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# Narrow- and Broad-Host-Range Symbiotic Plasmids of *Rhizobium* spp. Strains That Nodulate *Phaseolus vulgaris*

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***Agrobacterium*** **transconjugants containing symbiotic plasmids from different** ***Rhizobium*** **spp. strains that nodulate** ***Phaseolus vulgaris*** **were obtained. All transconjugants conserved the parental nodulation host range. Symbiotic (Sym) plasmids of** ***Rhizobium*** **strains isolated originally from** ***P. vulgaris*** **nodules, which had a broad nodulation host range, and single-copy nitrogenase genes conferred a Fix$^+$ phenotype to the** ***Agrobacterium*** **transconjugants. A Fix$^-$ phenotype was obtained with Sym plasmids of strains isolated from** ***P. vulgaris*** **nodules that had a narrow host range and reiterated** ***nif*** **genes, as well as with Sym plasmids of strains isolated from other legumes that presented single** ***nif*** **genes and a broad nodulation host range. This indicates that different types of Sym plasmids can confer the ability to establish an effective symbiosis with** ***P. vulgaris*****.**

Bacteria of the genus *Rhizobium* are capable of interacting with plant roots to form nitrogen-fixing nodules. *Rhizobium leguminosarum* biovar *phaseoli* effectively nodulates *Phaseolus vulgaris*. The classification of the biovar is based largely, but not entirely, on host plant specificity. Results of studies from several laboratories (2, 4, 16) suggest that bacteria capable of nodulating *P. vulgaris* form a very heterogeneous group. Native isolates from *P. vulgaris* nodules differ in their nodulation host range and in nitrogenase (*nif*) gene copy number (13). Type I strains have a narrow nodulation host range and repeated *nif* genes, while type II strains have a broad host range, which includes *Phaseolus* and *Leucaena*, and have single-copy *nif* genes (13, 14). In several instances strains isolated from other tropical legumes (*Dalea leporina* and *Pachyrhizus erosus*) are able to elicit an effective symbiosis with beans (13). Some isolates from *Clitoria ternantea* or *Glycine max* cv. Jupiter form an ineffective symbiosis with *Phaseolus vulgaris* (see below).

The genetic information required for nodulation and nitrogen fixation in *Rhizobium* strains is encoded in plasmids known as symbiotic (Sym) plasmids (5, 9, 10). The Sym plasmids of different strains have been transferred to another plant-interacting bacterium, *Agrobacterium tumefaciens*. Some of the transconjugants induced the formation of nodulelike structures (8, 11, 19, 20). Recently, it has been shown that an *Agrobacterium* transconjugant carrying the Sym plasmid of strain CFN299 (originally isolated from *P. vulgaris*) forms effective nodules on beans (12).

We present here results of an analysis of the Sym plasmids from eight *Rhizobium* strains isolated from *P. vulgaris* and other legumes. Each strain was able to nodulate and, in most cases, fix nitrogen with *P. vulgaris*. The Sym plasmids were transferred to an *Agrobacterium tumefaciens* plasmidless strain, and the symbiotic properties of the transconjugants were studied. These experiments allowed us to analyze the functional capacity of the Sym plasmids from the different strains with the same chromosomal background, devoid of the influence of other plasmids present in the *Rhizobium* parent strains.

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To isolate *A. tumefaciens* transconjugants containing *Rhizobium* Sym plasmids, the different *Rhizobium* strains (Table 1) were marked with Tn*5-mob* by mating them with strain S17-1(pSUP5011) (18) and selecting for nalidixic acid (20 μg/ml), kanamycin (30 μg/ml), and neomycin (30 μg/ml) resistances. Transconjugants were obtained by mobilization to *A. tumefaciens* GMI9023 (17) of random Tn*5-mob* mutants of each strain by using plasmid pJB3JI (3) as a helper for mobilization, except in the case of strain CFN299, whose Tn*5-mob*-marked plasmids were mobilized without a helper. The transconjugants were selected in LB medium containing rifampin (100 μg/ml), kanamycin (30 μg/ml), and neomycin (30 μg/ml). Transconjugants were used to inoculate *P. vulgaris* plants en masse. *P. vulgaris* cv. Negro jamapa seeds were surface sterilized as described previously (13) and grown in 250-ml Erlenmeyer flasks with the agar medium described by Fahraeus (7), without added nitrogen, at 28°C. Plants were grown for 15 days. The number of nodules per plant was very variable, probably due to the fact that a mixture of transconjugants was used for inoculation. Single colonies were isolated from the nodules after cells diluted in 10 mM MgSO4–0.01% (vol/vol) Tween 40 were plated.

The phenotypic markers of approximately 400 colonies isolated from nodules formed by each of the different strains were checked. All were rifampin, kanamycin, and neomycin resistant and produced 3-ketolactose. The 3-ketolactose production was assayed as described previously (1) by using liquid cultures in BYLA medium (13). This indicates that the cells that induced nodule formation were indeed the *Agrobacterium* transconjugants, as parental *Rhizobium* strains which failed to produce 3-ketolactose were never recovered from the nodules.

The plasmid profiles of the original *Rhizobium* strains (Fig. 1, lanes 1, 3, 5, 7, 10, 12, 14, and 16) and those of the transconjugants (Fig. 1, lanes 2, 4, 6, 8, 9, 11, 13, 15, 17, and 18) were determined by the procedure of Eckhardt (6). The Sym plasmid was identified because it conferred on the *Agrobacterium* transconjugants the ability to form nodules (see below) and also hybridized to *nif* gene sequences on Eckhardt (6)-type gels (data not shown). The Sym plasmid of some of the strains used was identified previously (12, 15).

TABLE 1. Bacterial strains and plasmids used in this study

| Strains and plasmids | Isolated from: | Source or reference[a] |
|---|---|---|
| *Rhizobium* | | |
| CFN42 | *Phaseolus vulgaris* | 16 |
| CFN285 | *Phaseolus vulgaris* | 13 |
| CFN299 | *Phaseolus vulgaris* | E. Martinez |
| CIAT899 | *Phaseolus vulgaris* | P. Graham |
| CFN249 | *Dalea leporina* | 13 |
| CFN400 | *Clitoria ternantea* | E. Martinez |
| CFN401 | *Pachyrhizus erosus* | E. Martinez |
| USDA191 | *Glycine max* cv. Jupiter | H. H. Keyser |
| *Agrobacterium* | | |
| GMI9023 | | 17 |
| GMI9023(pCFN42*d*) | | This work |
| GMI9023(pCFN285*b*) | | This work |
| GMI9023(pCFN299*a*,*c*) | | This work |
| GMI9023(pCIAT899*b*) | | This work |
| GMI9023(pCIAT899*a*,*b*) | | This work |
| GMI9023(pCFN249*a*) | | This work |
| GMI9023(pCFN400*a*) | | This work |
| GMI9023(pCFN401*b*) | | This work |
| GMI9023(pUSDA191*b*) | | This work |
| *Escherichia coli* | | |
| S17-1(pSUP5011) | | 18 |
| 1830(pJB3JI) | | 3 |

[a] Sources: E. Martinez, Departamento de Genetica Molecular, Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Cuernavaca, Morelos, Mexico; Peter Graham, Centro Internacional de Agricultura Tropical, Cali, Colombia; and Harold H. Keyser, Nitrogen Fixation and Soybean Genetics Laboratory, Beltsville Agricultural Research Center, Beltsville, Md.

*Agrobacterium* transconjugants derived from strains CFN 42, CFN285, CFN249, and CFN401 contained only the Sym plasmid. Some of the strain CIAT899 transconjugants contained another plasmid, in addition to the Sym plasmid, while others contained the Sym plasmid alone. All the transconjugants of strain CFN299 contained both the Sym and another plasmid. The symbiotic properties of strain CFN299 have been analyzed previously (12). It was shown that the same phenomenon of plasmid coinheritance was observed in all cases after the selection of *Agrobacterium* transconjugants through plant passage. Note that the *Agrobacterium* transconjugants of this particular strain were constructed without a helper plasmid. A possibility is that one of the other indigenous plasmids of the *Rhizobium* strain mobilizes the Sym plasmid.

In the case of *Rhizobium fredii* USDA191, some transconjugants with deletions in the Sym plasmid (Fig. 1, lane 18) were recovered. This deleted Sym plasmid hybridized with *nif* gene sequences on Eckhardt (6)-type gels (data not shown).

The *Rhizobium* strains and *Agrobacterium* transconjugants were assayed for their capacity to form nodules on *P. vulgaris* and *Leucaena leucocephala* var. K-8. The nodulation assay used for *L. leucocephala* was similar to that used for *P. vulgaris*.

All transconjugants were able to nodulate *P. vulgaris* (Table 2), although the number of nodules was always 70 to 80% lower than those in the parental strains. The nodules produced by strain GMI9023(pCFN42*d*) were analyzed by light microscopy. Infected cells, vascular bundles, and starch grains were observed (results not shown). The transconjugants conserved the host range of the *Rhizobium* parent. Transconjugants from strains CFN249, CFN299, CIAT899, and CFN400 nodulated *L. leucocephala*, while transconjugants from CFN42, CFN285, and CFN401 were unable to form nodules on this plant. The only exception was strain USDA191, whose transconjugants did not nodulate *L. leucocephala*. This may have been due to the poor nodulation shown by the parental *Rhizobium* strain (approx. 1 nodule per plant).

*P. vulgaris* whole nodulated roots were assayed for acetylene reduction at day 15 after inoculation, as described previously (13) (Table 2). Transconjugants carrying the Sym plasmids of type II strains CFN299 and CIAT899 were able to fix nitrogen. The results from strain CFN299 are in complete agreement with those previously reported by Martinez et al. (12). This nitrogen-fixing capacity was seen only when the plants were incubated at 28°C. At 21°C, nodulation was greatly diminished and the acetylene reduction capacity disappeared (data not shown) (12). We found that *Agrobacterium* sp. strain CIAT899 transconjugants resembled those of strain CFN299 in some characteristics such as their acetylene reduction activity and the frequent coinheritance of a 185- to 200-kilobase-pair plasmid.

On the other hand, nodules formed by transconjugants of type I strains CFN42 and CFN285 had a barely detectable acetylene reduction activity. The nodules formed by the

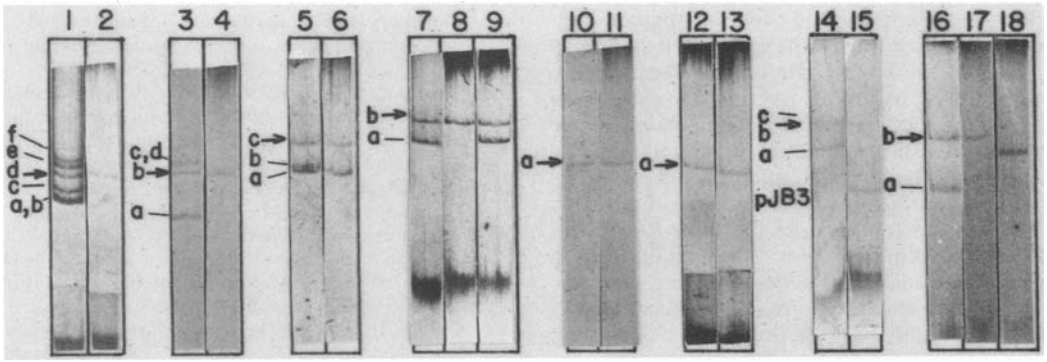


FIG. 1. Plasmid electrophoretic pattern of *Rhizobium* strains and *Agrobacterium* transconjugants on 0.7% agarose gels stained with ethidium bromide. Lanes: 1, CFN42; 2, GMI9023(pCFN42*d*); 3, CFN285; 4, GMI9023(pCFN285*b*); 5, CFN299; 6, GMI9023(pCFN299*a*,*c*); 7, CIAT899; 8, GMI9023(pCIAT899*b*); 9, GMI9023(pCIAT899*a*,*b*); 10, CFN249; 11, GMI9023(pCFN249*a*); 12, CFN400; 13, GMI9023(pCFN400*a*); 14, CFN401; 15, GMI9023(pCFN401*a*); 16, USDA191; 17, GMI9023(pUSDA191*b*); 18, GMI9023(pUSDA191*b*). Arrows indicate localization of the Sym plasmids.

TABLE 2. Nodulation and nitrogen fixation in *P. vulgaris* and *L. leucocephala* by *Rhizobium* strains and *Agrobacterium* transconjugants isolated from *P. vulgaris* nodules

| Strain | Nodulation in: | | Nitrogen fixation in *P. vulgaris*[c] |
|---|---|---|---|
| | *P. vulgaris*[a] | *L. leucocephala*[b] | |
| CNF42 | + | − | 100% |
| GMI9023(pCFN42*d*) | + | − | 2% |
| CFN285 | + | − | 100% |
| GMI9023(pCFN285*b*) | + | − | 1% |
| CFN299 | + | + | 100% |
| GMI9023(pCFN299*a*,*c*) | + | + | 21% |
| CIAT899 | + | + | 100% |
| GMI9023(pCIAT899*b*) | + | + | 9% |
| GMI9023(pCIAT899*a*,*b*) | + | + | 11% |
| CFN249 | + | + | 100% |
| GMI9023(pCFN249*a*) | + | + | ND |
| CFN400 | + | + | ND |
| GMI9023(pCFN400*a*) | + | + | ND |
| CFN401 | + | − | 100% |
| GMI9023(pCFN401*a*) | + | − | ND |
| USDA191 | + | + | ND |
| GMI9023(pUSDA191*b*) | + | − | ND |

[a] Nine plants were tested.
[b] Three plants were tested.
[c] Total acetylene reduction activity per plant in the transconjugants is reported compared with the corresponding values for the parental strain. The total activity of strain CFN42 was defined as 1: the relative activities of the other parental strains were as follows: CFN285, 1.2; CFN299, 3.6; CIAT899, 1.8; CFN249, 1.5; CFN401, 7.8. ND, Not detectable.

transconjugants of strains CFN249, CFN400, CFN401, and USDA191 (originally isolated from other legumes) had no reducing activity, although strains CFN249 and CFN401 formed nitrogen-fixing nodules on *P. vulgaris*. *Leucaena* nodules were not assayed for acetylene reduction.

The Sym plasmids of strains that nodulate *P. vulgaris* differ in *nif* gene copy number and nodulation host range (13, 14). The data presented here indicate that the plasmids also differ in the functional capacity conferred on the *Agrobacterium* transconjugants that carry them. Strains that were isolated originally from *P. vulgaris* nodules were of two types. Plasmids of type I strains have reiterated *nif* genes and a narrow host range (13) and confer only a barely detectable nitrogen fixation ability on the *Agrobacterium* transconjugants. Type II strains have single-copy *nif* genes and a broad nodulation host range (13) and confer a significant nitrogen fixation activity on transconjugants. The Sym plasmids of strains isolated from other legumes differed among themselves in the capacity of effective nodulation in *P. vulgaris*. They resembled those of type II strains, because they carry single *nif* genes (13), but differed because no nitrogenase activity was exhibited by the *Agrobacterium* transconjugants. Taken together, these data indicate that the genes necessary for the establishment of an effective symbiosis with *P. vulgaris* are found in different types of plasmids. Two of them were found in strains isolated originally from *P. vulgaris* nodules, and the others were found in *Rhizobium* sp. strains isolated from other legumes. It will be interesting to know whether these different types of Sym plasmids require a specific chromosomal background, a plasmid background, or both to optimally express themselves.

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