JOURNAL OF BACTERIOLOGY, Oct. 1984, p. 483–487
0021-9193/84/100483-05$02.00/0

Vol. 160, No. 1

Mobilization of a Sym Plasmid from a Fast-Growing Cowpea *Rhizobium* Strain

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A large Sym plasmid from a fast-growing cowpea *Rhizobium* species was made mobilizable by cointegration with plasmid pSUP1011, which carries the *oriT* region of RP4. This mobilizable Sym plasmid was transferred to a number of *Rhizobium* strains, in which nodulation and nitrogen fixation functions for symbiosis with plants of the cowpea group were expressed.

Rhizobium sp. strain NGR234 is unusual in that it is a fast-growing strain able to nodulate a very broad range of plants, including *Parasponia andersonii*, a nonlegume (12, 13). Strain NGR234 shares physiological characteristics with fast-growing *Rhizobium* strains from the tropical tree species *Leucaena leucocephala* and has a symbiotic host range which overlaps slow-growing *Rhizobium* species from the cowpea group of plants.

Genetic investigations of the symbiotic relationship between legumes and rhizobia have relied largely on *Rhizobium trifolii*, *Rhizobium leguminosarum*, and *Rhizobium meliloti*. It has been shown in these fast-growing rhizobia from temperate legumes that large Sym plasmids encode genes essential for nodulation and nitrogen fixation (2, 4, 7, 10). To date, such Sym plasmids have not been reported in slow-growing rhizobia of any type. Strain NGR234 has a large Sym plasmid which carries genes for nodulation and nitrogen fixation. Although this plasmid is not self-transmissible, it has been mobilized at low frequency by the IncP1 group plasmid RP1::Tn*501* (9).

The aim of this study was to construct a high-frequency mobilizable Sym plasmid labeled with antibiotic resistance markers. This plasmid was then used as a genetic tool to determine whether fast-growing *Rhizobium* species, such as *R. meliloti*, *R. trifolii*, and *R. leguminosarum*, could nodulate cowpea-type plants if they were supplied with the Sym plasmid genes. The bacterial strains and plasmids used are shown in Table 1.

The mobilizable Sym plasmid was constructed in vivo by cointegration with plasmid pSUP1011, a small vector normally used for Tn*5* mutagenesis. Plasmid pSUP1011 is a pACYC184 derivative containing a gene for high frequency mobilization ability (*mob*) and the transposon Tn*5* (11). Although this plasmid can be transferred to *Rhizobium* strains, it cannot be maintained. However, the transposon Tn*5* can "escape" by transposition to another replicon, giving a strain which has inherited kanamycin resistance (Km^r) coded by Tn*5* but which has not acquired the other markers on pSUP1011; namely, chloramphenicol resistance (Cm^r) and mobilization ability (*mob*). When pSUP1011 was used for transposon mutagenesis in strain ANU240 (a streptomycin-resistant derivative of strain NGR234), about 20% of the putative Tn*5* derivatives had inherited Cm^r. These strains could have arisen by cointegrative "rescue" of pSUP1011, since Tn*5* is known to mediate cointegration events (6). As well as rescuing Km^r and Cm^r, cointegration would result in the insertion of the mobilization gene. If such cointegrations were in the chromosome, it could provide a site for mobilizing the chromosome at high frequency. If the cointegration was in the Sym plasmid, then this could create a mobilizable Sym plasmid-pSUP1011 cointegrate.

Plasmid pSUP1011 was conjugated from the *Escherichia coli* SM10-1011 to *Rhizobium* sp. strain ANU269, a rifampin-resistant (Rif^r) mutant of NGR234 which also carries the IncP1 plasmid pJB3JI (3). This plasmid was used to provide the necessary transfer functions for the mobilization of possible cointegrates. Transconjugant ANU269 colonies were plated on Cm-containing media to ensure that only possible cointegrates were selected. Rifampin (Rif) was used to select against the *E. coli* donor. The rate of transfer of Cm^r was 5×10^{-7}. Over 3,000 Cm^r ANU269 transconjugants were subcultured three times onto selective media by replica plating. No contamination of the *E. coli* donor could be detected after this purification method was carried out.

The plant selection method of Brewin et al. (3) was used to select for the transfer of nodulation ability (Nod^+) from the purified Cm^r ANU269 clones to the heat-cured Nod^- mutant ANU265. Strain ANU265 is rifampin sensitive (Rif^s), streptomycin resistant (Sm^r), and spectinomycin resistant (Sp^r). The mating was set up by replica plating of the Cm^r ANU269 clones onto lawns of ANU265 on TY medium. Conjugation was allowed to proceed overnight before the transfer of Km^r and Cm^r was selected in ANU265 by replica plating onto appropriate selective media. A very high rate of transfer of both Km^r and Cm^r was found. Transconjugant ANU265 colonies were purified by replica plating onto TY medium containing appropriate levels of Sp, Sm, and Km (Km^r was used due to poor expression of Cm^r in the presence of Sp). After three selective subcultures, no contaminating ANU269 cells could be detected by replica plating onto TY Rif medium. Purified Km^r ANU265 cells were washed off selective plates en masse and used to inoculate siratro (*Macroptilium atropurpureum*) plants. After 2 weeks, 14 nodules had developed on 2 plants out of 10 that were inoculated with the undiluted cell suspension. Nodules did not develop on plants inoculated with diluted cell suspensions. Bacteria were reisolated from the nodules and tested for various characteristics. All had the distinctive colony morphology of strain NGR234 derivatives, and their Nod^+ ability was stable in subculture. These strains were able to initiate a normal

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TABLE 1. Bacterial strains and plasmids used

Strains and plasmids	Relevant characteristics	Source or reference
Strains		
NGR234	Wild-type, broad-host-range, fast-growing cowpea *Rhizobium*	12
ANU269	Rif^r NGR234 derivative carrying plasmid pJB3JI	This laboratory
ANU265	Sm^r Sp^r nonnodulating (Nod$^-$) NGR234 derivative which has lost the Sym plasmid	This laboratory
ANU271	Sm^r Sp^r Rif^s Nod$^+$ Fix$^+$ ANU265 derivative carrying the cointegrate plasmid, pNM4AN	This study
6015	Nod$^-$ *nif* deletion of *R. leguminosarum*	3
ZB157	Nod$^-$ deletion mutant of *R. meliloti* 41	2
ANU1064	Nod$^-$, heat-cured, Rif^r *R. trifolii* 5 from Rothamsted collection	This laboratory
C58	Virulent *A. tumefaciens* strain	15
A136	C58 cured of its Ti plasmid; avirulent	15
SM10-1011	*E. coli* donor strain carrying pSUP1011	
Plasmids		
pJB3JI	Km^s R68:45 *tra*$^+$ IncP1 Tc^r Ap^r	3
pSUP1011	Tn*5* mutagenesis vector	11
pNM4AN	Mobilizable cointegrate between pSUP1011 and the NGR234 Sym plasmid, Km^r Cm^r, confers Nod$^+$ ability	This study

nitrogen-fixing (Fix$^+$) response on siratro, which was indistinguishable from that of the wild-type strain NGR234. All strains were Sm^r and Sp^r (characteristic of strain ANU265), Tc^r (carried on pJB3JI), Km^r, and Cm^r (carried on pSUP1011). All strains were sensitive to Rif. When plasmid profiles were checked, all had the 38-megadalton (Mdal) plasmid pJB3JI and a band corresponding to the size of the NGR234 Sym plasmid (data not shown). It was assumed that this plasmid was indeed a cointegrate between pSUP1011 and the NGR234 Sym plasmid. The cointegrate plasmid was designated pNM4AN.

One of these transconjugants (designated strain ANU271) was used as a donor in conjugation experiments with the following strains: the Nod$^-$ *R. meliloti* ZB157 (2), the Nod$^-$ *R. leguminosarum* 6015 (3), strain ANU1064 (Nod$^-$ heat-cured *R. trifolii* 5 from the Rothamsted collection). ANU271 was able to transfer Km^r to all these strains at a frequency of about 10^{-2}, a rate comparable to the transfer of pJB3JI (selected by Tc^r). Ten transconjugants from each strain were purified twice on selective media and tested for nodulation on siratro and on their normal hosts (Table 2).

Strain ZB157(pNM4AN, pJB3JI) did not nodulate lucerne (*Medicago sativa*) but was able to nodulate siratro, forming normal-sized effective nodules after 2 weeks (Fig. 1A). The interiors of the nodules were pink, indicating that leghemoglobin was present. Acetylene reduction assays on pot-grown siratro showed that ZB157(pNM4AN, pJB3JI) was partially effective, having 30 to 40% of the activity of the Nod$^+$ Fix$^+$ strain ANU240 after 12 weeks. Microscopic examination of nodule tissue showed a normal nodule morphology with bacteroid-packed plant cells (Fig. 2B). Bacteria were reisolated from the nodules, and the identity of ZB157(pNM4AN, pJB3JI) was verified by culture tests.

TABLE 2. Nodulation of various plants by *Rhizobium* and *Agrobacterium* strains carrying the cointegrate plasmid pNM4AN

pNM4AN transconjugant	Nodulation of the following plants:			
	Clover	Peas	Lucerne	Siratro
Rhizobium sp. ANU271	Nod$^-$	Nod$^-$	Nod$^-$	Nod$^+$ Fix$^+$
R. meliloti ZB157	Nod$^-$	Nod$^-$	Nod$^-$	Nod$^+$ Fix$^+$
R. trifolii ANU1064	Nod$^-$	Nod$^-$	Nod$^-$	Nod$^+$ Fix$^-$
R. leguminosarum 6015	Nod$^-$	Nod$^-$	Nod$^-$	Nod$^+$ Fix$^-$
A. tumefaciens C58, A136	Nod$^-$	Nod$^-$	Nod$^-$	Nod$^+$ Fix$^-$

The presence of pNM4AN and pJB3JI in the transconjugant strain ZB157(pNM4AN, pJB3JI) was verified by using the Ekhardt gel technique (5) (Fig. 3). This gel shows that strains ANU240 and ANU265 both have a large megaplasmid of unknown size. This megaplasmid was not previously detected (9); however, it is clear that strain ANU265 has lost the smaller Sym plasmid. Strain ZB157(pNM4AN, pJB3JI) clearly contains pNM4AN and pJB3JI as two distinct entities. This is verified by the observation that Km^r and Cm^r

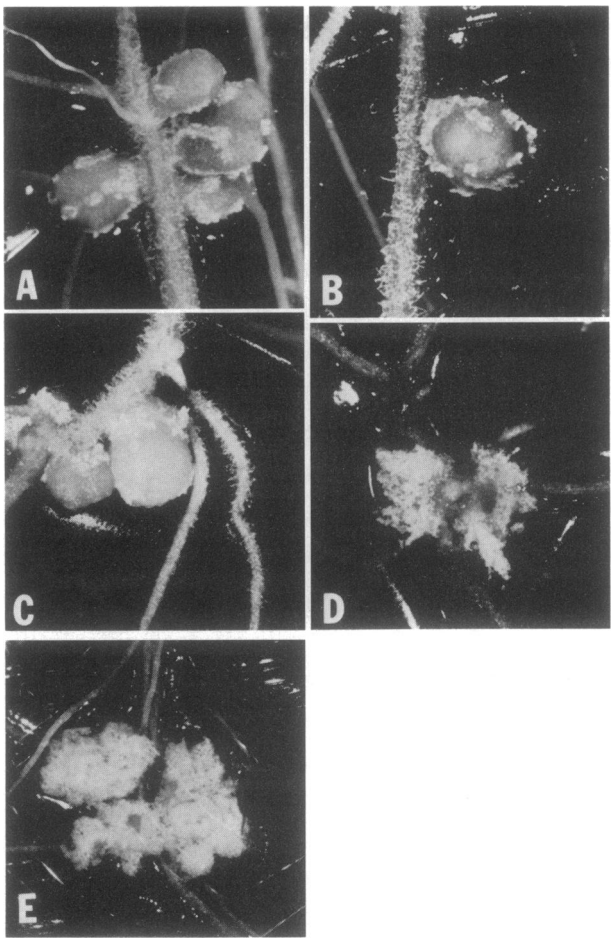

FIG. 1. Nodules induced on siratro by various strains after five weeks: (A) NGR234; (B) ZB157(pNM4AN, pJB3JI); (C) 6015(pNM4AN, pJB3JI), normal-looking nodule on the tap root; (D) same strain as in C, nodule with epidermal outgrowths; (E) A136(pNM4AN, pJB3JI).

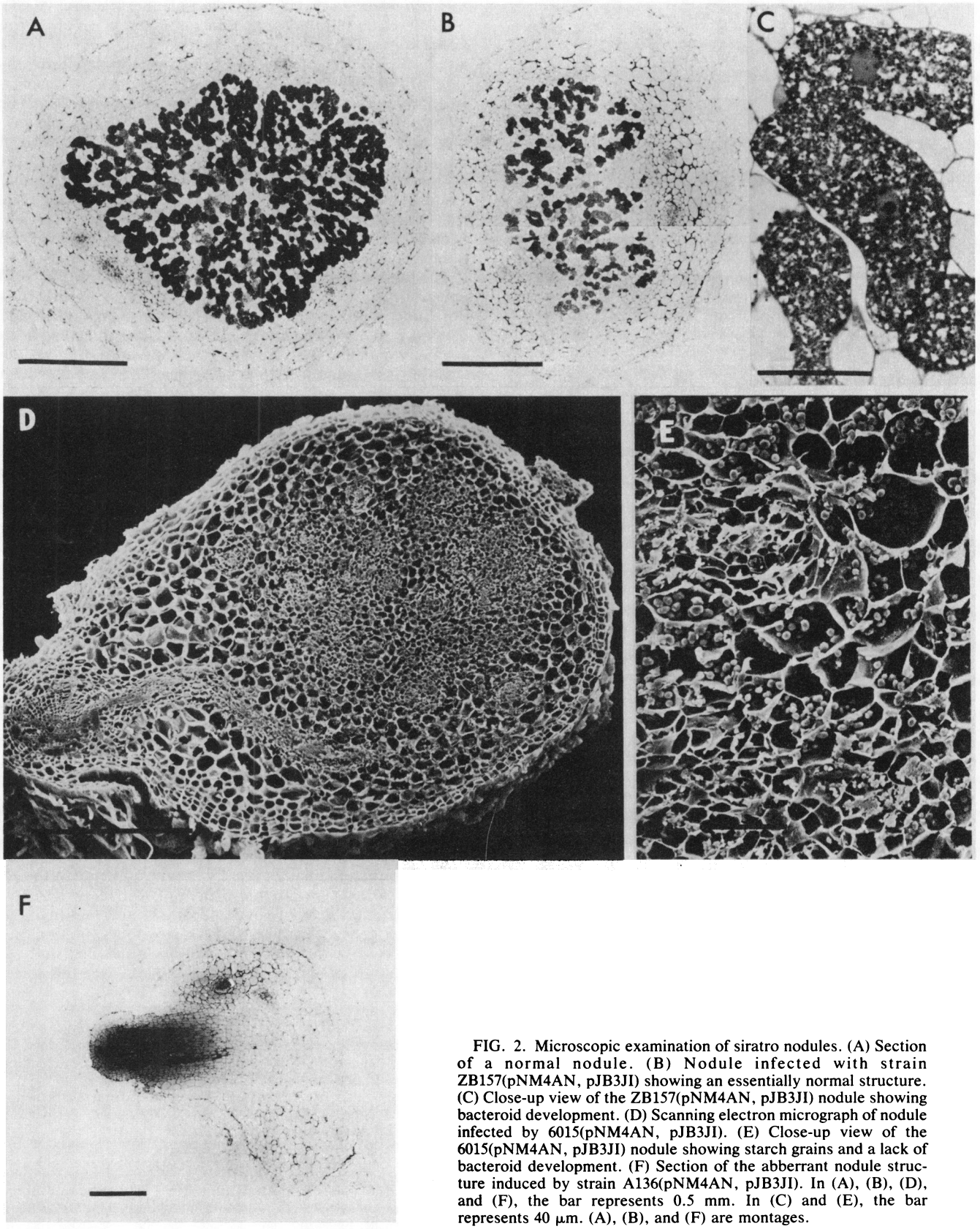

FIG. 2. Microscopic examination of siratro nodules. (A) Section of a normal nodule. (B) Nodule infected with strain ZB157(pNM4AN, pJB3JI) showing an essentially normal structure. (C) Close-up view of the ZB157(pNM4AN, pJB3JI) nodule showing bacteroid development. (D) Scanning electron micrograph of nodule infected by 6015(pNM4AN, pJB3JI). (E) Close-up view of the 6015(pNM4AN, pJB3JI) nodule showing starch grains and a lack of bacteroid development. (F) Section of the abberrant nodule structure induced by strain A136(pNM4AN, pJB3JI). In (A), (B), (D), and (F), the bar represents 0.5 mm. In (C) and (E), the bar represents 40 μm. (A), (B), and (F) are montages.

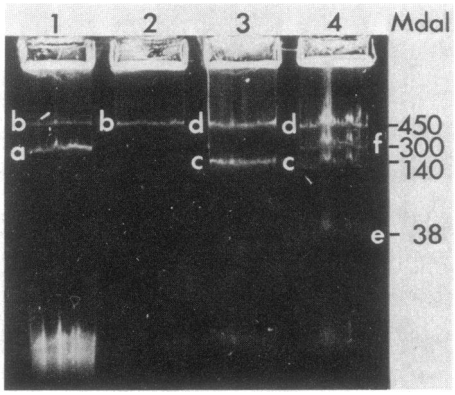

FIG. 3. Visualization of pNM4AN in *R. meliloti* ZB157. Lane 1, strain NGR234, which has two plasmids: the Sym plasmid (a) and the megaplasmid (b); lane 2, Nod$^-$ heat-cured derivative of NGR234, strain ANU265, which has only the megaplasmid (b); lane 3, *R. meliloti* 41 (the parent strain to ZB157), which has two plasmids: the 140-Mdal cryptic plasmid pRme41*a* (c) and the megaplasmid pRme416*b* (which carries the symbiotic gene region of this *Rhizobium* strain) (d); lane 4, strain ZB157(pNM4AN, pJB3JI), which has four plasmids: pJB3JI (e), pRme41*a* (c), pNM4AN (f), and pRme41*b* (d). The approximate molecular masses of these plasmids are shown: pRme41*a* has been reported to be 140 Mdal (2) and pRme41*b* has been reported to be 450 Mdal (A. Kondorosi, personal communication; J. Plazinski, unpublished data). The NGR234 Sym plasmid has been quoted previously as being about 200 Mdal (9); however, a figure of 300 Mdal is more appropriate. pJB3JI is about 38 Mdal (3). Notice that pNM4AN appears to be the same size as the NGR234 Sym plasmid, although it is a cointegrate between the NGR234 Sym plasmid and pSUP1011. Since pSUP1011 is about 8 Mdal, compared with 300 Mdal for the NGR234 Sym plasmid, the relative increase in size of the cointegrate molecule is not detectable. Our clone of the *R. meliloti* mutant ZB157 has deletions in the two native *R. meliloti* plasmids pRme41*a* and pRme41*b* (2). The deletion in pRme41*b* has been described by Banfalvi et al. (2), and the deletion in pRme41*a* is apparently present only in our strain. Since pRme41*a* has no known function, this deletion is not important. These deletions are responsible for the slightly increased mobility of these plasmids in lane 4 compared with the parent *R. meliloti* 41 (lane 3).

can be maintained, whereas Tc^r (encoded on pJB3JI) can be lost. Such strains with Km^r Cm^r Tc^s phenotypes can still nodulate but cannot transfer Km^r and Cm^r. Reintroduction of pJB3JI reinstates the mobilizability of pNM4AN.

The transconjugant strains *R. leguminosarum* 6015(pNM4AN, pJB3JI) and *R. trifolii* ANU1064(pNM4AN, pJB3JI) were able to nodulate siratro, forming normal-looking nodules as well as some nodules with white epidermal outgrowths (Fig. 1C and D). Both strains were Fix$^-$, indicated by a lack of acetylene reduction activity, poor growth of the plants, and a lack of pink color inside the nodule. Nodules of strain 6015(pNM4AN, pJB3JI) were examined by scanning electron microscopy and had no bacteroids and very few bacteria in the nodule (Fig. 2C and D). Thus, a defective bacterial release (Bar$^-$) phenotype probably explains the Fix$^-$ responses of these transconjugants on siratro. Strains ZB157(pNM4AN, pJB3JI), 6015(pNM4AN, pJB3JI), and ANU1064(pNM4AN, pJB3JI) were all able to retransfer pNM4AN back to ANU265 at a rate of about 10^{-2}. ANU265 transconjugants gave a completely normal Nod$^+$ Fix$^+$ response, indicating that the symbiotic properties of pNM4AN were not altered as a result of passage through these strains. Strain ANU271 could transfer pNM4AN at a frequency of 10^{-2} to *Agrobacterium tumefaciens* C58 and its avirulent mutant A136 (15). There was no difference in the expression of pNM4AN in C58 compared with its Ti plasmid-cured mutant A136. These strains formed nodules with white epidermal outgrowths (Fig. 1F) mostly on the lateral roots of the plant, occasionally forming small nodules on the tap root (Fig. 1E). The association with siratro was ineffective. Microscopic examination of these furry nodules revealed a complete lack of bacterial infection of plant cells (Fig. 2G). Strains A136(pNM4AN, pJB3JI) and C58(pNM4AN, pJB3JI) could retransfer pNM4AN back to strain ANU265, in which a totally normal Nod$^+$ Fix$^+$ symbiotic response was regained. Strain C58(pNM4AN, pJB3JI) could still induce crown gall tumors on *Datura* plants when tested by stab wounds.

The results reported in this paper describe the in vivo construction of a cointegrate-mobilizable Sym plasmid by a novel method. This plasmid can be stably maintained and express nodulation functions in fast-growing rhizobia which normally nodulate temperate legumes and in *Agrobacterium* strains. This is the first time that genes for symbiosis with cowpea-type plants have been introduced into other bacteria.

The fact that strain ZB157(pNM4AN, pJB3JI) could fix nitrogen in symbiosis with siratro is surprising given that siratro and lucerne are not closely related plants and have entirely different nodule structure and physiology (1). It is known that strain ZB157 retains at least *nifH* and *nifD* but is suspected to have lost some other gene essential for nitrogen fixation (2), so it is likely that pNM4AN *nif* genes are functioning. Kondorosi et al. (8) transferred a mobilizable derivative of the *R. meliloti* 41 Sym plasmid to strain NGR234. The NGR234 transconjugant strain produced tumor-like growths on lucerne. These data and the fact that the other transconjugants mentioned in this report fail to fix nitrogen suggest that there is a plant-determined host-range phenomenon controlling early events requisite to the onset of nitrogen fixation. This phenomenon is obviously not Sym plasmid related but depends on the strain carrying the plasmid. The event may be the release of bacteria from the infection thread since Wong et al. (16) and Van Brussel et al. (14) have observed a lack of bacterial release in similar experiments.

Although strain NGR234 has been reported as being Nod$^+$ on lucerne (12), we have not been able to repeat this finding. Banfalvi et al. (2) have reported that ZB157 can be complemented for lucerne nodulation by the introduction of the *R. leguminosarum* Sym plasmid pJB5JI (4). ZB157(pNM4AN, pJB3JI) cannot nodulate lucerne, so it may be that the nodulation genes of strain NGR234 are too evolutionarily diverged to allow this complementation. A detailed study of the nodulation genes of NGR234 should answer this question.

We thank Jan McIver for excellent technical assistance. J. Beringer, P. Hooykaas, A. Kondorosi, M. Trinick, J. Schell, and R. Simon are thanked for bacterial and plasmid strains. N.A.M. and H.C.C. are Australian National University Scholars.

This study was partially supported by a grant from Agrigenetics Research Associates Limited, an R & D Limited Partnership sponsored by Agrigenetics Corp.

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