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Increased Bean (*Phaseolus vulgaris* L.) Nodulation Competitiveness of Genetically Modified *Rhizobium* Strains

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Rhizobium leguminosarum **bv. phaseoli strain collections harbor heterogeneous groups of bacteria in which two main types of strains may be distinguished, differing both in the symbiotic plasmid and in the chromosome. We have analyzed under laboratory conditions the competitive abilities of the different types of *Rhizobium* strains capable of nodulating *Phaseolus vulgaris* L. bean. *R. leguminosarum* bv. phaseoli type I strains (characterized by *nif* gene reiterations and a narrow host range) are more competitive than type II strains (that have a broad host range), and both types are more competitive than the promiscuous rhizobia isolated from other tropical legumes able to nodulate beans. Type I strains become even more competitive by the transfer of a non-Sym, 225-kilobase plasmid from type II strain CFN299. This plasmid has been previously shown to enhance the nodulation and nitrogen fixation capabilities of *Agrobacterium tumefaciens* transconjugants carrying the Sym plasmid of strain CFN299. Other type I *R. leguminosarum* bv. phaseoli transconjugants carrying two symbiotic plasmids (type I and type II) have been constructed. These strains have a diminished competitive ability. The increase of competitiveness obtained in some transconjugants seems to be a transient property.**

Field inoculation with *Rhizobium* and *Bradyrhizobium* spp. may increase the yield of host legumes as a result of nodule formation in plant roots. In the nodules, the bacteria fix atmospheric nitrogen and export ammonium to the host legume, thus providing part of the nitrogen the plant requires.

Among other factors, field legume inoculation with *Rhizobium* and *Bradyrhizobium* spp. is mainly restricted by the presence in the soil of native strains capable of nodulating the host legumes (10, 27, 34). This is particularly evident in the sites of origin of host legumes, where specific rhizobia are abundant, e.g., *Rhizobium leguminosarum* bv. phaseoli in Mexican soils (1). Failure to introduce *Rhizobium* strains may be overcome by the introduction of highly competitive strains or by using very high rates of application of inoculated strains (2, 22, 23). The latter is not economically feasible. The traditional method for obtaining *Rhizobium* strains with improved properties has been the selection of naturally occurring field isolates that best exhibit the trait desired. An alternative approach is to construct improved *Rhizobium* strains by genetic transfer of symbiotically favorable determinants.

Plasmid transfer may increase nodulation or nitrogen fixation in *R. leguminosarum* bv. viciae strains (7), and there is one report of a plasmid loss that improves symbiotic properties in *Rhizobium loti* (28). In *Rhizobium meliloti*, a nonsymbiotic plasmid enhances nodulation of the strains harboring it. This plasmid may be related to a modification of exopolysaccharides and confers sensitivity to some phages (36). Succinate-sensitive strains of *Rhizobium* spp. demonstrate increased symbiotic properties and increased competitiveness for nodulation (39).

The analysis of *Rhizobium* isolates from *Phaseolus vulgaris* nodules revealed two distinct types of strains (24, 26). The genetic relatedness of both types of strains has been established (3, 29). *R. leguminosarum* bv. phaseoli type I strains have multiple copies of the nitrogenase structural genes (26, 30), produce melanine, and hybridize to the *psi* gene originally described in *R. leguminosarum* bv. phaseoli (5). Type II strains have an extended host range for nodulation. They nodulate beans as well as *Leucaena* species (26). In this paper we analyze the competitive abilities of both types of strains originally isolated from bean nodules and present the genetic construction of *R. leguminosarum* bv. phaseoli derivatives that demonstrate enhanced competitiveness for nodule formation.

MATERIALS AND METHODS

Bacterial strains. The bacteria used are listed in Table 1. Transconjugant strains were obtained as described earlier (25) by matings of donor strains (Tn*5-mob* derivatives) (35) in a 1:1 ratio with recipients with or without the helper strain HB101(pRK2013) (15). The transconjugants were selected for their resistance to neomycin (60 or 80 μg/ml) and purified as single colony isolates.

Nodulation assays. Seeds of *P. vulgaris* cv. Negro Jamapa or Negro Argel were surface sterilized and germinated on 0.8% (wt/vol) agar-water plates. Plant assays were performed at 28°C in 250-ml agar flasks or in vermiculite jars with Fahraeus medium (14). Bacteria for inocula were grown in solid individual PY plates (26) and suspended in 10 mM $MgSO_4$. Inoculum concentrations were determined by A_{600} and by total bacterial counts by use of a Neubauer microscope counting chamber. Cell numbers were adjusted in suspensions to formulate the mixtures of inoculum strains, and final cell numbers were verified by serially diluting the inocula in 10 mM $MgSO_4$–0.01% (vol/vol) Tween 40 and plating on PY medium to count CFU. About 10^7 bacteria were added to each plant rootlet. Nitrogenase activity was measured by the acetylene reduction assay of whole roots (26), and total numbers of nodules were obtained from plants inoculated with individual strains.

Identification of bacteria in competition assays. Surface-sterilized nodules were crushed in PY plates, and single

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TABLE 1. Bacterial strains used in this study

Strain	Host plant	Relevant characteristics[a]	Source or reference[b]
R. leguminosarum bv. phaseoli type I			
CFN42	*P. vulgaris*	Sm^r derivative (100 μg/ml)	30
CFN42pb		CFN42(pCFN299b::Tn*5-mob*)	This study
CFN42pa		CFN42(pCFN299a::Tn*5-mob*)	
CFN279	*P. vulgaris*		26
CFN279pb		CFN279(pCFN299b::Tn*5-mob*)	This study
Viking I	*P. vulgaris*	Sm^r derivative (100 μg/ml)	31
Viking Ipb		Viking I(pCFN299b::Tn*5-mob*)	This study
TAL182	*P. vulgaris*		B. B. Bohlool
TAL182pb		TAL182(pCFN299b::Tn*5-mob*)	This study
CFN402	*P. vulgaris*		This study
CFN42-18		CFN42::Tn*5-mob* (in chromosome)	S. Brom
R. leguminosarum bv. phaseoli type II			
CFN299	*P. vulgaris*	Sp^r (100 μg/ml)	25
CIAT899	*P. vulgaris*	Rif^r (100 μg/ml)	40
C-05 I	*P. vulgaris*		M. Tsai
Car22	*P. vulgaris*		M. Tsai
CFN2993		Donor strain CFN299, with Tn*5-mob* in plasmid pb (225-kilobase non-Sym plasmid)	This study
Rhizobium strain			
CFN244	*M. gibbosifolium*	Nod^+ Fix^+ in *P. vulgaris*	26
CFN249	*D. leporina*	Nod^+ Fix^+ in *P. vulgaris*	26
CFN265	*L. esculenta*	Nod^+ Fix^+ in *P. vulgaris*	26
CFN401	*C. ternantea*	Nod^+ Fix^- in *P. vulgaris*	3

[a] Sm, Streptomycin; Rif, rifampin; Sp, spectinomycin.
[b] Sources: B. B. Bohlool, NifTAL Project, Paia, Hawaii; S. Brom, Departamento de Genética Molecular, Centro de Investigación sobre Fijación de Nitrógeno, Cuernavaca, Morelos, México; M. Tsai, Universidade de Sao Paulo, Sao Paulo, Brazil.

colonies were picked and tested for growth in selective media for strain identification as previously described (25).

Statistical analysis. Four replicate jars (two plants per jar) were used per treatment, and these were arranged in a completely randomized design. Twenty randomly selected nodules from each jar were analyzed (10 per plant). In competition experiments, analyses of variance were done to compare the percentage of nodules formed by the original and the derivative strains, using angular transformation of percentage data.

Profiles, purification, and hybridization of plasmids. Plasmid patterns were visualized by the procedure of Eckhardt (13). Plasmid b from type II strain CFN299 was isolated from *Agrobacterium tumefaciens* A2 (25) by the procedure of Hirsch et al. (20). The plasmid was purified by CsCl density gradient centrifugation and was used as a probe for hybridization against blots of plasmid profiles as described previously (16).

RESULTS

Competitive abilities of strains of *Rhizobium* spp. that nodulate *P. vulgaris*. Table 2 shows the percentage of *P. vulgaris* L. bean nodules formed by type I and type II strains inoculated at different ratios. At a 1:1 ratio of inoculation, more than 90% of the nodules in the Negro Jamapa and in the Negro Argel bean cultivars were formed by type I strains. Inocula with greater numbers of organisms of type II strain CFN299 are needed to increase its percent nodule occupancy. The minimal number of organisms in the inoculum required to form one nodule in beans is similar for both types, around 4 to 20 bacteria per plant, as determined by inoculating serial dilutions of bacteria from both types of strains independently. Nodules appeared on day 4 after inoculation for both types of strains, and there was no significant difference in total number of nodules formed by type I or type II strains when tested independently (data not shown).

There are various *Rhizobium* sp. strains isolated from tropical legumes not closely related to beans that are able to form effective nodules in beans (26). These strains are genetically different from type I and type II isolates (29), and they may be recovered from bean nodules in field cultures (26). We tested the competitive abilities of some of these strains. Strains CFN244, CFN249, CFN401, and CFN265,

TABLE 2. Competition of type I and type II strains for nodule occupancy in *P. vulgaris* L. bean

Ratio inoculated (type I:type II)	Strains in inoculum		Strains occupying nodules (%)		
	Type I	Type II	Type I	Type II	Types I + II
1:1	CFN42	CFN299	90	10	0
	CFN42	CIAT899	100	0	0
	CFN279	CIAT899	97	0	3
	CFN402	CIAT899	77	10	12
	Viking I	C-05 I	100	0	0
	Viking I	Car22	75	0	25
	Viking I	CFN299	98	2	0
	Viking I	CIAT899	100	0	0
1:5	CFN42	CFN299	62	38	0
1:15	CFN42	CFN299	42	42	16
1:150	CFN42	CFN299	2	90	8

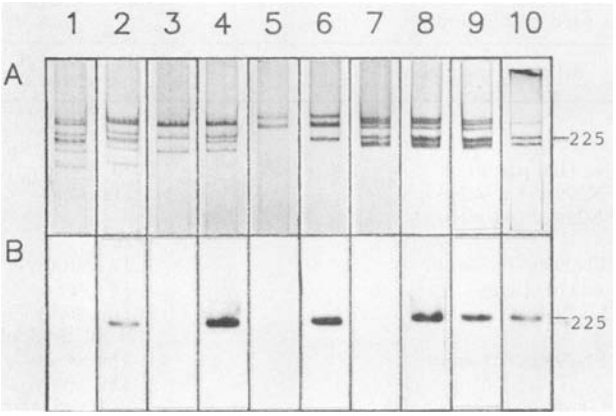

FIG. 1. (A) Plasmid profiles of parental type I and type II *R. leguminosarum* bv. phaseoli strains and transconjugants. Lanes: 1, CFN279; 2, CFN279pb; 3, TAL182; 4, TAL182pb; 5, Viking I; 6, Viking Ipb; 7, CFN42; 8, CFN42pb; 9, CFN42pb (7-month subculture); 10, type II donor strain CFN299. (B) Autoradiogram of the plasmid profiles hybridized with purified plasmid b (225 kilobases) of CFN299. Molecular weight markers are indicated in kilobases.

originally isolated from *Macroptilium gibbosifolium*, *Dalea leporina*, *Clitoria ternantea*, and *Leucaena esculenta*, respectively, did not form any nodules when tested in a 1:1 ratio in competition with any of the type I or type II strains (data not shown).

Nodulation and competitive abilities of type I transconjugant strains. Type I *R. leguminosarum* bv. phaseoli CFN42, Viking I, TAL182, and CFN279 were genetically modified by the transfer of a 225-kilobase plasmid from type II strain CFN299. The frequency of transfer of plasmid b from CFN2993 to type I strains was around 10^{-3} transconjugants per recipient when using Tn*5-mob*. The plasmid patterns of the parental strains and transconjugants are presented in Fig. 1. All transconjugants carried an additional plasmid corresponding to plasmid pb of CFN299.

Sixteen days after inoculation, the average numbers of nodules of 11 plants were 51.9 for parental strains and 72.7 for transconjugant strains (per plant) in flasks. On average, therefore, 40% more nodules were obtained with the transconjugants on *P. vulgaris* both in flasks and in jars. Analysis of variance showed that the difference between the total number of nodules induced by transconjugants and the total number induced by parental strains was highly significant ($P \leq 0.01$). The increase in nodulation efficiency may explain the significantly greater percentage of nodules formed by the transconjugants when tested in competition with the original strains in cultivars Negro Jamapa and Negro Argel (Table 3).

In another type of experiment, the parental strain and the modified strain were each tested against a third strain (Table 4). For example, while strain CFN42 did not form any nodules when tested with Viking I in a 1:1 ratio, strain CFN42pb formed approximately 15% of the total number of nodules when tested with Viking I under the same conditions. The ratio of CFN42 to Viking I must be increased 10- to 100-fold for CFN42 to form approximately 10% of the total nodules in the presence of Viking I. Similar results were obtained with Viking I and Viking Ipb against the more competitive strain CFN279pb (Table 4).

Other transconjugants were obtained by the transfer of the symbiotic plasmid from *R. leguminosarum* bv. phaseoli type II strain CFN299 to type I strains CFN42 and Viking I. Nine donor CFN299 derivatives were used, each harboring a different Tn*5-mob* insertion in the symbiotic plasmid. All nine of the different transconjugants with double symbiotic plasmids (CFN42pc) strains had significantly reduced competitive abilities (Table 5). Plasmid a of CFN299 appeared to be more or less neutral in a type I background, since it did not significantly change the competitive ability of the strain harboring it, i.e., CFN42pa. This was also the case with a Tn*5* insertion in the chromosome, indicating that Tn*5* is not responsible for the effects on competition.

TABLE 3. Competition of transconjugants and parental strains for nodule occupancy in *P. vulgaris* L. bean

Ratio inoculated (parent: transconjugant)	Strains in inoculum		Strains occupying nodules (%)			
	Parent	Transconjugants	Parent	Transconjugants	Both	SE
1:1	CFN42	CFN42pb	18	67	15	±8.4[b]
	CFN42	CFN42pb[a]	53	39	8	±3.5
	CFN279	CFN279pb	26	66	8	±9.5[c]
	TAL182	TAL182pb	35	60	5	±4.0[b]
2:1	CFN42	CFN42pb	38	55	7	±3.7
	Viking I	Viking Ipb	15	70	15	±3.2[c]

[a] Transconjugant tested after a 7-month subculture.
[b] Significantly more nodules were formed by the transconjugant strain at the 99% confidence level.
[c] Significantly more nodules were formed by the transconjugant strain at the 95% confidence level.

TABLE 4. Bean nodule occupancy of strains and transconjugants

Strains in inoculum		Ratio inoculated	% of nodules showing occupancy by:			
Parent-reference	Transconjugant-reference		Parent or transconjugant strain	Reference strain	Both	SE
CFN42-Viking I		1:1	0	100	0	±0.0
CFN42-Viking I		10:1	2	98	0	±2.7
CFN42-Viking I		100:1	34	66	0	±3.3
Viking I-CFN279pb		1:1	9	91	0	±1.8
	CFN42pb-Viking I	1:1	17	83	0	±10.4
	Viking Ipb-CFN279pb	1:1	26	62	12	±12.5

TABLE 5. Competition of derivatives and parental strains for nodule occupancy in *P. vulgaris* (bean)

Strains in inoculum[a]		% of nodules showing occupancy by:			
Parent	Derivative	Parent	Derivative	Both	SE
CFN42	CFN42pc[b]	95	4	1	±1.4[c]
CFN42	CFN42pa	41	59	0	±3.1
CFN42	CFN42-18	50	40	10	±10.0
CFN42	CFN42pb	18	67	15	±8.4[c]

[a] Ratio inoculated (parent:derivative), 1:1.
[b] Nine different derivatives were tested, each harboring the symbiotic plasmid of CFN299 with different Tn*5-mob* insertions.
[c] The derivative strain was significantly different from the parent strain at the 99% confidence level.

Instability of transconjugants. After a 7-month subculture, the genetically modified strain CFN42pb no longer had enhanced competitiveness for nodule formation (Table 3). This instability was not related to the loss of plasmid b since both the resistance to neomycin and the plasmid profile in the transconjugant were maintained (Fig. 1). A new transconjugant was constructed which again had the previously observed advantage for nodule formation (data not shown).

DISCUSSION

A majority (around 90%) of strains isolated from bean nodules in agricultural fields correspond to what we have designated *R. leguminosarum* bv. phaseoli type I strains (24, 26). This greater prevalence may be due to the greater competitive ability of type I strains as compared with type II strains, as reported here. The greater competitive ability of type I over type II strains cannot be explained by a bacteriocinogenic or bacteriostatic effect of type I over type II strains (data not shown).

There is a wide range of competitive abilities among type I strains. Strain CFN42 is a poor competitor, especially against strain Viking I (Table 4). Viking I has been described as a very good competitor in U.S. soils (31), and TAL182 is a good competitor in Hawaiian soils (B. B. Bohlool, personal communication).

The competition of strains for nodulation on beans has been evaluated by de Oliveira and Graham by a different approach which uses a Fix^- natural mutant as a reference strain (7a). Of 62 strains, 7 were identified as both superior in competitive ability and in nitrogen-fixing activity (7a). We analyzed five of these seven strains (data not shown) and found that all five correspond to type I strains.

Interstrain competition for nodule formation may not be a permanent or universal characteristic of a strain but rather a result of interactions among biotic (4, 10, 18, 33) and abiotic factors (6, 22, 42) which allow for the selection of particular genotypes (strains) best suited for nodule formation under certain conditions. Thus, inoculants derived from indigenous strains have an advantage over introduced strains (9). A general characteristic of type II isolates is their resistance to acid conditions. *Rhizobium* strains are normally very sensitive to acid conditions (40). In addition, some of the type II strains are able to grow at 37°C. At this temperature, most type I isolates do not grow (data not shown). One may predict that, in acid conditions or at high temperatures, type II strains will have an advantage over type I strains in competition assays.

In another approach, it has been shown that the production of a toxin by *Rhizobium* strains allows them to be highly competitive by inhibiting the growth of other *Rhizobium* strains (37, 38). A nodulation inhibition effect has been observed among *R. leguminosarum* bv. viciae strains, and nodulation genes from the inhibiting bacteria seem to be responsible for this phenomenon (11, 12). It is important to identify other genetic markers that can give strains a selective advantage for competition. One of them seems to be plasmid b of strain CFN299.

The biological explanation of how plasmid pb from an inherently less competitive type II strain could improve nodulation competitiveness of a more competitive type I strain must await further research. Type I and type II strains differ greatly both chromosomally and plasmidwise, and they may have different strategies for plant interaction and for nodulation. Whether plasmid b increases the copy number of any gene(s) involved in the nodulation or differentiation process or whether it alters the regulation or function of those genes or their products in type I strains is at present only a matter of speculation.

In an *A. tumefaciens* plasmidless strain, plasmid b allows transconjugants carrying the symbiotic plasmid of CFN299 to nodulate faster, to form more nodules, and to fix more nitrogen in *P. vulgaris* L. bean. *A. tumefaciens* transconjugants with only the symbiotic plasmid have a 4-day delay for nodulation, form 30% of nodules, and fix around 10% of the nitrogen as compared with the original CFN299 strain. With plasmid b, on the other hand, there is only a 2-day delay before nodulation and transconjugants make 50% of the nodules and fix around 25% of the nitrogen that the original strain does (25). When plasmid b is transferred to type I *R. leguminosarum* bv. phaseoli strains, nodulation efficiency and competitiveness for nodule formation improve. The nitrogen-fixing capacity of the nodules formed by the transconjugants remains unchanged or improves slightly (data not shown).

We have previously shown that type I and type II symbiotic plasmids belong to different incompatibility groups since both plasmids are stably maintained in *A. tumefaciens* (25). *R. leguminosarum* bv. phaseoli type I strains, with two symbiotic plasmids with the same specificity (for bean nodulation), have a diminished competitive ability. Other reports indicate that there may be symbiotic interference in strains with double symbiotic plasmids (8, 19, 21, 32, 41).

Genomic rearrangements have been reported to occur frequently in *R. leguminosarum* bv. phaseoli (17). At present we do not know if such genomic instability could be related to the unstable enhanced competitiveness of transconjugants harboring plasmid pb. This instability may be an advantage from an ecological point of view because it allows further introductions of *Rhizobium* strains into the soil.

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