pCI6 and used as a hybridization probe. All *Rhizobium* strains have three plasmids, of 525, 420 and 315 kb. The 315 kb plasmid in each strain was identified as the Sym plasmid (Higashi *et*

Fig. 4. Agarose gel electrophoresis and Southern hybridization of DNAs from *Rhizobium* strains probed with the *attP* fragment and pSUP202. DNAs from *Rhizobium* strains and plasmid pCI6 were digested with *EcoRI* and separated on a 0.7% agarose gel (a). Southern-blotted filters were hybridized with the *attP* fragment (b) and pSUP202 (c). Lane M, λ phage *HindIII* fragments as molecular mass markers; lanes 1, strain 4S; lanes 2, strain 4S(ϕ U); lanes 3, strain CI101; lanes 4, strain CI301; lanes 5, plasmid pCI6.

al., 1983; Uchiumi *et al.*, 1989). No plasmid bands corresponding to pCI6 could be detected in total cellular DNAs from strains CI101 and CI301 (Fig. 3a, b, lanes 3 and 4). This indicated that pCI6 did not exist in CI strains as an autonomously replicating plasmid. Besides the fragmented chromosomal DNA, many hybridized bands were detected in strain 4S(ϕ U) (Fig. 3b, lane 2). These were assumed to be vegetative phage ϕ U DNA in strain 4S(ϕ U) cells. The *attP* probe hybridized with fragmented chromosomal DNA in strains CI101 and CI301, and did not hybridize with plasmids and chromosomal DNAs from strain 4S (Fig. 3b, lanes 1, 3 and 4). These data suggest that plasmid pCI6 integrated into the chromosome in strains CI101 and CI301.

To confirm the chromosomal integration of pCI6 and locate its site of integration, total cellular DNAs from *Rhizobium* strains were digested with *EcoRI*, and hybridized with the labelled *attP* fragment and pSUP202 (Fig. 4). Plasmid pCI6 was digested into two fragments of the *attP* fragment and pSUP202 with *EcoRI* (Fig. 4a, lane 5). Southern blots probed with the *attP* fragment are shown in Fig. 4(b). Three bands were detected in strain 4S(ϕ U) DNA (Fig. 4b, lane 2). The longest and the

shortest bands were identified as *attL* and *attR* fragments. The middle band was derived from the *attP* fragment of vegetative phage ϕ U DNA in cells of strain 4S(ϕ U). In DNAs from strains CI101 and CI301, only two bands corresponding to *attL* and *attR* were detected (Fig. 4b, lanes 3 and 4). A very faint band was detected between the *attL* and *attR* fragments of strain CI301 (Fig. 4b, lane 4). This band may be a partially digested fragment, because its length was slightly different from that of the *attP* fragment. No hybridizing band could be detected in DNA from wild-type strain 4S (Fig. 4b, lane 1).

Southern hybridization was also performed using pSUP202 as a probe (Fig. 4c). The hybridized band identical to pSUP202 could be detected in both CI101 and CI301 strains. The very faint band of approximately 3.0 kb in all *Rhizobium* strains may be an *Ap^r* gene which is analogous to that on pSUP202.

These hybridization results support the proposal that plasmid pCI6 integrates entirely into the chromosome of CI strains by integrative site-specific recombination between *attP* on pCI6 and *attB* on the *Rhizobium* chromosome.

Discussion

Two types of lysogenization have been reported. One type is the integration of the phage genome into the host bacterial chromosome as represented by phage λ . The other type is plasmid-like replication in the host bacteria as represented by phage P1 (Ikeda & Tomizawa, 1968). In a lysogen of the former type, the *attP* fragment of lysogenic phage DNA is split, generating *attL* and *attR* fragments as a result of integrating the phage DNA into the bacterial host chromosome. Southern hybridization using a 6.0 kb *EcoRI* fragment (the putative *attP* fragment) of phage ϕ U as a probe was performed and two fragments of *attL* and *attR* could be detected in the lysogenic *Rhizobium* strain (Fig. 1). This indicates that the *EcoRI* 6.0 kb fragment is the *attP* fragment of phage ϕ U DNA.

The *attP* fragment of phage ϕ U was cloned into an *EcoRI* site of pSUP202 to construct a chromosome integrative vector system. The resultant recombinant plasmid, pCI6, was transferred from *E. coli* S17-1 into wild-type and lysogenic *R. leguminosarum* biovar *trifolii* strains by conjugation using *Tc^r* as a selection marker. *Tc^r* transconjugants appeared at high frequency for both recipient *Rhizobium* strains. The transfer frequency of plasmid pCI6 from *E. coli* to *R. leguminosarum* biovar *trifolii* strains was significantly higher than that reported for transfer of the wide host range pKT230 and pKT231 plasmids into *R. meliloti* (10^{-5} ; Donnelly *et al.*, 1987).

By Southern hybridization analyses of transconjugant *Rhizobium* strains, it was revealed that pCI6 integrates into the bacterial chromosome by site-specific recombination between *attP* on pCI6 and *attB* on the bacterial chromosome. The integration reaction is mediated by the phage-encoded protein integrase (Int) and the IHF (integration host factor) of host bacteria [see Sadowski (1986) for review]. Leong *et al.* (1986) have reported the 1728 bp DNA sequence encoding the 19 bp *attP* core sequence and the Int protein. The termination of the Int protein is located only 16 bp upstream of the *attP* core sequence. If the structure of the *attP* region and the integration mechanism of phage ϕ U are similar to those for phage λ , the *int* gene may be located on the 6.0 kb *EcoRI* *attP* fragment of phage ϕ U DNA. Plasmid pCI6 integrates into the chromosome of *R. leguminosarum* biovar *trifolii* at a high frequency at the site of phage ϕ U integration. This indicates that the integrase gene on the *attP* fragment functions efficiently in *Rhizobium* host cells.

The excisionase gene (*xis*) which mediates excision of prophage DNA from the chromosome is located near the *attP* site (Leong *et al.*, 1986). There is a possibility that *xis* resides on pCI6 and functions in the same way as *int*. The excision of pCI6 from the chromosome of the

transconjugants has not been observed so far when tetracycline is present in the medium. However, it is very important to estimate the frequency of pCI6 excision from the integrated state, when considering the application of pCI6 for accurate analyses on gene expression in *Rhizobium*.

When pCI6 was introduced into the lysogenic strain 4S(ϕ U), the resulting transconjugants of CI300 series had lost immunity against phage ϕ U and ability to produce phage ϕ U. These results suggest that phage ϕ U is no longer present in the CI300 series. Transfer of pCI6 into strain 4S(ϕ U) cells may cause exclusion of prophage DNA from the chromosome of strain 4S(ϕ U) by the integration and excision mechanism. Plasmid pCI6 will become a useful tool to study the mechanism of integration and excision of lysogenic phage in *Rhizobium* and also be useful as a chromosome integrative vector, like the vectors reported by Hermes *et al.* (1992).

The entire 40 kb phage ϕ U genome is able to integrate into the *Rhizobium* host chromosome. Plasmid pCI6 is also expected to integrate large DNA fragments into the *Rhizobium* chromosome without any deletions. The insertion into pCI6 of the *cos* site for packaging and a multi-cloning site will make this possible. The application of pCI6 as a chromosome integrative vector for other *Rhizobium* species should also be investigated.

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