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Integration of the temperate phage ϕU into the putative tRNA gene on the chromosome of its host *Rhizobium leguminosarum* biovar *trifolii*

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The plasmid pCI6, carrying the *attP* site of the temperate phage ϕU, integrates into the *attB* site on the chromosome of *Rhizobium leguminosarum* biovar *trifolii* strain 4S. The 4 kb *Eco*RI-*Hin*dIII region of pCI6 involved in site-specific integration was subcloned as the *attP* fragment of phage ϕU and sequenced. The *attL* fragment, one of the new DNA junctions generated from the insertion of pCI6 into the chromosome of the host *Rhizobium*, was used as a hybridization probe for isolation of the *attB* fragment of strain 4S. The nucleotide sequence of the 2 kb *Pst*I fragment of strain 4S, which hybridized with the *attL* fragment, was decided and compared with that of the *attP* fragment. A 53 bp common sequence was expected to be the core sequence of site-specific integration between phage ϕU and strain 4S. One of the ORFs on the *attP* fragment, which was located adjacent to the core sequence, had structural homology to the integrase family. However, the *attB* fragment showed high homology with the tRNA genes of *Agrobacterium tumefaciens* and *E. coli*. A 47 bp sequence of the 53 bp core sequence overlapped with this tRNA-like sequence. This indicates that the target site of phage ϕU integration is the putative tRNA gene on the chromosome of the *Rhizobium* host.

Key Words——DNA sequence, integrase, integration, *Rhizobium leguminosarum* biovar *trifolii*, temperate phage, tRNA

Temperate phages, represented by the λ phage of *Escherichia coli*, integrate their genome site-specifically into the chromosome of host bacteria. The integration reaction occurs between *attP* on the phage genome and *attB* on the host bacterial chromosome, and requires the integrase protein coded by the *int* gene on the phage genome (Sadowski, 1986 for review). The phage genome integrates via conservative site-specific recombination similar to other recombination systems mediated by proteins belonging to the integrase family. A plasmid vector carrying the *attP* and *int* genes will enable the introduction of genetic materials in single copy at a specific site on the host chromosome without disrupting any host genes. Hermesz et al. (1992) constructed several vectors utilizing the *attP* site of rhizobiophage 16-3, and they were efficiently integrated into the chromosome of *Rhizobium meliloti* strain Rm21.

Temperate phage ϕU lysogenizes *Rhizobium leguminosarum* biovar *trifolii* strain 4S (Uchiumi et al., 1989). The 6 kb *Eco*RI fragment, which carried the *attP* of phage ϕU was cloned into the suicide plasmid pSUP202. The resulting recombinant plasmid, pCI6, could integrate into the chromosome of *R. leguminosarum* biovar *trifolii* strain 4S and its derivatives, and *R. leguminosarum* biovar *viciae* strain K5 at high frequency (Uchiumi et al., 1993a, b). Another chromosome integrative plasmid, pCINod1, carrying *nod* genes was constructed. Plasmid pCINod1 carries a 4 kb *Eco*RI-*Hin*dIII fragment derived from the 6 kb *Eco*RI fragment of phage ϕU. All the genes required for site-specific integration were expected to be located on this 4 kb *Eco*RI-*Hin*dIII fragment because the *nod* genes were efficiently integrated into the chromosome of *Rhizobium* and were completely functional (Uchiumi et al., 1995). The chromosome integrative plasmid also made it possible to make the deduced restriction map including the *attB* site on the chromosome of the host *Rhizobium* (Uchiumi et al., 1993b). The usefulness of chromosome integrative vectors is

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Table 1. Phage, plasmids, and bacterial strains.

Phage/Plasmid/Strain	Relevant characteristics	Reference/Source
Phage		
φU	Temperate phage of *Rhizobium*	Uchiumi et al. (1989)
Plasmids		
pCI6	pSUP202 carrying *attP* of phage φU	Uchiumi et al. (1993a)
pATL1	pUC118 carrying *attL* of strain CI101	This work
pATB1	pUC118 carrying *attB* of strain 4S	This work
pATP1	pUC118 carrying *attP* of phage φU	This work
pUC118	Cloning vector	Vieira and Messing (1987)
pCRII	Cloning vector for PCR product	Invitrogen
Bacterial strains		
R. leguminosarum biovar *trifolii*		
4S	Wild-type	Higashi and Abe (1980)
CI101	pCI6::chromosome of strain 4S	Uchiumi et al. (1993a)
E. coli		
InvαF′	Cloning host	Invitrogen

thus quite clear, but there is a need to know the exact location of the *attP* and *int* genes for construction of a minimum integrative module and for understanding the molecular mechanism of integration.

In this study, we have cloned and sequenced two DNA fragments which are involved in the site-specific integration of phage φU and its host *Rhizobium* (i.e., a 4 kb *Eco*RI-*Hin*dIII fragment (*attP* fragment) of phage φU and a 2 kb *Pst*I fragment (*attB* fragment) of *R. leguminosarum* biovar *trifolii* strain 4S). The DNA sequence of *attL* and *attR* fragments, which were border fragments generated by site-specific recombination, were also decided. By analyzing the nucleotide sequences of the four fragments, the *int* gene on phage φU genome and the core sequence for integration which is common to the all fragments, were identified. The relationship between the core sequence and a tRNA gene on the chromosome of the host *Rhizobium* is also discussed.

Materials and Methods

Phage, plasmids, and bacterial strains. Phage φU is a temperate phage of *Rhizobium leguminosarum* biovar *trifolii* strain 4S (Uchiumi et al., 1989). The attachment site of phage φU (*attP*) is known to be located on a 4 kb *Eco*RI-*Hin*dIII fragment of the phage genome (Uchiumi et al., 1995). Plasmid pCI6, which carries the 6 kb *Eco*RI fragment including the 4 kb *Eco*RI-*Hin*dIII fragment of phage φU, is able to integrate into the chromosome of strain 4S in the same manner as phage φU integration (Uchiumi et al., 1993a). Plasmids pUC118 (Vieira and Messing, 1987) and pCRII (Invitrogen) were used for cloning and sequencing. *R. leguminosarum* biovar *trifolii* strain 4S is a wild-type strain (Higashi and Abe, 1980). *R. leguminosarum* biovar *trifolii* strain CI101 is a derivative of strain 4S, carrying pCI6 on its chromosome (Uchiumi et al., 1993a). *E. coli* INVαF′ was used as a cloning host. The relevant features of phage, plasmids and bacterial strains used in this study are shown in Table 1.

Media. *Rhizobium* strains were usually maintained on mannitol-yeast agar plates (Keele et al., 1969). For total cellular DNA isolation, TY medium (Beringer, 1974) was used. *E. coli* strains harboring the recombinant plasmids were cultured in LB medium containing ampicillin (50 μg ml^{-1}).

DNA manipulations. The total cellular DNA of *Rhizobium* was isolated by the method of Higashi et al. (1983). Plasmid isolation from *E. coli* was done according to Birnboim and Doly (1979) for routine analysis. Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and used as specified by the supplier. The conditions for agarose gel electrophoresis and isolation of DNA from agarose gels were as described previously (Uchiumi et al., 1993a). The reagents for the labelling of probe DNA with digoxigenin and for colorimetric detection of the hybridized probe were purchased from Boehringer Mannheim Co., Ltd., Mannheim, Germany. Conditions for Southern hybridization were as described previously (Uchiumi et al., 1993a), and detection was performed according to the supplier's instructions. For DNA sequencing, the unidirectional nested deletions in recombinant plasmids were created by digestion with exonuclease III and mung bean nuclease (New England BioLabs Co., Ltd., Beverly, USA).

Polymerase chain reaction. For cloning of the DNA fragment containing the *attR* region, polymerase chain reaction (PCR) was employed. PCR was performed on 50 ng of total DNA from strain CI101. The two primers used for PCR were ATR1 GGATACAGAGGCGATCAACG and ATR2 CCAATGTCC-

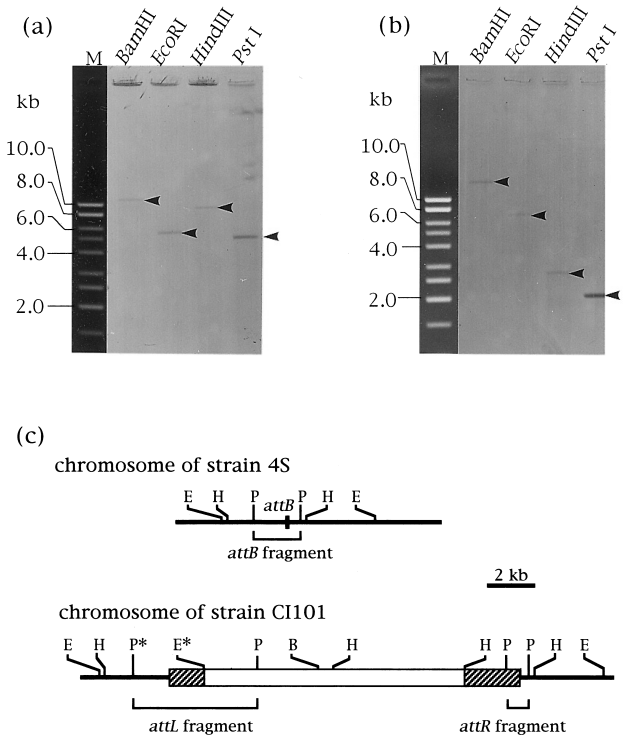

Fig. 1. Southern hybridization probed with the *attL* fragment and restriction map of rhizobial chromosomes containing *attL* and *attB*.

(a) Southern hybridization was made against restriction digests of the total cellular DNA of strain CI101. A part of the *attL* fragment of strain CI101 [the fragment lies between P* and E* in panel (c)] was used as a probe. The hybridized signals are indicated by arrowheads. (b) Southern hybridization was performed against strain 4S using the *attL* probe. The hybridized signals are indicated by arrowheads. Lane M in (a) and (b) is the molecular size marker. (c) Restriction map of the chromosome of *Rhizobium* strains. The accurate location of *attB* was demonstrated in this study. On the chromosome of strain CI101, the integrated pCI6 is indicated by open and hatched boxes. The *attP* fragment (4 kb *Eco*RI-*Hin*dIII fragment) on pCI6 was split and generated two fragments of *attL* and *attR* (indicated by hatched boxes flanking the thin lines).

GATAAGCACC. After 30 cycles of amplification (94°C for 30 s, 55°C for 30 s, 72°C for 1 min), the unique PCR product was ligated into cloning vector pCRII (Invitrogen).

DNA sequence analysis. The recombinant plasmids for sequencing were purified by the method of Hattori et al. (1985). The sequence of DNA was determined using both an Applied Biosystems 373A automated DNA sequencer and an Applied Biosystems 310 genetic analyzer. Database searches were performed using the programs FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990).

Results

Cloning of attB fragment of host Rhizobium

R. leguminosarum biovar *trifolii* strain CI101 was generated by inserting plasmid pCI6 into the chromosome of *R. leguminosarum* biovar *trifolii* strain 4S (Uchiumi et al., 1993a). The *attL* and *attR* fragments were detected among the restriction fragments of strain CI101 by Southern hybridization probed with the *attP* fragment of phage ϕU. For cloning the 5 kb *Pst*I fragment (*attL*, Fig. 1c) of strain CI101, the total cellular DNA of strain CI101 was digested with restriction endonucleases *Hin*dIII, *Bam*HI, and *Pst*I, ligated with the *Pst*I site of pUC118, and then transformed into *E. coli* INVαF′. The *E. coli* clone carrying the *attL* fragment was screened by Southern hybridization probed with the 6 kb *Eco*RI fragment of phage ϕU. The recombinant plasmid in positive *E. coli* clones was referred to as pATL1. Plasmid pATL1 had a 5 kb insert which could be digested with *Eco*RI but not with *Hin*dIII and *Bam*HI. When Southern hybridization was performed against the restriction digests of the total cellular DNA of strain CI101 using a part of the 5 kb insert as a probe, single fragments could be detected in each digest (Fig. 1a, c). The molecular size of these fragments was identical to that of the *attL* fragments detected among the restriction fragments of strain CI101 by Southern hybridization probed with a 6 kb *Eco*RI fragment of phage ϕU. These results indicate that the 5 kb insert in pATL1 is the *attL* fragment of strain CI101.

The restriction map of the *attB* region of strain 4S has been deduced from the restriction maps of pCI6 and the pCI6-integrated chromosome of strain CI101, as shown in Fig. 1c. Southern hybridization was performed against the restriction fragments of strain 4S using purified *attL* fragments in pATL1 as the probe (Fig. 1b). The molecular size of the detected fragments was identical to the expectations from the map of the *attB* region. The results of this hybridization justified the deduced map of the *attB* region. In this study, the 2 kb *Pst*I fragment of strain 4S (indicated by an arrow in Fig. 1b) was cloned as the *attB* fragment into pUC118 and the recombinant plasmid was referred to as pATB1.

Determination of core sequence for site-specific integration

It has already been known that the genes responsible for site-specific integration are located within the 4 kb *Eco*RI-*Hin*dIII region of the 6 kb *Eco*RI fragment of the phage ϕU genome (Uchiumi et al., 1995). For sequencing, the 4 kb *Eco*RI-*Hin*dIII fragment (*attP* fragment) was subcloned from pCI6 into pUC118. The resulting recombinant plasmid was referred to as pATP1, and the nucleotide sequence of the *attP* fragment was done.

The *attR* fragment (Fig. 1c) was cloned directly from the total DNA of strain CI101 by PCR. Two primers

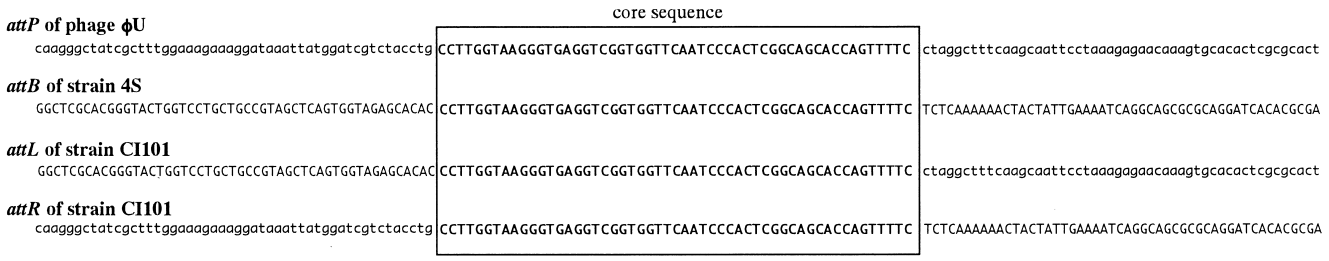

Fig. 2. Alignment of DNA sequences of *attP*, *attB*, *attL*, and *attR*.
The sequences of host bacterial chromosomes and the sequence derived from the phage ϕU genome are written in capital letters and small letters, respectively. The 53 bp core sequence is boxed. The nucleotide sequence data reported here will appear in the DDBJ, EMBL, and GenBank with the accession numbers AB004561, AB004562, AB010267, and AB010268.

were synthesized based on the sequences of the *attP* and *attB* fragments. The 0.6 kb PCR product, as the *attR* fragment, was cloned into pCRII and then sequenced.

A comparison of the nucleotide sequences of the four fragments, *attP*, *attB*, *attL*, and *attR*, revealed the existence of a 53 bp common sequence (Fig. 2). It is known that the site-specific integration of lysogenic phage takes place at the site of the common sequence between *attP* and *attB* (Sadowski, 1986). The 53 bp sequence was conserved in both the *attL* and *attR* fragments (Fig. 2). This suggests that the conservative site-specific recombination occurred within the 53 bp sequence. In the case of phage ϕU and its host *Rhizobium*, this 53 bp common sequence is expected to be a core sequence for integration.

Identification of integrase gene on the attP fragment of phage ϕU

A database search was performed for all of the amino acid sequences of the 21 possible ORFs on the *attP* fragment. One of the ORFs (the position from 64 to 681 in Fig. 3), which was located adjacent to the core sequence, showed significant homology with the integrase family. This ORF was referred to as *orf3* and was analyzed in detail. The nucleotide sequence containing *orf3* with the core sequence is shown in Fig. 3. *Orf3* initiates at ATG (position 64) and terminates at TAA (position 679). The termination codon is 19 bases upstream from the 5′ end of the core sequence. The transcript of *orf3* contains 205 amino acids with an estimated molecular size of 22.7 kDa and a pI value of 10.41. This protein is highly basic with 42 basic (20.5%) and 24 acidic (11.7%) residues. This is the same characteristic as the integrase of staphylococcal bacteriophage L54a (Ye and Lee, 1989). Abremski and Hoess (1992) reported about three regions which were highly conserved in the integrase family; a region containing Arg, a region containing the His-X-X-Arg motif and a region containing Tyr. It is possible to pick up many of the integrase family members from the SWISS-PROT database using the pattern Arg-X-

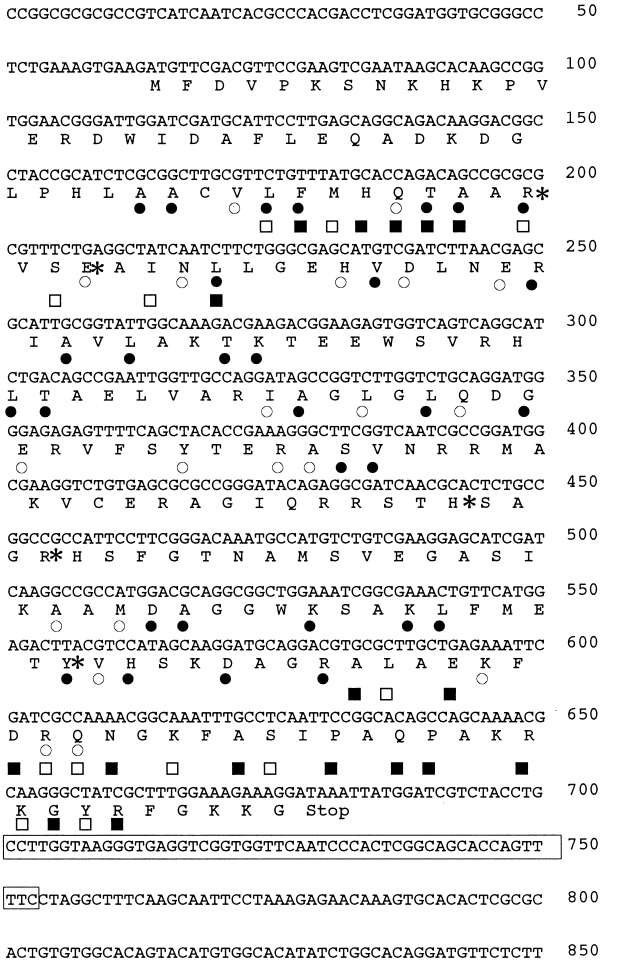

Fig. 3. Nucleotide sequences of the *attP* fragment of phage ϕU.
The nucleotide sequence of the 850 bp from the *Eco*RI side of the *attP* fragment is shown. The encoded amino acid sequence is indicated below the nucleotide sequence at *orf3*. The boxed sequence corresponds to the 53 bp core sequence which is common to the *attB* fragment of strain 4S. The amino acids with asterisks are highly conserved in the integrase family reported by Abremski and Hoess (1992). The amino acids homologous to shufflon-specific recombinase are indicated by circles (● and ○) and to the RYB-a protein by squares (■ and □). The filled symbols (● and ■) and open symbols (○ and □) indicate identical and functionally homologous amino acids, respectively. The nucleotide sequence data reported here will appear in the DDBJ, EMBL, and GenBank with the accession number AB004561.

X-[Asp, Glu, Asn] ⟨73, 131⟩ His-X-X-Arg ⟨31, 38⟩ Tyr. The transcript of *orf3* exhibited the same pattern except that the His-X-X-Arg motif has an insertion of one amino residue (i.e., Arg-Val-Ser-Glu-77 amino residues-His-Ser-Ala-Gly-Arg-33 amino residues-Tyr) (Fig. 3, amino acids with asterisk). Furthermore, a homology search using the program BLAST gave the highest score with the shufflon-specific recombinase of *Haemophilus influenzae* (Fleischmann et al., 1995) and significant homology with many integrase proteins. The database search also revealed that the C-terminal region was homologous to RYB-a protein, a DNA-binding protein of rat (Ito et al., 1994). The amino acids homologous to the shufflon-specific recombinase are indicated by ● and ○, and to RYB-a protein by ■ and □ in Fig. 3, respectively. These results suggest that *orf3* encodes the integrase of phage ϕU.

No other ORFs on the *attP* fragment could be found to show significant homology with any known proteins.

Overlap of core sequence with a putative tRNA gene of host Rhizobium

When database searches were carried out, the *FlaABC* genes of *Agrobacterium tumefaciens* (ac. no. X80701) and the tRNA genes of *E. coli* and other various organisms were found to be highly homologous to the 2 kb *Pst*I fragment (*attB* fragment) of *Rhizobium* strain 4S. Because part of the nucleotide sequence of the *A. tumefaciens FlaABC* gene, which gave the highest score of homology with the *attB* fragment, also has a cloverleaf folding structure, it is expected to be a putative tRNA gene of *A. tumefaciens*. A cloverleaf representation of the putative 75-base RNA transcript from the *attB* fragment of *Rhizobium* strain 4S is shown in Fig. 4a. The sequence GGU in the anticodon loop indicates that this tRNA is a putative $tRNA^{Thr}$. The 47 bp (89%) of the 53 bp core sequence overlaps the 3′ end of this putative tRNA gene (Fig. 4b). This indicates that the target site of phage ϕU integration is a putative $tRNA^{Thr}$ gene on the chromosome of its host *Rhizobium*.

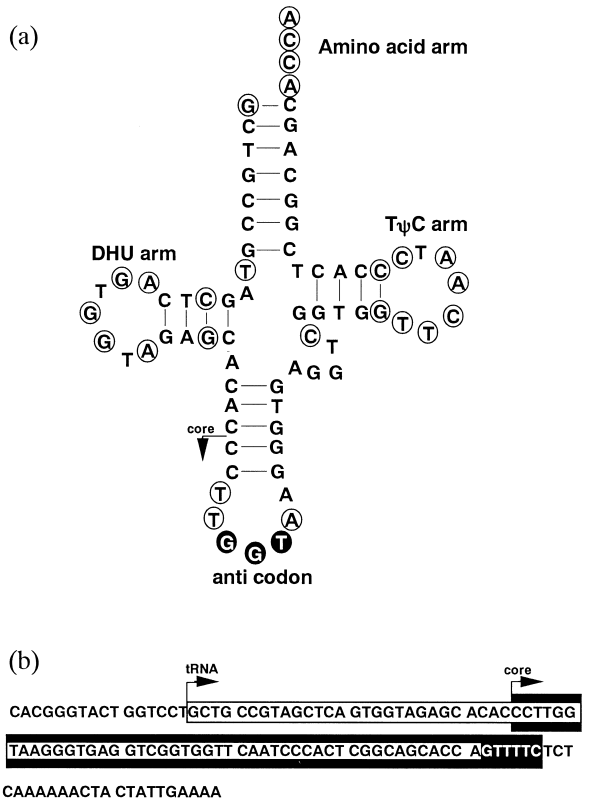

Fig. 4. Cloverleaf structure alignment and nucleotide sequence of the *attB* site of strain 4S with core sequence.

(a) Circled bases are conserved in tRNAs. The anticodons (GGT) are indicated by the closed circles with white letters. (b) The nucleotide sequence corresponding to tRNA is enclosed with an open box. The core sequence is written on a closed box. The core sequence overlaps the tRNA sequence.

Discussion

To understand the molecular mechanism of site-specific integration of phage ϕU and its host *Rhizobium*, two DNA fragments, the *attP* fragment of phage ϕU and the *attB* fragment of *R. leguminosarum* biovar *trifolii* strain 4S, were investigated in detail. The 4 kb *Eco*RI-*Hin*dIII fragment of phage ϕU and the 2 kb *Pst*I fragment of strain 4S had already been expected to be the *attP* and *attB* fragments, respectively (Uchiumi et al., 1993b). In this study, nucleotide sequence analyses of these two fragments revealed the region involved in site-specific recombination between the phage and host chromosome.

Orf3, one of the orfs on the *attP* fragment of phage ϕU, encoded a basic polypeptide of 205 amino acids and was located adjacent to the core sequence. The molecular size of the deduced product of *orf3* is relatively small compared with the known integrases of temperate phages; however, its location and homology to site-specific recombinases of the integrase family suggest strongly that *orf3* encodes the integrase of phage ϕU. In characterized site-specific integration systems so far studied, the integrase (*int*) and excisionase (*xis*) genes are located next to each other (Gregg et al., 1994; Leong et al., 1986). However, no ORF which has specific features for known excisionases could be found within the *attP* fragment sequenced in this study.

A comparison of the nucleotide sequences of the *attP* and *attB* fragments revealed a 53 bp common core sequence for integration. This 53 bp core sequence was conserved in both *attL* and *attR* fragments. Furthermore, the core sequence overlapped

the 3′ end of the tRNA gene of the host *Rhizobium*, as found in other phages of Gram-negative bacteria (Pierson III and Kahn, 1987; Reiter et al., 1989; Sun et al., 1991) and in the integrative plasmids of actinomycetes (Brown et al., 1990; Vrijbloed et al., 1994). Some of the tRNA genes are located on the 16S-23S rDNA spacer region (Gürtler and Stanisich, 1996). However, no characteristic sequence, including rRNA genes, could be found within the 2 kb chromosomal region containing the target tRNA gene for phage ϕU integration. Why is the target sequence for the phage integration the tRNA gene, and why does the phage have a sequence which is identical to the 3′ end of the tRNA gene of the host bacteria? One explanation for tRNA targeting is that integrases use the symmetric sequence in the tRNA gene as a recognition site (Campbell, 1992). Concerning horizontal gene transfer, genetic elements able to integrate into tRNA genes may spread easily among heterogeneous bacterial populations because of the high conservation of the structure of the tRNA gene. It is also possible to speculate that ancestors of temperate phages may have incorporated a tandemly arranged integrase-tRNA unit (e.g., a shufflon-specific integrase and tRNA) which has been found in the *Haemophilus influenzae* genome (Fleischmann et al., 1995). The spacer region between integrase and tRNA may have been deleted during the process of incorporation.

We have already reported that suicide plasmids carrying the *attP* fragment of phage ϕU are integrated into the chromosome of host *Rhizobium* strains at high frequency. Such integration is expected to require the core sequence and integrase coded on the fragment. In this study, the DNA sequence and exact location of the core sequence and integrase were shown to be on the 4 kb *Eco*RI-*Hin*dIII fragment. This result will help in the construction of a minimum chromosome-integrative vehicle for *Rhizobium*. Since the target sequence for integration is the tRNA gene, it is quite possible that many bacterial strains belonging to the family Rhizobiaceae may have the target sequence. In fact, the highest score of homology to the target sequence was obtained from a putative tRNA gene of *Agrobacterium tumefaciens*. It will be possible to construct wide-host range vectors based on a minimum chromosome integrative vector.

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