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Sym Plasmid Transfer to Various Symbiotic Mutants of *Rhizobium trifolii*, *R. leguminosarum*, and *R. meliloti*

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Two self-transmissible Sym(biosis) plasmids, one encoding pea-specific nodulation and nitrogen-fixation functions (plasmid pJB5JI) and the other encoding clover-specific nodulation and nitrogen-fixation functions (plasmid pBR1AN) were used to determine whether the symbiotic genes encoded on these plasmids are expressed in various members of the *Rhizobiaceae*. The host specificity of *Rhizobium trifolii* and *R. leguminosarum* Sym plasmid-cured strains could be directly determined by the transfer to these strains of the appropriate Sym plasmid. The nodulation of white clovers was restored by either plasmid pJB5JI or pBR1AN when these plasmids were transferred to two transposon Tn*5*-induced hair-curling (Hac$^-$) *R. trifolii* mutants. In addition, lucerne nodulation was restored to a Hac$^-$ *R. meliloti* mutant when either plasmid pBR1AN or pJB5JI was transferred to this strain. The phenotype of nonmucoid (Muc$^-$) *Rhizobium* mutants, which had altered cell surfaces, was not influenced by the transfer to these strains of plasmid pBR1AN or plasmid pJB5JI.

The *Rhizobium*-legume symbiosis involves a multistep process which leads to the formation of a nitrogen-fixing nodule (18, 23). Many of the symbiotic functions encoded by the bacteria are located on large plasmids. In particular, genes encoding root hair adhesion, infection thread formation, host specificity, and nitrogen fixation are located on one plasmid species in most *Rhizobium* strains (1, 3–5, 7, 8, 10, 13–15, 27, 28, 30), and these genes occur on a segment of this symbiosis (Sym) plasmid which spans approximately 20 to 30 kilobase pairs (kb) (12, 16).

We report here the isolation and characterization of a self-transmissible 140-megadalton (Mdal) Sym plasmid (derived from the *R. leguminosarum* Sym plasmid pJB5JI) which encodes clover nodulation and nitrogen-fixation functions. This plasmid was designated pBR1AN. Both the clover and pea Sym plasmids were transferred to various *Rhizobium* nodulation-defective mutants which were derived either from plasmid curing experiments or by transposon mutagenesis with the drug resistance transposon Tn*5*. This approach was used to determine (i) whether both plasmids could transfer to the same strain, (ii) whether both plasmids could restore the capacity for host-specific nodulation in the various mutants tested, and (iii) whether the loss of exopolysaccharide production (Muc$^-$) could be restored in those strains which had lost this capacity.

MATERIALS AND METHODS

Bacterial strains. A list of strains used is given in Table 1.

Media used. All media used have been described elsewhere (19).

Plasmid-curing/plant assays. Plasmid-cured *R. trifolii* mutants were obtained by prolonged incubation of various wild-type strains at elevated temperatures (35 to 37°C) by the method of Zurkowski and Lorkiewicz (29). Survivors were picked and screened for the ability to nodulate *Trifolium repens* (NZ white clover 5826) and *Trifolium subterraneum* (Mt. Barker variety), using the rapid plant assay technique (19). Nodulation tests on lucerne plants (*Medicago sativa*) were done with Hunter Valley variety seedlings, using the same plant assay technique as that used for clovers. Nodulation tests for *R. leguminosarum* strains were done on *Pisum sativum* seedlings (Green Feast variety) grown in 250-ml flasks as described previously (8). Transposon mutagenesis was achieved as described previously (2, 8). These derivatives were screened in the same manner as the putative plasmid-cured derivatives. Bacteria were isolated from single nodules as described elsewhere (19).

Bacteriocin determination. The method of Hirsch (11) was used to determine the type of bacteriocin produced by the bacterial strain tested, except that BMM medium (19) was used to grow the test and indicator strains. The bacteriocin types were classified in the way described by Hirsch (11): "small" bacteriocin production is indicated by a large zone of killing (15 to 30 mm), and "medium" bacteriocin production is indicated by an intermediate-size killing zone (5 to 10 mm).

Plasmid transfer. Plasmid transfer between *Rhizobium* strains was effected by a patch mating technique. Donor and recipient strains freshly grown on TMY media were suspended in sterile distilled water to give approximately 5×10^8 cells per ml. Samples (0.1 ml) of the donor and the recipient strains were dropped separately onto a nonselective TMY plate (19) while 0.1 ml of both the donor and the recipient strains were mixed together in a separate area on the same plate. After drying the plates and incubating for 18 h, the mating plate was then replica plated onto TMY media (or if the donor was auxotrophic, TM minimal media) containing the appropriate combination of antibiotics, which selected for (i) the donor only, (ii) the recipient only, and (iii) the transconjugants only. The transconjugants were picked and purified on the same selective medium before plant assays were conducted.

The transfer of plasmids pBR1AN and pJB5JI was detected by selecting for kanamycin-resistant transconjugants. When these plasmids were transferred to the kanamycin-resistant (Tn*5*-induced) Nod$^-$ or Muc$^-$ *R. trifolii* mutants, selection was made for pea or clover nodulation by the method of Brewin et al. (5). To ensure that no donor carryover had occurred during the mating, bacteria were isolated from the resulting nodules and retested for antibiotic resistance markers, nodulation capacity, and for the characteristic plasmid profile of the recipient strain.

Root hair examinations. Four 1-day-old white clover and two subterranean clover plants were placed on Fåhraeus plates (19) which had been inoculated with 0.1 ml of a bacterial suspension (approximately 10^8 cells per ml). A number of replicas were made, and microscopic assays on each plant on one plate were made at 2, 4, and 6 days after inoculation and incubation in plant growth chambers. The conditions used have been described previously (20). After removing the plants from the plates and immersing the root system in a small volume of liquid Fåhraeus medium on a glass slide, the area above the hypocotyl was removed to allow the roots to be covered completely by a large cover slip. General root hair observations were made with low magnification (×4) and dark-field conditions. Bacterial attachment and root hair-curling responses were observed by using Nomarski interference optics (×20 magnification). The response was graded similar to the system used by Yao and Vincent (25, 26). A marked root hair-curling response meant that root hairs curled through 360° were prominent and easily found.

Plasmid visualizations. Plasmids were visualized by a rapid screening technique which is a modification of the method of Eckhardt (9) adapted for horizontal gels. This method is described in detail elsewhere (8).

Filter hybridizations. Plasmid gels were illuminated with UV light (254 nm) at a distance of 8 cm for 10 to 15 min before DNA transfer was attempted by the method of Southern (12, 22). Radioactively labeled DNA probes were prepared by the random primer method (24). DNA-DNA hybridizations were done at 65°C after a 1- to 2-h prehybridization as described elsewhere (24).

Probes used. A 3.2-kb *Hin*dIII fragment derived from *R. trifolii* ANU794 was used as a *nif* probe. This sequence contained the entire *R. trifolii nifH* sequence, part of *nifD*, and 1.4 kb of the sequence to the 5′ side of *nif*H. This 1.4-kb sequence contained a Sym plasmid-specific repeat sequence which is conserved in many *R. trifolii* strains (J. Shine et al., *in* A. Puhler, ed., *Molecular Genetics of the Bacteria-Plant Interaction*, in press), but not in other heterologous *Rhizobium* strains.

A 7.2-kb *Eco*RI fragment derived from *R. trifolii* ANU843 was used as a nodulation probe (P. R. Schofield et al., Mol. Gen. Genet., in press). This fragment contains sequences surrounding the insertion site of the Tn*5* located in the *R. trifolii* ANU851 Nod$^-$ (see Tables 1 and 4).

RESULTS

Plasmid pBR1AN. Plasmid pJB5JI normally transfers to various derivatives of *R. leguminosarum* and *R. trifolii* at rates of about 10^{-2} to 10^{-4}. However, plasmid pJB5JI transferred to *R. trifolii* ANU6 at a rate of about 10^{-7}. The resultant kanamycin-resistant transconjugants were picked and purified. These were then tested for their capacity to nodulate clovers and peas. Two of three isolates were able to nodulate only clovers and not peas. The third isolate, however, was able to nodulate white clovers, subterranean clovers, and peas and could retransfer kanamycin-resistance to the Nod$^-$ *R. leguminosarum* strain ANU615. These resulting kanamycin-resistant ANU615 cells induced nitrogen-fixing nodules (Nod$^+$ Fix$^+$) on peas and also Nod$^+$ Fix$^+$ nodules on both white and subterranean clovers, indicating that a cotransfer of symbiotic information had occurred. Upon subculturing these broad-host-range ANU615 transconjugants through white clover nodules, symbiotic variants were isolated which had lost the ability to nodulate peas but had retained the ability to nodulate both white and subterranean clovers. One such isolate was purified and designated strain ANU618.

Strain ANU618 was able to cotransfer both kanamycin resistance and the ability to nodulate clovers. Transfer rates were comparable to those for plasmid pJB5JI. A plasmid gel profile (Fig. 1) revealed a plasmid similar in size to pJB5JI. This appeared to be a new plasmid and was designated pBR1AN. It consistently banded just above the position of plasmid pJB5JI, which is 130 Mdal. A comparison with other known genetic markers on pJB5JI indicated that the two plasmids were similar (Table 2). Both plasmids could not confer medium bacteriocin production on recipient strains (due to Tn*5* inactivation of medium bacteriocin production), but they could confer immunity to medium bacteriocins in these strains, indicating that Tn*5* was in the same position in both plasmids. This was confirmed by hybridizing a Tn*5*-containing probe to nitrocellulose filters containing *Eco*RI restriction endonuclease digests of total DNA of strains ANU618 and ANU617. These showed identical hybridization patterns (data not

TABLE 1. Strains commonly used

Strain	Phenotype[a]/genotype	No. of plasmids present	Source (reference)
Donors			
ANU617	ANU615(pJB5JI) Km^r	8	8
ANU618	ANU615(pBR1AN) Km^r	8	17
ANU850	ANU845(pJB5JI) Km^r	5	This paper
ANU870	ANU845(pBR1AN) Km^r	5	This paper
R. trifolii			
ANU6	Wild-type Sp^r	2	E. Schwinghamer
ANU843	Wild-type	5	J. M. Vincent
ANU845	Plasmid-cured Nod^- mutant Sp^r	4	This paper
ANU851	Tn*5*-induced Nod^- mutant Km^r	5	This paper
ANU871	Plasmid-deleted ANU851 derivative Km^s	5	This paper
ANU794	Wild-type Sm^r	3	8
ANU453	Tn*5*-induced Nod^- mutant Sm^r Km^r	3	This paper
ANU437	Muc^- Tn*5*-induced Fix^- mutant Sm^r Km^r	3	6
ANU815	Plasmid-deleted Nod^- mutant	3	This paper
R. leguminosarum			
ANU300	Wild-type	7	15
ANU615	Plasmid-deleted ANU300 derivative *trp phe* Sm^r Rf^r	7	15
ANU53	Wild-type Sm^r Rf^r	3	P. Albersheim
ANU54	Muc^- Nod^- plasmid-cured ANU53 Sm^r Rf^r	2	P. Albersheim
A. tumefaciens			
ANU1019	Ti-plasmid-cured C58 derivative	2	13
R. meliloti			
ANU1004-1008	Plasmid-deleted Nod^- L5-30 derivatives	2	21
ANU1000	Plasmid-deleted Nod^- Rm41 derivative	2	1

[a] Abbreviations: Sp^r, spectinomycin resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Rf^r, rifampin resistant. The following strains are also known by other designations (in parentheses): ANU843 (SU843), ANU794 (SU794), ANU300 (strain 300), ANU615 (strain 6015), ANU1000 (ZB157), ANU1019 (LBA288).

shown). In addition, both plasmids possessed the ability to inhibit both the production of, and the self-immunity to, small bacteriocin production in all the *R. trifolii* strains tested that produce small bacteriocins. Thus, a wild-type *R. trifolii* strain producing small bacteriocins could kill a derivative of the same strain if it possessed either plasmid pBR1AN or pJB5JI (Fig. 2).

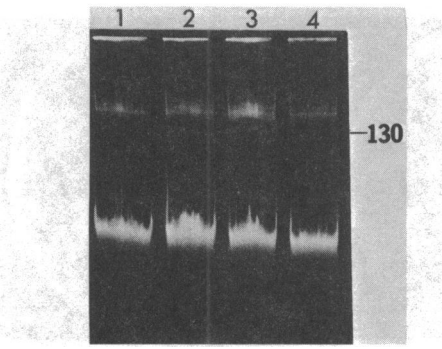

FIG. 1. Plasmid profile of strains ANU794 (lanes 1 and 4), ANU801 (lane 2), and ANU820 (lane 3). Strain ANU820 is strain ANU453(pBR1AN). Plasmid pBR1AN migrates to a position just above the banding position of plasmid pJB5JI, which is 130 Mdal.

Differences between these two plasmids were shown when specific DNA hybridization probes were hybridized to nitrocellulose filters containing restriction endonuclease digests of the total DNA of strains which contained either pBR1AN or pJB5JI (Fig. 3). Digests were included from *R. trifolii* ANU6, the strain from which the clover sequences present in plasmid pBR1AN were derived. These results show that pBR1AN-derived fragments hybridized strongly to *R. trifolii*-specific nodulation and *nifH* probes, whereas pJB5JI-derived fragments either did not show detectable hybridization or responded differently to the probe used (Fig. 3). Significantly, the responses of the DNAs of strains ANU6 and ANU870 were identical for each probe used, indicating that these hybridizing fragments had a common origin.

***R. trifolii* mutants.** A series of nodulation-defective *R. trifolii* mutants derived from wild-type strains ANU843 and ANU794 were isolated by either plasmid-curing techniques or transpo-

TABLE 2. Comparison of the known genetic markers[a] on Sym plasmids pBR1AN and pJB5JI

Genetic markers	Plasmid pJB5JI	Plasmid pBR1AN
Nodulation ability		
Root adhesion	+[b]	+
Hair curling	+[b]	+
Nodule initiation	+	+
Host specificity		
Peas	+	−
White clover	−	+
Subterranean clover	+	+
Nodule function (Fix^+)	+	+
Self-transmissibility	+	+
Presence of Tn*5*	+	+
Medium bacteriocin production	−	−
Immunity to medium bacteriocins	+	+
Suppression of small bacteriocins	+	+
Suppression of small bacteriocin immunity	+	+
Hybridization to:		
R. trifolii Nod probe[c]	—[d]	+
R. trifolii nifH probe[c]	+	+

[a] The plasmids were in the common genetic background of ANU615.

[b] Observed on subterranean clover plants.

[c] The *R. trifolii nifH* sequence cross-hybridizes to the *R. leguminosarum nifH* sequence encoded on plasmid pJB5JI.

[d] Very poor hybridization of this probe was observed after prolonged exposure of autoradiographs.

son mutagenesis. The 110-Mdal Sym plasmid from strain ANU843 could be cured at low rates (<1%) after incubation at 37°C, whereas only deletion mutants of the 220-Mdal strain ANU794 Sym plasmid could be obtained under the same conditions (Fig. 4). Three transposon-induced (Tn*5*) nodulation mutants were isolated from these strains. In two of these strains, ANU851 and ANU453, DNA hybridization to Tn*5* sequences demonstrated that the transposon was located on the respective Sym plasmids in these strains (data not shown). The third Tn*5*-induced mutant, ANU437, was a Muc^- invasive strain that did not induce nitrogen-fixing nodules (see below). Two spontaneous *R. trifolii* mutants derived from strain NA34 were also used. Details of the plasmid-cured mutants used are given in Tables 1 and 3, and nonmucoid mutants are described in Table 5.

Sym plasmid transfer. Strains ANU617 and ANU618, which possess plasmids pJB5JI and pBR1AN, respectively, were used as donors in all cases, except where indicated. Transfer of pJB5JI to *R. trifolii* and to a *R. leguminosarum* plasmid-cured strain resulted in transconjugants which were able to nodulate peas and subterranean clovers but not white clovers, whereas the corresponding pBR1AN transconjugants were able to nodulate both white and subterranean clovers but not peas (Table 3).

Sym plasmid transfers to Tn*5*-induced Nod^- *R. trifolii* strains. Although the transfer of pJB5JI or pBR1AN to the Tn*5*-induced mutant *R. trifolii* strains ANU453 and ANU851 could not be directly selected for by using antibiotics, it was reasoned that a correction of the defects in these two Nod^- strains could be effected by the corresponding nodulation genes encoded on these Sym plasmids. If this were so, the white clover plants would screen the mating mixture for the appropriate invasive bacteria. Although at least 60 white clover plants were used in two separate experiments, only one putative ANU851(pJB5JI) nodule was formed and only two putative ANU453(pJB5JI) nodules were produced. The roots of the white clover plants had small swellings, and the root hairs of these plants upon microscopic observation had a

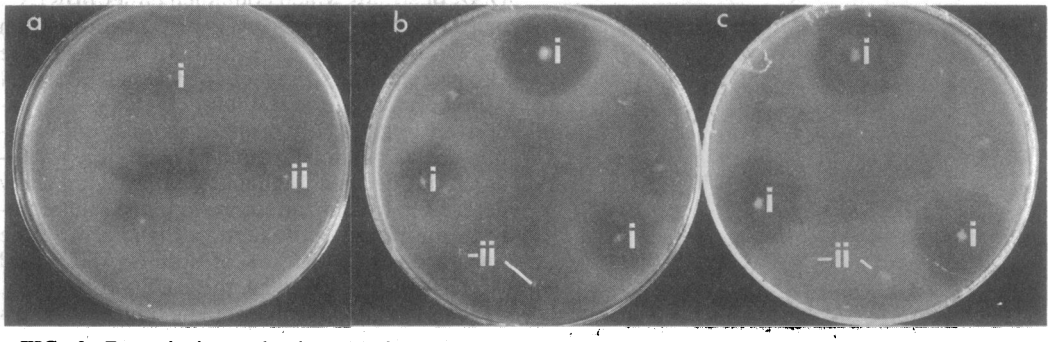

FIG. 2. Bacteriocin production. (a) Control containing an ANU794 overlay with test spots of ANU794, ANU794(pJB5JI), and ANU453(pBR1AN). (b) ANU794(pJB5JI) overlay. The areas of clearing in plate b were caused by ANU794 spots (i), whereas ANU794(pJB5JI) (ii) and ANU453(pBR1AN) (unmarked) control spots induced no killing zone. (c) The same arrangement of strains as in plate b except that the overlay was strain ANU453(pBR1AN).

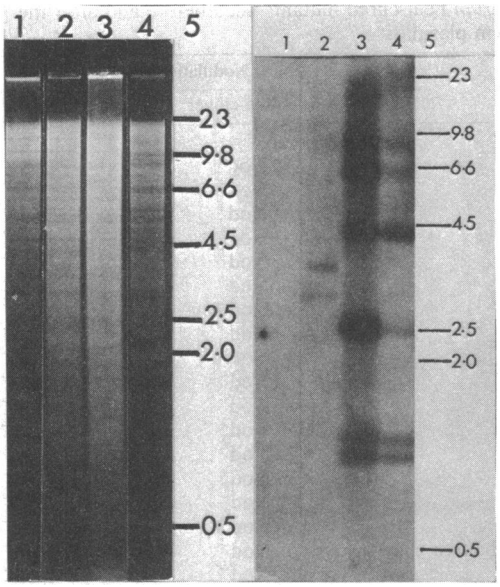

FIG. 3. *Hin*dIII-restricted *R. trifolii* DNAs and the corresponding autoradiogram. Probes made from the *R. trifolii* DNA isolated from the *nod* and *nif* gene regions were hybridized to the following DNAs: ANU845, a Sym plasmid-cured *R. trifolii* strain (lane 1); ANU845(pJB5JI) (lane 2); ANU845 (pBR1AN) (lane 3); ANU6 (lane 4). The Sym plasmid-cured strain shows no hybridization to either probe used. The responses of the DNAs in lanes 3 and 4 were almost identical. This indicates that the symbiotic sequences occurring on plasmid pBR1AN and those on the Sym plasmid in strain ANU6 are similar. The response of the DNA in lane 2 (containing plasmid pJB5JI) differed from that in lanes 3 and 4. The 8.5-kb bands in lanes 3 and 4 are due to hybridization to the nodulation gene probe. (A faint 8-kb band which hybridizes to the *nod* gene probe in lane 2 becomes discernible after prolonged exposure of the autoradiogram.) Other bands appearing in lanes 3 and 4 are either due to *nifH*- and *nifD*-specific hybridization (2.4 and 1.2 kb) or due to hybridization to the repeated sequence present on this probe. Size standards are given in kb.

marked curling response. Bacteria isolated from the stray nodules could reinfect and nodulate white clover plants and possessed the genetic markers of the recipient strains.

Plasmid pJB5JI encodes the ability to nodulate subterranean clovers (and thus overlaps in host specificity with plasmid pBR1AN). Hence, it was not surprising when each subterranean clover plant screened (50 plants) with putative ANU453 or ANU851 cells containing pJB5JI possessed at least four nodules. These bacteria were extracted from individual nodules, screened for the appropriate markers, and reinoculated onto white and subterranean clovers to ensure that the total population of cells inoculated onto plants contained plasmid pJB5JI in the background of two Tn*5*-induced Nod$^-$ *R. trifolii* strains. These bacteria could markedly distort the roots of white clover plants upon microscopic observation as before. However, nodules were produced after 10 to 14 days (7 to 8 days delay when compared with the appropriate controls). The number of nodules per plant was reduced compared with controls, but each plant tested (25 plants) did give a Nod$^+$ response (Table 4).

Although it was difficult to screen for nodulating cells which had received plasmid pJB5JI with white clovers, putative ANU453(pBR1AN) and ANU851(pBR1AN) cells were easily selected from the original mating mix by using this system, and all white and subterranean clover plants screened (50 tested) possessed nodules (Table 4).

Nonmucoid (Muc$^-$) *R. trifolii* mutants. The transfer of plasmid pJB5JI to the non-nitrogen-fixing (Tn*5*-induced) Muc$^-$ mutant strain ANU437 did not result in the correction of the Muc$^-$ phenotype of this strain. Strain ANU437(pJB5JI) was able to nodulate peas and clover, but the nodules produced on both host plants were blocked at an early development stage (Table 5). The transfer of plasmid pBR1AN to strain ANU437 could not be selected. However, no mucoid colonies were noted when pBR1AN transfer was attempted by selecting for kanamycin-resistant "transconjugants" and assuming a transfer rate of 10^{-2} (which is the transfer rate of pBR1AN into strain ANU815).

Neither plasmid pBR1AN nor pJB5JI was able to correct the Muc$^-$ phenotype of the spontaneous *R. trifolii* NA34 mutant, ANU846. Both transconjugant types were blocked at an early development stage, and no nitrogen was fixed. However, when either plasmid pBR1AN or pJB5JI was transferred to ANU847 (the Nod$^-$

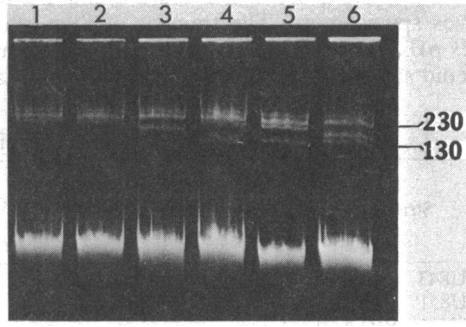

FIG. 4. Plasmid gel profile of various derivatives of strain ANU794. Lane 1, ANU794; lane 2, ANU453, the Tn*5*-induced Nod$^-$ strain; lane 3, ANU815 (Sym plasmid deleted); lane 4, ANU801 [ANU794(pJB5JI)]; lane 5, ANU820 [ANU453(pBR1AN)]; lane 6, ANU816 [ANU815(pBR1AN)]. The smallest resident plasmid in ANU794 is the Sym plasmid, and it is approximately 220 Mdal. Plasmid sizes are in Mdal.

TABLE 3. Phenotypes of *R. trifolii* and *R. leguminosarum* heat-cured mutants used before and after the introduction of Sym plasmids[a]

Strain	Mutant phenotype	Nodulation response on:	
		White clover	Peas
R. trifolii			
ANU843	Wild type	Nod^+	Nod^-
ANU845	Sym plasmid cured	Nod^-	Nod^-
ANU845(pBR1AN)		Nod^+	Nod^-
ANU845(pJB5JI)		Nod^-	Nod^+
ANU871[b]	Sym plasmid deletion	Nod^-	Nod^-
ANU871(pBR1AN)		Nod^+	Nod^-
ANU871(pJB5JI)		Nod^-	Nod^+
ANU794	Wild type	Nod^+	Nod^-
ANU815	Sym plasmid deletion	Nod^-	Nod^-
ANU815(pBR1AN)		Nod^+	Nod^-
ANU815(pJB5JI)		Nod^-	Nod^+
ANU12[c]	Wild type	Nod^+	Nod^-
ANU13[c]	Heat-cured ANU12	Nod^-	Nod^-
ANU13(pBR1AN)		Nod^+	Nod^-
ANU13(pJB5JI)		Nod^-	Nod^+
ANU846[c]	Muc^- NA34 derivative	Nod^+	Nod^-
ANU847[c]	Nod^- Muc^+ NA34 derivative	Nod^-	Nod^-
ANU847(pBR1AN)		Nod^+	Nod^-
ANU847(pJB5JI)		Nod^-	Nod^+
R. leguminosarum			
ANU300	Wild type	Nod^-	Nod^+
ANU615	Sym plasmid deleted	Nod^-	Nod^-
ANU615(pBR1AN)		Nod^+	Nod^-
ANU615(pJB5JI)		Nod^-	Nod^+

[a] Ten to fifteen transconjugants were checked in each case.

[b] ANU871 was derived from ANU851 after heat-curing tests. The deletion in ANU871 extends from the left arm of Tn*5* for a distance of approximately 35 kb. *nifH* hybridization is lost (M. A. Djordjevic, manuscript in preparation).

[c] Strains ANU12, ANU13, ANU846, and ANU847 are also known as T12, T12 Nod^-, SU846, and SU847, respectively (see references 18 and 27).

derivative of strain NA34), effective nodulation resulted, thus bypassing the stage at which the corresponding ANU846 transconjugant derivatives were blocked (Table 5).

One particular set of heat-cured mutants deserves special note. The transfer of Sym plasmids pJB5JI and pBR1AN to a conditional nonmucoid strain, ANU14, resulted in a differential expression of the nodulation functions encoded by these plasmids. Strain ANU14 was derived from a heat-cured Sym plasmid-deficient strain ANU13 via a second exposure to elevated growth temperatures such as those used in heat-curing experiments. Nonmucoid colonies were picked and analyzed. The ANU14 cells appeared Muc^- only when cultured on defined

TABLE 4. Transfer of Sym plasmids to Tn*5*-induced Nod^- *R. trifolii* strains

Strain	Phenotype	Hair curling and nodulation response		
		Clover		Peas
		White	Subterranean	
ANU843	Wild type	Nod^+ (Hac^+)	Nod^+ (Hac^+)	Nod^-
ANU851[a]	Nod^- (Tn*5*)	Nod^- (Hac^-)	Nod^- (Hac^-)	Nod^-
ANU851(pBR1AN)		Nod^+ (Hac^+)	Nod^+ (Hac^+)	Nod^-
ANU851(pJB5JI)		Nod^+ (Hac^+)	Nod^+ (Hac^+)	Nod^{+b}
ANU794	Wild type	Nod^+ (Hac^+)	Nod^+ (Hac^+)	Nod^-
ANU453[a]	Nod^- (Tn*5*)	Nod^- (Hac^-)	Nod^- (Hac^-)	Nod^-
ANU453(pBR1AN)		Nod^+ (Hac^+)	Nod^+ (Hac^+)	Nod^-
ANU453(pJB5JI)		Nod^+ (Hac^+)	Nod^+ (Hac^+)	Nod^{+b}

[a] Both ANU851 and ANU453 are defective in capsule synthesis.

[b] Root hair curling assays were not attempted with peas.

TABLE 5. Transfer of Sym plasmids pJB5JI and pBR1AN to nonmucoid *R. trifolii* and *R. leguminosarum* strains

Strain[a]	Mucoidy	Nodulation response on:		
		White clover	Subterranean clover	Peas
R. trifolii				
ANU12	+	Nod^+	Nod^+	Nod^-
ANU13	+	Nod^-	Nod^-	Nod^-
ANU13(pJB5JI)	+	Nod^-	Nod^+	Nod^+
ANU13(pBR1AN)	+	Nod^+	Nod^+	Nod^-
ANU14	−	Nod^-	Nod^-	Nod^-
ANU14(pJB5JI)	−	Nod^-	Nod^+	Nod^+
ANU14(pBR1AN)	−	Nod^-	Nod^-	Nod^-
ANU34	+	Nod^-	Nod^-	Nod^-
ANU34(pBR1AN)	+	Nod^+	Nod^+	Nod^-
ANU34(pJB5JI)	+	Nod^-	Nod^+	Nod^+
ANU846	−	Nod^+	Nod^+	Nod^-
ANU847	+	Nod^-	Nod^-	Nod^-
ANU846(pBR1AN)	−	Nod^+	Nod^+	Nod^-
ANU846(pJB5JI)	−	Nod^+	Nod^+	Nod^+
ANU847(pBR1AN)	+	Nod^+	Nod^+	Nod^-
ANU847(pJB5JI)	+	Nod^-	Nod^+	Nod^+
ANU794	+	Nod^+	Nod^+	Nod^-
ANU437[b]	−	Nod^+	Nod^+	Nod^-
ANU437(pJB5JI)	−	Nod^+	Nod^+	Nod^+
R. leguminosarum				
ANU53	+	Nod^-	Nod^+	Nod^+
ANU54	−	Nod^-	Nod^-	Nod^-
ANU54(pBR1AN)	−	Nod^+	Nod^+	Nod^-
ANU54(pJB5JI)	−	Nod^-	Nod^+	Nod^+

[a] Ten to 15 individual transconjugants were checked in each case.

[b] ANU437 is a Tn*5*-induced Muc^- rough mutant which has been shown to have a substantial reduction in the amount of cell surface polysaccharide (6).

media which contained either mannitol, sorbitol, or fructose as carbon sources but were Muc^+ when cultured on the same medium containing either glucose, sucrose, galactose, arabinose, inositol, or dulcitol. The transfer of pJB5JI or pBR1AN to ANU13 (Nod^-) resulted in the ability of strain ANU13 to nodulate peas or clovers, respectively. However, pJB5JI could confer the ability to nodulate peas when transferred to strain ANU14 (Nod^- Muc^-), whereas the transfer of pBR1AN to ANU14 could not induce nodulation to occur on clovers (Table 5). The retransfer of kanamycin resistance from putative ANU14(pBR1AN) cells into Sym plasmid-cured *R. trifolii* recipients resulted in the transconjugants being able to nodulate clovers, suggesting that the nodulation functions encoded on plasmid pBR1AN were intact. Selection of Muc^+ revertants of strain ANU14 on media containing mannitol occurred at a rate of $<10^{-6}$. Subsequent transfer of plasmid pBR1AN to this strain, ANU34, resulted in the ability to nodulate clovers (Table 5), indicating that the Muc^- phenotype of ANU14 was responsible for the apparent lack of expression of plasmid pBR1AN.

***R. leguminosarum* mutants.** The transfer of either pBR1AN or pJB5JI from strain ANU845(pBR1AN) or ANU845(pJB5JI) to the *R. leguminosarum* plasmid deletion mutant ANU615 conferred the ability to nodulate the appropriate host plant (strain ANU845 is a Sym plasmid-cured *R. trifolii* strain). ANU615(pBR1AN) nodulated clovers, whereas ANU615(pJB5JI) nodulated peas and subterranean clovers (Table 3). The transfer of these Sym plasmids to the nonnodulating Muc^- *R. leguminosarum* strain ANU54 resulted in the restoration of the ability to nodulate either clovers (pBR1AN) or peas (pJB5JI) (Table 5). The many small nodules produced, however, did not develop fully. No nitrogen was fixed, and the Muc^- phenotype of the bacterium was not corrected.

***R. meliloti* mutants.** When either of the Sym plasmids was transferred to the various nonnodulating *R. meliloti* strains derived from temperature-curing experiments, none of the transconjugants could nodulate either peas or clovers (Table 6). In addition, with the exception of one strain, the various transconjugants isolated could not nodulate lucerne. The capacity of the nonnodulating *R. meliloti* strain ANU1000 to nodulate lucerne could be restored by the introduction of either pBR1AN or pJB5JI to this strain. The nodules were abundant but smaller, and no nitrogen was fixed after 4 weeks. Strain ANU1000 is thought to possess a comparatively small deletion in the Sym plasmid (A. Kondorosi, personal communication), and unlike the other *R. meliloti* strains used in this study, the deletion does not extend into the *nifH* gene (1). A plasmid gel profile of these transconjugant strains (Fig. 5) showed the presence of an additional band corresponding to pJB5JI. However, an additional plasmid band corresponding to the size of pBR1AN could not be detected. But upon hybridization of a Tn*5*-specific DNA probe to a nitrocellulose filter of the same plasmid gel containing strain ANU1000(pBR1AN) (Fig. 5), a hybridizing band was detected which was in a position that corresponded to a plasmid size of 140 Mdal. This coincides with the size of the smallest resident plasmid in this strain, and it seems likely that plasmid pBR1AN comigrates with this endogenous plasmid (1). Retransfer of kanamycin resistance, host-specific clover nodulation, and nitrogen-fixation genes could be effected from this strain to *R. leguminosarum* ANU615.

Transfer to *A. tumefaciens*. The transfer of pBR1AN to *Agrobacterium tumefaciens* ANU1019 resulted in the ability of this strain to

TABLE 6. Nodulation response of *R. meliloti* mutants and *A. tumefaciens* strains before and after Sym plasmid transfer

Strains[a]	Phenotype	Nodulation response on:		
		Clovers	Peas	Lucerne
R. meliloti				
ANU1000	Nod^- Hac^-	Nod^-	Nod^-	Nod^-
ANU1004-ANU1008	Nod^- Hac^-	Nod^-	Nod^-	Nod^-
ANU1000(pBR1AN)[b]		Nod^-	Nod^-	Nod^+
ANU1000(pJB5JI)[b]		Nod^-	Nod^-	Nod^+
ANU1004-8(pBR1AN)		Nod^-	Nod^-	Nod^-
ANU1004-8(pJB5JI)		Nod^-	Nod^-	Nod^-
A. tumefaciens				
ANU1019	Ti plasmid cured	Nod^-	Nod^-	ND[c]
ANU1019(pBR1AN)		Nod^+	Nod^-	ND
ANU1019(pJB5JI)		Nod^-	Nod^-	ND

[a] At least 10 transconjugants were tested in each case.
[b] These strains were able to markedly curl the root hairs of white and subterranean clovers. No infection threads were seen.
[c] ND, Not done.

nodulate clovers. The nodules were poor, and they resembled the nodules produced by the Muc^- *R. leguminosarum* and *R. trifolii* derivatives into which pBR1AN had been transferred. In contrast, pJB5JI could not induce strain ANU1019 to nodulate peas even after 5 weeks (Table 6). Both pJB5JI and pBR1AN transferred to strain ANU1019 at low rates ($<10^{-6}$).

To check whether plasmid pJB5JI had transferred to strain ANU1019, plasmid profiles of six putative kanamycin-resistant transconjugants were tested. All transconjugants contained an additional plasmid band (data not shown); however, this band did not possess the expected mobility of plasmid pJB5JI. Estimates of the size of this plasmid were 180 to 200 Mdal. To check whether the nodulation functions encoded on plasmid pJB5JI were present on this larger plasmid, all six derivatives were inoculated onto subterranean clover plants to check for pronounced root hair distortions. All transconjugants markedly curled the root hairs of subterranean clover plants, whereas the root hairs of plants inoculated with strain ANU1019 alone were not affected when compared with uninoculated control plants. In addition, pea nodulation capacity could also be retransferred from these transconjugants to *R. leguminosarum* recipients. The basis of this increase in the plasmid size of pJB5JI was not pursued.

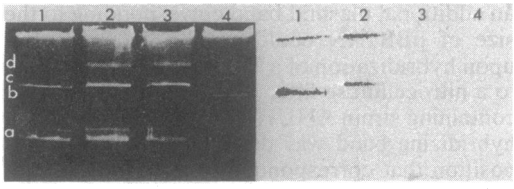

FIG. 5. Plasmid gel profile of various *R. meliloti* derivatives and the corresponding autoradiograph of this plasmid gel probed with Tn*5*-specific sequences. Lane 1, strain ANU1000(pJB5JI); lane 2, strain ANU1000(pBR1AN); lane 3, strain ANU1000; lane 4, strain L5-30. Bands c and d represent the resident plasmids in ANU1000. Band b is pJB5JI (130 Mdal), and band a is the linear chromosome. A plasmid band corresponding to pBR1AN could not be detected after gel electrophoresis; however, plasmid pBR1AN was found to comigrate with the smaller resident plasmid in strain ANU1000, which is 141 Mdal (1).

DISCUSSION

Although there is no direct molecular evidence available, the most likely mechanism for the derivation of plasmid pBR1AN is that an unstable cointegrate was formed between plasmid pJB5JI and the resident Sym plasmid of *R. trifolii* ANU6. The reluctance of pJB5JI to transfer to strain ANU6 suggests an incompatibility phenomenon, and this may have facilitated cointegrate formation. Upon transfer of this cointegrate to strain ANU615, variants were readily isolated from white clover nodules where the cointegrate had abnormally resolved. The end result was the formation of the plasmid designated pBR1AN.

Hybridization data suggest that plasmid pBR1AN encodes the nodulation and nitrogen-fixation genes originally present on the resident Sym plasmid in strain ANU6. It no longer encodes pea nodulation capacity or the nitrogenase genes originally encoded on plasmid pJB5JI. Physical data on plasmid size suggest that plasmid pBR1AN is probably 10 Mdal larger than plasmid pJB5JI, since it comigrates with the smallest plasmid in strain ANU1000, which has

been shown to be 141 Mdal (1).

In contrast, plasmids pBR1AN and pJB5JI probably share common transfer functions, transfer frequencies, and bacteriocin markers. The location of Tn*5* in both plasmids is retained. These results indicate that differences between these plasmids could be limited to the region encoding symbiotic information.

Both Sym plasmids could be transferred at high rates to various *R. trifolii* and *R. leguminosarum* mutant strains, resulting in the expression of the symbiotic information encoded by these two plasmids. *R. leguminosarum* and *R. trifolii* mutant derivatives to which plasmid pBR1AN was transferred behaved like *R. trifolii* strains, whereas the same derivatives to which plasmid pJB5JI was transferred behaved like wild-type *R. leguminosarum* strains. The transfer of the capacity for host-specific nodulation was also obtained by Beynon et al. (3) and Johnston et al. (15) with plasmid pJB5JI and by Hooykaas et al. (13, 14).

Considering that *R. leguminosarum* and *R. trifolii* strains are usually regarded as being closely related, particularly because they overlap in their host specificity (on subterranean clovers) and because they have been shown by Yao and Vincent (25, 26) to cause marked root hair curling (and occasionally nodules) on their respective heterologous hosts, it was surprising that plasmid pJB5JI did not more readily correct the nodulation defects in the *R. trifolii* Hac$^-$ strains ANU453 and ANU851. Possible reasons are that strains ANU851 and ANU453 compete for binding sites on the root hairs and reduce the ability of white clover plants to "sieve" out the subpopulation of ANU453 or ANU851 cells that had received plasmid pJB5JI. A more likely reason is that the subpopulations of ANU453(pJB5JI) or ANU851(pJB5JI) cells were killed by the small bacteriocins produced by either ANU453 or ANU851 cells. Recall that plasmid pJB5JI (and plasmid pBR1AN) suppresses both the production of and the immunity to small bacteriocins. Nevertheless, white clover plants displayed no corresponding difficulty in sieving out ANU453(pBR1AN) or ANU851(pBR1AN) cells, even though plasmid pBR1AN has the same effect upon small bacteriocin production. This may indicate that plasmid pJB5JI can only poorly correct the defects in this strain. This is supported by the observation that a homologous population of either ANU453(pJB5JI) or ANU851(pJB5JI) cells (obtained from subterranean clover nodules) displayed a relatively poor and delayed nodulation response on white clover plants.

Neither Sym plasmid was able to correct the gross appearance of a nonmucoid Muc$^-$ phenotype when transferred to the various Muc$^-$ *R. trifolii* and *R. leguminosarum* strains. The ability to nodulate either peas or clovers could be restored to one Muc$^-$ *R. leguminosarum* derivative, ANU54 (Exo-1), when the appropriate Sym plasmid was transferred to this strain. These derivatives formed poor nodules on either peas or clovers which were blocked during early nodule development. The poor nodulation ability of the Muc$^-$ *R. trifolii* derivative ANU846 (20) could not be improved by the transfer of plasmid pBR1AN. The transfer of plasmid pJB5JI to both *R. trifolii* Muc$^-$ strains ANU846 and ANU437 enabled them to nodulate peas, but again the nodules produced were poor and were blocked at an early development stage. This indicates that the genes coding for mucoid production are not encoded on Sym plasmid pJB5JI or pBR1AN. Moreover, it seems that the genes responsible for this Muc$^-$ phenotype are needed for proper nodule development (6).

The differential phenotypic expression of Sym plasmids pJB5JI and pBR1AN occurs when these plasmids are transferred to the conditional Muc$^-$ *R. trifolii* strain, ANU14. It is clear that the conditional Muc$^-$ phenotype of this strain masks the expression of the nodulation functions encoded on plasmid pBR1AN but not those encoded on plasmid pJB5JI. It seems likely that some alteration in the cell surface chemistry specifically alters the ability of plasmid pBR1AN to confer the capacity to nodulate clovers. This suggestion is also supported by the finding that the reversion of this conditional Muc$^-$ phenotype also restores the ability of pBR1AN to confer a nodulation capacity to strain ANU34.

When the Sym plasmids were transferred to more distantly related members of the *Rhizobiaceae* (*R. meliloti* and *A. tumefaciens*), variation in the apparent expression of the symbiotic functions occurred. Neither plasmid could induce any of the *R. meliloti* mutant derivatives to nodulate peas or clovers. However, both Sym plasmids could induce one particular mutant of *R. meliloti* 41 (ANU1000), which possesses a Hac$^-$ phenotype, to nodulate lucerne. Banfalvi et al. (1) also noted this result with plasmid pJB5JI in the same *R. meliloti* derivative. It is unusual that two heterologous Sym plasmids can complement the Nod$^-$ defect in this strain, particularly since it has been reported (1) to possess a Sym plasmid deletion. A possible explanation is that strain ANU1000 is an insertion element-induced Nod$^-$ mutant such as those found by Long et al. (16) rather than a Sym plasmid-deleted mutant. Alternately, if this was not the case, this result would suggest that certain functions involved in the nodulation process are common to *R. trifolii*, *R. leguminosarum*, and *R. meliloti*.

Variations in the expression of the information encoded on the Sym plasmids used also occurred when these plasmids were transferred to the *A. tumefaciens* ANU1019. Plasmid pBR1AN enabled this strain to nodulate white clovers, whereas the same strain with pJB5JI could not nodulate peas. The presence of a plasmid corresponding to the expected size of plasmid pJB5JI (which is 130 Mdal) was not found. Instead, in the six putative transconjugants tested, an additional plasmid of approximately 180 to 200 Mdal was found. Similar overall results have been obtained by Hooykaas et al. with the same recipient strain, using pRtr5a (14) and another *R. leguminosarum* plasmid (pSym1) (13), although no plasmid alteration occurred. Although plasmid pSym1 could confer vetch nodulation to *A. tumefaciens*, nodulation tests on *P. sativum* were not conducted. All of the six *A. tumefaciens* transconjugants tested in this study could markedly distort subterranean clover root hairs and could retransfer pea nodulation capacity to *R. leguminosarum* recipients, indicating that pea nodulation functions were still present on some of these abnormally large plasmids. This result, coupled with the finding that specific Hac$^-$ mutants could be complemented by heterologous nodulation information, casts doubt on the hair-curling step as being host specific.

The Sym plasmids pBR1AN and pJB5JI offer a unique opportunity to study the genetic analysis of host-specific nodulation because the differences in the two plasmids seem to be limited to a particular region where the symbiotic genes are located. It is apparent that these Sym plasmids encode functions which allow *Rhizobium* strains to attach, invade, and initiate a nodule on specific host plants, as well as late functions involved with nitrogen fixation. The self-transmissibility of these plasmids allows the study of the expression of host specificity functions in different members of the *Rhizobiaceae* and offers clues to the evolution of genes which enable *Rhizobium* strains to invade various legumes.

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