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Host Plant Effects on Hybrids of *Rhizobium leguminosarum* Biovars *viceae* and *trifolii*

By C. L. WANG,[1]† J. E. BERINGER[2] AND P. R. HIRSCH[1]*

[1]*Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, UK*

[2]*Unit of Molecular Genetics, University of Bristol, Woodland Road, Bristol BS8 1UG, UK*

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The *Rhizobium leguminosarum* biovar *viceae* host-range plasmid pJB5JI was transferred to six *R. leguminosarum* biovar *trifolii* strains. Inheritance of pJB5JI enabled the *R. leguminosarum* biovar *trifolii* strains to nodulate both peas and clover species. Bacteria were isolated from nodules formed on peas (the 'correct' host for *R. leguminosarum* biovar *viceae*) and the hosts for *R. leguminosarum* biovar *trifolii* (white, red and subterranean clover). Isolates from peas and white clover appeared to have lost or changed the host-range plasmid conferring the ability to nodulate white clover or peas respectively. Isolates from subterranean and red clover could often nodulate all host plants. These results show that the host exerts some form of functional incompatibility when interacting with hybrid *Rhizobium* strains and that some hosts are more stringent than others.

INTRODUCTION

The Rhizobiaceae is a family of Gram-negative micro-organisms whose taxonomy is based primarily on their ability to interact with plants. Thus the genus *Rhizobium* is split into species and further subdivided into biovars on the basis of the legume plants on which particular isolates form N_2-fixing nodules. For example, *R. leguminosarum* biovar *trifolii* nodulates clover (*Trifolium* spp.), biovar *phaseoli* nodulates *Phaseolus* spp. and biovar *viceae* nodulates pea (*Pisum* spp.), *Vicia*, *Lens* and *Lathyrus*. Until the recent re-classification of the Rhizobiaceae (Jordan, 1984), these biovars were defined as the three separate species *R. trifolii*, *R. phaseoli* and *R. leguminosarum* respectively. This classification is very useful in agriculture because the groupings determine whether a particular strain of *Rhizobium* will be suitable for inoculating a particular legume crop.

The value of plant nodulation responses as a taxonomic criterion was first seriously challenged by the observation by Higashi (1967) that the ability to nodulate clover could be transferred from *R. leguminosarum* biovar *trifolii* to *R. leguminosarum* biovar *phaseoli*. Later Johnston *et al.* (1978) reported that host range determination was plasmid-borne and could be transferred from *R. leguminosarum* biovar *viceae* to *R. leguminosarum* biovar *trifolii* and *R. leguminosarum* biovar *phaseoli*. This observation was expected because Johnston & Beringer (1977) had previously shown that the chromosomes of these three biovars were apparently identical.

Beynon *et al.* (1980) reported that hybrids of *R. leguminosarum* biovar *phaseoli* carrying *R. leguminosarum* biovar *viceae* host-range genes could only nodulate peas (the 'correct' host for *R. leguminosarum* biovar *viceae*) if they had lost a plasmid carrying *R. leguminosarum* biovar *phaseoli* host-range genes, or if the genes were lost due to deletions in the plasmid. This work suggested that there was a functional incompatibility which prevented bacteria that carried, and presumably expressed, genes determining two different host ranges from nodulating particular hosts. Interestingly, when *Phaseolus* plants growing on agar were inoculated with the hybrids,

† Present address: Huazhong Agricultural University, Wuhan, Hubei, Peoples Republic of China.

nodules were formed and contained bacteria which retained both host-range plasmids. The *R. leguminosarum* biovar *viceae* strain forms non-N_2-fixing (Fix^-) nodules on *Phaseolus* grown under these conditions and thus it was assumed that *Phaseolus* was a less discriminating host than the pea, which is not nodulated by *R. leguminosarum* biovar *phaseoli*.

Djordjevic *et al.* (1982), using the same *R. leguminosarum* biovar *viceae* host range plasmid (pJB 5JI) as we have used in this study, reported that the host influenced the stability of pJB5JI in *R. leguminosarum* biovar *trifolii* and that derivatives unable to nodulate peas had suffered a deletion of about 30 MDa of pJB5JI DNA. Their work also indicated that the Fix^+ phenotype was less readily maintained than the broadened host range ability conferred by the presence of pJB5JI. Christensen & Schubert (1983) have also studied the fate of pJB5JI in *R. leguminosarum* biovar *trifolii* strain T37, and have shown that it can interact with a host-range plasmid already present in this strain to produce strains with different plasmid profiles and symbiotic properties. They also reported that after isolation of *R. leguminosarum* biovar *trifolii* strains from clover nodules, over 30 isolates from 80 examined had lost kanamycin resistance conferred by pJB5JI.

It is clear from these studies that pJB5JI can confer an increased host range on *R. leguminosarum* biovar *trifolii* strains and that the plasmid, or most probably the expression of certain host range genes on it, is selected against when strains carrying two sets of host range genes nodulate clover plants. However, previous studies on the genetics and host plant responses to *R. leguminosarum* biovar *trifolii* strains carrying plasmids conferring two different host ranges have been restricted in the *R. leguminosarum* biovar *trifolii* strains used and clover species examined. While *R. leguminosarum* biovar *trifolii* strains usually nodulate most clover species, it is relatively unusual to find strains that are able to form N_2-fixing nodules on all, indicating a clear genotype × genotype interaction between *R. leguminosarum* biovar *trifolii* and *Trifolium* species.

The purpose of this research was to determine whether the existing genotype interactions between *R. leguminosarum* biovar *trifolii* and clover species would affect the response of plants to inoculation with *R. leguminosarum* biovar *trifolii* strains containing *R. leguminosarum* biovar *viceae* host range genes. For host plants we used the pea (*Pisum sativum* cv. Dark Skinned Perfection), which is the 'correct' host for *R. leguminosarum* biovar *viceae*; and white clover (*Trifolium repens*, cv. S100), red clover (*T. pratense* cv. S123) and subterranean clover (*T. subterranean* cv. Mount Barker), which are the 'correct' hosts for *R. leguminosarum* biovar *trifolii*. The different clover species were chosen because they differ in their ability to form N_2-fixing nodules with the six *R. leguminosarum* biovar *trifolii* strains used in this study (Table 1). Subterranean clover also had the advantage for this study in that it is readily nodulated by *R. leguminosarum* biovar *viceae* strains, though the nodules never fix N_2.

METHODS

Rhizobium strains and plasmids. The properties of the *Rhizobium* strains and plasmids and their plant nodulation features are listed in Table 1. Media and culture conditions were as described by Beringer (1974). Membrane crosses were as described by Beringer *et al.* (1978). Transconjugants were selected for the inheritance of kanamycin resistance (determined by transposon Tn*5* present on pJB5JI and then subcultured three times on minimal medium agar containing kanamycin before plants were inoculated.

Plant inoculation and nodule analysis. Seeds were surface-sterilized with 70% ethanol for 20 s, followed by sodium hypochlorite (8%, w/v, available chlorine) for 5–10 min according to seed size and then washed 10 times in sterile water before they were transferred to 1% yeast mannitol (YM) agar (Vincent, 1970) for germination. The pea seeds were incubated for about 4 d in the dark at 28 °C. Sterilized clover seeds were kept at 4 °C overnight, then the plates were inverted and incubated at 28 °C. Pea plant growth conditions were as described by Beringer (1974) except that Perlite was used with Fahraeus solution (Fahraeus, 1957). Clover plants were grown on 1% agar Fahraeus slopes under the same conditions as for peas. Nodules were usually visible 1–2 weeks after infective bacteria had been added. Nodule nitrogenase activity was measured by C_2H_2 reduction as described by Johnston & Beringer (1975). Between 10^8 and 10^9 rhizobia, washed from TY slopes in sterile water, were added to each plant.

Strain re-isolation. Full-size nodules were excised from the root and surface-sterilized by washing in 95% ethanol for 15–30 s before transfer to 6% hydrogen peroxide (20 vols H_2O_2) for 2–5 min (according to nodule size). Nodules were rinsed in sterile distilled H_2O, then crushed and streaked on YM agar. Phenotypes of the re-isolated bacteria were checked by plating on suitable selective media and by analysis of plasmid profiles using agarose gel

Table 1. *Bacterial strains and plasmids*

Strain	Symbiotic properties with legume hosts* Pea	White clover	Red clover	Subterranean clover	Reference/source
R. leguminosarum biovar *trifolii*					
RCR5	N^-	F^+	F^+	F^-	Field isolates from the Rothamsted Culture Collection
RCR32	N^-	F^+	F^+	F^-	
RCR46	N^-	F^+	F^+	F^+	
RCR221	N^-	F^+	F^+	F^+	
RCR226	N^-	F^-	F^-	F^+	
RCR227	N^-	F^-	F^-	F^+	
R. leguminosarum biovar *viceae*					
T83K3	F^+	N^-	N^-	F^-	Johnston *et al.* (1978)

Strain	Relevant properties and plasmids	Reference
R. leguminosarum biovar *viceae*		
JI6015	Phe$^-$ Trp$^-$ Str^R Rif^R Nod$^-$, produces *small* bacteriocin	Johnston *et al.* (1978)
T83K3	JI6015 carrying pJB5JI, a transmissible symbiotic plasmid marked with Tn*5* conferring kanamycin resistance	Johnston *et al.* (1978)
B151	Non-nodulating derivative of strain 128C53 cured of its Sym plasmid	Brewin *et al.* (1982)
E. coli		
JI1830	Carries pJB4JI, a suicide vector for Tn*5*	Beringer *et al.* (1978)

* F^+, Fix^+; F^-, Fix^-; N^-, Nod^-.

electrophoresis (Hirsch *et al.*, 1980). Kanamycin-sensitive isolates which appeared to carry deleted derivatives of pJB5JI were investigated further by hybridizing blots from agarose gels with Tn*5* and *Klebsiella nif* DNA probes as described by Hombrecher *et al.* (1981). Such deleted strains were also screened for bacteriocin production and sensitivity as described by Hirsch (1979).

RESULTS

Transfer of plasmids between R. leguminosarum biovars viceae and trifolii

Crosses were done using *R. leguminosarum* biovar *viceae* strain T83K3 as the donor of pJB5JI to the six wild-type strains of *R. leguminosarum* biovar *trifolii*. The transfer frequency (per recipient) of pJB5JI was 10^{-4} to RCR46 and 10^{-3} to the other five strains. Five single colonies were taken from each cross and, after purifying by subculturing three times on selective medium, the appropriate transconjugants were inoculated on both pea and clover plants. These plant tests showed that the pJB5JI derivatives of five of the six strains had inherited the ability to nodulate peas (Table 2), forming many small nodules; two of them formed N_2-fixing (Fix^+) nodules, while at the same time retaining their original symbiotic properties on clover plants. Compared to *R. leguminosarum* biovar *trifolii* controls, the rate of N_2 fixation on clover plants was not significantly altered by the presence of pJB5JI in any of derivative strains (data not shown).

To test the stability of the kanamycin resistance determinant, all 30 transconjugants were subcultured three times on minimal medium agar without kanamycin. About 100 colonies for each were checked and none was found to have become kanamycin-sensitive. Thus the presence of Tn*5* in pJB5JI was judged to be stable in laboratory cultures. When these strains were used to inoculate peas and clovers and were isolated from nodules, all isolates from peas were still kanamycin-resistant. However, kanamycin-resistance was frequently absent in transconjugants isolated from clover nodules (Table 2).

Table 2. *Symbiotic properties of R. leguminosarum biovar trifolii strains isolated from nodules*

Strain	Initial host*	Initial plant response†	Km^R isolates‡	Plant responses to isolates from initial hosts†			
				Pea	White clover	Red clover	Sub. clover*
RCR5(pJB5JI)	Pea	F^-	5/5	$5F^-$	$4F^+$, $1F^-$	$4F^+$, $1F^-$	$5F^-$
	White clover	F^+	4/5	$4N^-$	$4F^+$	$3F^+$, $1F^-$	$4F^-$
	Red clover	F^+	5/5	$2F^-$, $3N^-$	$4F^+$	$5F^+$	$5F^-$
	Sub. clover	F^-	3/3	$3F^-$	$3F^+$	$3F^+$	$3F^-$
RCR32(pJB5JI)	Pea	F^-	5/5	$3F^-$	$4F^+$	$4F^+$	$4F^-$
	White clover	F^+	2/5	$3N^-$, $2F^-$	$5F^+$	$5F^+$	$5F^-$
	Red clover	F^+	5/5	$1N^-$, $4F^-$	$2N^-$, $3F^+$	$5F^+$	$1N^-$, $3F^-$
	Sub. clover	F^-	3/4	$4F^-$	$3F^-$, $1F^+$	$1F^-$, $3F^+$	$4F^-$
RCR46(pJB5JI)	Pea	F^+	5/5	$5F^+$	$4F^-$	$5F^+$	$5F^+$
	White clover	F^+	5/5	$5N^-$	$1N^-$,$4F^+$	$5F^+$	$5F^+$
	Red clover	F^+	5/5	$1N^-$, $3F^+$	$4F^+$	$4F^+$	$4F^+$
	Sub. clover	F^+	4/5	$2N^-$, $3F^+$	$5F^+$	$5F^+$	$5F^+$
RCR221(pJB5JI)	Pea	F^-	5/5	$4F^-$	$5F^+$	$5F^+$	$5F^+$
	White clover	F^+	4/5	$3N^-$, $2F^-$	$5F^+$	$5F^+$	$2F^-$, $3F^+$
	Red clover	F^+	4/4	$1N^-$, $4F^-$	$5F^+$	$5F^+$	$2F^-$, $3F^+$
	Sub. clover	F^+	4/5	$4F^-$	$4F^+$	$4F^+$	$4F^+$
RCR226(pJB5JI)	Pea	F^+	5/5	$4F^+$	$2N^-$, $3F^-$	$2N^-$, $2F^-$	$3F^+$
	White clover	F^-	2/4	$2N^-$, $2F^-$	$3F^-$	$2N^-$, $2F^-$	$3F^+$
	Red clover	F^-	2/3	$3F^+$, $1F^-$	$3F^-$	$1N^-$, $2F^-$	$1F^+$
	Sub. clover	F^+	5/5	$5F^+$	$1N^-$, $4F^-$	$3N^-$, $2F^-$	$2N^-$, $2F^+$
RCR227(pJB5JI)	Pea	N^-	–	–	–	–	–
	White clover	F^-	3/4	$2N^-$, $2F^-$	$4F^-$	$4N^-$	$4F^-$
	Red clover	F^-	4/5	$3N^-$, $2F^-$	$5F^-$	$2N^-$, $2F^-$	$2F^-$, $3F^+$
	Sub. clover	F^+	3/4	$4F^-$	$4F^-$	$3N^-$, $1F^-$	$2F^-$, $2F^+$

* Sub., subterranean.

† F^+, Fix^+; F^-, Fix^-; N^-, Nod^-. The plant response results show the number of plants used and the number of isolates from the initial hosts that were re-tested.

‡ The proportion of isolates, from the initial hosts, that were still resistant to kanamycin. One nodule per plant was sampled.

Inoculation tests using isolates from nodules

In the second nodulation tests, pea and clover plants were inoculated by those transconjugants which were isolated from nodules (Table 2). All six strains isolated from white clover had changed in their ability to nodulate peas, especially isolates from strains RCR5(pJB5JI) and RCR46(pJB5JI) where all replicates failed to nodulate peas. The nodule isolates for the four other strains varied in their ability to nodulate peas. However, with the exception of one clone of RCR46(pJB5JI), these white clover isolates had not changed in their ability to nodulate white clovers (Table 2).

Study of the deleted plasmids

Agarose gel electrophoresis. Plasmid profiles of transconjugant strains isolated from nodules are shown in Fig. 1. Some profiles, notably of pea nodule isolates, are identical to those of the inoculant strains. However, plasmid rearrangements have occurred, and loss of all or part of pJB5JI is apparent in many clover nodule isolates.

Re-isolates from clover nodules found to have lost kanamycin resistance were investigated further. In strain RCR32(pJB5JI) derivatives, the white clover selection had resulted in the total loss of pJB5JI in all three kanamycin-sensitive isolates tested, which accounts for the Nod^+ phenotype observed in the next plant inoculation tests. But derivatives of RCR226(pJB5JI) were found to have deletions in pJB5JI instead of total loss in all five of the kanamycin-sensitive isolates tested. Interestingly, in some of the RCR226(pJB5JI) derivatives, pJB5JI had small

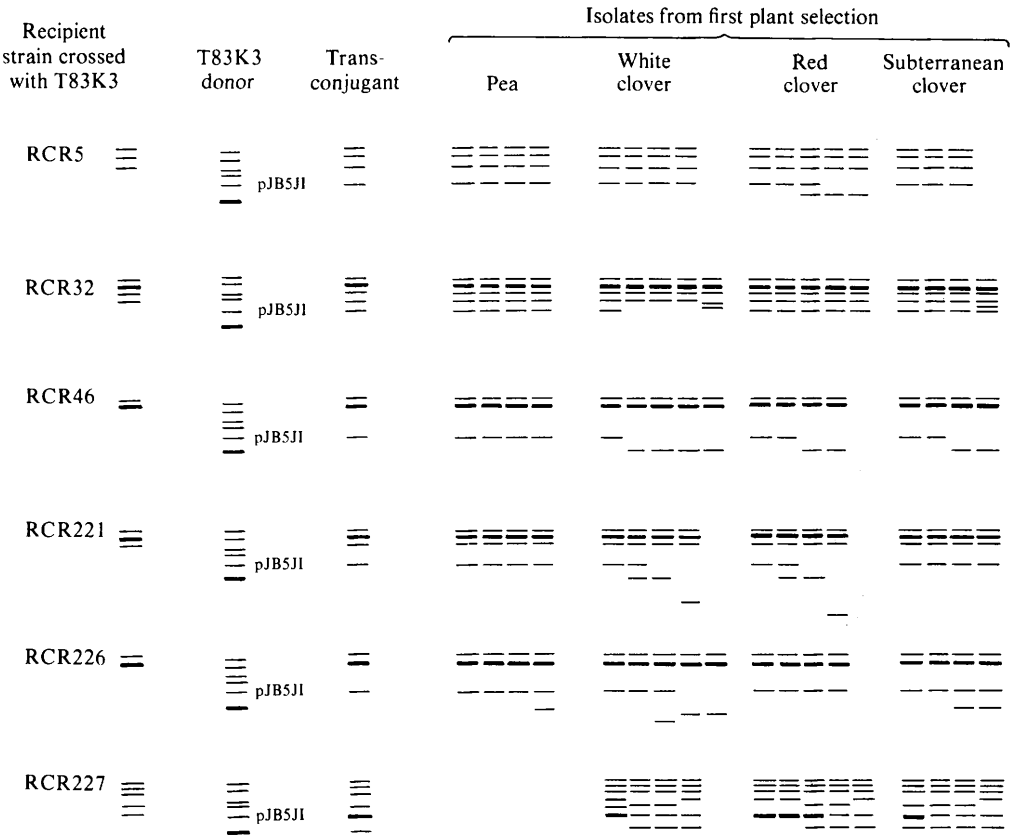

Fig. 1. Plasmid profiles of transconjugants after plant selection. The profiles of all the isolates tested are shown. RCR227 transconjugants acquired pJB5JI and one of the two smallest plasmids from T83K3. Heavy bands represent two plasmids of similar size.

deletions after the first white clover plant selection and such strains could infect peas, forming nodules which were Fix$^-$. The size of four of the deleted plasmids was about 100 MDa (about 30 MDa less than pJB5JI) and less than 100 MDa for the other plasmid. However, in the isolates from subsequent white clover nodule infections, the size of the deleted pJB5JI in the five isolates tested was only about 55 MDa. These derivatives had lost the ability to nodulate peas. This might mean that the entire nodulation and N_2 fixation region is functionally incompatible, and under continued selective pressure loss of all of the non-selected genes will be observed.

One of the 55 MDa deleted plasmids was marked by introducing Tn*5* into it from the suicide plasmid pJB4JI. In crosses with *R. leguminosarum* biovar *viceae* strain B151 the deleted plasmid was transferred at a frequency of 10^{-4} per recipient, showing that the *tra* genes on pJB5JI were still present.

Gel blots and DNA hybridization using Tn5 and nif DNA probes. An autoradiogram of a gel blot of kanamycin-sensitive derivatives of RCR226(pJB5JI) probed with Tn*5* is illustrated in Fig. 2. The Tn*5* probe hybridized to the deleted pJB5JI plasmids, the intensity of hybridization to the appropriate bands being similar to that to pJB5JI from T83K3. This suggested that the deletion involved only part of Tn*5*. No hybridization with these deleted plasmids by the *nif* probe (which contains the structural genes for nitrogenase) was obtained, although hybridization to pJB5JI in the *R. leguminosarum* biovar *viceae* control strain T83K3 was always observed. The results indicate that the deletion started in Tn*5*, leaving part of the transposon intact, and included *nod* (resulting in the failure to nodulate peas) and *nif* genes with homology to the *nif* probe.

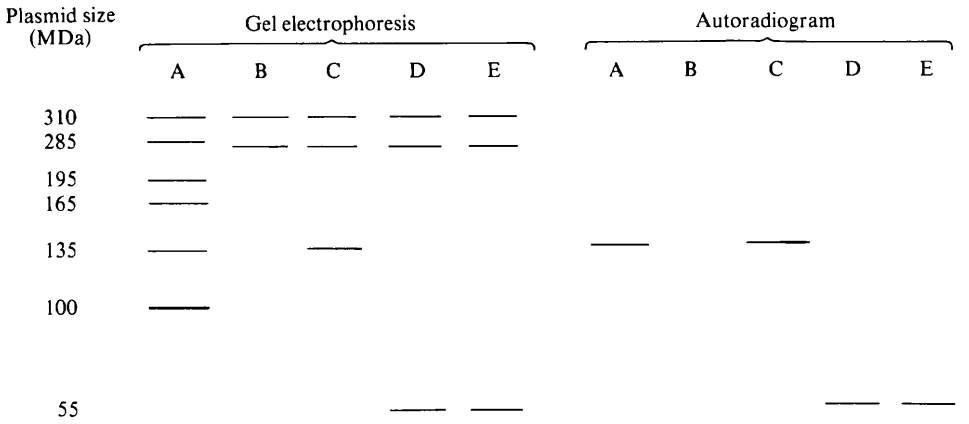

Fig. 2. Deletions in pJB5JI after plant selection. Lanes A, B and C are control strains T83K3, RCR226, RCR226(pJB5JI) respectively. Lanes D and E are derivatives of RCR226(pJB5JI), isolated from white clover nodules, with plasmid deletions. Blots of the gels were hybridized to Tn*5* DNA.

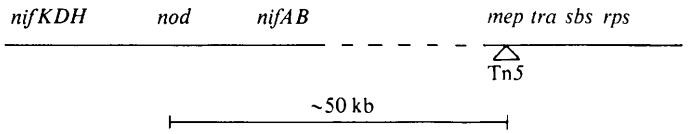

Fig. 3. Linkage of bacteriocin and *tra* genes in pJB5JI (C. A. Wijffelman, personal communication).

Some pea isolates from the first plant inoculation which failed to nodulate white clover were also investigated, but no physical differences were found between them and their Nod$^+$ parent strains (Fig. 1). However, RCR32(pJB5JI) derivatives that had been isolated from pea nodules and were used to re-infect peas were found, after the second isolation, to have lost or suffered deletions in two of the plasmids present in RCR32 (which were still present after the first isolation). One isolate had lost both plasmids, the other two tested had lost one and suffered a deletion in the other. The two plasmids both had homology to the *nif* probe and so are assumed to be Sym plasmids. The other *R. leguminosarum* biovar *trifolii* strains carrying pJB5JI showed no apparent change in plasmid content after the first or second isolation from pea nodules.

Bacteriocin prodution. R. leguminosarum biovar *trifolii* strain RCR32 is not sensitive to the *small* bacteriocin produced by *R. leguminosarum* biovar *viceae* strain JI6015. A pJB5JI derivative of RCR32 was examined and found to have become sensitive, demonstrating that the 'small bacteriocin sensitivity' (*sbs*) gene on pJB5JI is expressed in this strain.

R. leguminosarum biovar *trifolii* strain RCR226 produces a *small* bacteriocin to which strain RCR32(pJB5JI) is sensitive. Derivatives of RCR226 carrying pJB5JI no longer produced the *small* bacteriocin, demonstrating that the 'repression of small bacteriocin' (*rps*) gene on pJB5JI was expressed in these strains. Strains of RCR226 isolated from nodules that carried deleted derivatives of pJB5JI which had lost kanamycin resistance and *nod* and *fix* genes were still suppressed for *small* bacteriocin production and were still conjugative.

Plasmid pJB5JI is a derivative of plasmid pRL1JI carrying Tn*5* inserted within a gene involved in *medium* bacteriocin production (Johnston *et al.*, 1978). The observation that deletions of pJB5JI that removed Tn*5*, *nod* and *fix* genes did not affect *tra* or *rps* is consistent with the mapping of these genes in pRL1JI (see Fig. 3) by Priem & Wijffelman (1984). RCR226(pJB5JI) strains did not become sensitive to the *small* bacteriocin and thus the effect of the deletion on the *sbs* gene could not be tested in these strains.

DISCUSSION

This study confirms previous observations that host-range genes in *Rhizobium* can be functionally incompatible, even though the plasmids which carry them are apparently compatible during growth of the rhizobia in laboratory media. The loss or deletion of the incorrect host nodulation plasmids after plant selection was frequently observed and successive plant selection appeared to result in more extensive deletions. This phenomenon seems to be determined by the host plants: white clover appeared to be very discriminating. Of the 27 re-isolates from white clover nodules, 80% lost the ability to nodulate peas. In the less discriminating hosts, subterranean and red clover, less than 20% of nodule re-isolates had lost pea nodulation ability. About 10% of pea re-isolates could not form nodules on either white clover or red clover. In some cases the plant appeared to select those transconjugants which had undergone deletions and this selective pressure varied according to the 'stringency' of the plant.

However, the investigation shows that nodulating ability on peas varies according to the *Rhizobium* strain. In contrast to some previous reports, two of the *R. leguminosarum* biovar *trifolii* derivatives formed N_2-fixing nodules on peas after transfer of pJB5JI, and this property was stably inherited.

Beringer (1982) and others have reported that some members of the *Rhizobiaceae* are closely related to each other. Biovars *trifolii*, *viceae* and *phaseoli* are now classified as members of the same species (Jordan, 1984). Indeed Prakash *et al.* (1981) has shown that plasmids in *Agrobacterium* which are involved in tumour formation contain sequences of DNA that are also found in host-range plasmids in *R. leguminosarum* biovars *viceae*, *phaseoli* and *trifolii*, and *R. meliloti*. It is likely that different host plant responses, which are the criterion for speciation, can be elicited by strains that only have a few genes involved in host discrimination differing betwen them. If this interpretation is correct it should be possible to isolate or produce strains which are not clearly one biovar or another.

The observation that *R. leguminosarum* biovar *trifolii* strain RCR32 has two plasmids which hybridize to the *Klebsiella nif* DNA probe is the first that we know of for this biovar. More than one copy of *nifH* has been reported for some strains of *R. leguminosarum* biovar *phaseoli* (Quinto *et al.*, 1982) and *Anabaena* (Rice *et al.*, 1982; Kallas *et al.*, 1983). Our results do not show whether other genes involved in symbiosis are found on both plasmids, although the observation that strains nodulating peas have undergone deletions in both plasmids, or deletion in one and loss of the other, suggests that at least one gene whose function is incompatible with the nodulation of peas is present on each.

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