GENETIC ENGINEERING ON NODULATION GENES OF RHIZOBIUM

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GENETIC ENGINEERING ON NODULATION GENES OF *RHIZOBIUM*

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GENETIC ENGINEERING ON NODULATION GENES OF

RHIZOBIUM

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根粒菌の根粒形成に関する遺伝子工学的研究

内海俊樹

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CONTENTS

I. INTRODUCTION

1. GENERAL ASPECTS

(Brady)Rhizobia have the unique ability to fix nitrogen gas, comprising 78% of the atmosphere into ammonia only at the bacteroids in the root nodule cells. The fixed product, ammonia is excreted and quickly assimilated into glutamine or glutamate, which are subsequently converted to other amino acids and organic compounds in the nodule tissue. The other unique abilities of the group of bacteria are to select host plant (the host specificity) and to induce nodules on the conformable host roots (and stem) [32].

The effective nodule organogenesis requires a series of complex processes, including root adhesion (Roa), root hair curling (Hac), infection thread formation (Inf), bacterial release into plant cortical cells (Bar), and differentiation into nitrogen fixing nodules (Nif). Thus nodule organogenesis requires a genetic intimate collaboration between bacteria and their host plants.

The early stage of infection processes of *(Brady)Rhizobium* have been clarified on the dynamic interactions between bacterial nodulation (*nod*) genes and responding metabolism of the host plant [32]. The host plant secretes flavonoids from roots, and subsequently *nod* genes in rhizobial cells respond to these low chemical molecules. The flavonoids secreted from the roots of the host plant pull the trigger on the expression of the *nod* genes of *(Brady)Rhizobium*, resulting in the synthesis of specific lipo-oligosaccharides named Nod factors.

These Nod factors are secreted to the rhizosphere of the host plant. After recognizing the factors, the plant roots respond rapidly and produce nodule-specific proteins as early nodulins within a very short time [30]. Such interactions between *nod* genes of *(Brady)Rhizobium* and the host root tissues are existing on the complex communications.

(Brady)Rhizobia are classified into two groups depending on the growth rate; the fast growing group is classified as *Rhizobium* and the slow growing group as *Bradyrhizobium*. Generally, in genus *Rhizobium*, *nod* (nodlulation) genes and *nif* (nitrogen fixation), *fix* (fixation) genes are located at a large symbiotic (Sym) plasmid. In genus *Bradyrhizobium*, all these symbiotic genes are localized on the chromosome. However, some unique *Rhizobium* strains have been reported. *R. fredii* USDA 205 has the *nod* genes located on 112 Md plasmid and *nif*, *fix* genes located on 195 Md plasmid separately [68]. *R. loti* strains NZP2037 and NZP2213 which nodulate on *Lotus* species are expected to have chromosomal symbiotic genes as same as *Bradyrhizobium* [16]. The exact reasons why rhizobial species have different location of the symbiotic genes, Sym-plasmid or chromosome, remain unknown. Does the different localization of *nod* genes influence the symbiotic communications between *Rhizobia* and the host plants ? If a cloning vector system which can integrates symbiotic genes into the chromosome of *Rhizobium* (chromosomal integrative vector system) is developed, the system will play an important roll to answer the problem.

The chromosomal integrative vector system will also be useful on another point of view. Many different wide host range cloning vectors have contributed to the molecular genetic analysis of the *(Brady)Rhizobium*-legume symbiosis [9, 52, 61, 62]. Although interactions among genes need to be investigated, it is difficult to stably maintain multiple plasmids in a *(Brady)Rhizobium* cell. Loss of vectors and cloned inserts during nodulation is a problem for functional studies in many strains of *(Brady)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 *(Brady)Rhizobium*. Marker exchange of insertions flanked by a neutral sequence resident in the chromosome [58] and homologous recombination using suicide plasmids [82] were proposed as methods for introducing stable genomic copies of new or added genes. The repeat sequence of *Bradyrhizobium japonicum* may be useful for the integration of cloned genes into the chromosome [2]. Kunnimalaiyaan *et al.* [56] reported integration of the cloned *hup* (uptake-hydrogenase) gene into the chromosome of *Rhizobium* strain G36-84 by homologous recombination using transposon Tn5. The integrated *hup* gene was stably maintained and expressed under symbiotic conditions [55]. Hermesz *et al.* [31] reported another plasmid vector system which integrated into the chromosome of *Rhizobium meliloti*. They used the bacterial attachment site for lysogenic phage 16-3 as a target for gene integration, utilizing the site-

specific recombination process of lysogenic phage 16-3. The system is elaborate and suitable for maintaining cloned genes stably in *Rhizobium* cells. It is also important that this system makes it possible to introduce cloned genes in a single copy into the host *Rhizobium*. 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.

2. THIS WORK

In this work, integration of *nod* genes from the Sym plasmid into the chromosome was examined in *R. leguminosarum* biovar *trifolii* to compare the gene expression of the same *nod* genes under the different location. For integration of cloned *nod* genes into the bacterial chromosome, the chromosomal integrative vector system was developed using lysogenic phage ϕU which was isolated from *Rhizobium* in a wild grown clover nodule.

In Chapter II, isolation and characterization of phage ϕU are described. The gene expression of phage ϕU in both the free-living and the symbiotic form of the host *Rhizobium*, is also reported. Phage ϕU integrated its genome into the host bacterial chromosome by site-specific recombination between *attP* (phage) and *attB* (host bacterial chromosome). I hypothesized that if a plasmid carrying the *attP* site of phage ϕU could be constructed and introduced into *Rhizobium* cell, the recombinant plasmid might integrate into the host chromosome by the same mechanism as phage ϕU integration. The identification of *attP* site of phage

ϕU and the development of the chromosomal integrative vector system are described in Chapter III. The application of the chromosomal integrative vector for other strains of Rhizobiaceae is assessed and the chromosomal location of *attB* site is discussed in Chapter IV. The construction of the chromosomal integrative vector carrying *nod* genes and isolation of the *Rhizobium* transconjugant strains harbouring chromosomal *nod* genes are described in Chapter V. The nodulation ability and nodulation process of the *Rhizobium* transconjugant are also discussed.

Several parts of this work have already been published [86, 87, 88].

II. PHAGE INDUCTION OF LYSOGENIC *RHIZOBIUM LEGUMINOSARUM* BIOVAR *TRIFOLII* IN BOTH THE FREE-LIVING AND THE SYMBIOTIC FORM

1. INTRODUCTION

Bacteriophages of *Rhizobium* (rhizobiophages) have been isolated from soils and root nodules [18,54]. Kleczkowska [49,50] and Gupta & Kleczcowska [27] suggested a role for rhizobiophages in the evolution of ineffective rhizobial strains. Barnet [6] and Raleigh & Signer [72] reported that symbiotic-defective mutants were isolated as surviving cells after exposure to specific virulent phages. They also referred to changes in other morphological and physiological properties but the mechanism of loss of symbiotic properties was unknown.

Transduction system using lysogenic phages of *R. meliloti* or *R. leguminosarum* have been established and used to analyze *Rhizobium* genes [14, 25, 53, 66, 81]. In addition, genetic analyses of phage 16-3, the lysogenic phage of *R. meliloti*, have been done in detail and its possibility as a useful cloning vector has been discussed [21, 22, 70]. In view of the symbiotic nitrogen fixation ability of *Rhizobium*, it is important to study phage gene expression in members of this genus under both free-living and symbiotic conditions.

I report here the isolation and properties of a lysogenic phage of *R. leguminosarum* biovar *trifolii*. Its role in symbiotic effectiveness was also assessed.

2. MATERIALS AND METHODS

Bacterial strains and media. Rhizobium leguminosarum biovar *trifolii* UK-1(ϕU) (original host strain of phage ϕU) was isolated from a nodule of a wild grown white clover plant (*Trifolium repens*) in the campus field and lysogeny detected according to Ördögh & Szende [71]. Wild-type *Rhizobium leguminosarum* biovar *trifolii* strain 4S [34] and its derived mutants, A1 [34] and H1 [35], were used as indicators or hosts of phage ϕU. These strains were maintained on mannitol/yeast agar slants [47]. For phage induction and plasmid isolation, TY medium [8] was used to avoid excess production of exopolysaccharides. *Escherichia coli* RR1 carrying plasmid pRt032, which has *nod* genes of *R. leguminosarum* biovar *trifolii* [77] was cultured in LB medium.

Preparation of phage ϕU lysates. R. leguminosarum biovar *trifolii* UK-1(ϕU) was grown at 28 °C in 100 ml TY liquid medium to about 5 x 10^8 cells ml^{-1} and mitomycin C was added to a final concentration of 1 μg ml^{-1}. After 6-18 h incubation at 28 °C with vigorous shaking, 0.5-1.0 ml chloroform was added and incubation continued for 30 min. Bacterial cells and cell debris were removed by centrifugation at 10000 g for 10 min. The supernatant obtained was dialysed against phage buffer [81] overnight to remove chloroform.

Phage sensitivity test (spot test). Phage lysates of phage ϕU or virulent phage 4S-phage [33], and kp1 and kp2, isolated from soil samples using *R.*

leguminosarum biovar *trifolii* 4S as a host, were spotted on test strains. The plates were examined for bacterial lysis after 3 d incubation at 28 °C.

Lysogenization of R. leguminosarum biovar trifolii 4S with phage ϕU. Phage ϕU was spotted on plates of strain 4S, and incubation for 7 d at 28 °C. Colonies that had grown on the lytic area were isolated and purified by clonal isolation. Phage induction by UV irradiation (15W at 50 cm height for 30 sec) and immunity against phage ϕU were demonstrated with each isolate.

Isolation of phage ϕU DNA and plasmid DNAs. Phage ϕU DNA was prepared as described by Yamamoto *et al.* [93] and Dallmann *et al.* [17], using polyethylene glycol 6000 and Actinase E (Kaken Seiyaku Co., Japan) followed by phenol extraction. For plasmid isolation from *Rhizobium*, clear lysates were prepared as described by Casse *et al.* [15] except that the SDS concentration was 4% (w/v). Plasmid pRt032 was purified according to Birnboim & Doly [12]. Prepared DNAs were digested with restriction endonucleases according to standard methods.

Agarose gel electrophoresis. Phage ϕU DNA and plasmid DNAs were analysed on 0.7 % agarose gels. Electrophoresis was performed at 100 V for 4 h for restriction fragments and at 50 V for 14 h for plasmids, using TB buffer (89 mM-Tris base, 2 mM-EDTA, 89 mM-boric acid, pH 8.4).

Southern hybridization. After agarose gel electrophoresis, the gels were treated with hydrochloric acid solution followed by sodium hydroxide solution. Denaturated DNAs in the gels were electrophoretically transferred to Millipore GVHP304F0 filters employing a Trans-Blot cell (Bio-Rad). Phage ϕU DNA restriction fragments were labelled with ^{32}P using the Multiprime DNA labelling system (Amersham) and used as probes. Hybridization was performed at 60 °C for 18 h. Then the filters were washed with SSC solution as described by Southern [83] and autoradiographed. Southern hybridization using pRt032 as the probe was done in the same way.

Nodulation test. Nodulation and nitrogen fixation tests of strains UK-1(ϕU) and 4S(ϕU) were done as described elsewhere in detail [35].

Electron microscopy. Phage specimens obtained by glycerol stepwise density-gradient centrifugation [89] were negatively stained with 2 % (w/v) uranyl acetate. Nodules inoculated with strain UK-1(ϕU) or strain 4S(ϕU) were observed by scanning electron microscopy and transmission electron microscopy [36].

3. RESULTS AND DISCUSSION

Characteristics of Rhizobium leguminosarum biovar trifolii UK-1(ϕU)

To obtain lysogenic strains, 15 strains of *R. leguminosarum* biovar *trifolii* were isolated from nodules of wild grown white clovers and tested for lysogeny using *R. leguminosarum* biovar *trifolii* 4S as an indicator. One strain, UK-1(ϕU), indicated lysogeny. This strain also formed effective nodules on white clover (*Trifolium repens* cv. Ladino). A cell homogenate of UK-1(ϕU) was prepared and examined by polyacrylamide gel electrophoresis. The activity staining patterns of esterase, acid phosphatase and basic phosphetase of strain UK-1(ϕU) were very similar to those of strain 4S (data not shown). A clear lysate of strain UK-1(ϕU) was prepared and separated on 0.7 % agarose gel. Three mega-plasmids, of 525, 420 and 315 kb were detected (see Fig. 5 a, lane 1, and Fig. 7 a, lane 2).

Characteristics of phage ϕU

Table 1 shows the host range of phage ϕU determined by spot tests using ϕU lysate (see MATERIALS AND METHODS). Phage ϕU indicated lysogeny on *Rhizobium leguminosarum* biovar *trifolii* 4S and its derived mutants A1 and H1.

Electron microscopy of phage ϕU (Fig. 1) showed it to have an icosahedral head (diameter, 40 nm) and a short tail (length, 9 nm) resembling that of Salmonella phage P22 [13] and rhizobiophage MM1H reported by Werquin *et al.* [92]. The tail has three parts, collar (diameter, 10 nm), rigid short tail, and thin fibres (length, 15 nm). The thin fibres, which have a spherical molecules at the

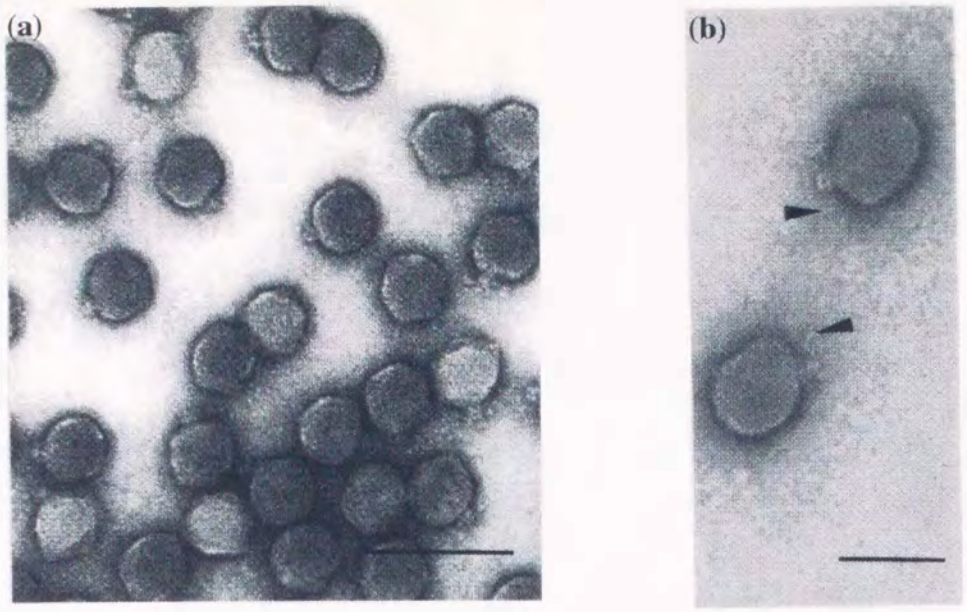

Fig. 1. Electron micrographs of lysogenic phage ϕU, negatively stained with uranyl acetate. (a) Bar, 100 nm. (b) The arrows indicate the spherical molecule of tail fibre. Bar, 50 nm.

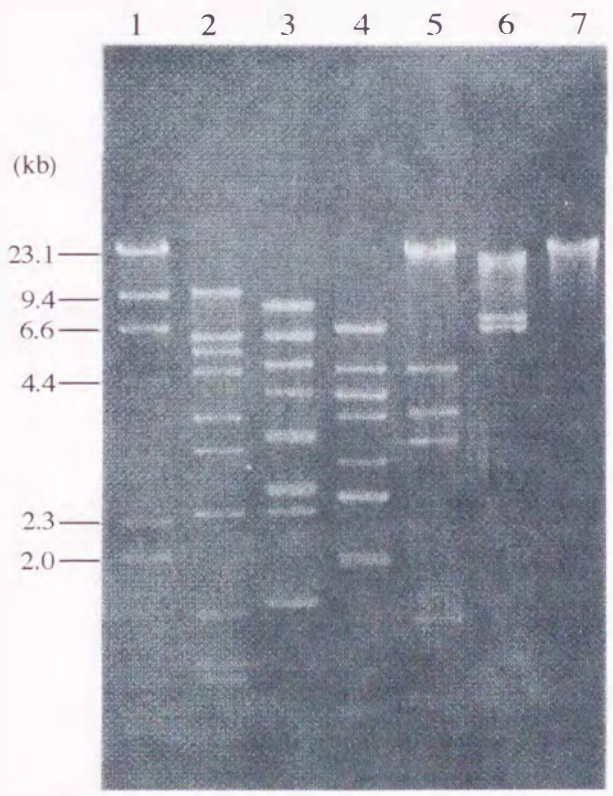

Fig. 2. Restriction endonuclease patterns of phage ϕU DNA on 0.7 % agarose gel. Lane 1, λ phage DNA digested with *Hin*dIII; lanes 2-7, phage ϕU DNA digested with *Hin*dIII, *Eco*RI, *Pst*I, *Sma*I, *Bam*HI and *Kpn*I, respectively.

end (Fig. 1 b), seem to extend from the neck-collar region. According to the morphological classification of Bradley [13], phage ϕU is a member of group C, *Podoviridae*.

DNA isolated from phage ϕU particles was digested with restriction endonucleases *Hin*dIII, *Eco*RI, *Pst*I, *Sma*I, *Bam*HI and *Kpn*I (Fig. 2). *Hin*dIII, *Eco*RI and *Pst*I gave at least ten fragments (lanes 2-4). *Sma*I and *Bam*HI gave six and four fragments, respectively (lanes 5 and 6). *Kpn*I did not digest the DNA (lane 7). The genomic size of phage ϕU was estimated from these digests to be about 40 kb.

Nodules formed by UK-1(ϕU)

The nodulation process of *R. leguminosarum* biovar *trifolii* UK-1(ϕU) was followed by light microscopy. As with the other *R. leguminosarum* biovar *trifolii* wild-type strains, root hair curling and infection thread formation were observed. Several nodules were visible on clover roots at 5 d after inoculation. The nodulation ability of strain UK-1(ϕU) was the same as that of strain 4S as to the numbers of nodules formed. These nodules were functional, as indicated by acetylene reduction ability and growth of host plants.

The nodule exudate prepared form a surface sterilized nodule formed a large number of plaques on indicator plates. This indicates that phage induction should occur both in bacteria and in bacteorids within host plant cells. Nodules were incubated overnight at 28 °C in sterilized water containing mitomycin C

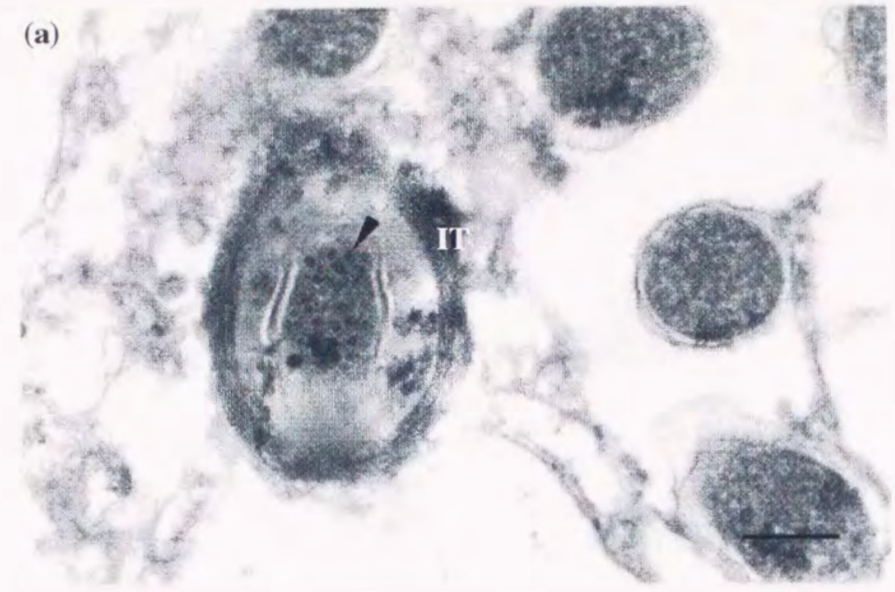

Fig. 3. Transmission electron micrographs of phage ϕU in host plant cells. Nodules formed by *R. leguminosarum* biovar *trifolii* UK-1(ϕU) were treated overnight with mitomycin C before sectioning. (a) *R. leguminosarum* biovar *trifolii* UK-1(ϕU) in an infection thread (IT). The arrow indicates phage ϕU particles. Bar 0.5 μm. (b) phage ϕU particles (arrowed) in the host plant cell. Bar, 1 μm. CW, host plant cell wall; Bd, bacteroid; PBM, peribacteroidal membrane.

(10 μg ml-1), then sectioned and examined by transmission electron microscopy. *Rhizobium* cells invade the host plant cells using infection threads, and then change their form to a bacteroid. Fig. 3(a) shows a bacterium in an infection thread and Fig. 3(b) shows a bacteroid surrounded by a peribacteroidal membrane. Highly electron-dense particles (arrowed in Fig. 3) were observed in both cases. In Fig. 3(b), the particles are apparently free in the host cell cytoplasm. Observation of these particles at higher magnification revealed them to be the same size and shape as phage ϕU heads. These particles appear to be phage ϕU. Although phage ϕU is expected to be induced spontaneously in host plant cells, the frequency of such spontaneous induction was not determined.

As phage ϕU gene expression is induced in strain UK-1(ϕU) under both symbiotic and free-living conditions, this phage may became a useful tool for gene transfer in a rhizobial symbiosis system.

Lysogeny and symbiotic ability

To lysogenize strain 4S, phage ϕU lysates were spotted on spread plates of strain 4S (see MATERIALS AND METHODS). Bacteria isolated from turbid plaques were purified and examined for lysogeny. One of these isolates, strain 4S(ϕU), was used for further investigation.

The phage sensitivity of strain 4S(ϕU) is summarized in Table 1. Strain 4S(ϕU), like its parent strain, was sensitive to all three virulent phages tested, but resistant to phage ϕU. The morphology of phages induced in strain 4S(ϕU)

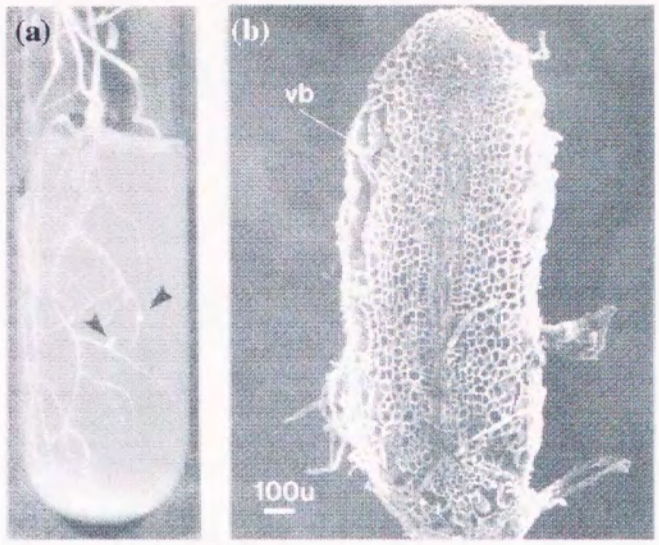

Fig. 4. Root protrusions induced by *R. leguminosarum* biovar *trifolii* 4S(ϕU). (a) White clover roots inoculated with *R. leguminosarum* biovar *trifolii* 4S(ϕU) after 2 months incubation. The arrows indicate root protrusions. (b) Scanning electron micrograph of a longitudinal section of the root protrusion in (a). Bar, 100 μm. VB, vascular bundles.

Table 1. *Characteristics of R. leguminosarum biovar trifolii strains against phage ϕU and the other virulent phages*

Strain	Lysogenic phage	Virulent phage			Symbiotic
	ϕU	4S-phage	kp1	kp2	ability
UK-1(ϕU)	R	S	S	R	+
4S(ϕU)	R	S	S	S	-
4S	L	S	S	S	+
H1	L	S	S	S	-
A1	L	S	S	S	-

R, Resistant; L. Lysogeny; S, Sensitive.
+, Effective nodule formation; -, no nodule formation.

by either UV irradiation or mitomycin C treatment was identical to that of phage ϕU. The growth rates of 4S(ϕU) in YM medium and TY medium were the same as those of 4S.

Strain 4S(ϕU) was inoculated onto 200 clover seedlings and incubated for about 2 months. These inoculated seedlings did not form nodules, and only two root protrusions were induced on one plant (Fig. 4 a). These protrusions were very similar in shape to those described by Hirsch *et al.* [37]. Fig. 4(b) shows a scanning electron micrograph of a cross-section of one of the protrusions. The vascular bundles are at the centre and the cells are empty. It appeared that normal nodulation processes might have been inhibited by lysogenization, through an unknown mechanism.

Hybridization analyses

Plasmid DNAs of strains UK-1(ϕU), 4S(ϕU) and 4S were compared on agarose gel (Fig. 5). Strains UK-1(ϕU) and 4S both carried three plasmids, of 525, 420 and 315 kb. The 315 kb plasmids in each strain were identified as Sym plasmids [35]. This 315 kb plasmid could not be detected in strain 4S(ϕU), although the two larger plasmids were present (Fig. 5 a, lane 3; Fig. 7 a, lane 4).

It was not clear whether the Sym plasmid of 4S(ϕU) had been cured or whether phage ϕU DNA had integrated into the *nod* region of the Sym plasmid. If phage ϕU DNA was integrated into Sym plasmid, the mobility of the plasmid should be decreased on agarose gel. Crude plasmid DNAs (Fig. 5) and *Hin*dIII

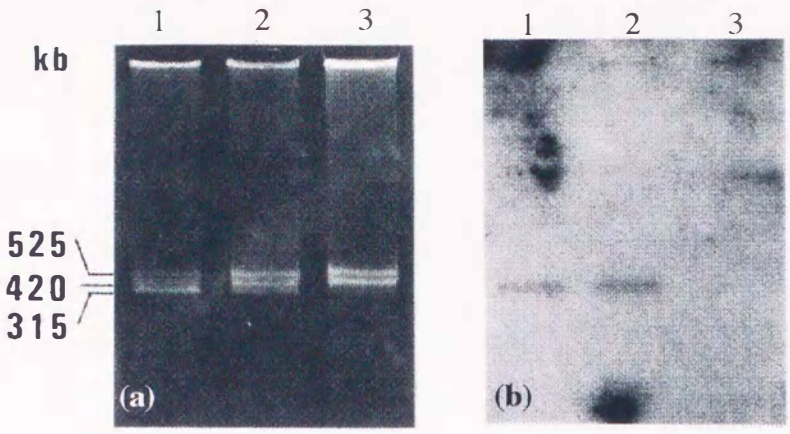

Fig. 5. (a) Crude plasmids from *R. leguminosarum* biovar *trifolii* strains prepared according to Casse [15] and separated on 0.7 % agarose gel; (b) Southern hybridization under stringent conditions using ^{32}P-labelled *Hin*dIII digest of pRt032 as a nod probe. Lanes 1, UK-1(φU); lanes 2, 4S; lanes 3, 4S(φU).

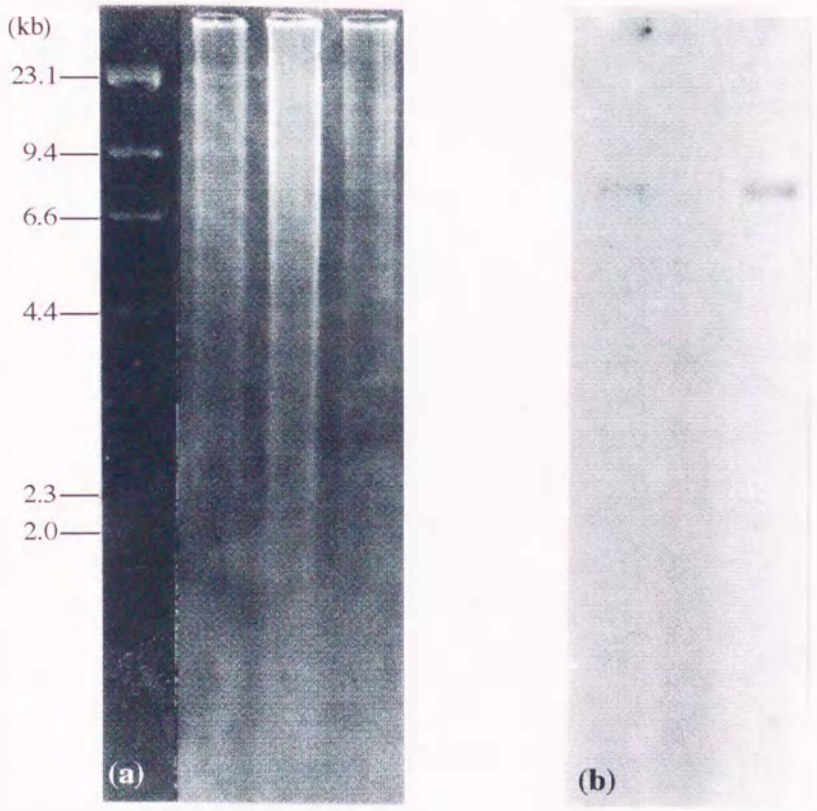

Fig. 6. (a) Restriction endonuclease patterns of *R. leguminosarum* biovar *trifolii* strains digested with *Hin*dIII; (b) Southern hybridization using ^{32}P-labelled *Hin*dIII digest of pRt032 as a *nod* probe. Lane λ, *Hin*dIII fragments of λ DNA; lanes 1, UK-1(φU); lanes 2, 4S(φU); lanes 3, 4S. A 7.2 kb fragment in UK-1(φU) and 4S hybridized with the *nod* probe. No hybridization was observed with 4S(φU).

digests of total DNA (Fig. 6) from each strain were hybridized with pRt032, as a *nod* gene probe. In strains UK-1(ϕU) and 4S, the 315 kb plasmid (Fig. 5 b, lanes 1 and 2) and a 7.2 kb *Hin*dIII fragment (Fig. 6 b, lanes 1 and 3) hybridized with the *nod* probe. However, no hybridization was detected in strain 4S(ϕU) (Fig. 5 b, lane 3; Fig. 6 b, lane 2). These results support the hypothesis that the Sym plasmid was cured in strain 4S(ϕU).

Additional broad bands were, however, detected in strain 4S(ϕU). These new bands (arrowed in Fig. 7 a) were always detected, independent of mitomycin C treatment. These additional bands also appeared in strain UK-1(ϕU), but only when the cells were incubated with mitomycin C (1 μg ml^{-1}). The bands were first detected after 20 min incubation with mitomycin C. Their staining intensity on agarose gel increased with increasing length of incubation time with mitomycin C (data not shown). These bands were presumed to be phage DNA. An *Eco*RI digest of phage ϕU DNA was labelled and hybridized with plasmid DNA of the lysogenic strains treated or not treated with mitomycin C (Fig. 7 b). The additional bands in UK-1(ϕU) treated with mitomycin C and in 4S(ϕU) with or without mitomycin C treatment all hybridized with phage ϕU DNA. In UK-1(ϕU) not treated with mitomycin C (Fig. 7, lanes 2), the phage ϕU probe hybridized with fragmented chromosomal DNA. These results suggest integration of phage ϕU into the chromosome. To investigate this further, *Eco*RI or *Hin*dIII fragments of total DNA of UK-1(ϕU) and 4S(ϕU) were hybridized and compared with *Eco*RI or *Hin*dIII fragments of phage ϕU DNA isolated from phage ϕU particles (Fig. 8). The 7.4 kb and 5.0 kb fragments in the *Eco*RI

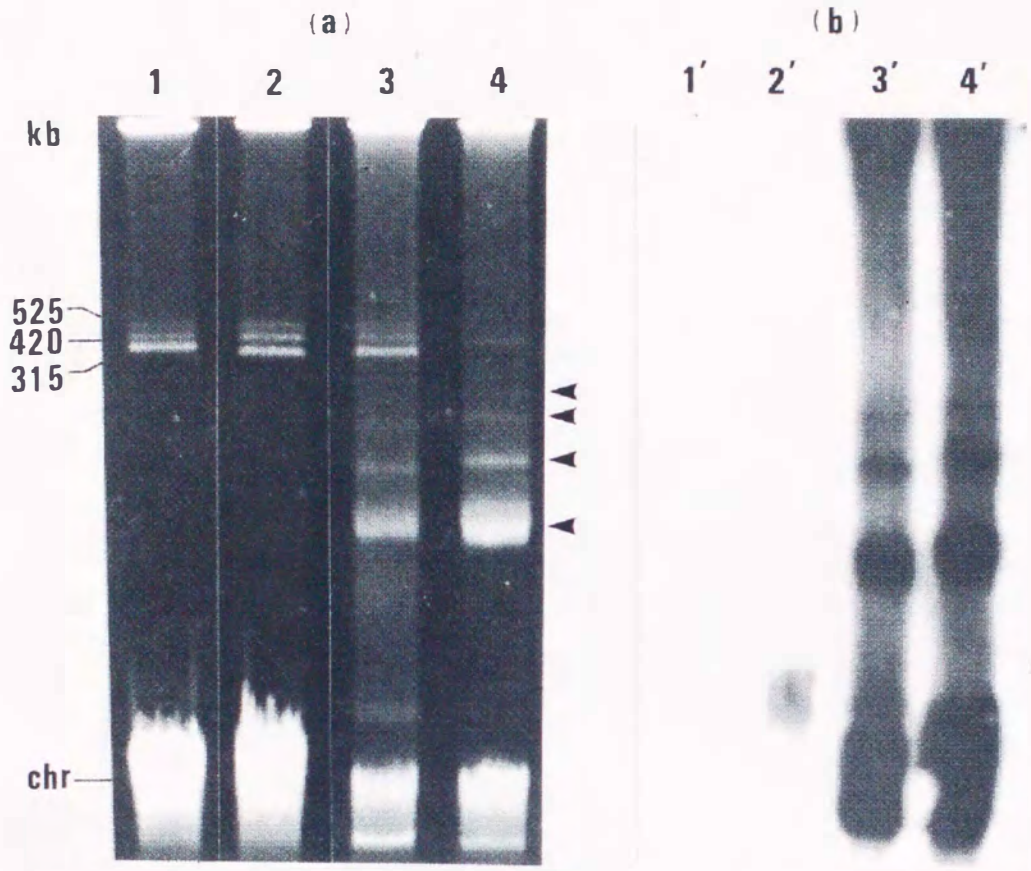

Fig. 7. (a) Crude plasmids of *R. leguminosarum* biovar *trifolii* strains separated on 0.7 % agarose gel. The arrows indicate additional bands (see RESULTS AND DISCUSSION); chr, fragmented chromosomal DNA. (b) An *Eco*RI digest of phage φU DNA was used as a probe for Southern hybridization. Lanes 1, 4S; lanes 2, UK-1(φU); lanes 3, UK-1(φU) incubated with mitomycin C (1 μg ml^{-1}) for 4h; lanes 4, 4S(φU).

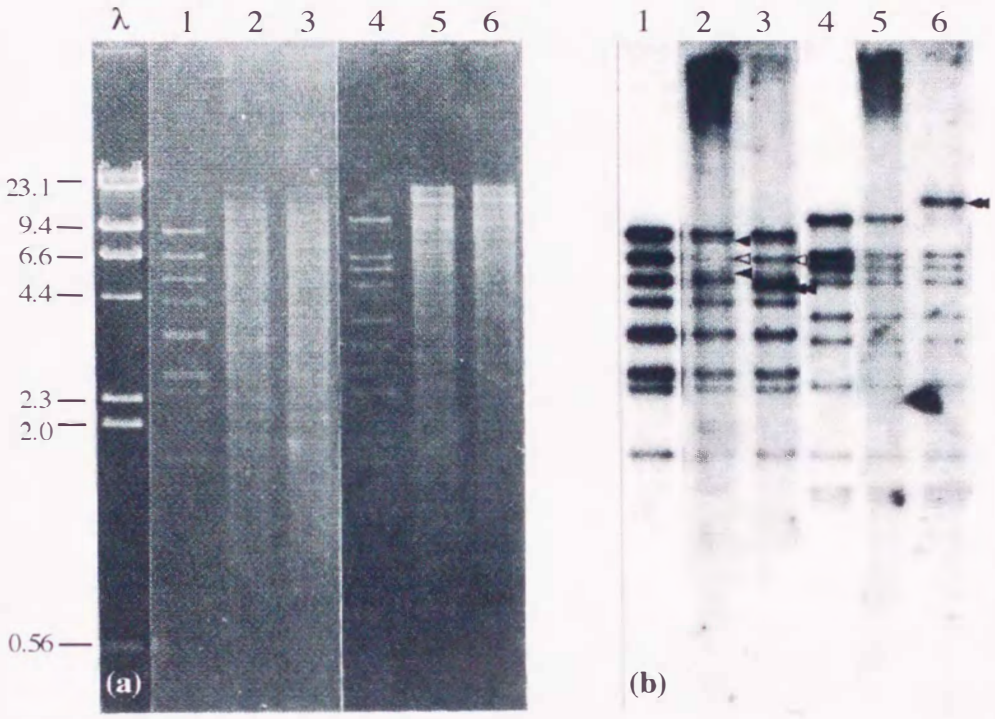

Fig. 8. (a) Restriction endonuclease patterns of phage ϕU and of *R. leguminosarum* biovar *trifolii* strains digested with *Eco*RI or *Hin*dIII. (b) Southern hybridization using ^{32}P-labelled *Eco*RI or *Hin*dIII digests of phage ϕU as probe. The symbols ◁, ◀, and ◀◀ are explained in RESULTS AND DISCUSSION. Lane λ, *Hin*dIII fragments of λ DNA; lanes 1, *Eco*RI fragments of phage ϕU DNA; lanes 2, *Eco*RI digests of strain UK-1(ϕU); lanes 3, *Eco*RI digests of strain 4S(ϕU); lanes 4, *Hin*dIII fragments of phage ϕU DNA; lanes 5, *Hin*dIII digests of strain UK-1(ϕU); lanes 6, *Hin*dIII digests of strain 4S(ϕU).

digests of strain UK-1(ϕU) (Fig. 8 b, lane 2, indicated by ◀)could not be detected in the *Eco*RI digest of ϕU DNA (Fig. 8 a, b, lanes 1) and the 6.0 kb fragment in the UK-1(ϕU) digest (Fig. 8b, lane 2, indicated by ◁) was very faint. These results indicate that phage ϕU DNA integrated in strain UK-1(ϕU) chromosome at the 6.0 kb *Eco*RI fragment. All three fragment were detected in the *Eco*RI digest of strain 4S(ϕU) (Fig. 8 b, lane 3), but the 6.0 kb fragment (indicated by ◁) was more strongly labelled than that in the UK-1(ϕU) digest, suggesting that phage ϕU DNA might replicate autonomously without integration into the chromosome in strain 4S(ϕU). Furthermore, fragments which were characteristic to strain 4S(ϕU) were detected (Fig. 8 b, lane 3 and 6, indicated by ◀◀). The origin of these fragments is not clear, but they may relate to Sym plasmid curing.

The molecular mechanism of Sym plasmid curing caused by lysogenization is incompletely understood. Incompatibility may occur between unintegrated phage ϕU DNA and the Sym plasmid of strain 4S(ϕU). Lysogenic phage may also effect the symbiotic properties of *Rhizobium* in a manner like that of virulent phages.

4. SUMMARY

A lysogenic strain, *Rhizobium leguminosarum* biovar *trifolii* UK-1(ϕU), was isolated from a wild white clover nodule. It was symbiotically effective on white clover. A lysogenic phage (ϕU) was induced from this strain by treatment with either UV irradiation or mitomycin C. Phage ϕU had an icosahedral head

(40 nm wide), a short tail (9 nm long) and tail fibres (15 nm long). Phage ϕU was induced with mitomycin C both from bacterial cells in infection threads and from bacteroids in nodules. Southern hybridization using *Eco*RI fragments of phage ϕU as probe indicated that phage ϕU DNA was integrated into the chromosome of strain UK-1(ϕU) at a site involving the 6.0 kb *Eco*RI fragment of phage ϕU. Phage ϕU also lysogenized the wild-type strain *R. leguminosarum* biovar *trifolii* 4S. The resulting lysogenized strain, 4S(ϕU), had lost its 315 kb Sym plasmid and its nodulation ability.

III. A CHROMOSOMAL INTEGRATIVE VECTOR SYSTEM UTILIZING DNA FRAGMENTS OF A LYSOGENIC PHAGE OF *RHIZOBIUM LEGUMINOSARUM*

1. INTRODUCTION

The lysogenic phages of *Rhizobium* have been contributed to analysis of *Rhizobium* genes [14, 25, 53, 66, 81]. Phage 16-3, a lysogenic phage of *Rhizobium meliloti*, has been investigated in detail [21, 22] and Hermesz *et al.* [31] constructed cloning vectors using attachment site (*attP*) of phage 16-3. These vectors integrated into the chromosome of *R. meliloti* by site specific recombination between *attP* site on the vectors and *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 *Rhizobium*-legume symbiosis, because loss of vectors and cloned genes during nodulation has been a problem [62].

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.* [90] distinguished *attL* and *attR* fragments among the restriction fragments prepared from lysogenic strain of *Haemophilus influenzae* L-10. When the 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 lysogen. Duchrow & Giffhorn [23] also reported the decision of *attL*, *attR*, and *attP* fragments of 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 [Chapter II]. It was suggested that phage ϕU integrates its genomic DNA into the host chromosome during lysogeny, and that the *attP* site may locate on an 6.0 kb *Eco*RI fragment of phage ϕU DNA [Chapter II]. The DNA fragments corresponding to *attL* and *attR* were thought to be 7.4 kb and 5.0 kb *Eco*RI fragments also found in the lysogen. I 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. I describe 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.*

2. MATERIALS AND METHODS

Phage, plasmids and bacterial strains . The relevant characteristics of the phage, plasmids, and bacterial strains used in this study are shown in Table 2. The lysogenic strain *R. leguminosarum* biovar *trifolii* 4S(ϕU), which can form nitrogen fixing nodules on the roots of white clover, 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 [47]. TY medium [8] 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 [80] 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 μl^{-1}.

Table 2. Bacterial strains, phage and plasmids.

	Relevant characteristics*	Reference or source
***Rhizobium* strains**		
4S	wild type	Higashi & Abe [34]
4S(ϕU)	lysogen of strain 4S	Chapter II
CI101	transconjugant, Tc^r	This work
CI301	transconjugant, Tc^r	This work
***E. coli* strains**		
S17-1	*tra* gene on chromosome	Simon *et al*. [82]
S17-1(pCI6)	S17-1 harboring pCI6	This work
JM109	cloning host for pUC18	Yanisch-Perron *et al* . [94]
LE392	cloning host	Sambrook *et al*. [76]
VCS257	cloning host	Stratagene
Phage		
ϕU	lysogenic phage of *Rhizobium*	Chapter II
Plasmids		
pSUP202	*mob* $^+$, Tc^r , Ap^r , Cm^r	Simon *et al*. [82]
pCI6	pSUP202 carrying *attP* of phage ϕU	This work
pUC18	Ap^r, *lacZ'*	Yanisch-Perron *et al*. [94]

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

Isolation of DNA and restriction endonuclease digestion . Plasmid isolation from *E. coli* was done on a small scale according to Birnboim & Doly [12] for routine analysis. For cloning vector and template for hybridization probes, the plasmid was purified according to Hattori *et al* . [28]. Isolation of phage ϕU DNA was performed as described by Yamamoto *et al.* [93] and Dallmann *et al* . [17]. Total cellular DNA from *Rhizobium* was prepared by the method of Casse *et al.* [15] as modified by Higashi *et al.* [35]. Isolated DNAs were digested with appropriate restriction endonucleases according to standard methods.

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 manufacture. 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 by 200 mM hydrochloric acid followed by 1 M sodium hydroxide. DNAs were then transferred to a Nytran NY13N filter (Shleicher & Shüll) by vacuum blotting. The blotted filters were irradiated by 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 ϕU DNA, which was a putative *attP* fragment, 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 ϕU was referred to as plasmid pCI6. The construction of pCI6 is shown in Fig. 9.

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

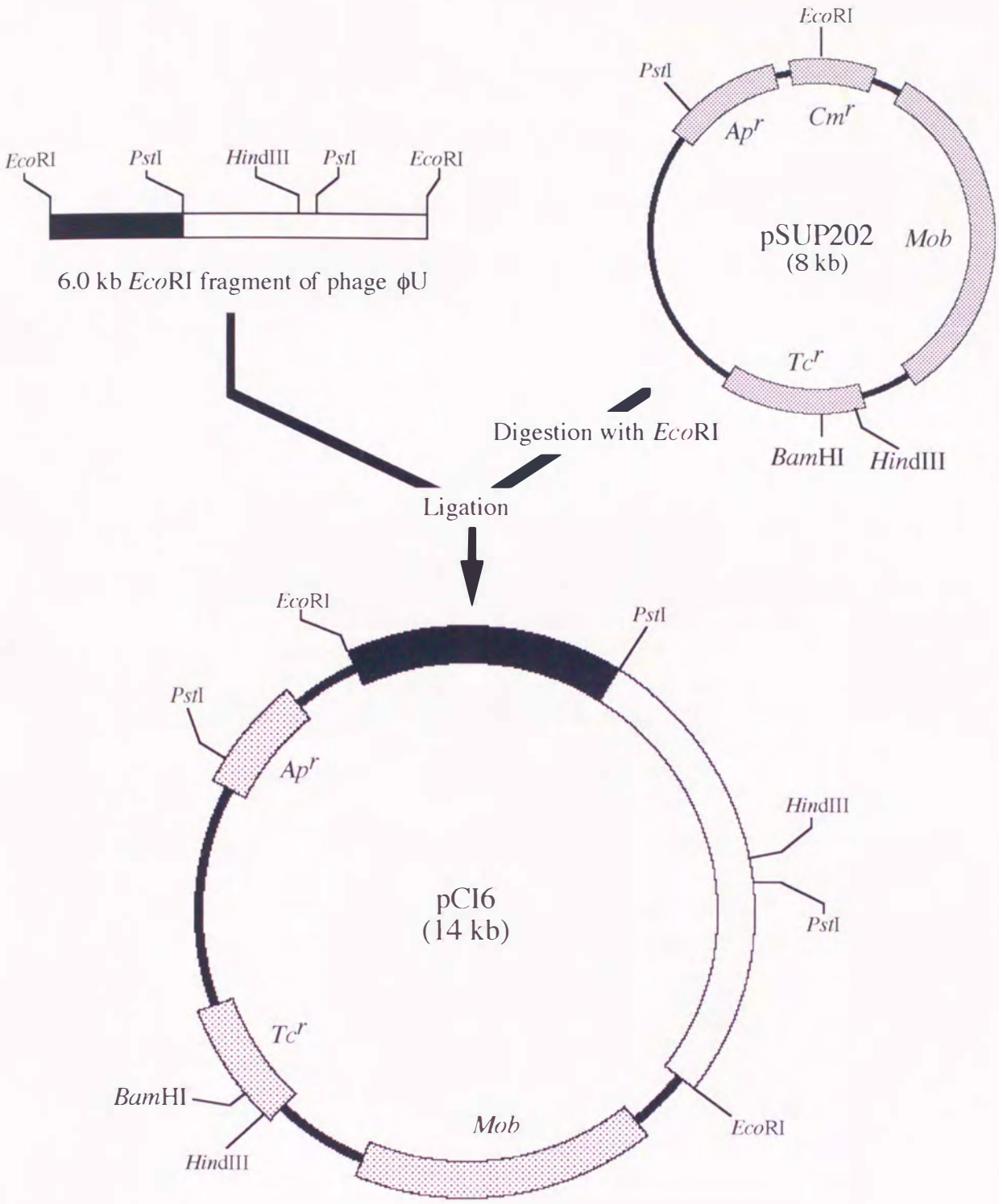

Fig. 9. Construction of chromosome integrative vector pCI6. The putative *attP* region of phage ϕU (6.0 kb *Eco*RI fragment) was cloned into *Eco*RI site of suicide plasmid pSUP202. The 2.3 kb *Eco*RI-*Pst*I fragment is indicated by black box.

TY medium for 24 h, respectively. A 100 μl sample (ca. 5 X 10^7 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 [Chapter II].

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.* [35].

3. RESULTS

Identification of the attP fragment of phage ϕU DNA

If phage ϕU integrates into the chromosome of *Rhizobium* at the site of 6.0 kb *Eco*RI 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 6.0 kb *Eco*RI fragment as a probe. Total cellular DNA was prepared from *R. leguminosarum* biovar *trifolii* lysogenic strain 4S(ϕU), and digested with *Eco*RI and *Bam*HI. Southern hybridization of fragments of phage ϕU and lysogenic strain 4S(ϕU) DNAs probed with the digoxigenin labelled putative *attP* fragment (6.0 kb *Eco*RI fragment) of phage ϕU DNA are shown in Fig. 10. Three hybridized bands were detected in fragmented DNA of strain 4S(ϕU), whereas only one band was detected in phage ϕU DNA. This indicates that the 6.0 kb *Eco*RI fragment of phage ϕU DNA is split in the lysogen. Two fragments which did not exist in phage ϕU DNA (indicated by large arrow heads in lanes 2') were judged to carry *attL* and *attR* in lysogenic strain 4S(ϕU). The other fragment (indicated by small arrow heads in lanes 2') was interpreted as the *attP* fragment itself derived from vegetative phage ϕU DNA in strain 4S(ϕU) cells, because corresponding fragments could be detected by agarose gel electrophoresis of phage ϕU 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.* [90] and Duchrow & Giffhorn [23]. 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 lysogenic strain. When Southern hybridization was performed using other *Eco*RI fragments of phage ϕU DNA as probes, they hybridized with only one fragment

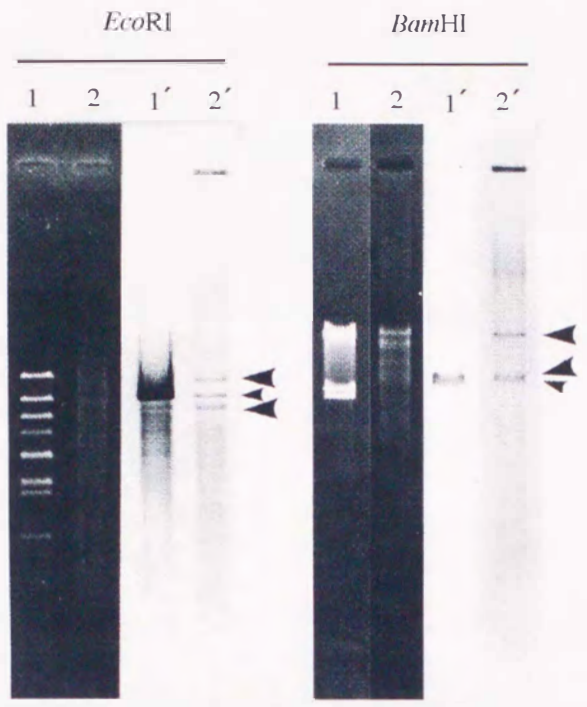

Figure 10. Southern hybridization for identification of the *attP* fragment of phage ϕU DNA. *Eco*RI- and *Bam*HI- digested DNA from *R. leguminosarum* biovar *trifolii* strain 4S(ϕU) were hybridized with a digoxigenin-labelled *Eco*RI 6.0 kb putative *attP* fragment of phage ϕU DNA. Lanes 1, phage ϕU DNA digested with *Eco*RI and *Bam*HI, respectively; lanes 2, strain 4S (ϕU) DNA digested with *Eco*RI and *Bam*HI, respectively; lanes 1', phage ϕU DNA hybridized with putative phage ϕU *attP* fragment; lanes 2', strain 4S(ϕU) DNA hybridized with putative phage ϕU *attP* fragment. For explanation of bands indicated by large and small arrowheads, see text.

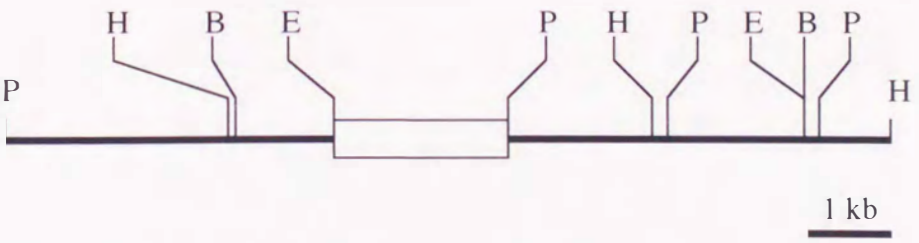

Figure 11. Restriction map of the putative *attP* region of phage ϕU DNA. This map was prepared by summarizing the results of Southern hybridization against restriction fragments of phage ϕU and the lysogenic *Rhizobium* strain 4S(ϕU) probed with the *attP* fragment of phage ϕU. The open box is a 2.3 kb *Eco*RI-*Pst*I fragment which contains the *attP* site. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I.

corresponding to themselves, in lysogenic strain 4S(ϕU) (data not shown). These hybridization data support the designation of the 6.0 kb *Eco*RI fragment as the *attP* fragment of phage ϕU DNA.

Restriction map of attP fragment

Phage ϕU DNA prepared from phage particles was single- and double-digested with restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I, then hybridized with the 6.0 kb *Eco*RI *attP* fragment for restriction mapping. Fig. 11 shows the restriction map of an 11.7 kb *Pst*I-*Hin*dIII 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(ϕU) DNA using a 2.3 kb *Eco*RI-*Pst*I fragment (indicated as an open box in Fig. 11.) as a probe, two fragments of *attL* and *attR* were detected (data not shown). This indicates that the integration might occur within the region of a 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 ϕU DNA was cloned into the *Eco*RI site of suicide plasmid pSUP202 and referred to as pCI6 (Fig. ?). *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 [82].

R. leguminosarum biovar *trifolii* strain 4S and its lysogenic derivative strain 4S(ϕU) 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(ϕU) 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(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 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 ϕU productivity, sensitivity to phage ϕU, and symbiotic phenotypes were investigated in ten transconjugants of 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 CI100 series retained the characteristics of its parent strain 4S, which had no phage ϕU and was sensitive to phage ϕU infection. Transconjugants of CI300 series lost phage ϕU productivity and was sensitive to phage ϕU, whereas its parent strain 4S(ϕU) had phage ϕU and resistant to phage ϕU infection. This indicates that CI300 series have apparently lost the integrated phage ϕU present in the parent strain 4S(ϕU). Transfer of pCI6 into the lysogenic strain 4S(ϕU) may exclude prophage from the chromosome by the integration and exicision 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. 12). 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 [35, Chapter II]. No plasmid bands corresponding to pCI6 could be detected in total cellular DNAs from strain CI101 and CI301 (Fig. 12 a, 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

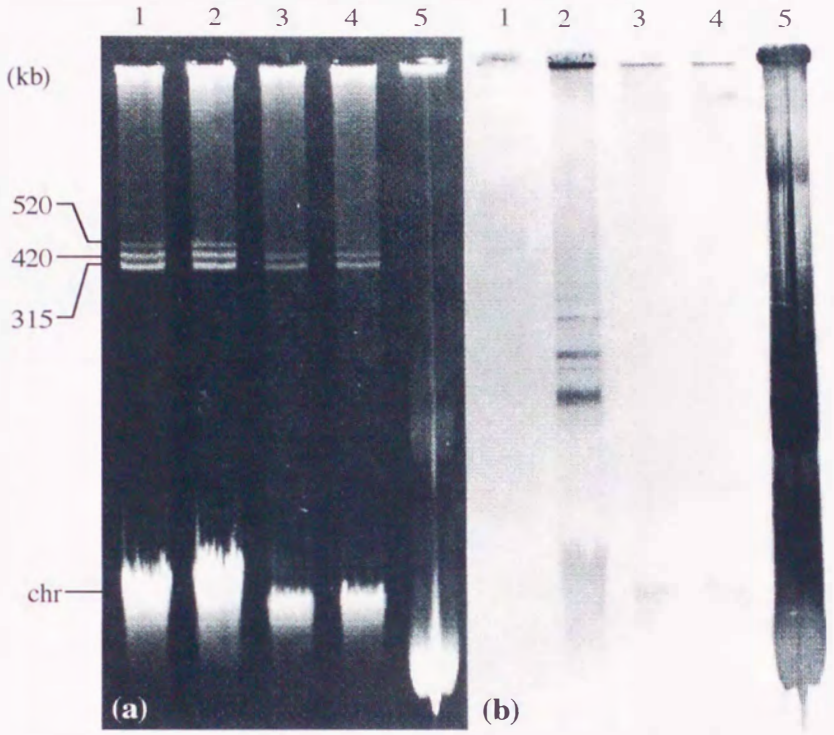

Figure 12. Agarose gel electrophoresis (a) and Southern hybridization (b) of total cellular DNAs from *Rhizobium* strains and plasmid pCI6. Digoxigenin-labelled pCI6 was used as a hybridization probe. Lanes 1, strain 4S; lanes 2, strain 4S(ϕU); lanes 3, strain CI101; lanes 4, strain CI301; lanes 5, plasmid pCI6 from *E. coli* VCS257 harbouring pCI6. chr, Fragmented chromosome DNA.

hybridized bands were detected in strain 4S(ϕU) (Fig. 12 b, lane 2). These were assumed to be vegetative phage ϕU DNA in strain 4S(ϕU) 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. 12 b, 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 *Eco*RI, and hybridized with the labelled *attP* fragment and pSUP202 (Fig. 13). Plasmid pCI6 was digested into two fragments of the *attP* fragment and pSUP202 with *Eco*RI (Fig. 13 a, lane 5). Southern blots probed with the *attP* fragment are shown in Fig. 4 (b). Three bands were detected in strain 4S(ϕU) DNA (Fig. 13 b, 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(ϕU). In DNAs from strains CI101 and CI301, only two bands corresponding to *attL* and *attR* were detected (Fig. 13 b, lane 3 and 4). A very faint band was detected between the *attL* and *attR* fragments of strain CI301 (Fig. 13 b, 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. 13 b, lane 1).

Southern hybridization was also performed using pSUP202 as a probe (Fig. 13, c). The hybridized band identical to pSUP202 could be detected in both

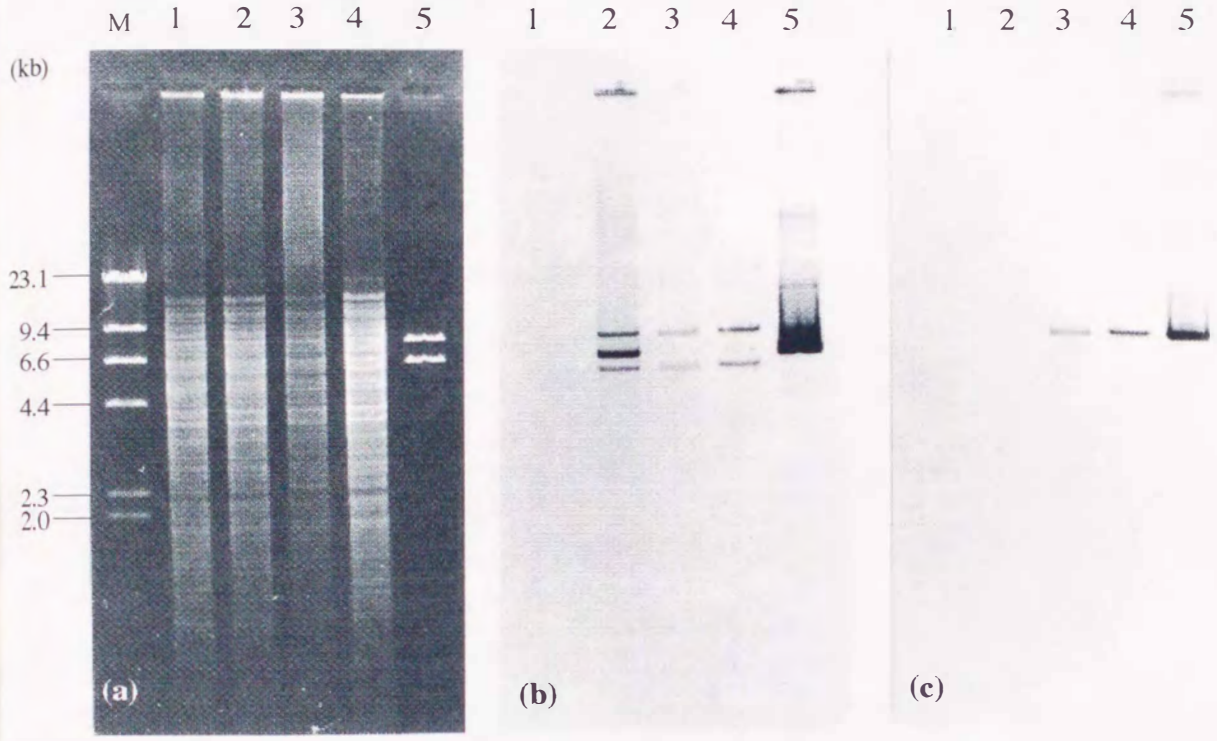

Figure 13. Agarose gel electrophoresis and Southern hybridization of DNAs from *Rhizobium* strains probed with the *attP* fragment and pSUP202. DNAs from *Rhizobium* strains and plasmid pCI6 were digested with *Eco*RI and separated on 0.7 % agarose gel (a). Southern-blotted filters were hybridized with the *attP* fragment (b) and pSUP202 (c). Lane M, λ phage *Hin*dIII fragments as molecular mass markers; lanes 1, strain 4S; lanes 2, strain 4S(ϕU); lanes 3, strain CI101; lanes 4, strain CI301; lanes 5, plasmid pCI6.

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.

4. 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 P1 [41]. 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 *Eco*RI fragment (the putative *attP* fragment) of phage ϕU as a probe was performed and two fragments of *attL* and *attR* could be detected in the lysogenic *Rhizobium* strain (Fig. 10). This indicates that the 6.0 kb *Eco*RI fragment is the *attP* fragment of phage ϕU DNA.

The *attP* fragment of phage ϕU was cloned into an *Eco*RI site of pSUP202 to construct a chromosomal 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 significantlly higher than that reported for transfer of the wide host range pKT230 and pKT231 plasmids into *R. meliloti* (10^{-5}, Donnelly *et al.* [19]).

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 (Sadowski [75] for review). Leong *et al.* [60] has reported the 1728 bp DNA sequence coding 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 ϕU are similar to those for phage λ, the *int* gene may be located on the 6.0 kb *Eco*RI *attP* fragment of phage ϕU DNA. Plasmid pCI6 integrates into the chromosome of *R. leguminosarum* biovar *trifolii* at a high frequency at the site of phage ϕU integration. This indicates that the integrase gene (Int) 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 [60]. 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(ϕU), the resulting transconjugants of CI300 series had lost its immunity against phage ϕU and ability to produce phage ϕU. These results suggest that phage ϕU is no longer present in CI300 series. Transfer of pCI6 into strain 4S(ϕU) cells may cause exclusion of prophage DNA from the chromosome of strain 4S(ϕU) 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 chromosomal integrative vector, like as vectors which Hermesz *et al.* [31] reported.

The entire 40 kb phage ϕU 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 chromosomal integrative vector for other *Rhizobium* species should also be investigated.

5. SUMMARY

The attachment site (*attP*) of phage ϕU, 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(ϕU) using tetracycline resistance (Tc^r) as a selection marker. The Tc^r transconjugants of *R. leguminosarum* biovar *trifolii* appeared at high frequency (10^{-3}-10^{-4} per recipient cell in both matings). Southern hybridization probed 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.

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

1. INTRODUCTION

Many different wide host range cloning vectors have contributed to the molecular genetic analysis of the *Rhizobium* - legume symbiosis [9, 52, 61, 62]. 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 [74] and homologous recombination using suicide plasmids [82] 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.* [56] reported integration of the cloned *hup* (*up*take-*h*ydrogenase) 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 [55]. Hermesz *et al.* [31] 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, I cloned the *attP* fragment of lysogenic phage ϕU, which lysogenizes *Rhizobium leguminosarum* biovar *trifolii* strain 4S, into suicide vector pSUP202 [Chapter III]. The recombinant plasmid pCI6 could be transferred from *E. coli* S17-1 to *R. leguminosarum* biovar *trifolii* strain 4S and its lysogenic strain 4S(ϕU) 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 ϕU integration, and could be stably maintained in

Rhizobium under conditions of selective pressure. After integration, the *attP* fragment on pCI6 was devided 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 [Chapter III]. No helper phage was required for pCI6 integration into the chromosome of *Rhizobium*.

In this chapter, I describe integration of plasmid pCI6 into the chromosome of a *Rhizobium* strain which is not susceptible to phage ϕU infection. The restriction map of the *attB* region for phage ϕU integration on the *Rhizobium* chromosome is also reported.

2. MATERIALS AND METHODS

Phage, plasmid and bacterial strains. Lysogenic phage ϕU lysogenizes wild type *Rhizobium leguminosarum* biovar *trifolii* strain 4S. Isolation and characterization of phage ϕU were described previously [Chapter II]. Plasmid pCI6 was constructed by insertion of the 6.0 kb *Eco*RI fragment (*attP* fragment) of phage ϕU into the *Eco*RI site of the suicide vector pSUP202 [Chapter III]. Plasmid pCI6 can integrate into the chromosome of strain 4S and its lysogenic strain 4S(ϕU) by site specific recombination. *R. leguminosarum* biovar *trifolii* strain CI101 is one of the pCI6-integrated derivatives of strain 4S [Chapter III]. Bacterial strains used as conjugation recipients are listed in Table 3. *Escherichia coli* S17-1 harbouring pCI6 was used as a conjugation donor.

Table 3

Culture conditions. *Rhizobium* and *Bradyrhizobium* strains were maintained on mannitol-yeast agar medium [47]. For bacterial mating, plasmid isolation and phage sensitivity tests, they were cultured in TY medium [8]. *Agrobacterium* strains were grown in LB medium [51]. For screening of *Rhizobium* and *Bradyrhizobium* transconjugants after mating, Sherwood's minimal agar plates [80] were used. MinA agar plates [10] were employed for screening of *Agrobacterium* transconjugants. *E. coli* S17-1 harbouring pCI6 were cultured in LB medium containing ampicillin (Ap, 50 μg ml^{-1}) and tetracycline (Tc, 20 μg ml^{-1}).

Phage sensitivity test. Phage ϕU lysate was prepared by phage induction of lysogenic strain *R. leguminosarum* biovar *trifolii* 4S(ϕU) with mitomycin C [Chapter II]. Phage ϕU 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 μl 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 μg ml^{-1} followed by overnight incubation. Cells were removed by centrifugation (5000 g, 3 min) and the supernatant was filtrated through a membrane filter (0.22 μm, Millipore HAWP01300), and then spotted onto soft

agar overlays containing *R. leguminosarum* biovar *trifolii* strain 4S to detect phage.

Phage ϕU adsorption test. The adsorption rate of phage ϕU 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 ϕU 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 Ap 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 μl of each) were mixed and washed twice with water. The washed bacteria were suspended in 100 μl of water, and 50 μl of the mixture was spotted onto a membrane filter (HAWPO1300, Millipore) on a TY agar plate, as described in Chapter III. 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^{-1} of Tc for selection of transconjugants. Frequency of transconjugants appearance was calculated after 4 d incubation at 28°C.

Isolation of DNA and restriction endonuclease digestion. Plasmids were isolated from *E. coli* according to Hattori *et al.* [28]. Total cellular DNA from *Rhizobium* strains were prepared as described previously [15, 35]. Restriction enzyme digestions of DNA were as described by Sambrook *et al.* [76].

Agarose gel electrophoresis and Southern hybridization. The conditions for agarose gel electrophoresis were described in Chapter III. Plasmid pCI6, the cloned *attP* fragment (6.0 kb *Eco*RI fragment) of phage ϕU, and pSUP202 [82] were labelled by random priming with Klenow fragment in the presence of digoxigenin [20] 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).

3. RESULTS

Susceptibility of Rhizobium, Bradyrhizobium, and Agrobacterium strains to phage ϕU infection

Phage ϕU lysate was spotted on top agar containing each of the bacteria listed in Table 3 and plaque formation was observed. The phage titer of the lysate (*ca.* 10^{10} plaque forming units ml^{-1}) was high enough to form a distinguishable clear zone on a lawn of bacteria which were susceptible (sensitive) to phage ϕU infection. *R. leguminosarum* biovar *trifolii* strains 4S and 4S(ϕU) were used as sensitive and resistant controls, respectively. All bacterial strains used as pCI6 recipients were resistant to phage ϕU infection.

Adsorption of phage ϕU

Adsorption experiments were performed and typical profiles are shown in Fig. 14. Phage ϕU efficiently adsorbed to *R. leguminosarum* biovar *trifolii* strain 4S, which is the original host strain of phage ϕU. 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 ϕU 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 ϕU could not adsorb to the other strains used as pCI6 recipients, as exemplified by *Agrobacterium tumefaciens* LBA4404.

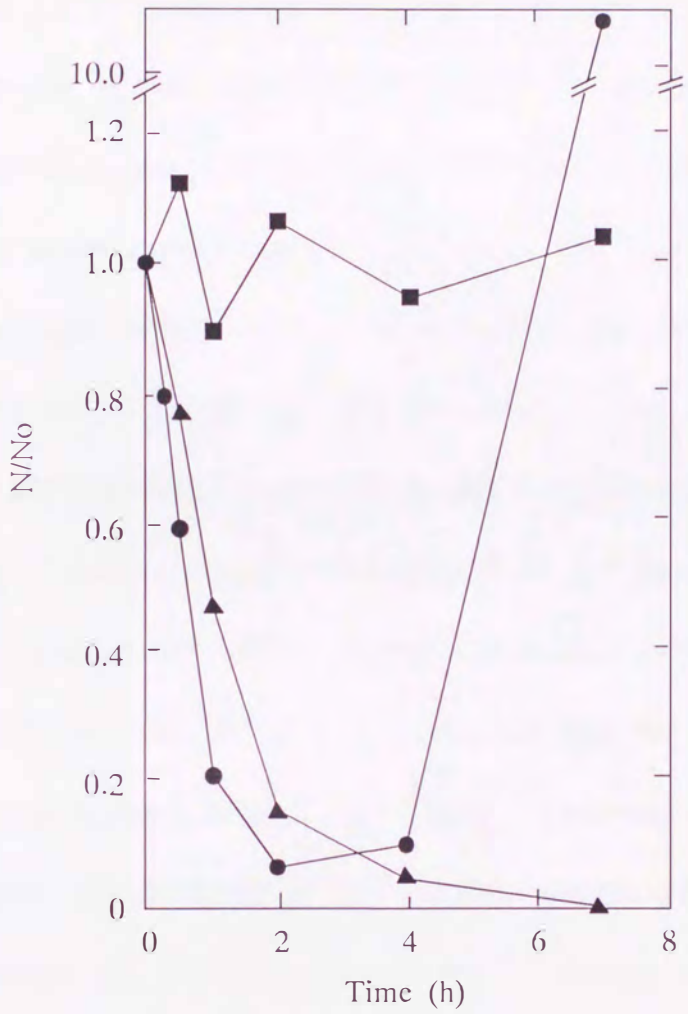

Fig. 14. Phage ϕU adsorption to different species of Rhizobiaceae. Bacterial cells and phage ϕU were mixed and kept at 30 °C. Free phages were titrated at the times indicated. No, initial phage titer; N, free phage titer in each sample. ●, *R. leguminosarum* biovar *trifolii* strain 4S; ▲, *R. leguminosarum* biovar *viciae* strain K5; ■, *A. tumefaciens* strain LBA4404.

Plasmid pCI6 transfer to strains of Rhizobiaceae which are resistant to phage ϕU

E. coli S17-1 harbouring pCI6 was mated with *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* strains which were resistant to phage ϕU 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 frequency of 4.41×10^{-3} (Table 3). 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 ϕU. If strain K5 was a lysogenic strain, this strain might be resistant to phage ϕU infection and pCI6 could integrate into the chromosome of the strain, the same as in the case of strain 4S(ϕU). However, phage induction test and genomic Southern hybridization using total phage ϕU DNA as a probe showed that strain K5 did not have a ϕU 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 analysed 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 correponding in size to plasmid pCI6 was detected (data not shown). The labelled *attP* 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 strain derived from *R. leguminosarum* biovar *trifolii* strain 4S, as shown in Chapter III), 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. 15 representing for transconjugant CIK50 series. Plasmid pCI6 yielded two fragments corresponding to the phage ϕU *attP* fragment (6.0 kb) and suicide plasmid pSUP202 (*ca.* 8 kb) by *Eco*RI digestion (Fig. 15, *Eco*RI digests, lane pCI6. See also Fig. 18). Two hybridizing fragments detected in strain CI101 by *attP* probe (Fig. 15, lane CI101) have already been identified as *attL* and *attR* [Chapter III]. The length of two fragments detected in strain CIK51 by *attP* probe (Fig. 15, lane CIK51) were indistinguishable from the two fragments (*attL* and *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, were detected (Fig. 15, lanes CI101 and CIK51. See also Fig. 18). These results of the hybridization experiments suggest that

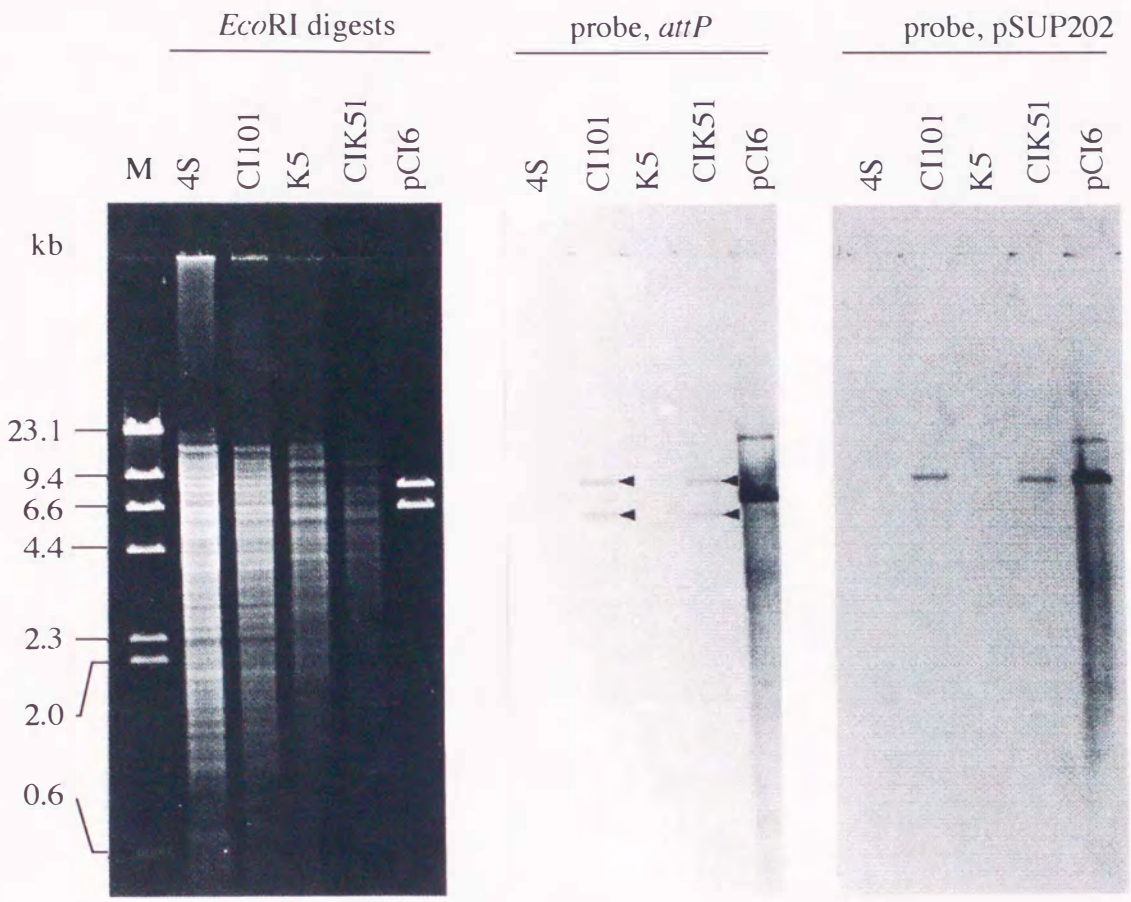

Fig. 15. Agarose gel electrophoresis and Southern hybridization of *Eco*RI digested DNA from *Rhizobium* strains. Total cellular DNA from strains 4S, CI101, K5 and CIK51, and plasmid pCI6 were digested with *Eco*RI and separated on 0.7 % agarose gel. Southern blotted filters were hybridized sequentially with the *attP* fragment of phage ϕU then with pSUP202. Hybridized fragments are indicated by arrow heads (◄). *Hin*dIII-digested λ DNA was used as the size marker (lane M).

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, *Hin*dIII, *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 arrow heads in Fig. 16. See also Fig. 18). In *Eco*RI digestion, the 6.0 kb *attP* fragment (Fig. 16, *Eco*RI, 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. 16, *Eco*RI, lane CI101). The 8.0 kb chimeric fragment (indicated by an arrow head with asterisk) electrophoretically overlapped with pSUP202 cut out from pCI6 integrated chromosome (See also Fig. 15). The *attP* site locates on the 9 kb *Hin*dIII fragments of pCI6 (Fig. 16, *Hin*dIII, the upper band in lane pCI6). This fragment split and generated two fragments of 9 kb and 2.9 kb *Hin*dIII fragments in strain CI101 (Fig. 16, *Hin*dIII, lane CI101), although the chimeric

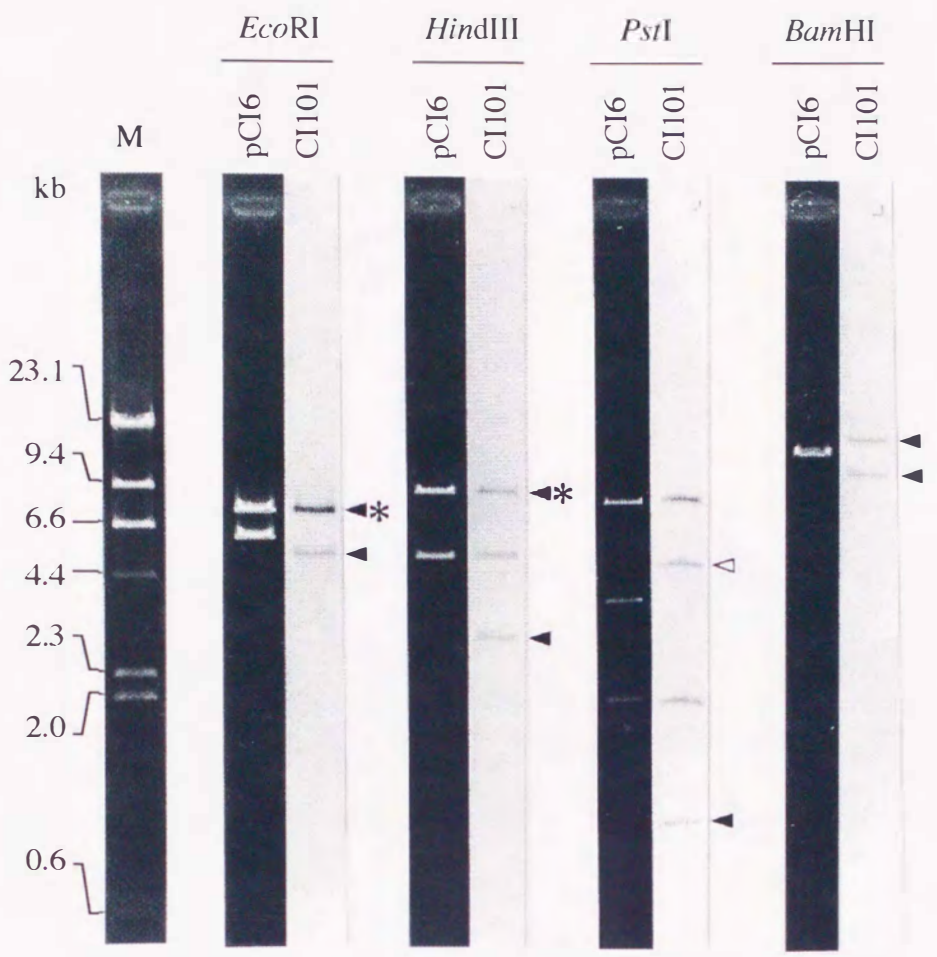

Fig. 16. Detection of *attL* and *attR* fragments in strain CI101. Restriction endonuclease digested DNA of strain CI101 was hybridized with digoxigenin labelled pCI6. Then, hybridized fragments detected in strain CI101 were compared with the restriction fragments of pCI6. The DNA fragments identified as containing *attL* and *attR* are indicated by closed arrow heads (◀). The 5.0 kb chimeric *Pst*I fragment which is indicated by a open arrow head (◁) was assigned as the left border. The chimeric 8 kb *Eco*RI and 9 kb *Hin*dIII fragments in strain CI101 (◀*) electrophoretically ovaerlapped with the *Eco*RI and *Hin*dIII fragments of pCI6, respectively (see RESULTS in detail).

9 kb *Hin*dIII fragment (indicated by an arrow head with asterisk) was not electrophoretically distinguishable from the *Hin*dIII fragment of pCI6. Two chimeric fragments of 5.0 kb and *ca.* 1 kb were detected in DNA from strain CI101 digested with *Pst*I (indicated by an open and a closed arrow heads in Fig. 16, *Pst*I, lane CI101). The 5.0 kb *Pst*I fragment (indicated by an open arrow head in Fig. 16, *Pst*I), one of the chimeric fragments, was assigned as the left border (*attL*) even though complete genetic and physical maps of phage ϕU and ϕU prophage had not yet been constructed. The *Bam*HI 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 *Bam*HI. It was deduced that the *attB* site must be located on an 18 kb *Bam*HI 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. 17). 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 arrow heads in Fig. 17) 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 *Bam*HI-*Eco*RI 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

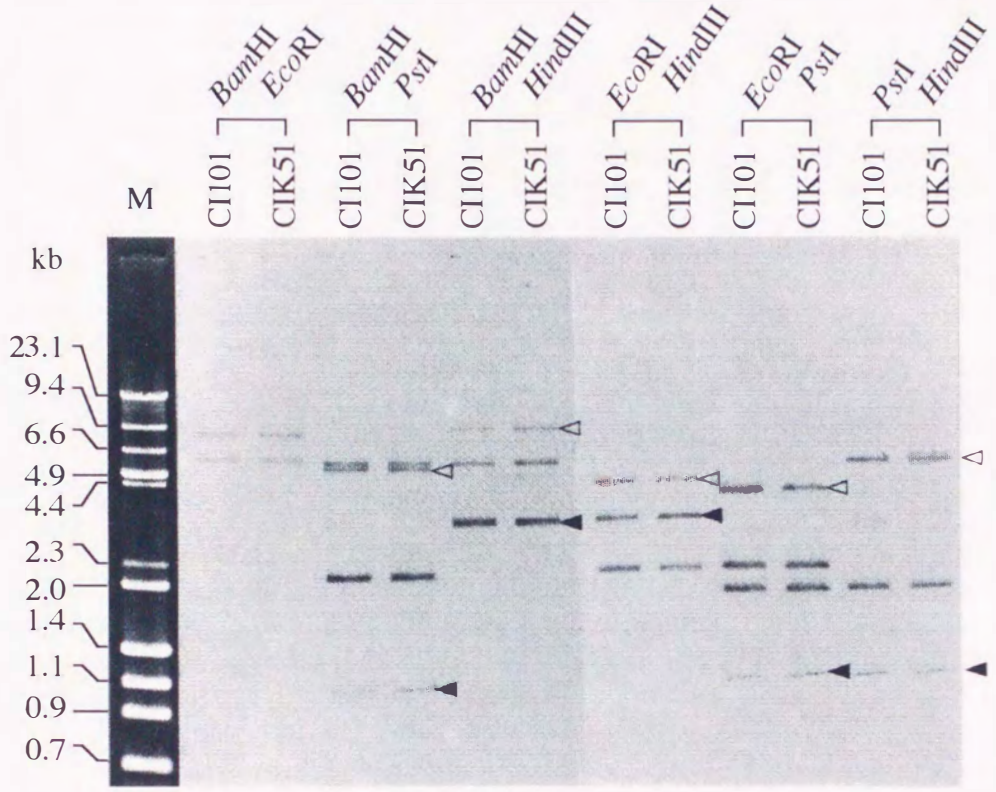

Fig. 17. Southern hybridization of double restriction endonuclease digests of DNA from strains CIK51 and CI101. Total cellular DNA from strains CIK51 and CI101 were digested with two different restriction endonucleases and hybridized with the *attP* fragment of phage ϕU. *Hin*dIII-digested λ DNA mixed with pHY marker (Takara, Japan) was used as size marker (lane M). The *attL* (◅) and *attR* (◀) were assigned as described in RESULTS. The *attL* and *attR* could not be decided among the *Eco*RI-*Bam*HI doule digested DNA fragments.

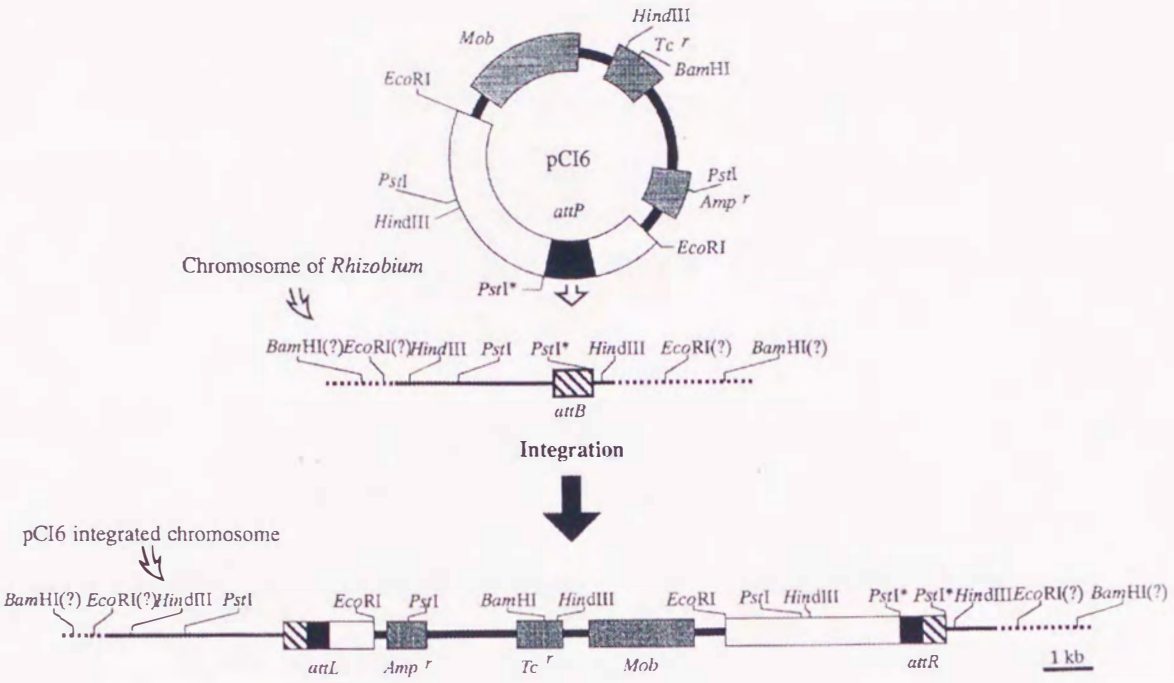

Fig. 18. Schematic drawing of pCI6 integration into the chromosome of *Rhizobium* by *attP-attB* recombination. The restriction map of the *attB* region on the *Rhizobium* chromosome was deduced from the restriction maps of pCI6 and pCI6-integrated chromosome. The *Bam*HI and *Eco*RI sites whose exact locations could not be determined are shown as dotted lines with question marks. The *Bam*HI(?)-*Bam*HI(?) fragment and the *Eco*RI(?)-*Eco*RI(?) fragment in the map of the *attB* region are 18 kb and 7.7 kb in length, respectively. The *attP* fragment of phage ϕU 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 close and hatched boxes, respectively. The restriction sites of *Pst*I* on pCI6 and on chromosome are described in RESULTS.

hybridization data (Fig. 18). The exact location of the flanking chromosomal *Eco*RI and *Bam*HI sites could not be determined and are drawn as dotted lines with question marks. Since the *Pst*I fragment corresponding to *attR* is 1.0 kb in length, the *attP* and *attB* must be located within approximately 1 kb of the *Pst*I sites (labelled *Pst*I* in Fig. 18) on pCI6 and bacterial chromosome, respectively.

4. DISCUSSION

Inter-specific DNA homologies between members of the Rhizobiaceae have been demonstrated using DNA reassociation methods. Heberlein *et al.* [29] confirmed the close relationship between the so-called "fast-growing" rhizobia and *Agrobacterium*. Jarvis *et al.* [44] 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 ϕU into the suicide vector pSUP202, could integrate into the chromosome of *R. leguminosarum* biovar *trifolii* strain 4S. If the *attB* site for phage ϕU 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 ϕU 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 recoverd from the conjugation performed with *R. leguminosarum* biovar *viciae* strain K5, which was not susceptible strain to phage ϕU 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 ϕU 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 [26, 75]. 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 *Hin*dIII 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 ϕU infection.

The host range of phage ϕU seemed to be limited to certain strains of *R. leguminosarum* biovar *trifolii* by a plaque formation test. However, phage ϕU adsorption experiments revealed that *R. leguminosarum* biovar *viciae* strain K5 had the ability to adsorb phage ϕU. This indicates that the gene for the specific receptor for phage ϕU 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 [73, 91]. The cloned *lamB* gene was transferred into *Rhizobium*, *Agrobacterium* and *Pseudomonas* [65]. *R. meliloti* carrying *lamB* could adsorb λ*c*I857 and was quantitatively transduced with *in vitro* λ–packaged cosmid. Analogously, phage ϕU 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 ϕU 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.

5. SUMMARY

Fourteen strains belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* were investigated for susceptibility to infection by a lysogenic phage (phage ϕU) from *Rhizobium leguminosarum* biovar *trifolii*. All strains tested were resistant to phage ϕU infection; however, the phage was able to adsorb to *R. leguminosarum* biovar *viciae* strain K5. Plasmid pCI6 carrying the attachment site (*attP*) of phage ϕU 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 ϕU indicates that pCI6 is integrated into the chromosome of ten 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 ϕU.

V. NODULE FORMATION BY CLOVER-*RHIZOBIUM* CARRYING CHROMOSOMAL *NOD* GENES

1. INTRODUCTION

In fast-growing *Rhizobium* spp. including *Rhizobium leguminosarum* biovars, the nodulation (*nod*) genes and nitrogen fixation (*nif*) genes are located on a large symbiotic (Sym) plasmid [5, 39, 40, 58]. Many genes have been introduced into *Rhizobium* using wide host range cloning vectors [62]. However, often the cloned genes and/or the vector were eliminated during the nodulation process [19, 42, 59, 62]. Incompatibility between the vector and endogeneous Sym plasmid will also occur [11]. To overcome these problems, cloning vectors that integrate into the chromosome of *Rhizobium* have been developed using transposable elements [7, 55, 56], repeat sequence [2] and the attachment site (*attP*) of a lysogenic phage [31].

In Chapter III, I developed the chromosomal integrative vector system using the *attP* site of a lysogenic phage ϕU. Here, I describe on the integration of *nod* genes into the chromosome of *Rhizobium* using the chromosomal integrative vector system and the nodulation phenotype of the *Rhizobium* transconjugant strain.

2. MATERIALS AND METHODS

Phage, plasmids and bacterial strains. Phage ϕU, a lysogenic phage of *Rhizobium leguminosarum* biovar *trifolii* 4S, can integrate its genome into the chromosome of the host *Rhizobium* strains by site-specific recombination between *attP* (phage) and *attB* (host bacteria) [Chapter II]. The *attP* locates on a 2.0 kb *Eco*RI-*Pst*I fragment of phage ϕU DNA [Chapter III and IV]. Plasmid pSUP203 is the suicide plasmid constructed by Simon *et al* [82]. Although pSUP203 can be transferred from *E. coli* to *Rhizobium* by conjugation, it is unable to replicate in *Rhizobium*. Plasmid pCI6 is the derivative of pSUP202 and carries the 2.0 kb *Eco*RI-*Pst*I fragment containing *attP* site of phage ϕU, as described in Chapter III. Plasmid pRt032 has a 14 kb *Hin*dIII fragment derived from the Sym plasmid of *Rhizobium leguminosarum* biovar *trifolii* ANU843 [77]. This *Hin*dIII fragment encodes *nodD*, *nodA*, *nodB*, *nodC*, *nodF*, *nodE*, *nodL*, *nodM*, *nodN*, *nodI*, *nodJ*, and a part of *nodT* (Fig. 20). *R. leguminosarum* biovar *trifolii* H1 is a symbiotically defective mutant derived from a wild type strain 4S [34]. Strain H1 has lost its 315 kb Sym plasmid and cannot form nodules on white clover roots [35]. *R. leguminosarum* biovar *trifolii* H1t1 is a transformant harbouring pRt032 [85]. Strain H1t1 induces nodules on white clover roots but does not fix nitrogen. *R. leguminosarum* biovar *trifolii* CI101, derived from strain 4S, integrates pCI6 into the chromosome by site specific recombination between *attP* on pCI6 and *attB* on the chromosome, as described in Chapter III. Strain CI101 does not exhibit any changes in symbiotic

Table 4. Bacterial strains, phage and plasmids

	Relevant characteristics[a]	Reference
Rhizobium strains		
4S	Wild-type	[34]
H1	pSym cured strain derived from strain 4S	[35]
H1t1	H1 harbouring pRt032	[85]
CI101	pCI6 :: chromosome of 4S, Tc^r	
CNH1	*nod* genes :: chromosome of H1	This work
E. coli strains		
S17-1	*tra* gene :: chromosome	[82]
S17-1(pCINod1)	S17-1 harbouring pCINod1	This work
Phage		
ϕU	Lysogenic phage of *Rhizobium*	Chapter II
Plasmids		
pRt032	pKT240 carrying *nod* genes	[77]
pSUP203	*mob*$^+$ Tc^r Ap^r Cm^r	[82]
pCI6	pSUP202 carrying *attP* of phage ϕU,	Chapter III
pCI3	pSUP203 carrying *attP* of phage ϕU	This work
pCI4	deletion mutant derived from pCI3, Tc^s Ap^r	This work
pCINod1	pCI4 carrying *nod* genes, Ap^r	This work

[a] *mob*$^+$, mobilizable by *tra* gene; Tc^r, tetracycline resistant; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Tc^s, tetracycline sensitive. The double colon (::) indicates integration.

properties. *Escherichia coli* S17-1 can mobilize pSUP203 and its derivatives to other bacteria by conjugation [82]. Phage, plasmids and bacterial strains used in this study are listed in Table 4.

Isolation of DNA. Plasmid DNA from *E. coli* was isolated using methods described by Birnboim and Doly [12], then purified according to Hattori *et al.* [28]. Total cellular DNA from *Rhizobium* was prepared by the method of Higashi *et al.* [35].

Restriction endonuclease digestion and agarose gel electrophoresis. DNA was digested with appropriate restriction enzymes according to standard method [76] and supplied to agarose gel electrophoresis. The running conditions and the detection of DNA fragments in the agarose gel were described in Chapter III.

Construction of pCINod1. The 6.0 kb *Eco*RI fragment (*attP* fragment) of phage ϕU was ligated into the *Eco*RI site on the suicide plasmid pSUP203 and the resulting recombinant plasmid was designated as pCI3. Two *Hin*dIII sites exist on pCI3; one is in the *attP* fragment and the other is in the tetracycline resistant (Tc^r) gene. To make a unique *Hin*dIII site, pCI3 was digested with *Hin*dIII and the self-ligated plasmid was screened using tetracycline sensitive (Tc^s) and ampicillin resistant (Ap^r) selective markers. The resulting plasmid, designated as pCI4, conferred Ap^r and had a unique *Hin*dIII site. The 14 kb

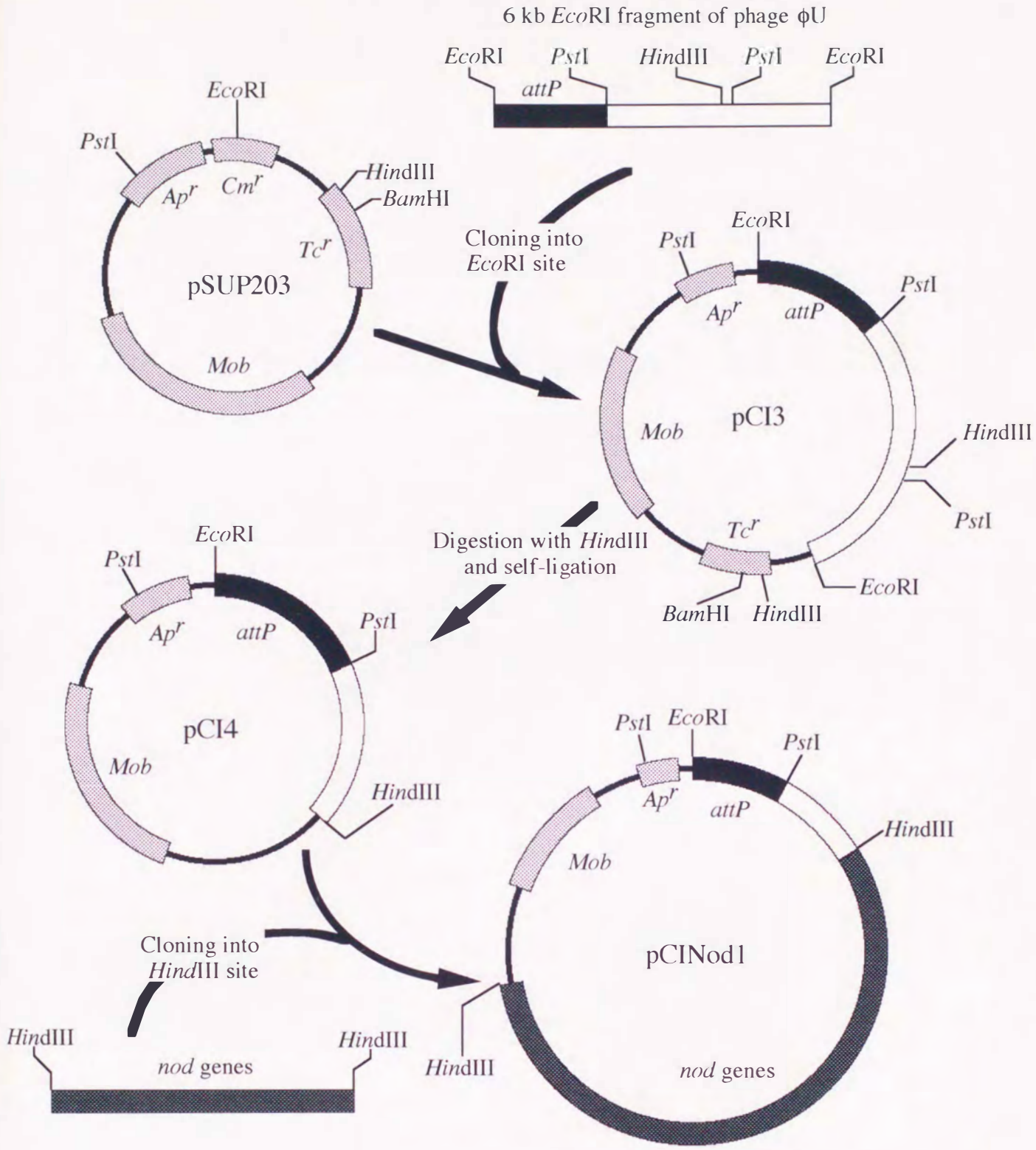

Fig. 19. Construction of pCINod 1. The *nod* genes derived from *R. leguminosarum* biovar *trifolii* strain ANU843 was cloned into the unique *Hin*dIII site of plasmid pCI4. The *Eco*RI-*Pst*I fragment on which *attP* is located is indicated by black box.

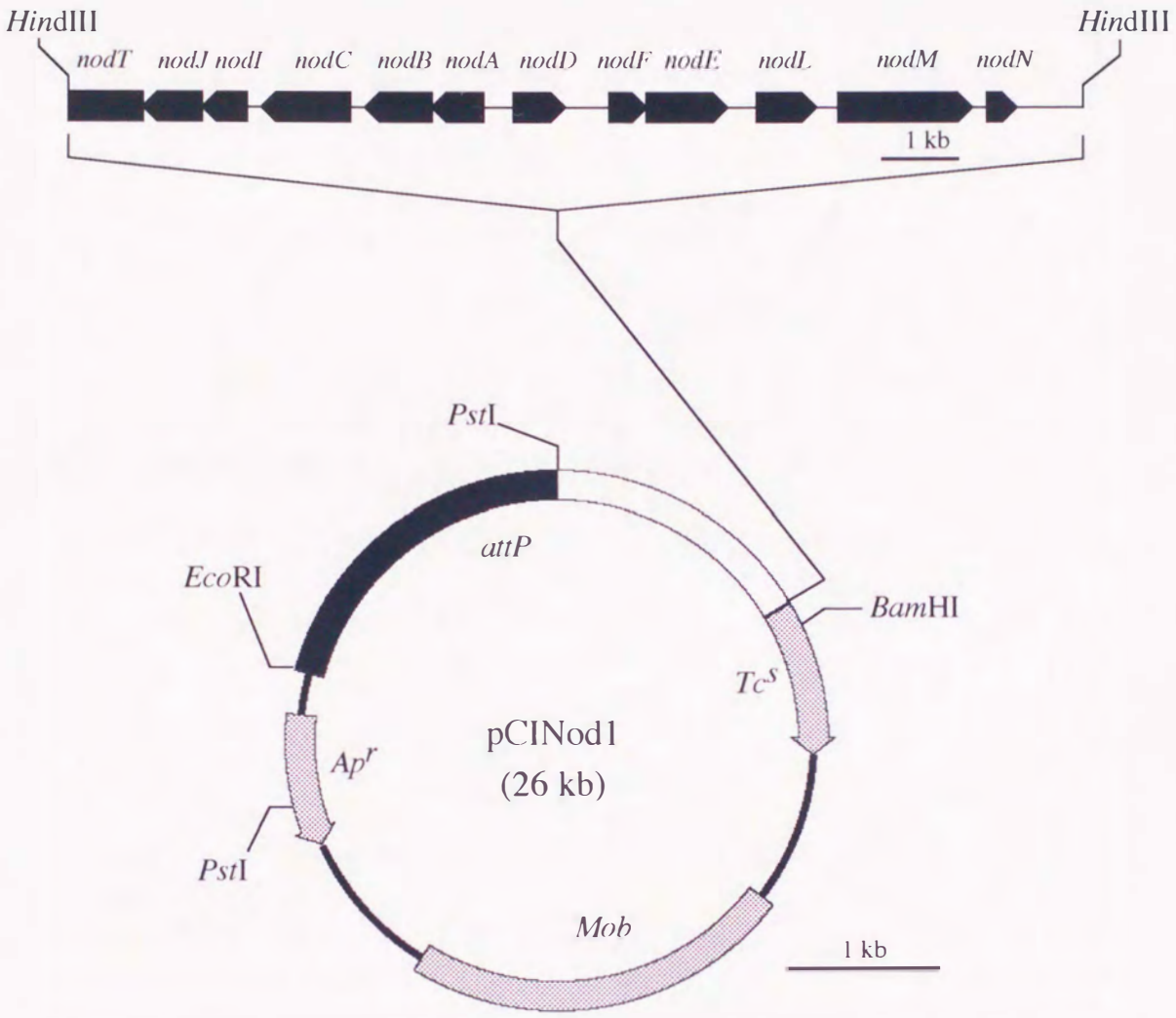

Fig. 20. Restriction map of pCINod1. The 14 kb *Hin*dIII fragment (linear map) derived from *R. leguminosarum* biovar *trifolii* ANU843 was cloned into the unique *Hin*dIII site on pCI4 (circular map). The restriction sites of *Eco*RI, *Pst*I and *Bam*HI on the 14 kb *Hin*dIII fragment coding *nod* genes are omitted. The black box on the circular map indicates the 2.0 kb *Eco*RI-*Pst*I fragment on which *attP* is located.

*Hin*dIII fragment encoding *nod* genes prepared from plasmid pRt032 was cloned into the unique *Hin*dIII site of pCI4 and designated as pCINod1 (Fig. 19 & 20).

Conjugative transfer of pCINod1 to Rhizobium. *E. coli* S17-1 harbouring pCINod1 was used as a conjugation donor. *R. leguminosarum* biovar *trifolii* strain H1, a pSym cured strain derived from *R. leguminosarum* biovar *trifolii* 4S, was used as a recipient. The conjugation was performed on TY agar plates as same method in Chapter III. After washing with sterilized water, the conjugation mixture of *E. coli* and strain H1 was diluted to 10^7-10^8 cells ml^{-1}. Two hundred µl of this mixture were inoculated on 8-10 clover seedlings (*Trifolium repens* L. cultivar Ladino), each grown axenically in separate test tubes on nitrogen-free medium [24], under the condition of 16 h light and 8 h dark.

Isolation of transconjugants from clover nodules. Four weeks after inoculation of roots with the conjugation mixture, nodules were removed and surface-sterilized as described by Long *et al.* [64]. The nodules were squashed and the macerates were spread on mannitol-yeast agar plates [47]. Four days after plating, many colonies appeared on the agar plates were used as transconjugants.

DNA labelling and Southern hybridization. Fragmented DNAs were transferred from agarose gels to Nytran 13N membrane filter (Shleicher and Shüll) using a vacuum blotting apparatus. The blotted filters were irradiated with

UV light (302 nm) to bind the DNA. The 14 kb *Hin*dIII fragment of pRt032 and 2.0 kb *Eco*RI-*Pst*I fragment of pCI4 were isolated from an agarose gel using Ultrafree-C3 (Millipore), then used as *nod* genes and *attP* probes, respectively. Labelling of the probes with digoxigenin and colorimetric detection of the hybridized probes were carried out under the conditions recommended by the supplier (Boehringer Mannheim).

Plant assays. Seeds of *Trifolium repens* cv. Ladino were sterilized and incubated at 25 °C for 2 d in dark. For observation of root hair deformation and infection thread formation, germinated seedlings were transferred into Petri dishes and floated on the bacterial suspension (ca. 1 X 10^6 cells ml^{-1} in distilled water). The seedlings were grown at 25 °C with 16h photoperiod per day and observed by light microscopy. For observatiron by transmission electron microscopy and scoring of nodule number, germinated seedlings were transferred in test tube (ϕ 25 mm) containing Jensen agar medium [45] and inoculated with 200 μl of bacterial suspension (ca. 1 X 10^6 cells ml^{-1} in distilled water). After 3 weeks incubation under the condition of 16 h light and 8 h dark, nodules were harvested and observed by transmission electron microscopy. The number of nodules was counted 8 weeks after inoculation.

3. RESULTS

Transfer of pCINod1 to Rhizobium and nodule induction by the conjugation mixture

Plasmid pCINod1 has *attP* derived from phage ϕU and the *nod* genes. Fig. 20 shows the restriction map of pCINod1. The *attP* locates on the 2.0 kb *Eco*RI-*Pst*I fragment (Fig. 20, indicated by the black box on the circular map) which is identical to the 2.0 kb *Eco*RI-*Pst*I fragment of pCI6. *Rhizobium leguminosarum* biovar *trifolii* strain H1 was conjugated with *E.coli* S17-1 harbouring pCINod1. Isolation of *Rhizobium* transconjugants using the ampicillin resistant (Ap^r) gene on pCINod1 would be difficult since the recipient strain H1 was Ap^r by nature. I expected that inoculation of the conjugation mixture would induce nodule formation on the clover roots, if pCINod1 integrates into the chromosome of strain H1 at a high frequency to that reported for pCI6 (which is pSUP202 carrying the *attP* of phage ϕU) [Chapter III]. Five days after inoculation of the conjugation mixture, nodule formation was initiated on the roots of white clover. Four weeks after inoculation, *Rhizobium* transconjugants were isolated from the nodules and referred to CNH strains.

Total DNA analysis of the transconjugant

Strains CNH1, one of the transconjugants isolated from the root nodules, was used as a representative transconjugant. Total DNA isolated from strain CNH1 was separated on 0.7 % agarose gel electrophoresis and compared with relevant *Rhizobium* strains (Fig. 21). Strain CNH1, H1, and H1t1 have lost pSym (pSym is indicated by asterisk in Fig. 21, lanes 4S and CI101) and harbour two large endogeneous plasmids. The molecular size of pCINod1 is estimated at 26 kb, which is as almost same size to pRt032 (27 kb). If pCINod1 replicates as a plasmid form in strain CNH1, a distinguishable band should be detected as pRt032 is detected in strain H1t1 (pRt032 is indicated by an arrow in Fig. 21, lane H1t1.). However, plasmid corresponding to pCINod1 could not be detected in strain CNH1 (Fig. 21, lane CNH1). This result indicates that pCINod1 did not exist in strain CNH1 as an autonomously replicating plasmid. I expected that pCINod1 integrated into the chromosome of strain CNH1, because it was already known that the plasmid carrying *attP* fragment of phage ϕU integrates into the chromosome of *Rhizobium* which are host strains of phage ϕU [Chapter III and IV].

Integration of the nod genes into the chromosome of the transconjugant

When the plasmid carrying *attP* fragment of phage ϕU integrated into the *Rhizobium* chromosome by *attP*/*attB*-mediated site-specific recombination, the *attP* fragment was devided into the two new DNA junctions (*attL* and *attR*). The

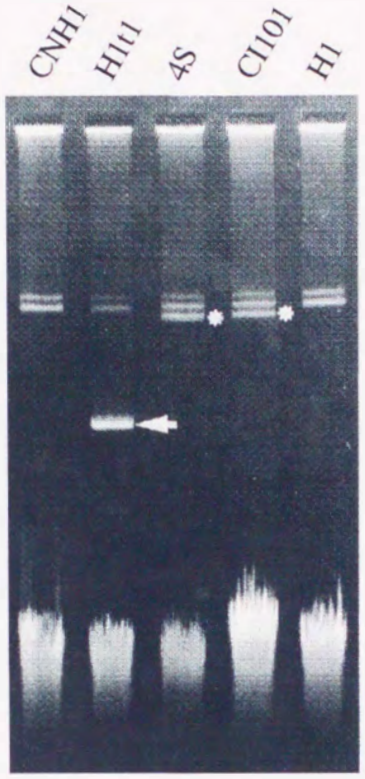

Fig. 21. Agarose gel electrophoresis of total DNA of *Rhizobium* strains. Total DNA from *Rhizobium* was prepared according to the method of Higashi *et al.* [35]. The asterisks in lanes 4S and CI101 indicate Sym plasmids. The arrow in lane H1t1 indicates pRt032. chr, fragmented chromosomal DNA.

two fragments of *attL* and *attR* could be detected by Southern hybridization probed with the *attP* fragment.

To confirm the integration of pCINod1 into the CNH1 chromosome, Southern hybridization was performed using the 2.0 kb *Eco*RI-*Pst*I fragment of phage ϕU which contains *attP* site [Chapter III] and the 14 kb *Hin*dIII fragment pSym *nod* genes as probes (Fig. 22). Total DNA of *Rhizobium* was digested by restriction endonucleases *Eco*RI mixed with *Pst*I (for *attL* and *attR* detection) or by *Hin*dIII alone (for *nod* genes detection). The *attP* located on the 2.0 kb *Eco*RI-*Pst*I fragment of pCINod1 (indicated by closed arrowhead in Fig. 22). Although this *Eco*RI-*Pst*I 2.0 kb fragment could not be detected in strain CNH1, two new fragments (indicated by open arrowheads in Fig. 22) were detected by the *attP* probe. Furthermore, molecular size of the two fragments were completely identical to that of *attL* and *attR* fragments which were detected in strain CI101. This indicated that the *attP* fragment on pCINod1 was split into two fragments corresponding to *attL* and *attR*. The 14 kb *Hin*dIII fragment was detected in strain CNH1 by the *nod* genes probe. No fragments could be detected in parent strain H1 by *attP* and the *nod* genes probes. When Southern hybridization probed with the *attP* and the *nod* genes were performed against 10 other CNH strains, the hybridization profiles were identical to that of strain CNH1 (data not shown). These hybridization data indicate that the pSym *nod* genes derived from pRt032 integrated into the chromosome of *R. leguminosarum* biovar *trifolii* strain CNH1 by a site-specific recombination between *attP* on pCINod1 and *attB* on the chromosome.

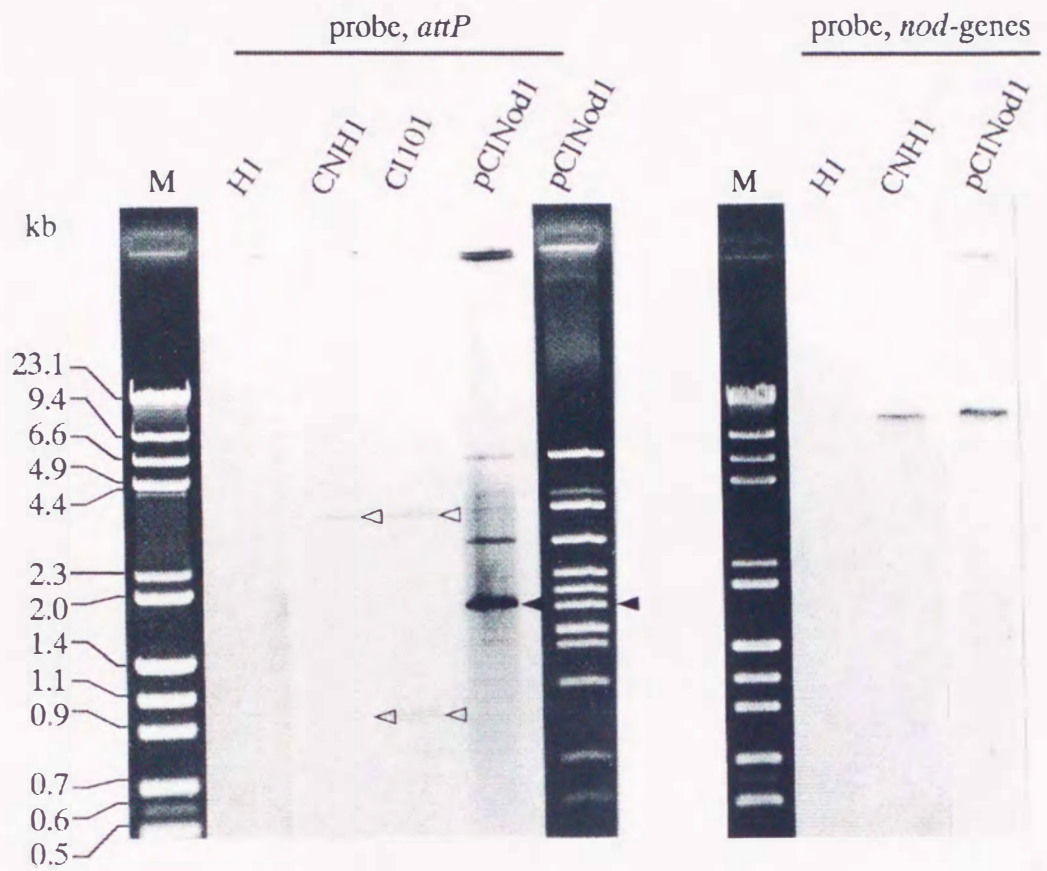

Fig. 22. Southern hybridization with *attP* and *nod* genes probes. Total DNA prepared from *Rhizobium* strains and pCINod1 were digested with *Eco*RI mixed with *Pst*I or *Hin*dIII alone. The 2.0 kb *Eco*RI-*Pst*I fragment of pCI4 and 14 kb *Hin*dIII fragment of pRt032 were used as *attP* and *nod* genes probe, respectively. The *attP* probe hybridized with the identical fragment of pCINod1 (indicated by closed arrowhead). The restriction fragments corresponding to *attL* and *attR* were detected in strain CNH1 (indicated by open arrowheads). The 14 kb *Hin*dIII fragment was also detected in strain CNH1 with the *nod* genes probe. M, molecular size marker (λ *Hin*dIII fragments mixed with pHY marker, TAKARA, Japan).

Nodulation process, nodule morphology, and nodule numbers induced by strain CNH1

Strain CNH1 was inoculated onto the seedlings of white clover, and plant response was observed by light microscopy. The nodulation defective mutant strain H1 was used as a control. Within 7 d after inoculation with strain CNH1, root hair deformation and infection thread formation were observed (Fig. 23, a and b). No root hair deformation was observed with the parent strain H1 (Fig. 23, c) and without inoculation (Fig. 23, d). Nodules which initiated by 3 weeks after inoculation developed well showing cylindrical nodule morphology. These nodules were observed by transmission electron microscopy. Infection threads were found in the infected cells (data not shown). Bacteria were released from the infection threads into cytoplasm of the host plant cell, enclosed by the peribacteroid membrane, and changed their form to bacteroid (Fig. 23, e). Some of the bacteroids showed typical Y-shape. Until 8 weeks after inoculation, many small spherical nodules were formed on the roots of clover (Fig. 23, f). All nodules did not fix nitrogen due to the lack of *nif* (nitorgen fixation) genes in strain CNH1. Table 5 shows the numbers of nodules induced by three strains of *Rhizobium* (8 weeks after inoculation). The average number of nodules developing on 300 clover plants was estimated as 2.46 ± 1.59 by inoculation of the wild type strain 4S, whereas it was estimated as 16.39 ± 6.08 by inoculation of strain CNH1 and as 16.30 ± 7.29 by inoculation of strain H1t1 which carrying pRt032, respectively. These data indicated that *nod* genes integrated into the

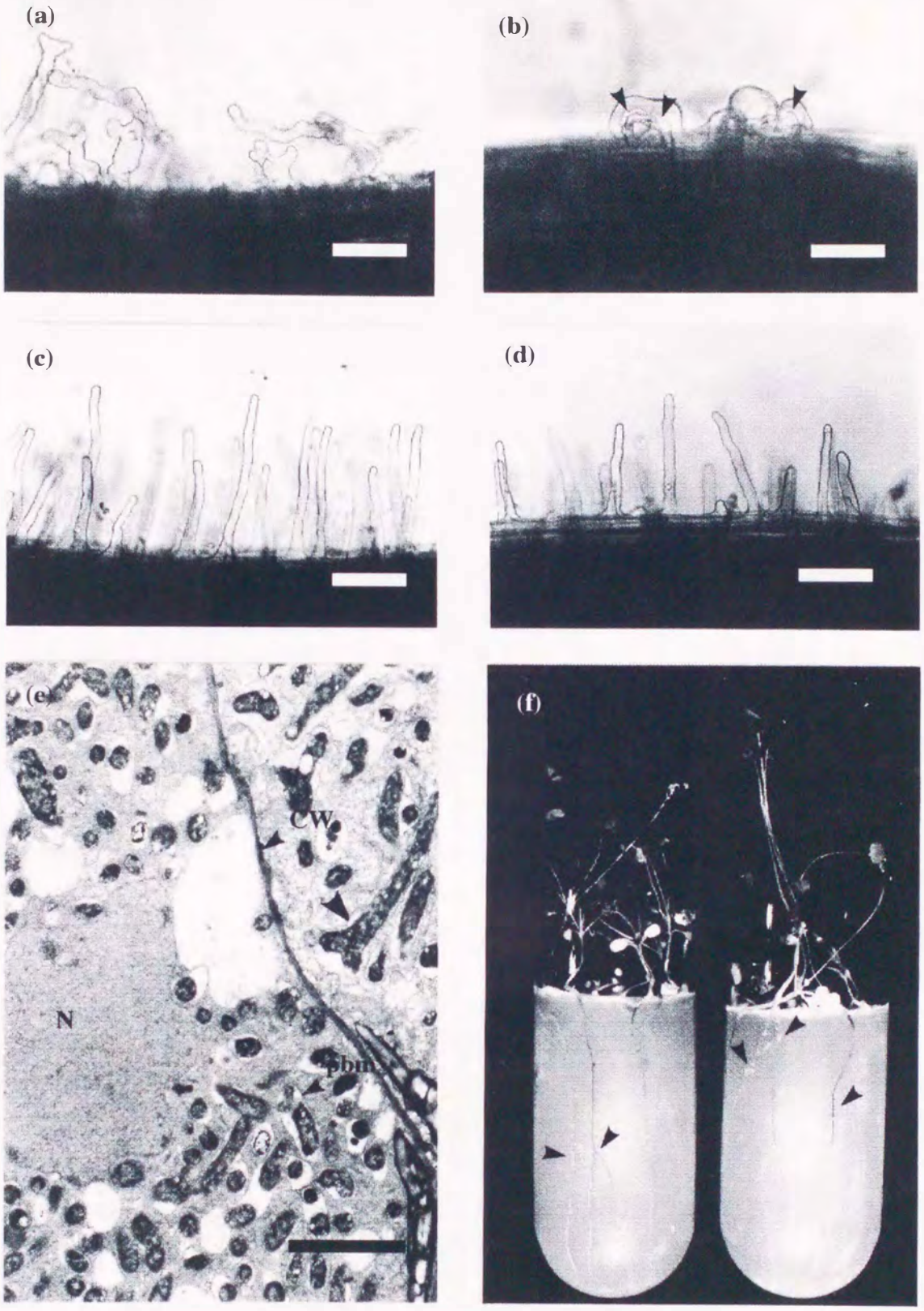

Fig. 23. Nodulation process of strain CNH1. Nodulation process of strain CNH1 was observed by light and transmission electron microscopy.
(a) Root hair deformation induced by strain CNH1. The bar indicates 25 μm. (b) Infection thread formation by strain CNH1. The arrowheads indicate infection threads. The bar indicates 25 μm. (c) Root hair strain H1 inoculated. The bar indicates 50 μm. (d) Root hair without inoculation. The bar indicates 50 μm. (e) Transmission electron micrograph of the nodules induced by strain CNH1(3 weeks after inoculation). The arrowheads indicate Y-shaped bacteroids. The bar indicates 2.5 μm. CW, cell wall; N, nucleus; pbm, peribacteroid membrane. (f) Nodulated clover plants (6 weeks after inoculation). The arrowheads indicate small spherical nodules.

Table 5. The numbers of nodules induced by *Rhizobium* strains

strain	nodule no. / plant	max. no.[a]	min. no.[a]	nodulated plants (%)
4S	2.46 ± 1.59	9	1	100
CNH1	16.39 ± 6.08	32	6	100
H1t1	16.30 ± 7.29	38	3	100

[a] The maximum and minimum numbers of nodules induced on a plant.

chromosome of CNH1 were completely expressed and functioned.

Re-inoculation of Rhizobium isolated from nodules induced by CNH1 inoculation

Strain CNH1 stably maintained nodulation ability for at least 10 generations. Single colony of strain CNH1 was inoculated onto the white clover seedlings and bacteria were isolated from the nodules 4 weeks after inoculation. One hundred colonies randomly selected were inoculated onto clover seedlings again. Twenty colonies of the 100 colonies could not induce root nodule on the clover roots. Southern hybridization, using *attP* and *nod* genes probes, was performed against the DNAs prepared from 5 colonies of the 20 non-nodulated colonies. The *attL* and *attR*, and *nod* genes could not be detected in these 5 non-nodulated colonies (data not shown). These data indicated that the chromosome-integrated pCINod1 could be excised from the chromosome during the nodulation process.

4. DISCUSSION

The cloned *nod* genes (14 kb *Hin*dIII fragment) derived from *Rhizobium leguminosarum* biovar *trifolii* strain ANU843 were integrated into the chromosome of the nodulation defective strain, *R. leguminosarum* biovar *trifolii* strain H1, utilizing the chromosomal integrative vector. The resulting transconjugant strain CNH1 acquired nodulation ability on white clover. The light and transmission electron microscopic observation revealed that the

nodulation process of strain CNH1 was very similar to that observed with wild type strains. The 14 kb *Hin*dIII fragment encodes root hair deformation, infection thread formation, nodule development, and bacterial release [77]. All these phenotypes were observed in the nodulation process of strain CNH1. This indicates that *nod* genes which integrated into the chromosome of *Rhizobium* were completely expressed and functioned.

Strain CNH1 maintained nodulation ability for at least 10 generations, however, 20 % of isolates from the nodules could not induce nodules due to the loss of pCINod1 from the chromosome. Leong *et al.* [60] reported that the excisionase gene (xis) which mediates excision of prophage DNA from the chromosome was located near the *attP* site in λ phage. If the genetic structure of the *attP* region of phage ϕU are similar to that of λ phage, there is a possibility that *xis* resides on pCINod1 and functions. To achieve the permanent integration of *nod* genes into the chromosome of *Rhizobium*, it is very important to identify the *xis* gene on the *attP* fragment of phage ϕU and to construct excisionase defective vector.

Nodule forming bacteria belonging to the family Rhizobiaceae can be devided into two groups depending on the location of the *nod* genes; in fast-growing species of *Rhizobium* the *nod* genes are located on a large Sym plasmid, whereas in *Bradyrhizobium* and *Azorhizobium* spp. including some tropical strains, the *nod* genes are located on their chromosome [4, 32, 63, 67]. It is known that the Sym plasmid in *R. leguminosarum* biovar *trifolii* was efficiently eliminated after incubation at elevated temperature [35, 95]. Zurkowski [96]

referred to the temperature-sensitive-replication of the Sym plasmid. Segovia *et al.* [79] investigated about the soil population of *Rhizobium leguminosarum* in a bean field. They reported a ratio of 1 symbiotic strain to 40 nonsymbiotic strains. The nonsymbiotic isolates restored symbiotic ability by transfer of the Sym plasmid. The spontaneous curing of the Sym plasmid may occur in the fields. In *Bradyrhizobium* including some tropical strains, *nod* genes are located on chromosome. It is an interesting hypothesis that the chromosomal *nod* genes are more stable than the *nod* genes on a large Sym plasmid. *Rhizobium* strain which carries *nod* genes on the chromosome will be a useful strain to compare the stability of *nod* genes between Sym plasmid and chromosomal location.

5. SUMMARY

Plasmid pCINod1 carrying *attP* of a lysogenic rhizobiophage and the *nod* genes was constructed. Inoculation of a conjugation mixture of *Escherichia coli* harbouring pCINod1 and a nodulation-defective *Rhizobium leguminosarum* biovar *trifolii* strain resulted in formation of root nodules on white clover. *Rhizobium* transconjugant strain CNH1 isolated from these nodules had integrated pCINod1 into its chromosome. The light and electron microscopic observation of the nodulation process revealed that the *nod* genes on the chromosome of strain CNH1 completely functioned.

VI. CONCLUDING REMARKS

In this thesis, the chromosomal integrative vector system for *Rhizobium leguminosarum* biovar *trifolii* has been established. The aim of this work is to integrate *nod* genes on the Sym plasmid into the chromosome and investigate the expression of the chromosomal *nod* genes.

In Chapter II, isolation and charaterization of the lysogenic phage ϕU and its lysogen *Rhizobium leguminosarum* biovar *trifolii* UK-1(ϕU) were described. The phage induction with mitomycin C was confirmed in both the free-living and the symbiotic form of the lysogen by electron microscopic observation. Phage ϕU has shown to lysogenize *R. leguminosarum* biovar *trifolii* strain 4S. One of the resulting lysogenic strains, strain 4S(ϕU), had cured the 315 kb Sym plasmid and lost symbiotic ability simultaneously. It was suggested that the lysogenization might cause the elimination of the Sym plasmid. However, molecular mechanism of the Sym plasmid curing could be uncompletely understood.

Southern hybridization probed with phage ϕU DNA was performed against restriction fragments of DNAs prepared from the lysogenic strains and ϕU itself. The hybridization profiles indicated that phage ϕU integrated into the chromosome of host *Rhizobium* at a site involving the 6.0 kb *Eco*RI fragment of phage ϕU. This 6.0 kb *Eco*RI fragment (*attP* fragment) was cloned into the unique *Eco*RI site of the suicide plasmid pSUP202 and the resultant recombinant plasmid was referred to pCI6. Plasmid pCI6 was transferred from *E. coli* S17-1

to *R. leguminosarum* biovar *trifolii* strains 4S and 4S(ϕU). As described in Chapter III, pCI6 was able to integrate into the chromosome of *Rhizobium* strains at high frequency in same manner as phage ϕU integration. It was shown that the suicide plasmid such as pCI6 would be a useful vehicle to integrate genetic materials into the bacterial chromosome.

The application of the chromosomal integrative vector was assessed in Chapter IV, using 14 strains of Rhizobiaceae as conjugation recipients. *R. leguminosarum* biovar *viciae* strain K5 is not host strain for phage ϕU, however, pCI6 could integrate into the chromosome of strain K5 at high frequency. Furthermore, the *attB* site and its adjacent region were conserved in *R. leguminosarum* biovar *trifolii* strain 4S and *R. leguminosarum* biovar *viciae* strain K5 independent of susceptibility to phage ϕU infection.

In Chapter V, *R. leguminosarum* biovar *trifolii* strain which carries chromosomal *nod* genes was generated utilizing chromosomal integrative vector system. Plasmid pCINod1 carrying *attP* from phage ϕU and *nod* genes from the Sym plasmid was constructed and integrated into the chromosome of *R. leguminosarum* biovar *trifolii* strain H1, a Sym plasmid cured mutant derived from a wild type strain 4S. The resultant strain CNH1 had chromosomal *nod* genes and acquired nodulation ability. The light and electron microscopic observation of nodulation process of strain CNH1 revealed that the chromosomal *nod* genes were expressed and functioned completely.

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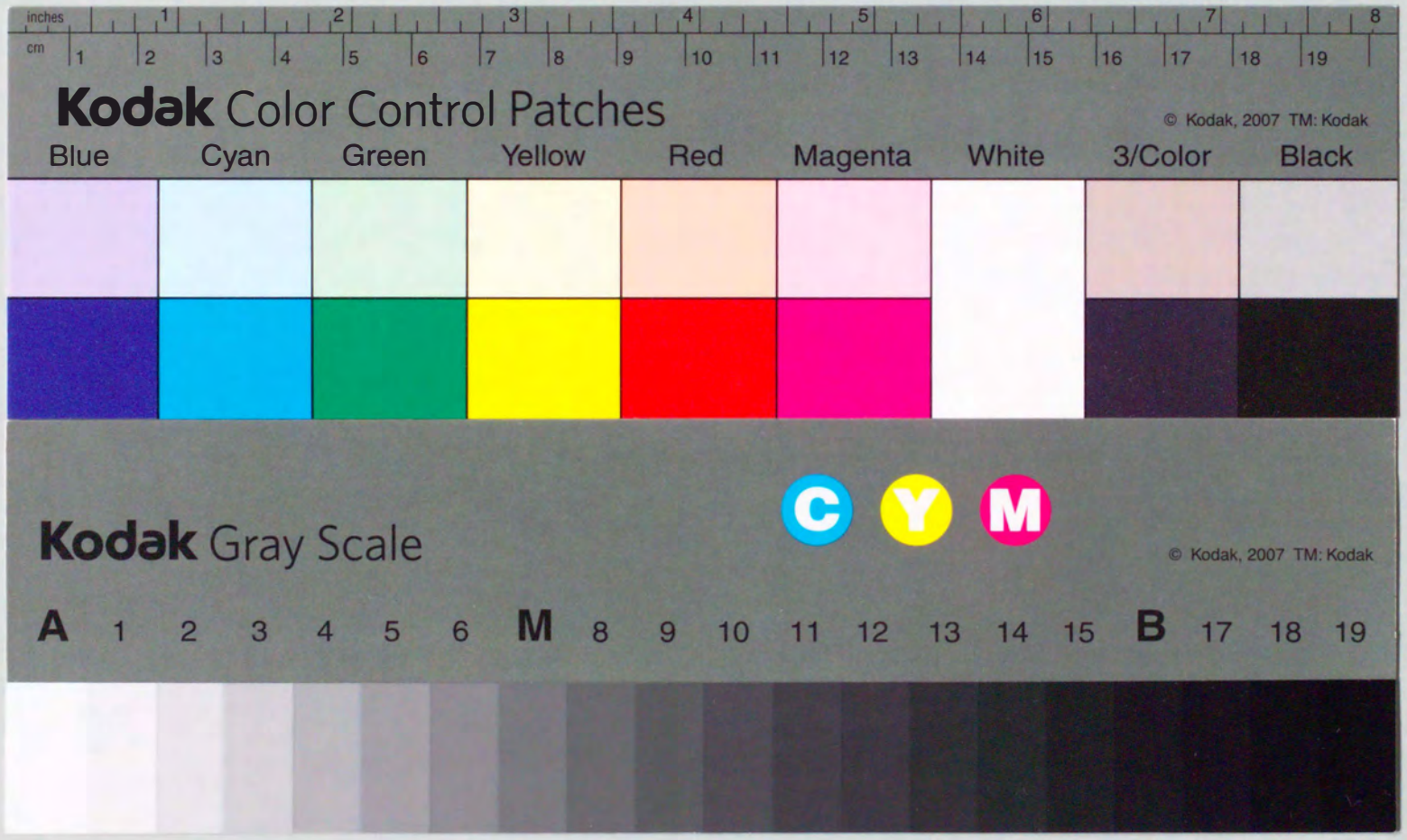
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