

## Evidence for Genetic Exchange and Recombination of *Rhizobium* Symbiotic Plasmids in a Soil Population

PETER R. SCHOFIELD,† ALAN H. GIBSON, WILLIAM F. DUDMAN, AND JOHN M. WATSON\*

Commonwealth Scientific and Industrial Research Organisation Division of Plant Industry, Canberra City 2601, Australia

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A soil population of 16 *Rhizobium leguminosarum* bv. *trifolii* isolates was characterized by using three Sym (for symbiotic) plasmid-specific DNA hybridization probes: (i) an *R. leguminosarum* bv. *trifolii*-specific, repeated-sequence probe; (ii) a *nifHDK* gene probe, and (iii) a *nod* gene probe. A predominant Sym plasmid family was identified among the isolates. Three other unrelated Sym plasmid families were also identified. The isolates were also classified either by using a chromosomal DNA hybridization probe or by serological relatedness to 25 different *R. leguminosarum* bv. *trifolii* antisera. With either method, it was possible to group the 16 soil isolates into identical or related families. However, the correlation between the two techniques was not high. Irrespective of the means used to classify the bacterial host strain, it was possible to identify the same Sym plasmids in unrelated strains, as well as unrelated Sym plasmids in identical host strains. These data indicate that, within this soil population, there has been genetic exchange of Sym plasmids, and in one instance the hybridization pattern indicates that *in vivo* recombination of two different Sym plasmids may have occurred. Symbiotic effectiveness tests on red, strawberry, and subterranean clovers clearly differentiated the isolates. In general, the pattern of response was similar within groupings on the basis of Sym plasmid and chromosomal profiles but different between such groups.

The ability of bacteria of the genus *Rhizobium* to fix nitrogen in symbiosis with legumes is of considerable agricultural significance. However, one of the major agronomic problems of applying *Rhizobium* strains as soil inoculants is that indigenous soil rhizobia are frequently more competitive than the inoculant strain (15). Ideally, an inoculant strain must be competitive as well as highly effective at nitrogen fixation.

In fast-growing *Rhizobium* species, genes that determine nodulation and nitrogen fixation functions are located on large Sym (for symbiotic) plasmids, and molecular linkage maps have been established in *Rhizobium leguminosarum* bv. *trifolii* (23), *R. leguminosarum* (10), *R. meliloti* (19, 21), and a cowpea *Bradyrhizobium* sp. (6). Other genes, involved in different stages of the symbiosis, are chromosomally located, such as those involved in heme biosynthesis (20) and C<sub>4</sub>-dicarboxylate transport (22). By constructing synthetic strains consisting of combinations of various chromosomal backgrounds and Sym plasmids, Brewin et al. (4) suggested, on the basis of competition studies, that the chromosomal and Sym plasmid genes contributed to the ability of a strain to form nodules.

In *Agrobacterium*, another genus of the *Rhizobiaceae*, the tumor-inducing (Ti) plasmid may be transferred between strains in planta (18). Conjugal transfer of *Rhizobium* Sym plasmids has been demonstrated previously under laboratory conditions (16, 17), and recombinant Sym plasmids have been observed (3, 7, 9). Results from field studies are consistent with the occurrence of genetic exchange within soil populations of *Rhizobium* spp. (5, 13). Recombinant Sym plasmid-containing strains have been constructed and shown by symbiotic effectiveness assays to improve the

effectiveness of nitrogen fixation by various recipient strains (8), suggesting that generation or isolation of recombinant Sym plasmids may be a useful approach to the development of superior inoculant strains.

We previously identified a portion of the *R. leguminosarum* bv. *trifolii* *nifHDK* promoter which hybridizes specifically to the Sym plasmid of *R. leguminosarum* bv. *trifolii* isolates. The characteristic hybridization pattern (profile) of each isolate allows taxonomic classification at the strain level (29).

In this report, we characterize 16 isolates of *R. leguminosarum* bv. *trifolii* obtained from nodules of white clover (*Trifolium repens* L.) growing within a limited area. The characterization was achieved on the basis of hybridization of total DNA to (i) a species-specific probe, (ii) a *nif* probe, (iii) a *nod* probe, and (iv) a chromosomal probe that encodes a *met* gene. The isolates were also characterized serologically on the basis of immune diffusion reactions with 25 different *R. leguminosarum* bv. *trifolii* antisera and on the basis of their symbiotic effectiveness with three *Trifolium* species which exhibit distinctly different symbiotic response patterns with different strains (28). The observation of similar Sym plasmids in different host chromosomal backgrounds and of different Sym plasmids in similar host chromosomal backgrounds, as well as the presence of a putative recombinant Sym plasmid, indicates that genetic exchange of Sym plasmids had occurred in this soil population.

### MATERIALS AND METHODS

**Strain isolation.** Four white clover (*T. repens*) plants were obtained from a 5- by 5-m area in a clover pasture (Canberra, Australia) not known to have been inoculated with *R. leguminosarum* bv. *trifolii*. The plants were located approximately 3 m apart, except for plants 2 and 3, which had intertwined root systems. Six nodules were excised from each plant, rinsed, and surface sterilized in 1% sodium hypochlorite for 15 min. Severe surface sterilization resulted

\* Corresponding author.

† Present address: Laboratory of Molecular Neuroendocrinology, ZmbH, Universität Heidelberg, D-6900 Heidelberg, Federal Republic of Germany.

in complete killing of the contents of two small nodules but probably ensured that the surviving isolates were bona fide nodule occupants. After two rinses in sterile water, each nodule was crushed in protoplast dilution buffer and the suspension was plated on BMM plates with added mannitol (36 g/liter) (14). A single-colony isolation was made from each nodule when growth occurred. Isolates 1 to 5 were derived from plant 1, isolates 6 to 9 were from plant 2, isolates 10 to 14 were from plant 3, and isolates 15 and 16 were from plant 4. For DNA isolation, isolates were grown in TY medium (2). However, two isolates from plant 2 and four from plant 4 failed to grow on this medium. DNAs were obtained from three of these isolates after growth in GMY medium (1).

**Molecular biology.** DNAs were obtained from the 16 isolates, restricted with *Hind*III, and electrophoresed on a 1% agarose gel before Southern blotting on nitrocellulose membrane (23). DNA hybridization probes were prepared by random priming (30). Four probes were used: (i) the species-specific, repeated-sequence (RtRS) probe pRt642, containing 232 base pairs of the *nifHDK* promoter (29); (ii) the *nif* gene probe pRt585, containing the entire *nifHDK* operon (23); (iii) the *nod* gene probe pRt587, containing a 14-kilobase (kb) insert carrying the clover-specific nodulation genes (23, 24), and (iv) the chromosomal probe pRt654, containing 10 kb of DNA flanking a Tn5-induced methionine auxotrophic mutation in a derivative of *R. leguminosarum* bv. *trifolii* ANU9000 (27). The hybridization probes were applied sequentially to the same Southern blot. To remove the previous hybridization probe, the nitrocellulose was washed in 20 mM NaOH for 20 min and checked by autoradiography for removal of the previous probe.

**Gel immunodiffusion.** Rabbit antisera against whole cells of 25 strains of *R. leguminosarum* bv. *trifolii* were prepared as described previously (26). Each of the isolates was examined against each of the antisera by immunodiffusion by using standard hexagonal arrays of wells in which each isolate was adjacent to a standard suspension of cells homologous to the antiserum being used. When two or more isolates in nonadjacent wells reacted with an antiserum, they were reexamined with the same antiserum, with the isolates placed in adjacent wells so that the junctions of the precipitin bands could be observed.

**Symbiotic assay.** The 16 isolates, together with two control strains (TA1 and CIF), were assayed for symbiotic effectiveness after inoculated plants were grown with their roots inside foil-capped test tubes and with their shoots exposed (12). Growth conditions inside the controlled-environment cabinet were 22°C for roots and 22 and 16°C for shoots on the basis of a 14-h daily light period providing an irradiance of 300  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . The species used were *T. subterraneum* L. (subterranean clover cv. Mt. Barker; 10 replicates), *T. pratense* L. (red clover; 18 replicates), and *T. fragiferum* L. (strawberry clover; 18 replicates). The shoots were excised, dried, and weighed 28, 28, and 42 days, respectively, after inoculation.

## RESULTS

**Hybridization analysis.** (i) *R. leguminosarum* bv. *trifolii*-specific RtRS probe. A Southern blot of DNAs isolated from the 16 field isolates of *R. leguminosarum* bv. *trifolii* was hybridized with the RtRS probe (29). Figure 1A shows the profiles of positively hybridizing fragments observed with each isolate. One Sym plasmid profile, as exemplified by isolate 1, predominated in this soil population. Isolates 1, 2,

3, 8, 11, and 15 had this pattern. Isolates 5, 9, and 16 also displayed the same pattern as isolate 1, except that each of these isolates had one hybridizing band that was uniquely different in size. Thus, of the 16 isolates, 6 had identical Sym plasmid profiles and a further 3 had closely-related profiles. These nine isolates were designated as belonging to Sym plasmid-specific RtRS family  $\alpha$ . Completely different profiles were found with other isolates. Isolates 4 and 6 were identical and constitute RtRS family  $\beta$ . Isolates 7, 12, and 14, which had related profiles, represent RtRS family  $\gamma$ . Isolates 13 (RtRS family  $\delta$ ) and 10 (RtRS family  $\xi$ ) had profiles which differed from each other and from those of the other isolates. Thus, in this sampling, RtRS family  $\alpha$  predominated over the other four families (see Table 1).

(ii) *nif* gene probe. The *nifHDK* operon probe pRt585 was used to examine the patterns of restriction fragments that hybridized to the *nif* structural genes (Fig. 1B). With this probe, the isolates were classified into the groups defined with the RtRS probe (see Table 1). The one exception to this rule was isolate 10, which exhibited a unique profile with the RtRS probe (RtRS family  $\xi$ ) and yet showed a *nif* hybridization profile identical with those of isolates 4 and 6 (Nif family *b*).

Isolate 12 was classified into RtRS family  $\gamma$ , although it possessed an additional positively hybridizing fragment not present in the other two members of this family (isolates 7 and 14; Fig. 1A). The similar *nif* hybridization profiles of each of these three isolates (Fig. 1B) supported the assignment of isolate 12 to RtRS family  $\gamma$ .

(iii) *nod* gene probe. The patterns of isolate hybridization to the *nod* gene probe (pRt587) are shown in Fig. 1C. Each of these patterns included several (three to five) weakly hybridizing bands. Plasmid pRt587 contains a 14-kb insert which carries most, if not all, of the *R. leguminosarum* bv. *trifolii* nodulation genes (24). The 14-kb insert of pRt587 has been shown to carry three copies of a 76-base-pair repeated sequence (25), and further copies of this sequence have been located elsewhere in the *R. leguminosarum* bv. *trifolii* genome (unpublished data). The weakly hybridizing bands observed with each isolate probably represent DNA fragments which carry this repeated sequence.

The soil isolates were classified such that Nod family A represented the predominant Sym plasmid class, as was done for the RtRS ( $\alpha$ ) and Nif (*a*) families. Seven isolates (1, 2, 3, 5, 8, 9, and 15) had identical *nod* profiles, whereas a further two isolates (11 and 16) had closely related profiles. These isolates were grouped as Nod family A.

Nod family B consisted of isolates 4 and 6, which were also grouped into RtRS family  $\beta$  and Nif family *b* (see Table 1). Isolates 7, 12, and 14, whose *nod* hybridization profiles had a number of similarly sized fragments in common (Fig. 1C), were grouped into Nod family C.

The two remaining isolates (10 and 13) each displayed uniquely different hybridization profiles in response to the *nod* probe (Fig. 1C). Isolate 13 was classified as the sole representative of Nod family D. With each of the Sym plasmid-specific probes used in the above analyses, isolate 13 consistently yielded hybridization patterns distinctly different from those of any of the other 15 isolates. These observations indicate that isolate 13 harbors a unique Sym plasmid.

Because of its unique *nod* hybridization profile, isolate 10 was classified as a representative of Nod family E. Isolate 10 was also uniquely classified into RtRS family  $\xi$ . However, the response to the *nif* probe grouped it into Nif family *b*, which also included isolates 4 and 6 (see Table 1). These data

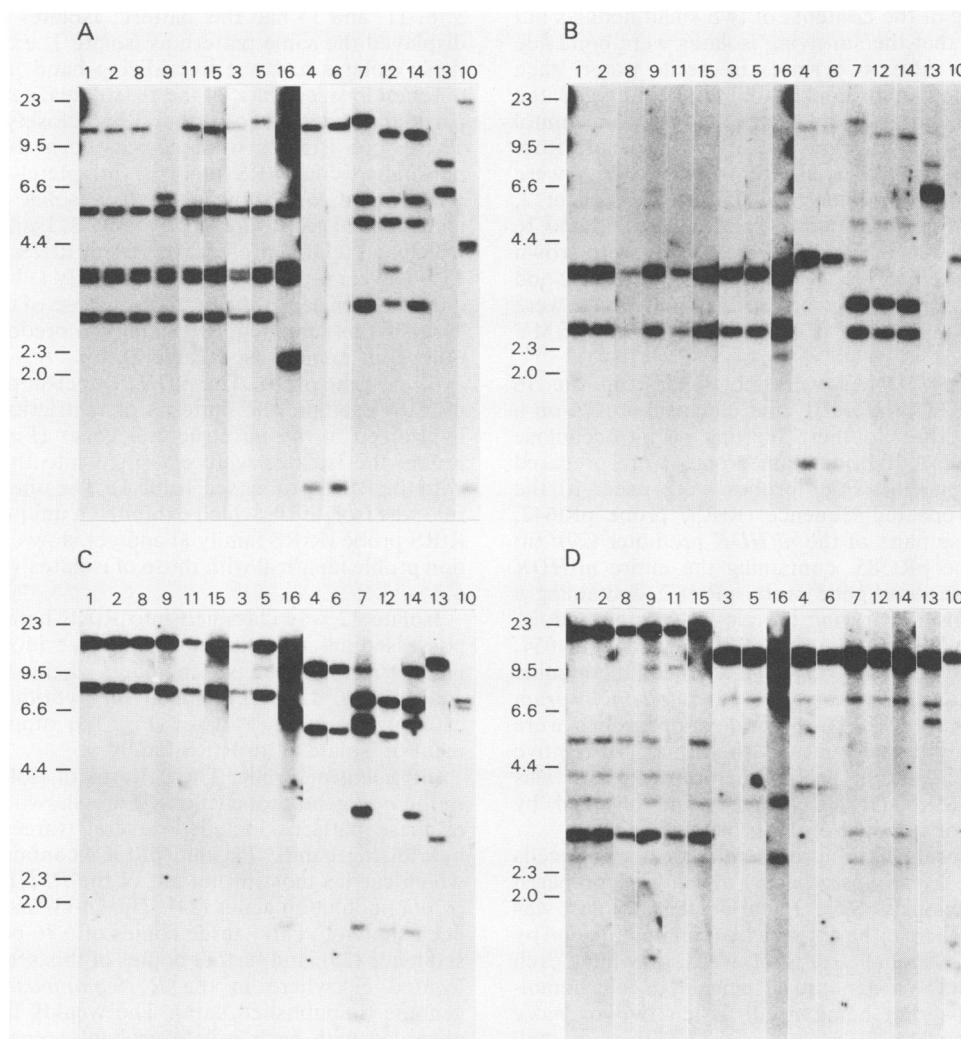


FIG. 1. DNA hybridization of *R. leguminosarum* bv. *trifolii* isolates. Total DNA was extracted from each of the isolates and subjected to Southern blot hybridization analysis as detailed in Materials and Methods. Four probes were sequentially hybridized to the same Southern blot (panels): A, pRt642 (RtRS probe); B, pRt585 (*nif* probe); C, pRt587 (*nod* probe); D, pRt654 (chromosomal probe). Isolate numbers are indicated across the top of each panel. The figures to the left of each panel indicate the sizes (in kilobases) and relative locations of *Hind*III restriction fragments of bacteriophage  $\lambda$  cI857 DNA.

suggest that isolate 10 harbors a Sym plasmid ( $\xi b E$ ) which arose by recombination between a  $\beta b B$  Sym plasmid and a hypothetical  $\xi e E$  Sym plasmid.

(iv) **Chromosomal probe.** The three hybridization probes used in the above analyses consist of DNA sequences specifically located on the Sym plasmid. To examine the chromosomal background of each of the 16 isolates, the recombinant plasmid pRt654 was used (Fig. 1D). In addition to strongly hybridizing fragments, this probe also revealed a number of weakly hybridizing fragments (not considered for isolate classification), suggesting that it also carries a repeated DNA sequence. As with the Sym plasmid-specific hybridization probes, this chromosomal probe also enabled the isolates to be classified. Chromosomal (Chm) family I comprised isolates 1, 2, 8, 9, 11, and 15, while Chm family II was represented by isolates 3, 4, 5, 6, 7, 10, 12, 13, 14, and 16 (Table 1).

**Antigenic analysis.** The availability of antisera prepared against 25 strains of *R. leguminosarum* bv. *trifolii* in the Commonwealth Scientific and Industrial Research Organisa-

tion Canberra collection made it possible to examine the antigenic relationships of the present isolates on the premise that antigenically identical isolates give rise to the same spectrum of reactions against the range of antisera. Four levels of response were recorded: no reaction, identity with an antiserum strain, cross-reaction without distinctive features, and cross-reactions distinguishable from each other with one or more given antisera (Table 2).

All of the isolates reacted with at least 20 antisera, and the remaining antisera reacted with two to seven isolates. Eight isolates gave reactions of identity with antiserum strains, but only two of these isolates (2 and 8) reacted in an identical manner against all of the antisera. Within two groups (isolates 4, 6, 10, 13, and 16 and isolates 9 and 11), the isolates were antigenically indistinguishable against the 25 antisera, although they failed to give a reaction of identity with any antiserum strain. A dendrogram of antigenic relationships of the isolates, constructed by nearest-neighbor analysis (11), indicated 10 serological groupings (Table 2).

**Symbiotic assays.** The symbiotic effectiveness of each

TABLE 1. Plasmid and chromosomal classifications, antigenic groupings, and symbiotic effectiveness data and classification for 16 field isolates and two control strains of *R. trifolii*

Isolate	Hybridization profile grouping	Antigenic group	Shoot dry wt (mg) <sup>a</sup>			Symbiotic effectiveness pattern <sup>b</sup>
			Subterranean clover	Red clover	Strawberry clover	
1	α a A I	5	43.5 (b)	11.0 (cd)	10.0 (f)	e i i
2	α a A I	10	45.7 (b)	11.2 (cd)	8.0 (f)	e i i
8	α a A I	10	49.5 (b)	7.6 (e)	9.0 (f)	e I i
9	α a A I	4	47.3 (b)	12.6 (bc)	7.9 (f)	e i i
11	α a A I	4	43.4 (b)	9.0 (de)	10.2 (f)	e I i
15	α a A I	3	41.0 (b)	10.4 (ce)	7.8 (f)	e i i
3	α a A II	8	33.3 (bc)	16.8 (b)	15.5 (de)	e i e
5	α a A II	2	39.6 (b)	11.7 (ce)	15.5 (de)	e i e
16	α a A II	1	42.7 (b)	45.8 (a)	23.6 (cd)	e E e
4	β b B II	1	47.7 (b)	45.3 (a)	27.9 (bc)	e E e
6	β b B II	1	34.7 (bc)	48.2 (a)	18.2 (de)	e E e
7	γ c C II	9	19.4 (de)	41.5 (a)	33.0 (ac)	i E e
14	γ c C II	7	16.1 (e)	39.2 (a)	28.2 (c)	i E e
12	γ c C II	6	106.3 (a)	36.1 (a)	46.0 (a)	E E E
13	δ d D II	1	28.3 (cd)	7.9 (de)	27.8 (c)	e I e
10 <sup>c</sup>	ξ b E II	1	94.2 (a)	44.3 (a)	42.4 (ab)	E E E
Strain TA1			102.3 (a)	30.2 (ab)	39.1 (ab)	E e E
Strain CIF			16.2 (e)	40.2 (a)	28.6 (c)	i E e

<sup>a</sup> Shoot weights were transformed (log<sub>e</sub>) and analyzed. Within a species, values followed by the same letter were not significantly different (*P* = 0.05).  
<sup>b</sup> E, Fully effective; e, about 50% of E; and i, significantly greater than I (completely ineffective) on subterranean, red, and strawberry clovers, respectively.  
<sup>c</sup> Putative recombinant Sym plasmid.

isolate was determined from the analysis of shoot dry weights (log<sub>e</sub> transformed) of subterranean, red, and strawberry clovers. With the exception of isolate 13, the responses of all of the isolates with red and strawberry clovers were similar (Table 1).

Within the four Sym plasmid chromosomal groups with two or more isolates, there was a strong similarity in the effectiveness response patterns and, in general, there were distinct differences among the groups. The differences between isolates 3 and 5 and isolate 16 (α a A II) and also the difference between isolate 12 and isolates 7 and 14 (γ c C II) could be due to single-point mutations that would not be detected by hybridization analysis. However, isolates with different backgrounds may achieve similar effectiveness patterns (isolates 12 and 10).

DISCUSSION

In a previous study (29), *R. leguminosarum* bv. *trifolii* strains of diverse origin were characterized by hybridization studies by using the Sym plasmid-specific repeated sequence RtRS1. By extending this approach to include two further plasmid-specific probes, we characterized the Sym plasmids of 16 soil isolates. Similarly, the bacterial host strain was characterized by chromosomal hybridization and serological analysis. By relating the bacterial host strain with its endogenous Sym plasmid, we have shown that different Sym plasmids can be found in identical backgrounds and also that identical Sym plasmids can be found in different backgrounds (Table 1). Since it is highly improbable that these combinations of bacterial host strains and Sym plasmids

TABLE 2. Summary of immunodiffusion reactions between *R. leguminosarum* bv. *trifolii* isolates and antisera against 25 known strains of *R. leguminosarum* bv. *trifolii*

Strain used for antiserum	Immunodiffusion reaction with <i>R. leguminosarum</i> bv. <i>trifolii</i> isolate(s) <sup>a</sup> :									
	1	2 and 8	9 and 11	15	3	5	4 <sup>b</sup>	7	14	12
TA1	I	C <sub>a</sub>	C <sub>a</sub>	C <sub>b</sub>	C <sub>b</sub>	C <sub>b</sub>	C <sub>b</sub>	C <sub>b</sub>	C <sub>c</sub>	C <sub>a</sub>
CC10 <sup>c</sup>	C	C	C	C	C	C	C	C	C	C
CC24	—	—	—	—	—	I	—	—	—	—
NA30	C	C	C	C	C	C	C	I	C	C
WA67	C <sub>d</sub>	C <sub>d</sub>	C <sub>d</sub>	C <sub>d</sub>	C <sub>d</sub>	C <sub>d</sub>	C <sub>d</sub>	C <sub>d</sub>	C <sub>e</sub>	C <sub>d</sub>
CC275a	C <sub>f</sub>	I	C <sub>g</sub>	C <sub>f</sub>	C <sub>f</sub>	C <sub>f</sub>	C <sub>f</sub>	C <sub>g</sub>	C <sub>f</sub>	C <sub>g</sub>
CC275b	C <sub>h</sub>	I	C <sub>h</sub>	C <sub>i</sub>	C <sub>i</sub>	C <sub>i</sub>	C <sub>i</sub>	C <sub>i</sub>	C <sub>j</sub>	C <sub>h</sub>
CC275c	C <sub>k</sub>	C <sub>l</sub>	C <sub>m</sub>	C <sub>l</sub>	C <sub>m</sub>	C <sub>m</sub>	C <sub>m</sub>	C <sub>l</sub>	C <sub>m</sub>	C <sub>k</sub>
CC275d	C <sub>n</sub>	I	C <sub>o</sub>	C <sub>o</sub>	C <sub>o</sub>	C <sub>o</sub>	C <sub>o</sub>	C <sub>o</sub>	C <sub>o</sub>	C <sub>n</sub>
WU290	C <sub>p</sub>	C <sub>p</sub>	C <sub>q</sub>	C <sub>q</sub>	C <sub>q</sub>	C <sub>q</sub>	C <sub>q</sub>	C <sub>q</sub>	C <sub>q</sub>	C <sub>q</sub>
CC2206f	C <sub>r</sub>	C <sub>s</sub>	C <sub>r</sub>	—	—	—	—	C <sub>t</sub>	—	C <sub>r</sub>
CC2206h	—	C <sub>u</sub>	—	—	—	—	—	C <sub>v</sub>	—	—
CC2238b <sup>d</sup>	C	C	C	C	I	C	C	C	I	C
CC2245a	C <sub>w</sub>	C <sub>w</sub>	—	I	—	—	—	C <sub>x</sub>	—	—
CC2247f	—	—	—	—	I	—	—	—	I	—

<sup>a</sup> I, Reaction of identity between isolate and appropriate homologous antigen; C, cross-reactions without distinctive features, common to all isolates; C<sub>a</sub>, C<sub>b</sub>, etc., cross-reactions distinguishable from one another with a given antiserum; —, no reaction.  
<sup>b</sup> Same reaction pattern as isolates 6, 10, 13, and 16.  
<sup>c</sup> Same reaction with antisera to strains UNZ29, CC275e, CC275f, CC276, CC2238c, CC2480a, and CC2483g.  
<sup>d</sup> Same reaction with antisera to strains CC2247b, CC2247d, and CC2258f.

arose independently, genetic exchange of *Rhizobium* Sym plasmids is the only hypothesis consistent with the available data. The demonstration that isolate 10 contains a putative recombinant Sym plasmid provides further support for this hypothesis. The only way that such a recombinant Sym plasmid could arise is by prior exchange of Sym plasmids. The conclusion that Sym plasmids are exchanged in soil populations of rhizobia is also supported by hybridization studies of the DNAs isolated from three of the isolates that were not able to grow on TY medium. This characteristic was taken as evidence that they represented a bacterial host background different from those represented by the 16 isolates able to grow on TY medium. The RtRS probe classified two of the isolates into RtRS family  $\beta$ . The other isolate possessed a profile identical to that of isolates in RtRS family  $\alpha$  (data not shown).

All of the strains were uniquely characterized by their hybridization profiles. Our results show that the Sym plasmid profile  $\alpha$  *a A* predominates among the population of *R. leguminosarum* bv. *trifolii* which nodulates white clover in this soil. Since we were able to identify five different Sym plasmid classes but only two different chromosomal classes, there must have been at least five individual strains present, at some stage, in this soil population. Whether the apparent loss of three bacterial host strain types was due to poor adaptation to this soil environment is a matter for investigation.

By definition, the most competitive strains generally occupy the greatest proportion of nodules. Hence, the analysis of bacteria isolated from white clover nodules would not be expected to reflect the actual population distribution of *R. leguminosarum* bv. *trifolii* isolates in the soil. However, only nodulating bacteria are of agronomic importance, and characterization of isolates from clover nodules constitutes a rational approach to the identification of competitive strains of *R. leguminosarum* bv. *trifolii*.

Isolate 10, which was classified into Nif family *b* and RtRS and Nod families  $\xi$  and *E*, respectively, is highly effective on all three plant hosts. As determined by both chromosomal hybridization and serology, the bacterial strain host is identical, or very closely related, to those of isolates 4 and 6, which possess the Sym plasmid profile  $\beta$  *b B*. Thus, by the criteria used, isolate 10 differs from isolates 4 and 6 by harboring a recombinant Sym plasmid. This plasmid appears to be unique among those present in the 16 isolates; however, like the Sym plasmids of isolates 4 and 6, it belongs to Nif family *b*. The symbiotic effectiveness of isolates 4 and 6 is moderate when compared with that of isolate 10. We suggest that isolate 10 achieves its greater symbiotic effectiveness by combining the nodulation (and possibly other) characteristics encoded on Sym plasmid Nod family *E* with more effective nitrogen fixation functions encoded on Sym plasmid Nif family *b*.

Isolates 7, 12, and 14 all possess the Sym plasmid profile  $\gamma$  *c C*, although isolate 12 differs from the other two by having an additional RtRS-hybridizing fragment of about 4 kb (Fig. 1A). Isolate 12 formed a fully effective symbiosis with the three clover species, whereas isolates 7 and 14 were only moderately effective with subterranean and strawberry clovers (Table 1). The possibility that the broader symbiotic effectiveness of isolate 12 is correlated with the presence of the additional fragment is under examination.

There is evidence that both Sym plasmid and chromosomal characteristics influence symbiotic effectiveness (Table 1). Among the nine  $\alpha$  *a A* isolates, only those in the Chm II group were effective with strawberry clover. Within the

Chm II group, the effectiveness response with subterranean and red clovers was dependent on the Sym plasmid type.

The serological data are puzzling. Within the  $\alpha$  *a A* I group of six isolates, which could reasonably be assumed to represent one strain, four antigenic groups were found (Table 1, groups 3, 4, 5, and 10). Antigenic group 1 contained five strains, all with Chm II profiles; five other strains with Chm II profiles gave serological reactions different from one another and from those of antigenic group 1. Although the antigens in *Rhizobium* strains are regarded as genetically stable, the present results show that antigenically identical strains cannot be presumed to be identical with respect to other characters. Serological characters of rhizobia should be used with caution in this type of study.

The data presented in this paper suggest that genetic exchange and recombination of *R. leguminosarum* bv. *trifolii* Sym plasmids occurs in field populations. As a consequence of such exchange and the demonstrated correlation between molecular constitution and symbiotic performance, we believe that it is now possible to characterize both the host cells and Sym plasmids of field isolates more precisely. Precise molecular characterization of *Rhizobium* Sym plasmids and chromosomal backgrounds by DNA hybridization analysis should also facilitate the study of a significant agricultural problem, namely, apparent temporal loss of inoculant strains in soil.

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