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

TOSHIKI UCHIUMI,* SHIRO HIGASHI and MIKIKO ABE

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

(Received 15 February 1993; revised 27 May 1993; accepted 7 June 1993)

The attachment site (attP) of phage φU, a lysogenic phage of *Rhizobium leguminosarum* biovar trifolii, was identified on a 6.0 kb EcoRI fragment of the phage DNA. Plasmid pCI6 was constructed by cloning this EcoRI fragment into the EcoRI site of suicide plasmid vector pSUP202. *Escherichia coli* S17-1 harbouring plasmid pC16 was mated with wild-type *R. leguminosarum* biovar trifolii strain 4S and its lysogenic strain 4S(φU) using tetracycline resistance (Tc') as a selection marker. The Tc' *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 φU.

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 Hermesz 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 influencae* 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 φRsG1 in the same way.

Lysogenic phage φU has been shown to lysogenize *Rhizobium leguminosarum* biovar trifolii strain 4S (Uchiumi et al., 1989. Phage φU was designated phage U-mole in the report.). It was suggested that phage φU integrates its genomic DNA into the host chromosome during lysogeny, and that the attP site may locate on a 60 kb EcoRI fragment of phage φU 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 EcoRI 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
construction of a suicide plasmid vector carrying the attP fragment of phage φU 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(φU), 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 (Keeler et al., 1969). TY medium (Beringer, 1974) was used for DNA preparation, phage φU 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 μg ml⁻¹; chloramphenicol (Cm) 30 μg ml⁻¹; ampicillin (Ap) 50 μg ml⁻¹; and mitomycin C for phage induction 0.1 μg 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.* (1979) as modified by Higashi *et al.* (1983). 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.

**Isolation of DNA fragments from agarose gel.** DNA and high frequency integration of this vector into the chromosome of *R. leguminosarum* biovar trifolii.

<table>
<thead>
<tr>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rhizobium strains</strong></td>
<td></td>
</tr>
<tr>
<td>4S</td>
<td>Wild-type</td>
</tr>
<tr>
<td>4S(φU)</td>
<td>Lysogen of strain 4S</td>
</tr>
<tr>
<td>CI101</td>
<td>Transconjugant, Te⁺</td>
</tr>
<tr>
<td>CI301</td>
<td>Transconjugant, Te⁺</td>
</tr>
</tbody>
</table>

| **E. coli strains**      |                     |
| 4S(φU)                   |                       |
| CI101                    |                       |
| CI301                    |                       |

| **Phage φU**             | Lysogenic phage of   |
|                         | *Rhizobium*          |

| **Plasmids**             |                     |
| pSUP202                  | mob⁺ Te⁺ Ap⁺ Cm⁺     |
| pCI6                     | pSUP202 carrying attP |
|                         | of phage φU          |
| pUC18                    | Ap⁺ lac⁺             |

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

**Aagarose 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-MC 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 EcoRI fragment of phage φU DNA, which was identified as an attP fragment in this work, was cloned into an EcoRI site of pUC18 and maintained in *E. coli* LE392. Purified pUC18 carrying the attP fragment and mobilizable (mob⁺) suicide plasmid pSUP202 were digested with EcoRI, ligated with T4 DNA ligase, and introduced into *E. coli* VCS257 by electroporation (Gene Pulser, Bio-Rad). Because the EcoRI site locates within the Cm⁺ gene on pSUP202, Ap⁺ Te⁺ Cm⁺ colonies were selected as transformants and their plasmids were confirmed. Plasmid pSUP202 carrying the attP fragment of phage φU 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 μl sample (ca. 5 x 10⁸ cells) of each culture was centrifuged, and washed twice with sterile distilled water, then 50 μl 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 μg 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 φU 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 φU DNA**

If phage φU integrates into the chromosome of *Rhizobium* at the site of the 6.0 kb EcoRI fragment of phage φU DNA, then attL and attR should be detectable among DNA fragments of lysogenic strain 4S(φU) by Southern hybridization using the EcoRI 6.0 kb fragment.
digoxigenin-labelled 60 kb EcoRI putative attP fragment of phage respectively; lanes against restriction fragments of phage localization of fragments of phage box is a 2.3 kb EcoRI-PstI fragment which contains the attP site. B, phage 4U attP fragment; lanes 2', strain 4S(#U) DNA hybridized with BamHI, respectively; lanes putative phage #U attP fragment. For explanation of bands indicated 4S(#U) DNA are shown in Fig. 2.

Fig. 1. Southern hybridization for identification of the attP fragment of phage #U DNA. EcoRI-and BamHI-digested DNA from R. leguminosarum biovar trifolii strain 4S(#U) were hybridized with a digoxigenin-labelled 60 kb EcoRI putative attP fragment of phage #U DNA. Lanes 1, phage #U DNA digested with EcoRI and BamHI, respectively; lanes 2, strain 4S(#U) DNA digested with EcoRI and BamHI, respectively; lanes 1', phage #U DNA hybridized with putative phage #U attP fragment; lanes 2', strain 4S(#U) DNA hybridized with putative phage #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 #U DNA. This map was prepared by summarizing the results of Southern hybridization against restriction fragments of phage #U and the lysogenic Rhizobium strain 4S(#U) probed with the attP fragment of phage #U. The open box is a 2.3 kb EcoRI–PstI fragment which contains the attP site. B, BamHI; E, EcoRI; H, HindIII; P, PstI.

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 EcoRI fragments of phage #U DNA as probes, they hybridized with only one fragment, corresponding to themselves, in lysogenic strain 4S(#U) (data not shown). These hybridization data support the designation of the 6.0 kb EcoRI fragment as the attP fragment of phage #U DNA.

Restriction map of the attP fragment

Phage #U DNA prepared from phage particles was single- and double-digested with restriction endonucleases BamHI, EcoRI, HindIII and PstI, then hybridized with the 6.0 kb EcoRI attP fragment for restriction mapping. Fig. 2 shows the restriction map of an 11.7 kb PstI–HindIII fragment on which the 6.0 kb EcoRI attP fragment is located. When Southern hybridization was performed against EcoRI and BamHI digests of lysogenic 4S(#U) DNA using a 2.3 kb EcoRI–PstI 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 EcoRI–PstI fragment.

Transfer of plasmid pCI6 into Rhizobium

The attP fragment of the 6.0 kb EcoRI fragment of phage #U DNA was cloned into the EcoRI site of suicide plasmid pSUP202 and referred to as pCl6. E. coli S17-1 transformed with pCl6 was used as a conjugation donor. Plasmid pCl6 encodes tetracycline resistance (Tc') and ampicillin resistance (Ap') 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 pCl6 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
lysogenic derivative strain 4S(φU) were mated with *E. coli* S17-1 harbouring pC16, respectively. *Rhizobium* transconjugants were selected on minimal agar plates containing tetracycline. Ap' was not used as a selection marker because strains 4S and 4S(φU) are intrinsically resistant to ampicillin. The frequency of appearance of Tc' transconjugants was estimated (average of three independent mating experiments). Tc' 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(φU) 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 pC16 also could not replicate in *Rhizobium*, pC16 was expected to integrate into the chromosome of *Rhizobium* by site-specific recombination between the attP and attB sites.

**Characteristics of CI strains**

Phage φU productivity, sensitivity to phage φU, and symbiotic phenotypes were investigated in ten transconjugants of the CI100 series (recipient, strain 4S) and ten transconjugants of CI300 series [recipient, strain 4S(φU)], 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 φU and was sensitive to phage φU infection. Transconjugants of the CI300 series lost phage φU productivity and were sensitive to phage φU, whereas the parent strain 4S(φU) had phage φU and was resistant to phage φU infection. This indicates that the CI300 series strains have apparently lost the integrated phage φU present in the parent strain 4S(φU). Transfer of pC16 into the lysogenic strain 4S(φU) 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 pC16 in Rhizobium**

To distinguish pC16 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 pC16 probe (Fig. 3). Plasmid pC16 (lanes 5) was prepared from *E. coli* VCS257 harbouring pC16 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(#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(#U) (Fig. 3b, lane 2). These were assumed to be vegetative phage φU DNA in strain 4S(#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(#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 φU DNA in cells of strain 4S(#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' 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 $\phi U$. 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 $\phi U$ 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 $\phi U$ DNA.

The attP fragment of phage $\phi U$ was cloned into an EcoRI site of pSUP202 to construct a chromosome integrative vector system. The resultant recombinant plasmid, pC16, was transferred from *E. coli* S17-1 into wild-type and lysogenic *R. leguminosarum* biovar *trifolii* strains by conjugation using Tc$^c$ as a selection marker. Tc$^c$ transconjugants appeared at high frequency for both recipient *Rhizobium* strains. The transfer frequency of plasmid pC16 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* ($10^{-3}$; Donnelly et al., 1987).

By Southern hybridization analyses of transconjugant *Rhizobium* strains, it was revealed that pC16 integrates into the bacterial chromosome by site-specific recombination between attP on pC16 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 $\phi U$ are similar to those for phage $\lambda$, the int gene may be located on the 6-0 kb EcoRI attP fragment of phage $\phi U$ DNA. Plasmid pC16 integrates into the chromosome of *R. leguminosarum* biovar *trifolii* at a high frequency at the site of phage $\phi U$ 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 pC16 and functions in the same way as int. The excision of pC16 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 pC16 excision from the integrated state, when considering the application of pC16 for accurate analyses on gene expression in *Rhizobium*.

When pC16 was introduced into the lysogenic strain 4S($\phi U$), the resulting transconjugants of C1300 series had lost immunity against phage $\phi U$ and ability to produce phage $\phi U$. These results suggest that phage $\phi U$ is no longer present in the C1300 series. Transfer of pC16 into strain 4S($\phi U$) cells may cause exclusion of prophage DNA from the chromosome of strain 4S($\phi U$) by the integration and excision mechanism. Plasmid pC16 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 Hermesz et al. (1992).

The entire 40 kb phage $\phi U$ genome is able to integrate into the *Rhizobium* host chromosome. Plasmid pC16 is also expected to integrate large DNA fragments into the *Rhizobium* chromosome without any deletions. The insertion into pC16 of the cos site for packaging and a multi-cloning site will make this possible. The application of pC16 as a chromosome integrative vector for other *Rhizobium* species should also be investigated.

We would like to thank Dr R. Simon and Professor A. Pühler for providing us with plasmid pSUP202 and *E. coli* S17-1. We also wish to thank Professor Frank Dazzo for critical reading of the manuscript and helpful comments.

References


Vector system for Rhizobium leguminosarum


