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Genetic Rearrangements of a *Rhizobium phaseoli* Symbiotic Plasmid

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Different structural changes of the Sym plasmid were found in a *Rhizobium phaseoli* strain that loses its symbiotic phenotype at a high frequency. These rearrangements affected both *nif* genes and Tn*5 mob* insertions in the plasmid, and in some cases they modified the expression of the bacterium's nodulation ability. One of the rearrangements was more frequent in heat-treated cells, but was also found under standard culture conditions; other structural changes appeared to be related to the conjugal transfer of the plasmid.

The genus *Rhizobium* comprises the gram-negative bacteria that form nodules on legumes. In this association, the bacteria fix atmospheric nitrogen that is then assimilated by the plant.

The genetic information controlling symbiotic activity in the fast-growing rhizobia is encoded in plasmids (10, 11, 13, 19). A symbiotic (Sym) plasmid has been defined as one that determines the plant species specificity for nodulation and contains the nitrogenase enzyme structural genes (*nif* genes) (12).

Plasmids participate very frequently in recombination events (14, 20). It has been proposed (27) that this plasticity enables the bacteria that harbor plasmids to adapt to different environmental changes and permits the rapid spread of newly created functions among very diverse bacteria. There is one report (24) of a change in the structure of a plasmid that resulted in modified metabolic activity in the recipient bacteria. This strongly suggests that plasmid plasticity is important in the generation of new functions in bacteria.

In *Rhizobium phaseoli*, the nitrogen fixation gene sequences are reiterated (25). In strain CFN42, there are three regions of the Sym plasmid that contain nitrogenase structural genes (*nif* regions) (22). These three regions contain the nitrogenase reductase gene (*nifH*); the nucleotide sequence of the three copies is identical (26). In addition, two of the regions contain also *nifD* and *nifK* genes. The identity of the *nifH* genes suggests that a recombination event could be involved in the generation or maintenance of their reiteration. Reiterated sequences are not common in bacteria, but they have been found in some strains of *R. phaseoli*, *Rhizobium trifolii*, and *Rhizobium japonicum* (2) and also *Streptomyces* sp. (21), *Halobacterium halobium* (29), and *Pseudomonas syringae* p.v. "*phaseolicola*" (32). The presence of these reiterations may be related to the instability and genetic rearrangements of these organisms (1, 6, 23).

We have found that symbiotically unstable isolates are very common among the *R. phaseoli* strains isolated in different regions of Mexico, and all have reiterated *nifH* genes (L. Castrejón and G. Soberón, manuscript in preparation). We suppose that the symbiotic instability of these *R. phaseoli* strains is due to genetic rearrangements caused by the presence of reiterated sequences. We report here that the loss of the symbiotic phenotype of an unstable *R. phaseoli* strain is due to changes in the structure of its Sym plasmid, and that this Sym plasmid can be involved in different rearrangements which modify the information it carries.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are shown in Table 1. The important characteristics of their isolation are described below.

Growth conditions. PY medium (18) was used to grow *R. phaseoli* strains, unless otherwise stated. *Agrobacterium tumefaciens* and *Escherichia coli* strains were grown in LB medium (17). Both *R. phaseoli* and *A. tumefaciens* strains were grown at 30°C, and *E. coli* strains were grown at 37°C. The antibiotic concentrations used were as follows: cloramphenicol, 30 μg/ml; erythromycin, 50 μg/ml; gentamicin, 40 μg/ml; kanamycin, 60 μg/ml; rifampin, 30 μg/ml; spectinomycin, 100 μg/ml; streptomycin, 200 μg/ml; and tetracycline, 10 μg/ml.

Construction of pLS151. Plasmid pLS151 is a intermediary vector derived from plasmid pSUP205 (31) carrying Sp^r. pLS151 was constructed by inserting a *Bam*HI fragment conferring resistance to spectinomycin from plasmid R702 (15) into the *Bgl*II site of the *nifH* coding frame from *R. phaseoli* in plasmid pEM15 (E. Morett, manuscript in preparation); pEM15 carries a 4.9-kilobase (kb) *Eco*RI fragment comprising most of *R. phaseoli* CFN42 *nifH* region a (25, 26) subcloned in the *Eco*RI site of pSUP205. Quinto et al. (26) described the use of intermediary vectors similar to pLS151 to obtain interrupted *nifH* genes.

Construction of strain CFN2414. Strain CFN2414 was constructed by mobilization of plasmid pLS151 in a triparental mating with strain CFN2314 (Table 1) by using plasmid pRK2013 (8) as a helper plasmid; kanamycin- and spectinomycin-resistant transconjugants were isolated and scored for tetracycline sensitivity. Since the origin of replication of plasmid pSUP205 is not functional in *R. phaseoli*, the kanamycin-, spectinomycin-, and tetracycline-resistant transconjugants have the pLS151 plasmid cointegrated by a single recombination event with one of the *nif* regions in the CFN2314 Sym plasmid, whereas the transconjugants that are tetracycline susceptible have one of the wild-type *nif* regions in the CFN2314 Sym plasmid substituted by the mutated fragment in pLS151 in a double recombination event.

One such tetracycline-susceptible transconjugant was further analyzed in Southern blot hybridization experiments of total DNA digested with *Bam*HI versus an *nifH* specific probe and found to have the spectinomycin resistance DNA

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TABLE 1. Bacterial strains and plasmids

Strains or plasmid	Relevant characteristics[a]	Source or reference
Bacteria		
R. phaseoli CFN23	Nod$^+$ *nifH*$^+$	Field isolate from Mexico
R. phaseoli CFN2370[b]	Nod$^-$ Δ*nifH* Str^r	This work
R. phaseoli CFN2315[b]	Nod$^+$ *nifH*$^+$ Rif^r Km^r	This work
R. phaseoli CFN2350[b]	Nod$^-$ Δ*nifH* Rif^r Km^s	This work
R. phaseoli CFN2314[b]	Nod$^+$ *nifH*$^+$ Rif^r Km^r	This work
R. phaseoli CFN2414[b]	Nod$^+$ *nifH*$^+$ Rif^r Km^r Sp^r	This work
R. phaseoli CFN2340[b]	Nod$^-$Δ*nifH* Rif^r Km^r Sp^r	This work
A. tumefaciens GMI9023	Vir$^-$ (without plasmids) Str^r Rif^r	(28)
A. tumefaciens CFN2302[c]	Nod$^+$ *nifH*$^+$ Rif^r Str^r Km^r	This work
A. tumefaciens CFN2303[c]	Nod$^-$ *nifH*$^+$ Rif^r Str^r Km^r	This work
A. tumefaciens CFN2304[c]	Nod$^+$ *nifH*$^+$ Rif^r Str^r Km^r	This work
A. tumefaciens C58C1	Vir$^-$ Ery^r Cam^r	(5)
E. coli HB101	*recA hsdR hsdM*	(3)
Plasmids		
pLS151[d]	Vector for *nifH* sequences; carries Sp^r	This work
pRK2013	Tra$^+$ Km^r (not transposable); unable to replicate in rhizobia	(8)
pSUP5011	pBR325::Tn*5 mob* Km^r Ap^r Cm^r	(30)
pJB3J1	Tra$^+$ Tc^r Gm^r Km^s	(4)

[a] Abbreviations used: Nod, ability to nodulate beans; Vir, tumor-forming ability; Tra, conjugal transfer ability; *mob*, gene encoding the ability to be mobilized by some transferable plasmids; *nifH*, nitrogenase reductase gene. Resistances to rifampin (Rif^r), kanamycin (Km^r), erythromycin (Ery^r), cloramphenicol (Cam^r), tetracycline (Tc^r), gentamicin (Gm^r), and spectinomycin (Sp^r) and susceptibility to kanamycin (Km^s) and spectinomycin (Sp^s) are indicated. *recA* mutants are unable to recombine; *hrdR* and *hsdM* mutants are unable to modify or restrict DNA.

[b] Derivatives of CFN23, described in the text.

[c] GMI9023 transconjugants in matings with CFN23 derivatives, described in the text.

[d] The construction of this plasmid is described in Materials and Methods.

inserted in the 9.8-kb *Bam*HI fragment that carries one of the *nifH* genes. This strain was called CFN2414 (see Results).

Heat treatment. *R. phaseoli* strains were heated by a modification of the procedure described by Zurkowski (33) to cure *R. trifolii* plasmids. A fresh culture of the bacteria was streaked in YM medium (33) and incubated for 5 days at 37°C. Single colonies were isolated at 30°C and tested for nodulation or antibiotic resistance. To determine the frequency of nonnodulating derivatives, 50 colonies were independently tested on bean plants; to test antibiotic susceptibility, 100 colonies were replicated onto agar plates with and without the antibiotic.

Matings. Matings were done as described by Quinto et al. (26). Tn*5 mob* insertions were isolated from a triparental mating between HB101(pRK2013), HB101(pSUP5011), and CFN23 Rif^r (Table 1).

The mobilization of the Tn*5 mob* insertions in the Sym plasmid was done by using HB101(pJB3JI) (Table 1) as a helper plasmid or with HB101(pRK2013) (Table 1) in a triparental cross. In the matings where Nod$^+$ transconjugants were infrequent, the nodulation of beans was used for selection; transconjugants were purified from nodules and tested again for their nodulation ability. When pJB3JI was used to mobilize plasmids marked with Tn*5 mob*, tetracycline-susceptible transconjugants were isolated to avoid the inheritance of the helper plasmid.

The frequency of CFN23 Sym plasmid transfer is about 10^{-6} per donor cell, but when the Sym plasmid Tn*5 mob* insertions were mobilized by pJB3JI or pRK2013 (Table 1), the frequency of kanamycin-resistant transconjugants was about 10^{-4} per donor cell.

Isolation and manipulation of DNA. Isolation of DNA, radioactive labeling, and Southern blot hybridization were carried out as reported by Quinto et al. (25). *nifH* and Tn*5* probes were those described previously (26). None of these probes hybridized with the total DNA of *A. tumefaciens* GMI9023 (Table 1).

Plasmid visualization. Plasmid visualization was done by the Eckhardt (7) procedure. The Sym plasmid was purified from strain CFN2302 (Table 1) by the Hirsch et al. (9) procedure and banded in a CsCl gradient containing ethidium bromide. The plasmid molecular weight was calculated from the comparison with *R. phaseoli* CFN42 plasmids run in the same agarose gel.

Nodulation. Conditions for nodulation of beans were as reported by Martínez et al. (16).

RESULTS

Instability of the symbiotic properties of *R. phaseoli* CFN23. *R. phaseoli* strains isolated in different regions of Mexico frequently lose their symbiotic phenotype at a high frequency after they are incubated at 37°C, a treatment that has been used to cure *Rhizobium* sp. plasmids (33). One of these strains, CFN23, was studied in detail in this work.

Strain CFN23 lost its symbiotic phenotype at a frequency of 62% after growth at 37°C for 5 days (Table 2). We asked whether the loss of the nodule-forming ability correlated with a loss of the symbiotic (Sym) plasmid, but the plasmid electrophoretic pattern of strain CFN23 was identical to the pattern of several of its nonnodulating (Nod$^-$) derivatives

TABLE 2. Percentage of loss by heat treatment[a]

Strain	% of strains with indicated phenotype		
	Km^s	Sp^s	Nod$^-$
CFN23			62
CFN2315	76		NT[b]
CFN2314	<1		74
CFN2414	<1	50	NT[b]

[a] Growth on petri dishes for 5 days at 37°C. See footnote *a* of Table 1 for abbreviations.

[b] NT, Not determined directly, but all the antibiotic-susceptible derivatives tested were Nod$^-$ and lacked the three *nifH* copies.

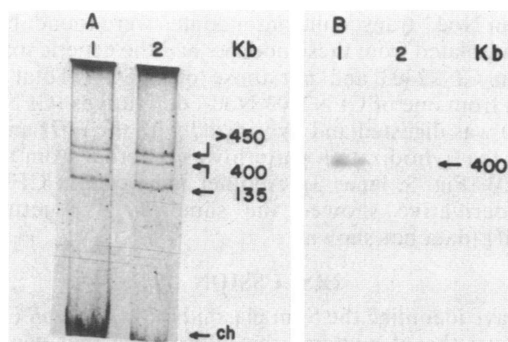

FIG. 1. (A) Plasmid electrophoretic pattern of wild-type and cured strains on 0.7% agarose gels stained with ethidium bromide and (B) hybridization of this plasmid profile with the 0.9-kb *nifH* probe. Lanes: 1, CFN23; 2, CFN2370. Numbers correspond to molecular sizes of plasmids in kb. ch, Chromosomal DNA.

(data not shown). Nevertheless, the loss of the *nifH* genes in all Nod$^-$ derivatives tested was demonstrated by DNA hybridization. An example of this is shown in Fig. 1, which shows the plasmid electrophoretic pattern and the absence of the *nifH* homologous sequences in the CFN23 Nod$^-$ derivatives CFN2370 (Table 1).

Deletion of the Sym plasmid in the CFN23 Nod$^-$ derivatives. We could not detect any change in the molecular weight of the CFN23 Sym plasmid when the three *nifH* genes were lost (Fig. 1). A deletion of up to 50 kb would not be seen in a 400-kb plasmid, so we looked for the presence of the replicon by using the whole Sym plasmid as a probe in hybridization experiments with the plasmid profiles of strains CFN23 and CFN2370 and in Southern blot experiments. We found that strain CFN2370 conserved a plasmid homologous to the CFN23 Sym plasmid (data not shown).

A Tn*5* *mob* insertion in the CFN23 Sym plasmid was isolated; the CFN23 derivative with this insertion is called CFN2315. The Sym plasmid location of this Tn*5* *mob* insertion is shown in Fig. 2, lanes 2. The frequency of loss of the kanamycin resistance of CFN2315 was determined after heat treatment, and a frequency of kanamycin-susceptible derivatives similar to that of loss of nodulation capacity in CFN23 was found (Table 2). In Fig. 2, lanes 4, the relevant characteristics of one of these kanamycin-susceptible derivatives (CFN2350, Table 1) are shown. By the criteria used, CFN2350 is identical to CFN2370 (Fig. 1, lanes 2). The frequency of kanamycin-susceptible derivatives of CFN2315 when grown at 30°C was 1%; this is a much lower value than at 37°C (Table 2) but sufficiently high to become a problem if CFN23 is used as an inoculant in the field.

When the plasmid marked with Tn*5* *mob* in CFN2315 was mobilized to a plasmidless *A. tumefaciens* strain (GMI9023, Table 1), the transconjugants gained the ability to nodulate beans. The nodules made by these transconjugants were similar to those made by CFN2315. Leghemoglobin was present in both instances (data not shown). One of these transconjugants (CFN2302, Table 1) was studied in detail; we found that it contains a single plasmid with the same molecular weight as the CFN23 Sym plasmid, and that *nifH* and Tn*5* probes gave the same hybridization pattern as CFN2315 DNA (Fig. 2, lanes 2 and 3).

Isolation of a stable Tn*5* *mob* insertion in the CFN23 Sym plasmid. A stable Tn*5* *mob* insertion in the Sym plasmid was isolated in strain CFN2314 (Table 2). We conclude that the Tn*5* *mob* insertion in CFN2314 was located in the Sym plasmid, because both Tn*5* and *nifH* probes hybridized with the same plasmid band as in CFN2315 (Fig. 2, lanes 2 and 5).

To show that the Tn*5* *mob* insertion in CFN2314 was indeed in the same plasmid as the *nifH* genes (i.e., the Sym plasmid), we determined the genetic linkage of these sequences. We constructed a derivative of CFN2314,

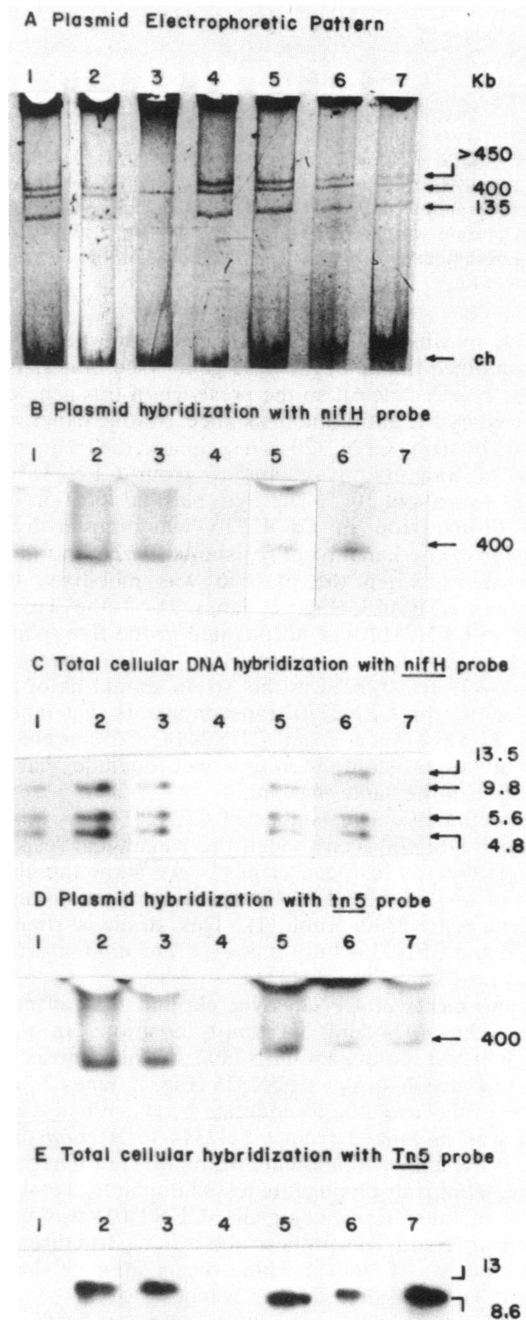

FIG. 2. Hybridization patterns of plasmid profiles and total cellular DNA digests of CFN23 and derivatives: (A) plasmid electrophoretic patterns on 0.7% agarose gels, Southern blot hybridization probed with *nifH* of (B) plasmid electrophoretic patterns and (C) total cellular DNA digested with *Bam*HI, and Southern blot hybridizations probed with Tn*5* of (D) plasmid electrophoretic patterns and (E) total cellular DNA digested with *Eco*RI. Lanes: 1, CFN23; 2, CFN2315; 3, CFN2302; 4, CFN2350; 5, CFN2314; 6, CFN2414; 7, CFN2340. Numbers correspond to molecular sizes of DNA sequences in kb. ch, Chromosomal DNA.

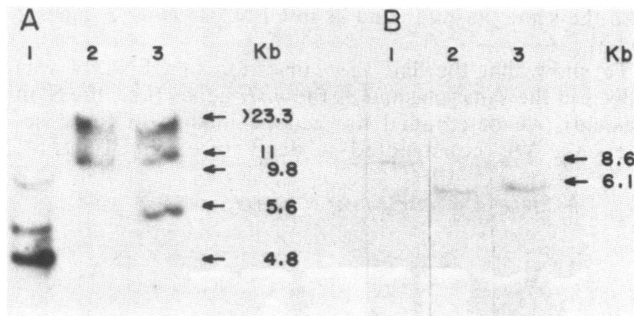

FIG. 3. Southern blot hybridization of CFN2314 and strains showing *nifH* rearrangements. (A) *nifH* was used as the probe, and DNA was digested with *Bam*HI; (B) DNA was digested with *Eco*RI and a Tn*5* probe was used. Lanes: 1, CFN2314; 2, CFN2303; 3, CFN2304. Numbers correspond to the sizes of the hybridizing fragments in kb.

CFN2414, in which spectinomycin resistance determinant was recombined in one of the *nifH* genes (see Materials and Methods; Fig. 2, lane 6), so the presence of this gene could be followed by the antibiotic resistance. Mating experiments were done by using CFN2370 as a recipient; the frequency of transfer of kanamycin resistance from CFN2315 and CFN2314 was about 10^{-6}. The Sym plasmid location of the Tn*5* *mob* insertion in CFN2315 was shown by the contransfer of the kanamycin resistance, *nifH*, and nodule-forming ability when the plasmid was mobilized to *A. tumefaciens* GMI9023 (Fig. 2, lanes 3). If the Tn*5* *mob* insertion in CFN2414 was not located in the Sym plasmid, then the frequency of contransfer of kanamycin and spectinomycin resistances in this strain should be of about 10^{-12}. Among the CFN2370 transconjugants that inherited kanamycin resistance from CFN2414, 76% were also spectinomycin resistant; therefore we conclude that both markers are in the same plasmid.

The spectinomycin resistance of CFN2414 was unstable after heat treatment even though the kanamycin resistance was not (Table 2). In Fig. 2, lanes 7, we show the characteristics of one of the CFN2414 spectinomycin-susceptible derivatives (CFN2340, Table 1). This strain is similar to CFN2370 and CFN2350 but retains the Tn*5* *mob* insertion in the same position as in CFN2314.

Rearrangements of CFN23 Sym plasmid that affect *nifH* position. The *nifH* and Tn*5* *mob* positions in the *A. tumefaciens* transconjugant CFN2302 are the same as in the parent *R. phaseoli* strain, CFN2315 (Fig. 2, lanes 2 and 3), and both strains are able to nodulate beans. When the Sym plasmid was mobilized from CFN2314 to *A. tumefaciens* GMI9023, the ability to nodulate beans was not inherited by 20 independent transconjugants tested in plants. Total DNA from one of these transconjugants (CFN2303) was hybridized with *nifH* and Tn*5* probes, and a new structure of the Sym plasmid was found in which the position of the *nifH* genes and Tn*5* was altered (Fig. 3, lanes 2).

CFN2303 was unable to nodulate beans, even though it contains at least part of the Sym plasmid as judged by the presence of *nifH* and Tn*5* *mob*. We decided to mobilize the CFN2303 plasmid to other genetic backgrounds to determine whether the nodulation ability might be regained. Unexpectedly, we found that the nodulation ability could be regained when CFN2303 was used as donor in mating experiments, although no transfer of genetic material was detected. Both CFN2303 and *A. tumefaciens* C58C1 are Nod$^-$ (Table 1); a mixture of these bacteria was used to inoculate beans and to isolate an Nod$^+$ transconjugant; nodules were found, but the bacteria isolated from these nodules had the genetic markers of strain CFN2303 and not those of C58C1. Total DNA isolated from one of CFN2303 Nod$^+$ derivatives (CFN2304, Table 1) was digested and hybridized with the *nifH* and Tn*5* probes; the hybridization pattern was different from that of CFN2303 (Fig. 3, lanes 3). Another independent CFN2303 Nod$^+$ derivative showed the same *nifH* structure as CFN2304 (data not shown).

DISCUSSION

We have identified the Sym plasmid of *R. phaseoli* CFN23 and shown that it contains the information that enables a plasmidless *A. tumefaciens* strain to form nodules in association with beans.

This Sym plasmid participates in recombination events which give rise to different molecular structures. The changes in structure modify the symbiotic phenotype of the bacteria. This high frequency of genetic rearrangements that affect the symbiotic information of *R. phaseoli* could make this bacteria a very adaptable symbiont.

We found that the most frequent genetic rearrangement is represented by the Nod$^-$ derivatives of CFN23. In these derivatives the three *nifH* genes are lost, but the Sym plasmid is not segregated, and the molecular weight of the plasmid is not appreciably affected (Fig. 1 and 2). These data could be explained as resulting from a small deletion which was not detected in the plasmid profile. Nevertheless, there is evidence in another *R. phaseoli* strain (CFN42) that the three *nif* regions present in the CFN42 Sym plasmid are not closely linked, and a deletion that would remove all the nitrogenase structural genes represents a loss of approximately 120 kb of DNA (R. Palacios, personal communication). We have evidence that the Nod$^-$ derivatives of CFN23 have not only *nifH* but also all of the structural genes of the nitrogenase; if the CFN42 Sym plasmid structure is conserved in CFN23, the most frequent rearrangement found in this molecule would require a large deletion of the plasmid and the substitution for this DNA by other DNA sequences so that the molecular weight of the plasmid could be conserved. We are carrying out experiments to test this hypothesis of deletion and substitution of DNA.

The different frequences at which kanamycin resistance was lost in strains CFN2315 and CFN2314 (Table 2) reflects the frequency at which the site of insertion of the transposon in each strain participates in the Sym plasmid rearrangement described above, i.e., the Tn*5* *mob* insert in CFN2315 was deleted when *nifH* genes were lost, but the one in CFN2314 was conserved in the deleted Sym plasmid.

The Sym plasmid structure in strain CFN2303 represents another genetic rearrangement. In this molecule, the positions of the *nifH* genes and of the Tn*5* *mob* insertion are different from those in the plasmid of the parent strain CFN2314, although this alteration cannot be explained by a single recombination event. It might be the product of multiple events which generate unstable intermediates. We do not know whether the CFN2303 Sym plasmid structure is formed during the conjugal transfer of this plasmid or whether it represents a small proportion of the plasmids present in the CFN2314 population that are more frequently transferred. If the second possibility is true we will be able to isolate a CFN2314 derivative that would be Nod$^-$ and retain the *nifH* genes. The Sym plasmid structure in CFN2303 could be also a product of rearrangements in *A. tumefaciens*. We are now looking for its presence in different rhizobial backgrounds.

The bacteria harboring the CFN2303 plasmid are unable to nodulate beans, but Nod$^+$ derivatives can be isolated in which the position of the *nifH* genes is altered (strain CFN2304, Fig. 3). This suggests that the Sym plasmid structure of strain CFN2303 does not allow the expression of the nodulation genes, although this information is present in the plasmid. The Nod$^+$ derivatives of strain CFN2303 could only be obtained when this strain was used as donor in a genetic cross, even though no transfer of genetic information was detected.

All of the rearranged Sym plasmid derivatives that hybridize with the *nifH* probe have more than one copy of this gene (CFN2303 and CFN2304, Fig. 3), and we found that the different arrangements of this gene are very similar to those present among wild-type *R. phaseoli* isolates (16). We conclude from these data that the *nifH* reiteration is a characteristic of *R. phaseoli* Sym plasmids and that the Sym plasmid rearrangements found under laboratory conditions also happen in nature.

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