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Cloning of *Rhizobium leguminosarum* Genes for Competitive Nodulation Blocking on Peas

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One type of competitive interaction among rhizobia is that between nonnodulating and nodulating strains of *Rhizobium leguminosarum* on primitive pea genotypes. *Pisum sativum* cv. Afghanistan nodulates effectively with *R. leguminosarum* TOM, and this can be blocked in mixed inoculations by *R. leguminosarum* PF_2, which does not nodulate this cultivar. We termed this PF_2 phenotype Cnb^+, for competitive nodulation blocking. Strain PF_2 contains three large plasmids including a 250-kilobase-pair symbiotic (Sym) plasmid. Transfer of this plasmid, $pSymPF_2$, to nonblocking rhizobia conferred the Cnb^+ phenotype on recipients in mixed inoculations on cultivar Afghanistan with TOM. A library of the PF_2 genome constructed in the vector pMMB33 was used to isolate two cosmid clones which hybridize to $pSymPF_2$. These cosmids, pDD50 and pDD58, overlapped to the extent of 23 kilobase pairs and conferred a Cnb^+ phenotype on recipient Cnb^- rhizobia, as did pSD1, a subclone from the common region.

Rhizobia used as inocula for legumes have to compete with indigenous rhizobia and other soil microorganisms for nodulation of a specific host. The outcome of interstrain competition determines the success or failure of legume inoculation. Although competitiveness as a strain characteristic has been observed for most *Rhizobium*-legume systems (for reviews, see references 12 and 30), its mechanisms are poorly understood. Most reports concern competition between specific strains nodulating the same plant and have investigated relative nodule occupancy as a measure of competitiveness (3, 27). Competition has also been observed between nodulating and nonnodulating rhizobia, and the nonnodulating strain may suppress nodulation of the host legume by the infective strain (11, 16). One such system is composed of nodulating and nonnodulating strains of *Rhizobium leguminosarum* on Pisum sativum cv. Afghanistan (23, 33), a primitive pea cultivar which generally fails to nodulate with European *R. leguminosarum* strains such as PF_2, but is nodulated effectively by those of Middle Eastern origin such as TOM. The ability of TOM to nodulate cultivar Afghanistan is due to a host-range gene (19) carried on the Sym plasmid (1). Strain PF_2 induces root hair curling and infection thread formation but not nodules on cultivar Afghanistan (10). In mixed inoculations it blocks nodulation of the cultivar by TOM. Neither bacterial antagonism nor preferential growth rate is responsible for blocking. Dead cells of PF_2 bind to roots but do not block TOM nodulation, suggesting that nodulation blocking by PF_2 is an active process (7, 8). We cloned genes from PF_2 which determine this competitive nodulation blocking of TOM on cultivar Afghanistan. Genetic and physical evidence described here indicates that these genes are linked to the Sym plasmid of PF_2.

The bacterial strains and plasmids used are described in Table 1. *R. leguminosarum* PF_2 was shown to have three large plasmids by Eckhardt in-well lysis gel analysis (14) (Fig. 1) or alkaline denaturation methods (18). The smallest of these plasmids, pPF_2a, 250 kilobase pairs (kbp) in size, was identified as a Sym plasmid (20) by hybridization and genetic techniques. Filters prepared from Eckhardt plasmid gels were hybridized with *Rhizobium meliloti nif* and *nod* probes. Both probes hybridized only to the smallest (250 kbp) plasmid of PF_2 (data not shown). To confirm this result, the indigenous plasmids of PF_2 were labeled by the introduction of pSUP5011 harboring the mobilizing transposon Tn*5*-Mob described by Simon (31). The kanamycin-sensitive plasmid RP4.4 was introduced into Tn*5*-Mob-labeled recipients by conjugation. Plasmids encoding kanamycin resistance were then mobilized by this IncP1 plasmid into *Rhizobium loti* NZP4010. Bacterial conjugations were done on sterile membrane filters (9), and transconjugants were analyzed for plasmid content on Eckhardt gels. Of 20 clones examined, 1, UG233, acquired only the 250-kbp pPF_2a plasmid, conferring on NZP4010 the ability to nodulate *P. sativum* cv. Rondo ineffectively (data not shown). When pPF_2a was retransferred by RP4.4 to 6015, a *nif-nod*-deleted strain of *R. leguminosarum*, it restored effective nodulation on *P. sativum* cv. Rondo. Taken together, these results show that pPF_2a encodes host specificity, nodulation, and nitrogen fixation genes. We therefore term it $pSymPF_2$.

An assay system devised by Lie et al. (23) allows reproducible detection of nodulation blocking by mixed inoculations on cultivar Afghanistan in nitrogen-free nutrient solution. Seeds of *P. sativum* cv. Afghanistan and cv. Rondo were surface sterilized as described previously (7) and germinated on nitrogen-free B&D medium (6) containing 1% agar. Three to five days after germination, seedlings were transferred aseptically to 250-ml Erlenmeyer flask assemblies containing 4× B&D solution (7). Assemblies were covered with a black cloth cape to shield the root system from light, and plants were grown at 25°C with a 15-h day and a light intensity of 300 einsteins m^{-2} s^{-1} (7). They were inoculated 5 days after planting with 2 ml (A_{600} = 1.0) of late-log-phase cells of PF_2 or *R. loti* transconjugants, followed 12 h later by reinoculation with an equivalent number of cells of strain TOM as previously described (7, 8, 33).

Typical nodulated and blocked 15-day-old (postinoculation) root systems of cultivar Afghanistan are shown in Fig. 2. Strain PF_2 completely inhibited nodulation by TOM of cultivar Afghanistan for a 15-day period, and thereafter one or two nodules sometimes formed 2 to 3 weeks later (50

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TABLE 1. Bacterial strains and plasmids

Bacterial strains and plasmids	Relevant characteristics	Reference or source
Rhizobium leguminosarum		
PF_2	Wild-type isolate (The Netherlands); Nod$^+$ Fix$^+$ *P. sativum* cv. Rondo; Nod$^-$ Cnb$^+$ *P. sativum* cv. Afghanistan[a]	33
TOM	Wild-type isolate (Turkey); Nod$^+$ Fix$^+$ *P. sativum* cv. Afghanistan	33
6015	Δ*nod-nif* 5-Fu^r Str^r Rif^r *phe trp*	21
Rhizobium loti		
NZP4010	Plasmid-free strain of NZP2037; Nod$^+$ Fix$^+$ *Lotus pedunculatus*; Str^r Rif^r	28
UG233	NZP4010(pSymPF_2::Tn*5*-Mob) Kan^r	This study
UG240	NZP4010(pDD50) Kan^r	This study
Escherichia coli		
S17.1	RP4::Tn*7* integrated chromosome; Str^r; mobilization strain	31
DH1	F$^-$ *recA1 end A1 thi-1 hsdR17*	24
JD75	DH1(R64.11) Tet^r mobilization strain	15
FM15	F$^-$ RecA$^-$ Δ*lac-pro* Δ*thi* Δ*lacZ*	R. Rodriguez, Stanford University, Stanford, Calif.
Plasmids		
pMMB33	*ori* (RSF1010) *cos* Km^r IncQ	15
pRK7813	*ori* (RK2) p*lac* Tc^r IncP1	J. D. G. Jones[b]
pRmR2	*R. meliloti nifDH* Tc^r	29
pEK5121	*R. meliloti nodABC* Ap^r	22
RP4.4	Tra$^+$ Ap^r Tc^r Km^s IncP1	17
pSUP5011	Tn*5*-Mob Km^r *oriT* (RP4)	31
pDD50, pDD58	Cnb$^+$ pMMB33 clones	This study
pSD1	*Hin*d III subclone of pDD50 in pRK7813	This study

[a] Cnb$^+$, Competitive nodulation blocking of strain TOM on pea cultivar Afghanistan
[b] Advanced Genetic Sciences, Oakland, Calif.

replicates). A competitive nodulation-blocking (Cnb$^+$) phenotype was exhibited by *R. loti* transconjugants carrying pSymPF_2 such as UG233 (Fig. 3).

Genomic DNA prepared from *R. leguminosarum* PF_2 was isolated by the method of Broughton et al. (5), and plasmid vectors were prepared by standard methods (26). PF_2 DNA was digested with *Sau*3AI, sized by the method of Maniatis et al. (26), and cloned into pMMB33 by the method of Frey et al. (15) to yield a library consisting of 1,200 *Escherichia coli* DH1 colonies containing cosmids with insertions of 30 to 35 kbp of PF_2 DNA. Since we had previously shown that Cnb$^+$ genes were encoded by the Sym plasmid, large plasmids of PF_2 were isolated by the method of Hirsch et al. (18), purified on CsCl-ethidium bromide gradients, and nick translated as a probe to screen the genomic library by colony hybridization (25) for clones containing plasmid sequences. Of 1,200 colonies, 181 hybridized strongly to the probe. This subset of the library was screened for Cnb$^+$ clones as follows. Cosmids were mobilized by the IncIa plasmid R64.11 in triparental matings with *R. loti* NZP4010. Kanamycin-resistant transconjugants were selected and purified on medium containing rifampin and kanamycin and tested directly in the cultivar Afghanistan assay system for the Cnb$^+$ phenotype. Of 100 tested clones, 2 were Cnb$^+$ (20 replicates); these were termed pDD50 and pDD58. *R. loti* transconjugants containing these clones behaved identically to UG233 or PF_2 itself for periods of 15 days (Fig. 3). Thereafter some nodules developed on about 25% of individual plants, while the rest of the plants remained unnodulated.

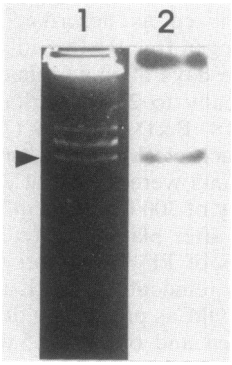

FIG. 1. Location of competitive nodulation-blocking genes on the Sym plasmid of PF_2. Lane 1, Eckhardt gel of PF_2 plasmids; lane 2, Southern filter hybridized with pDD50 probe. Hybridization conditions were as described previously (5). Arrow indicates Sym plasmid.

To confirm the origin of the Cnb$^+$ cosmids, pDD50 was nick translated and hybridized to a Southern transfer of an Eckhardt gel of PF_2 plasmids. Hybridization was found exclusively to pSymPF_2 (Fig. 1). To show that the cosmids were nonrearranged clones of the PF_2 genome, both were nick translated and hybridized to Southern filters of *Hin*dIII-digested genomic DNA (Fig. 4A and B). The pattern of hybridizing fragments observed, when compared with cosmid DNA digested with the same enzyme, showed that no rearrangement of the cloned sequences had occurred during library construction (Fig. 4A, lanes 1 and 3, and B, lanes 1 and 2). Using pDD50 as a probe against itself and pDD58 revealed a number of *Hin*dIII fragments ranging in size from 1.4 to 7.8 kbp which were common between pDD50 and pDD58. The reciprocal hybridization with pDD58 as a probe against pDD50 confirmed that about 23 kbp of DNA overlapped in the two cosmid clones. As *Hin*dIII digestion cleaves the vector internally generating

two fragments with cloned DNA attached, the identity of these fragments was confirmed by hybridization with a suitable probe (data not shown). A 4.4-kbp *Hin*dIII fragment in pDD50 and the 2.8-kbp band in pDD58 originated from the vector, while the largest band in pDD50 and in pDD58 also contained vector sequences. A *Hin*dIII partial subclone was made in pRK7813. This subclone, pSD1, contains no pMMB33 sequences, since the latter plasmid cross-hybridized only with its pRK7813 vector (data not shown). pSD1 contained the *Hin*dIII fragments of 10, 7.8, and 2.1 kbp from the overlapping region of pDD50 and pDD58 and conferred the Cnb^+ phenotype on *R. loti* NZP4010 transconjugants. The Sym plasmid $pSymPF_2$, pDD50, pDD58, and pSD1 conferred on NZP4010 the ability to block TOM nodulation completely for up to 15 days. Hence, the Sym plasmid genes concerned block nodulation competitively during the critical period of nodule initiation and development. Thereafter some instability of the cosmid phenotype was sometimes observed by 21 days, in that some nodules had formed on some individual plants of a replicate set (Fig. 3).

Our evidence is that pMMB33 cosmid clones are rather unstable and subject to deletion even in *E. coli rec* hosts (data not shown). In this context the small average number

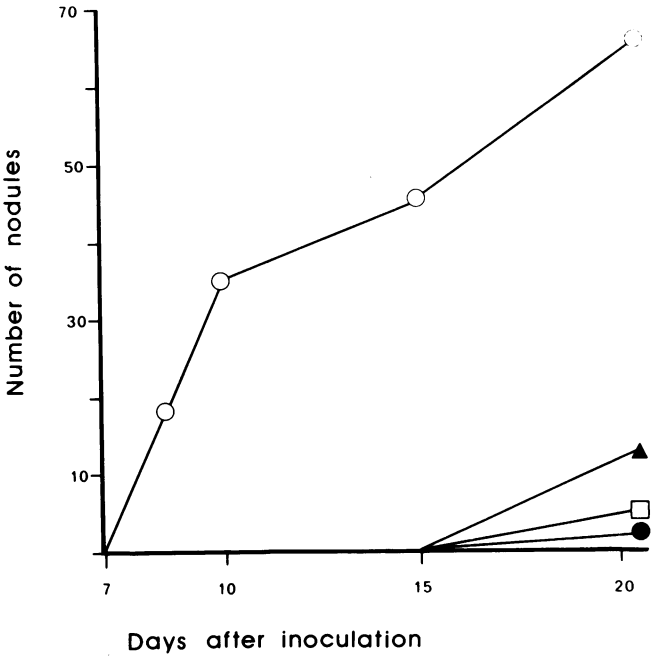

FIG. 3. Competitive nodulation blocking (Cnb^+) by *R. loti* NZP4010 transconjugants harboring $pSymPF_2$ or pDD50. Pea plants of the cultivar Afghanistan were inoculated as described in the text, and nodules were counted over a period of 21 days. Strain TOM can induce nodules in mixed inoculations with NZP4010 (○), whereas wild-type PF_2 can efficiently block nodulation by TOM (●). Strain UG233, which carries the Sym plasmid from PF_2 (□), and strain UG240, which carries the Cnb^+ cosmid pDD50 (▲), also block nodulation by TOM. The data present the average number of nodules from at least three experiments (20 replicates).

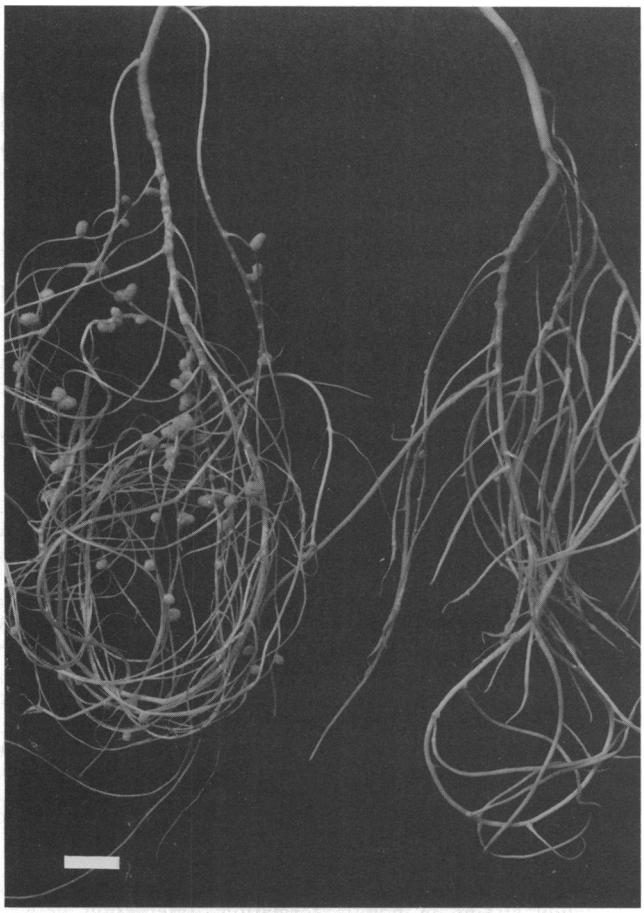

FIG. 2. Nodulated and nodulation-blocked root systems of *P. sativum* cv. Afghanistan. Effective nodules were produced by strain TOM with NZP4010 (left), and there was competitive nodulation blocking of TOM by a NZP4010(pDD50) transconjugant (right). Bar represents 1 cm.

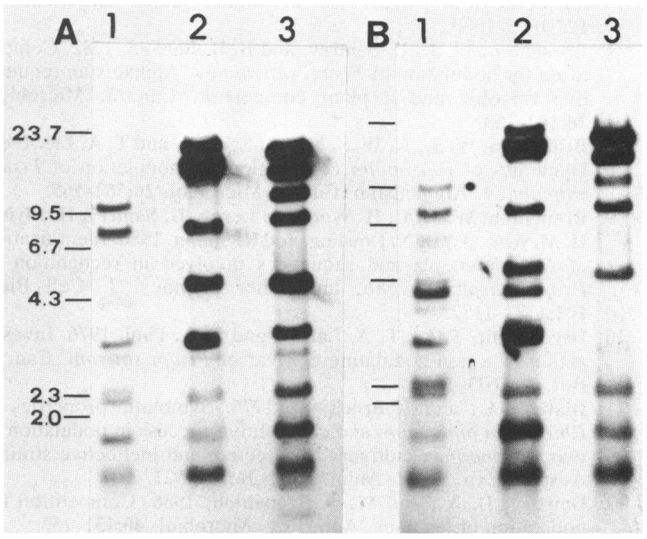

FIG. 4. (A) Hybridization of pDD50 against *Hin*dIII-digested DNA of strain PF_2 (lane 1), cosmid pDD58 (lane 2), and itself (lane 3). (B) Hybridization of pDD58 against *Hin*dIII-digested DNA of strain PF_2 (lane 1), itself (lane 2), and pDD50 (lane 3). Hybridization conditions were as described previously (32). Numbers on left are in kilobase pairs.

of nodules produced by 21 days on certain plants inoculated with TOM and NZP4010 transconjugants carrying pDD50 or pSD1 (Fig. 3) is comparable to nodulation of peas by reversions of Tn*5* insertion mutants in the *nod* genes of *R. leguminosarum* (13). It should be remembered that assay of the Cnb^+ phenotype takes place against a strong selective pressure for nodulation by the nitrogen-starved plant. It is interesting that the Cnb^+ phenotype is encoded by the Sym plasmid of PF_2. Large plasmids in other *Rhizobium* strains have been implicated in competition (2, 4). The precise mechanism of this blocking and the fine structure of the gene(s) concerned are the subject of ongoing investigation in our laboratory.

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LITERATURE CITED

1. **Brewin, N. J., J. E. Beringer, and A. W. B. Johnston.** 1980. Plasmid-mediated transfer of host-range specificity between two strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. **120**:413–420.
2. **Brewin, N. J., E. A. Wood, and J. P. W. Young.** 1983. Contribution of the symbiotic plasmid to competitiveness of *Rhizobium leguminosarum*. J. Gen. Microbiol. **129**:2973–2977.
3. **Bromfield, E. S. P., and D. G. Jones.** 1979. The competitive ability and symbiotic effectiveness of doubly-labelled antibiotic resistant mutants of *Rhizobium trifolii*. Ann. Appl. Biol. **91**:211–219.
4. **Bromfield, E. S. P., D. M. Lewis, and L. R. Barran.** 1985. Cryptic plasmid and rifampin resistance in *Rhizobium meliloti* influencing nodulation competitiveness. J. Bacteriol. **164**: 410–413.
5. **Broughton, W. J., B. B. Bohlool, C. A. Shaw, H. Bohnert, and C. E. Pankhurst.** 1985. Conserved plasmid/chromosome sequences in fast- and slow-growing rhizobia that nodulate the same plant. Arch. Microbiol. **141**:14–21.
6. **Broughton, W. J., and M. J. Dilworth.** 1971. Control of leghaemoglobin synthesis in snake beans. Biochem. J. **125**:1075–1080.
7. **Broughton, W. J., U. Samrey, and B. B. Bohlool.** 1982. Competition for nodulation of *Pisum sativum* cv. Afghanistan requires live rhizobia and a plant component. Can. J. Microbiol. **28**:162–168.
8. **Broughton, W. J., A. W. S. M. van Egeraat, and T. A. Lie.** 1980. Dynamics of *Rhizobium* competition for nodulation of *Pisum sativum* cv. Afghanistan. Can. J. Microbiol. **26**:562–565.
9. **Broughton, W. J., C. H. Wong, A. Lewin, U. Samrey, H. Myint, H. Meyer z. A., D. N. Dowling, and R. Simon.** 1986. Identification of *Rhizobium* plasmid sequences involved in recognition of *Psophocarpus*, *Vigna*, and other legumes. J. Cell Biol. **102**:1173–1182.
10. **Degenhardt, T. L., T. A. LaRue, and E. A. Paul.** 1976. Investigation of a non-nodulating cultivar of *Pisum sativum*. Can. J. Bot. **54**:1633–1636.
11. **Diatloff, A., and J. Brockwell.** 1976. Symbiotic properties of *Rhizobium japonicum* and competitive success in nodulation of two *Glycine max* cultivars by effective and ineffective strains. Aust. J. Exp. Agric. Anim. Husb.**16**:514–521.
12. **Dowling, D. N., and W. J. Broughton.** 1986. Competition for nodulation of legumes. Ann. Rev. Microbiol. **40**:131–157.
13. **Downie, J. A., C. D. Knight, A. W. B. Johnston, and L. Rossen.** 1985. Identification of genes and gene products involved in the nodulation of peas by *Rhizobium leguminosarum*. Mol. Gen. Genet. **198**:255–262.
14. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
15. **Frey, J., M. Bagdasarian, D. Feiss, C. H. Franklin, and J. Deshusses.** 1983. Stable cosmid vectors that enable the introduction of cloned fragments into a wide range of gram-negative bacteria. Gene **24**:299–308.
16. **Gibson, A. H.** 1968. Nodulation failure in *Trifolium subterraneum* L. cv. Woogenellup (syn. Marrar). Aust. J. Agric. Res. **19**:907–918.
17. **Hedges, R. W., and A. C. Jacob.** 1974. Transposition of ampicillin resistance from RP4 to other replicons. Mol. Gen. Genet. **132**:31–40.
18. **Hirsch, P. R., M. van Montagu, A. W. B. Johnston, N. J. Brewin, and J. Schell.** 1980. Physical identification of bacteriocinogenic nodulation and other plasmids in strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. **120**:403–412.
19. **Hombrecher, G., R. Götz, N. J. Dibb, J. A. Downie, A. W. B. Johnston, and N. J. Brewin.** 1984. Cloning and mutagenesis of nodulation genes from *Rhizobium leguminosarum* TOM, a strain with extended host range. Mol. Gen. Genet. **194**:293–298.
20. **Hooykaas, P. J. J., A. A. N. van Brussel, H. den Dulk-Ras, G. M. S. van Slogteren, and R. A. Schilperoort.** 1981. Sym plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*. Nature (London) **291**: 351–353.
21. **Johnston, A. W. B., J. L. Beynon, A. V. Buchanon-Wollaston, S. M. Setchell, P. R. Hirsch, and J. E. Beringer.** 1978. High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature (London) **276**:634–636.
22. **Kondorosi, E., Z. Banfalvi, and A. Kondorosi.** 1984. Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*. Identification of nodulation genes. Mol. Gen. Genet. **193**:445–452.
23. **Lie, T. A., R. Winarno, and P. C. J. M. Timmermans.** 1978. *Rhizobium* strains isolated from wild and cultivated legumes: suppression of nodulation by a non-nodulating *Rhizobium* strain, p. 398–401. *In* M. W. Lontit and J. A. R. Miles (ed.), Microbial ecology. Springer-Verlag KG, Heidelberg.
24. **Low, B.** 1968. Formation of d-merodiploids in matings with a class of recipient strains of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA **60**:160–167.
25. **Maas, R.** 1983. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid **10**:296–298.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual, p. 545. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **McLoughlin, T. J., L. M. Bordeleau, and L. K. Dunican.** 1984. Competition studies with *Rhizobium trifolii* in a field experiment. J. Appl. Bacteriol. **56**:131–135.
28. **Pankhurst, C. E., W. J. Broughton, and U. Wieneke.** 1983. Transfer of an indigenous plasmid of *Rhizobium loti* to other rhizobia and *Agrobacterium tumefaciens*. J. Gen. Microbiol. **129**:2535–2543.
29. **Ruvkun, G. B., and F. M. Ausubel.** 1980. Interspecies homology of nitrogenase genes. Proc. Natl. Acad. Sci. USA **77**:191–195.
30. **Schmidt, E. L., and F. M. Robert.** 1985. Recent advances in the ecology of *Rhizobium*, p. 379–385. *In* H. J. Evans, P. J. Bottomley, W. E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff, Dordrecht, The Netherlands.
31. **Simon, R.** 1984. High frequency mobilization of gram-negative bacterial replicons by the *in vitro* constructed Tn5-Mob transposon. Mol. Gen. Genet. **196**:413–420.
32. **Stanley, J., G. G. Brown, and D. P. Verma.** 1985. Slow-growing *Rhizobium japonicum* comprises two highly divergent symbiotic types. J. Bacteriol. **163**:148–154.
33. **Winarno, R., and T. A. Lie.** 1979. Competition between *Rhizobium* strains in nodule formation. Interaction between nodulating and non-nodulating strains. Plant Soil **51**:131–142.