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# Reiteration of Nitrogen Fixation Gene Sequences and Specificity of *Rhizobium* in Nodulation and Nitrogen Fixation in *Phaseolus vulgaris*

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We have previously reported that *Rhizobium phaseoli* has multiple copies of nitrogen-fixation gene sequences. In this work we extend our analysis to cover a broader range of *R. phaseoli* strains and other rhizobia isolated from different legumes. Our results indicate that most *R. phaseoli* strains have reiterated *nifH* sequences. Reiterations are also found in rhizobia from nodules of other *Phaseolus* species and from the close relative of *Phaseolus, Pachyrhizus*. However, *nifH* gene reiteration is not always found in the rhizobia able to nodulate and fix nitrogen in *Phaseolus*, since some strains isolated from nodules of *Phaseolus vulgaris* as well as from other legumes are able to establish an effective symbiosis with *Phaseolus vulgaris* and do not show reiterations. We propose that there are different evolutionary lines of *R. phaseoli*. The reiteration of *nif* genes may be considered a marker for the most abundant and specialized of these evolutionary lines.

## INTRODUCTION

Bacteria of the genus *Rhizobium* induce nodules and fix nitrogen in the roots of legumes. *Rhizobium* species have been defined according to the host they infect. This classification gathers into a group different bacteria that may share only a certain nodulation ability. *Rhizobium phaseoli*, the symbiont of *Phaseolus vulgaris*, common bean, has been catalogued as a very heterogeneous group (Beynon & Josey, 1980; Roberts *et al.*, 1980; Catteau *et al.*, 1984). We have described a peculiar organization of nitrogen fixation genes in *R. phaseoli* characterized by the presence of stable reiterations (Quinto *et al.*, 1982). In *R. phaseoli* strain CFN-42, there are three different regions in its symbiotic plasmid that contain identical and complete nitrogenase reductase coding sequences (*nifH*). Site-directed mutagenesis indicated that none of the three *nifH* gene copies is indispensable for nitrogen fixation during symbiosis with *Phaseolus vulgaris* (Quinto *et al.*, 1985). To establish how general *nifH* gene reiterations are in *R. phaseoli*, we extended our studies to strains isolated from diverse types of *Phaseolus vulgaris* and from other *Phaseolus* species. We analysed strains from different geographical origins with an emphasis on strains from Mesoamerica because *Phaseolus vulgaris* had its origin and was diversified in this region (Miranda, 1967; Gentry, 1969).

Reiterations of *nif* genes have been reported in bacteria other than rhizobia (Rice *et al.*, 1982; Kallas *et al.*, 1983; Scolnick & Haselkorn, 1984). To understand further *nif* gene reiterations, and because of a possible correlation between the evolutionary trends of plant and *Rhizobium*, fast-growing rhizobia were isolated from various legumes, the latter chosen because of their taxonomic relationships. In this paper we describe the symbiotic properties of these strains and analyse their *nifH* gene organization patterns.

## METHODS

*Bacterial strains. R. phaseoli* strains were isolated from bean nodules obtained from important native agricultural areas of Mexico: Jalisco, Hidalgo, Morelos and Guanajuato. *R. phaseoli* strains are listed in Table 1. *Rhizobium* strains from other legumes are listed in Table 2. To test for *Agrobacterium* contamination, all *Rhizobium* isolates were assayed for 3-ketolactose production (Bernaerts & De Ley, 1963) using Benedict's reagent and cultures grown in BYLA medium. BYLA contained, per litre, 10 g lactose, 0·5 g $K_2HPO_4$, 0·1 g $MgSO_4.7H_2O$, 0·2 g $CaCl_2$, 50 mg $FeCl_3.6H_2O$, 3 g yeast extract and 15 g agar. None of the *Rhizobium* strains gave a positive reaction.

*Isolation of Rhizobium strains.* Bacteria were isolated from active nitrogen-fixing nodules from legumes growing in the field or grown from seeds in the laboratory in soils, and under conditions, that resembled their natural habitats. Nodules were surface sterilized and tested for surface contamination. Nodule isolates were grown in peptone/yeast medium, PY (Noel *et al.*, 1984). Strains from *Centrosema* and *Vigna*, which do not grow well in PY, were grown in yeast/mannitol medium, YM (Vincent, 1970) that contained 1 g of yeast extract per litre. Isolated colonies were serially diluted in 10 mM-$MgSO_4$, 0·01% (v/v) Tween 40 (Kuykendall & Elkan, 1976) and plated for further colony purification.

*Nodulation assays.* Seeds of different legumes were surface sterilized (Wacek & Brill, 1976). Flasks of various sizes (depending on the size of the plants) were used, containing nitrogen-free plant nutrient solution (Wacek &

Table 1. *Rhizobium strains and pattern of nifH gene reiterations*

| Strain no. | Origin* | *nifH* gene hybridization pattern† | Strain no. | Origin* | *nifH* gene hybridization pattern† |
|---|---|---|---|---|---|
| *Strains isolated from cultivated Phaseolus vulgaris* | | | | | |
| CFN 1 | | I | Nitragin USA 8184 | Nitragin[a] | I |
| CFN 2 | | I | Nitragin USA 8251 | Nitragin[a] | I |
| CFN 5 | | I | Brazil 10 | Brazil | II |
| CFN 6 | | I | UMR1020 | Brazil[b] | – |
| CFN 7 | | I | UMR1026 | Brazil[c] | – |
| CFN 10 | | I | Viking 1 | USA[d] | III |
| CFN 17 | | I | CIAT 896 | Colombia | II |
| CFN 20 | | I | CIAT 899 | Colombia | – |
| CFN 22 | | II | Brazil 281 | Brazil[e] | II |
| CFN 23 | | I | | | |
| CFN 25 | | III | *Strains from other legumes‡* | | |
| CFN 36 | | I | CFN 226 | | II |
| CFN 38 | | II | CFN 227 | | I |
| CFN 39 | | I | CFN 238 | | IV |
| CFN 42 | | I | CFN 251 | | I |
| CFN 44 | | I | CFN 307 | | III |
| CFN 81 | | II | CFN 308 | | I |
| CFN 88 | | I | CFN 309 | | I |
| CFN 90 | | I | CFN 245 | | I |
| CFN 272 | | I | | | |
| CFN 273 | | I | | | |
| CFN 276 | | I | | | |
| CFN 277 | | I | | | |
| CFN 278 | | II | | | |
| CFN 279 | | II | | | |
| CFN 280 | | I | | | |
| CFN 281 | | I | | | |
| CFN 283 | | II | | | |
| CFN 284 | | II | | | |
| CFN 275 | | V | | | |
| CFN 285 | | IV | | | |
| CFN 286 | | IV | | | |

* Unless otherwise indicated, strains were isolated by us, and were from Mexico. *a*, obtained from Nitragin Co., Milwaukee, Wis., USA; *b*, *c*, obtained from Dr P. Graham, Dept of Soil Science, University of Minnesota St Paul, Minn., USA (*b* originally from Dr C. Vidor as C18 and *c* originally from Dr T. Saito as CENA 183); *d*, obtained from Dr E. Schmidt, Dept of Soil Science, University of Minnesota; *e*, obtained from Dr J. Döbereiner, EMBRAPA, SNLCS, Rio de Janeiro, Brazil.

† Hybridization patterns are defined in Fig. 1; – only a single *nifH* hybridization band observed.

‡ Includes strains from wild-type *Phaseolus vulgaris*. See Table 2 for original host plants.

Table 2. *Original hosts of Rhizobium strains isolated from legumes other than cultivated Phaseolus vulgaris*

| Strain no. | Original host legume |
|---|---|
| CFN 225 | *Acacia albida* |
| CFN 255 | *Centrosema pubescens* |
| CFN 264 | *Crotalaria mollicula* |
| CFN 241 | *Crotalaria pumila* |
| CFN 231 | *Dalea leporina* |
| CFN 232 | *Dalea leporina* |
| CFN 233 | *Dalea leporina* |
| CFN 242 | *Dalea leporina* |
| CFN 243 | *Dalea leporina* |
| CFN 249 | *Dalea leporina* |
| CFN 265 | *Leucaena esculenta* |
| CFN 234 | *Leucaena leucocephala* |
| CFN 244 | *Macroptilium gibbosifolium* |
| CFN 246 | *Medicago denticulata* |
| CFN 245 | *Pachyrhizus erosus* |
| CFN 226 | *Phaseolus acutifolius* |
| CFN 227 | *Phaseolus acutifolius* |
| CFN 251 | *Phaseolus acutifolius* |
| CFN 238* | *Phaseolus coccineus* |
| CFN 307 | *Phaseolus vulgaris*, wild-type |
| CFN 308 | *Phaseolus vulgaris*, wild-type |
| CFN 309 | *Phaseolus vulgaris*, wild-type |
| CFN 253 | *Vicia sativa* |
| CFN 254 | *Vigna vexillata* |

* Obtained from Dr N. Amarger, Laboratoire de Microbiologie des Sols, INRA, Dijon, France, as DC 11; isolated in France.

Brill, 1976) and 0·8% (v/v) agar. Inoculation was as described by Noel *et al.* (1984). Sometimes modified Leonard 'bottle jars' (Vincent, 1970) with vermiculite were used. All controls were devoid of nodules.

*Acetylene reduction assays.* Nitrogenase activity was measured by acetylene reduction (Wacek & Brill, 1976). Whole root systems were incubated for 1 h in sealed 25 ml vials containing 2% (v/v) acetylene. Samples were injected into a Packard 430 gas chromatograph with a Poropak N 80–100 column.

*DNA hybridization.* DNA was obtained from the different *Rhizobium* strains and digested with *Bam*HI restriction endonuclease. Total DNA digests were subjected to electrophoresis in 1% agarose gels and then transferred to nitrocellulose filters (Southern, 1975). Hybridization was performed under low stringency conditions, as reported previously (Quinto *et al.*, 1982). A recombinant plasmid (pCQ152) carrying a 300 bp sequence from the coding region of the *nifH* gene from *R. phaseoli* CFN-42 was used as probe; this fragment codes for amino acids 28 to 119 of nitrogenase reductase (Quinto *et al.*, 1985).

## RESULTS

*Most R. phaseoli strains have nifH gene reiterations.* Total DNA obtained from the strains isolated from *Phaseolus vulgaris* nodules was digested with *Bam*HI. Total genomic digests were subjected to agarose gel electrophoresis, blotted and hybridized with a *nifH*-specific probe from *R. phaseoli* CFN-42 (Quinto *et al.*, 1985). The strains tested included bacteria from both bush *Phaseolus vulgaris* and climbing cultivars (the latter are considered to be better nitrogen fixers: Graham, 1981), and from *Phaseolus vulgaris* wild-type. Fig. 1 shows the hybridization patterns of these strains. Pattern 1, with three bands of 9·8, 5·6 and 4·0 kb was the most common. We have also observed other triple-band patterns (Fig. 1, patterns II, III and IV) that have differences in the sizes of the bands. Pattern V shows only two bands.

In a first survey of 40 strains of *R. phaseoli*, only one did not show reiteration of *nifH*. Since this strain, CIAT 899, is aluminium and acid tolerant, we investigated whether this type of *R. phaseoli* was common in acid soils. We analysed three other acid-tolerant strains; one of them (CIAT 896) showed reiterations, the others (UMR 1020 and UMR 1026) did not. UMR 1020, UMR 1026 and CIAT 899 each have the same size *Bam*HI-generated *nifH* hybridization band.

*nifH gene reiterations are found in bacteria isolated from other species of Phaseolus and from the close relative Pachyrhizus.* Fig. 2 shows the taxonomic relationships of the legumes (Heywood, 1971) from which we isolated fast-growing strains of *Rhizobium* (Table 2). Emphasis was given

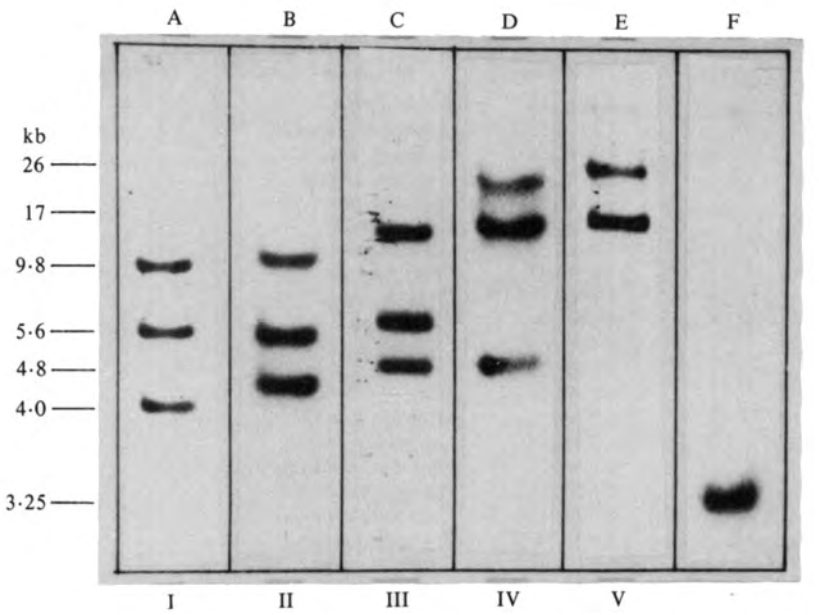


Fig. 1. Autoradiograms of Southern blots of *Bam*HI-digested genomic DNA of different *R. phaseoli* strains after hybridization with a 300 bp *nifH* internal sequence from *R. phaseoli* CFN 42. Hybridization patterns I to V are defined by the sizes of the fragments. Strains: A, CFN 42; B, CFN 279; C, CFN 25; D, CFN 285; E, CFN 275; F, CIAT 899.

to the isolation of bacterial strains from plants related to *Phaseolus vulgaris* (Marechal *et al.*, 1978). Strains from *Phaseolus coccineus, Phaseolus acutifolius* and from *Pachyrhizus* nodules showed reiterated *nifH* sequences and were able to nodulate and fix nitrogen in *Phaseolus vulgaris* (Fig. 2). The patterns of reiteration fall within the patterns obtained for *R. phaseoli* strains (Table 1). No reiterations were found among *Rhizobium* strains from other legumes including some *Phaseolus* relatives such as *Vigna* and *Macroptilium*.

*The capacity to nodulate P. vulgaris effectively is widespread among rhizobia.* Various strains from different legumes were able to nodulate and fix nitrogen in *Phaseolus vulgaris*; this capacity did not correlate with the taxonomic position of the legumes from which the bacteria were isolated (Fig. 2). Strains isolated from the subfamily Mimosoideae, from the genera *Leucaena* and *Acacia*, were able to nodulate *Phaseolus vulgaris*. Furthermore, strains from *Leucaena leucocephala* and *L. esculenta* were able to fix nitrogen in *Phaseolus vulgaris*. In contrast, strains from *Vigna*, which belongs to the same subtribe as *Phaseolus*, formed only small ineffective nodules from which bacteria could not be recovered.

*Non-specific strains that are able to fix nitrogen in bean do not have reiterated nif sequences.* Strains isolated from *Macroptilium gibbosifolium, Crotalaria pumila, Dalea leporina, Leucaena leucocephala* and *L. esculenta* established effective nodules in *Phaseolus vulgaris*, did not show reiterated sequences when probed with a *R. phaseoli* internal *nifH* fragment, and displayed different hybridization patterns that distinguished them from each other (Fig. 3). When reisolated from *Phaseolus vulgaris* nodules, the strains proved to be the same as the inoculum by the *nif* hybridization pattern (Fig. 3), the total DNA digestion pattern, and the ability to nodulate the original host legume (data not shown). The nitrogen-fixing activities of these strains in *Phaseolus vulgaris*, measured by the acetylene reduction assay, were in some cases similar to those of the *R. phaseoli* strains (data not shown).

*'Non-reiterated' R. phaseoli strains have a broad infection spectrum.* We tested the symbiotic properties of different *Rhizobium* strains using *Leucaena leucocephala* as a host. *L. leucocephala* has been described as specific for its *Rhizobium* requirements (Norris, 1967; Trinick, 1968).

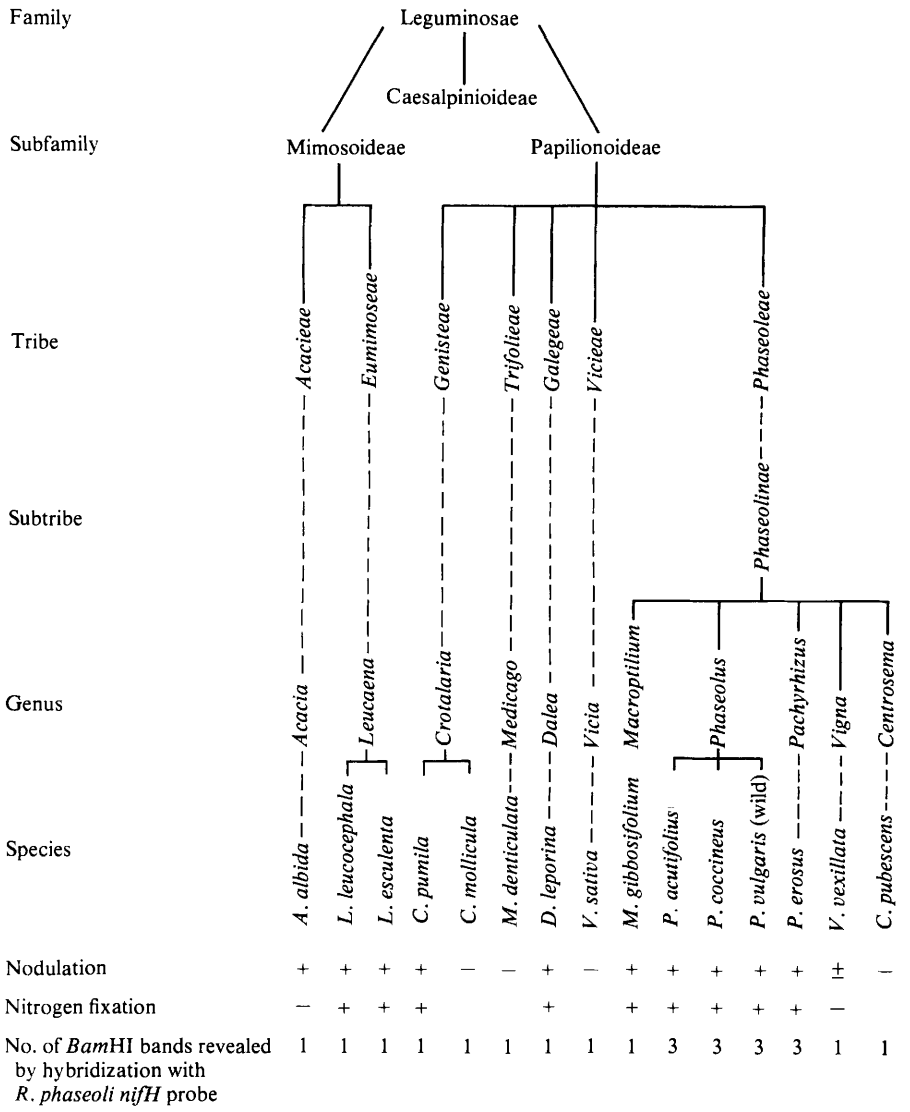


Fig. 2. Nodulation and nitrogen fixation in *Phaseolus vulgaris*, and *nifH* reiterations in rhizobia isolated from various legumes, whose taxonomic relations are shown.

'Non-reiterated' *R. phaseoli* strains, nodulated *L. leucocephala*, although not effectively: CIAT 899 (M. Megias, unpublished observation), UMR 1020 and UMR 1026, (this study). None of the '*nifH*-reiterated' *R. phaseoli* strains we tested (CFN 1, CFN 5, CFN 23, CFN 25, CFN 42) nodulated *L. leucocephala*. The non-specific *Rhizobium* strains that nodulated and fixed nitrogen in *Phaseolus vulgaris* (strains from *Dalea leporina*, *Crotalaria pumila* and *Macroptilium gibbosifolium*) nodulated *L. leucocephala* effectively, and may be considered broad-host-range strains. These strains showed single *nifH* hybridization bands.

*Under natural conditions Phaseolus vulgaris may be nodulated by strains that nodulate other legumes.* To test whether *Rhizobium* from other legumes could nodulate *Phaseolus vulgaris* under natural conditions, we grew beans in soils where wild or cultivated *Leucaena* trees grew. We isolated 12 strains from these bean nodules. About half of these strains were able to nodulate *L leucocephala* effectively and did not have reiterations of *nifH* (Fig. 4); the other isolates only nodulated bean and had the reiterated pattern of *R. phaseoli* (Fig. 4).

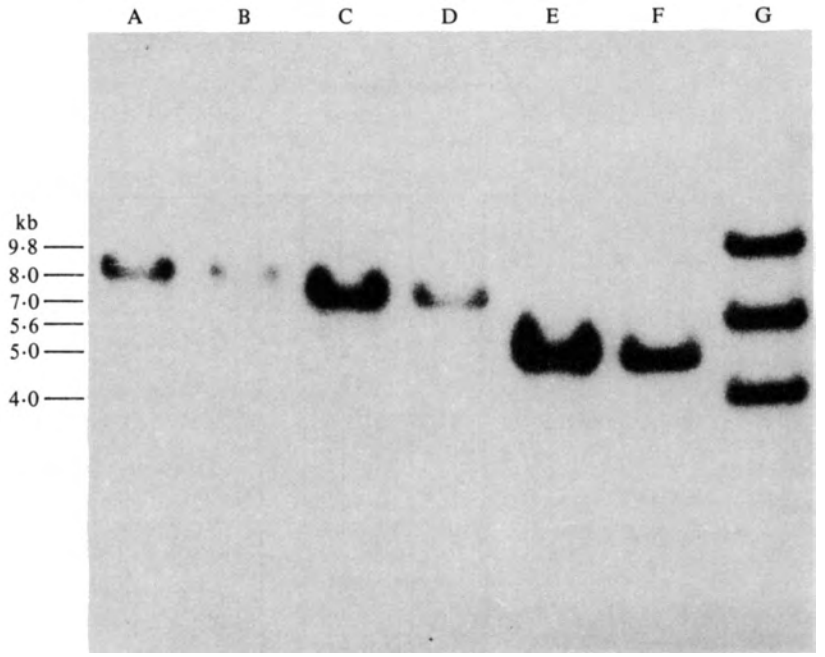


Fig. 3. Autoradiograms of Southern blots of *Bam*HI-digested genomic DNA of different *Rhizobium* strains hybridized with a 300 bp *nifH* internal sequence from *R. phaseoli* CFN 42. A, CFN 241 (from *Crotalaria pumila*); B, CFN 241 after reisolation from bean nodules; C, CFN 249 (from *Dalea leporina*); D, CFN 249 after reisolation from bean nodules; E, CFN 243 (from *Dalea leporina*); F, CFN 243 after reisolation from bean nodules; G, CFN 42, reference *R. phaseoli* strain.

## DISCUSSION

*R. phaseoli* has been defined as the *Rhizobium* that forms nitrogen-fixing nodules in *Phaseolus vulgaris*. It comprises a very heterogeneous group on the basis of protein patterns (Roberts *et al.*, 1980), plasmid profiles (Martínez & Palacios, 1984), antibiotic resistance (Beynon & Josey, 1980) and numerical taxonomy (Catteau *et al.*, 1984). Our results indicate that it is a more homogeneous group on the basis of *nif* gene reiterations. In *R. phaseoli* strain CFN 42, the three copies of *nifH* are part of a large 270 MDal plasmid (Quinto *et al.*, 1982) that also carries other symbiotic determinants (unpublished). Thus the reiterated *nif* genes are linked to the symbiotic information for bean nodulation as part of a plasmid that could be contained in different bacterial backgrounds. We have observed that randomly selected cloned fragments from the *R. phaseoli* CFN 42 symbiotic plasmid show a higher degree of conservation than cloned chromosomal fragments from the same strain when hybridized to total genome digests from other *R. phaseoli* strains (unpublished results).

Our screening of fast-growing rhizobia from different legumes of the subfamilies Papilionoideae and Mimosoideae was not intended to be comprehensive. *R. leguminosarum* and *R. trifolii* were not included, as their *nifH* gene organizations have already been established (Ruvkun & Ausubel, 1980). Our results suggest that reiterations of the *nifH* gene are restricted to rhizobia isolated from some genera of the tribe *Phaseolineae*. Results from other groups support this proposal. Strain ANU 240, a streptomycin-resistant derivative of NGR 234, has two *nifH* copies (Morrison *et al.*, 1983). This broad-host-range strain (Broughton *et al.*, 1984) was originally isolated from *Lab-lab purpureus* which belong to the tribe *Phaseolineae*. Strain NGR 234 does not nodulate *Phaseolus vulgaris* (Trinick, 1980). Recently, reiterations of *nif* genes have been reported in fast-growing *R. japonicum* (Prakash & Atherly, 1984); *Glycine max*, its host, also belongs to the tribe *Phaseolineae*. We have tested these *R. japonicum* strains for nodulation in *Phaseolus vulgaris*, and have found that they form small ineffective nodules.

Nodulation of *Phaseolus vulgaris* by a wide range of strains from tropical legumes was reported

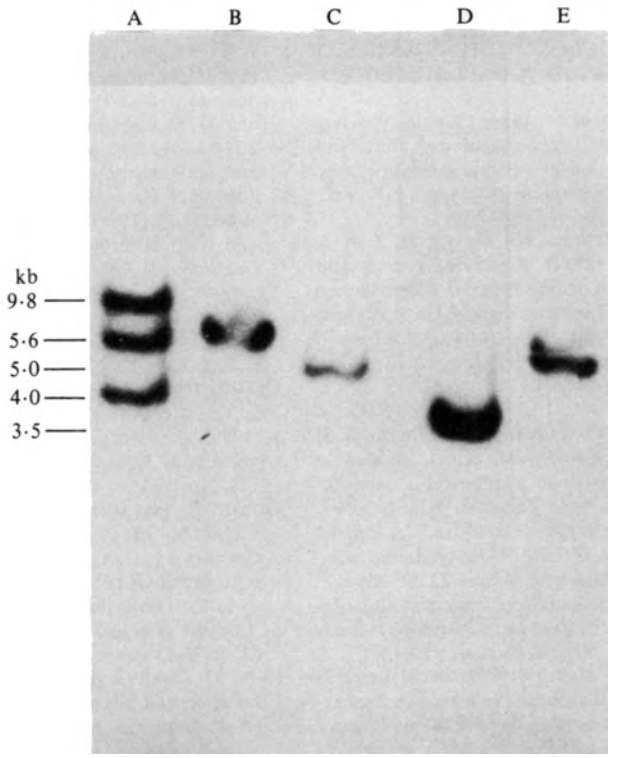


Fig. 4. *nifH* gene hybridization patterns of five strains of *Rhizobium* isolated from nodules of *Phaseolus vulgaris* grown in soils where *Leucaena* spp. grew. A, CFN 313 (this strain behaves like other '*nifH*-reiterated' *R. phaseoli*); B, CFN 288; C, CFN 289; D, CFN 311; E, CFN 290 (these strains resemble *Leucaena* symbionts in their ability to nodulate *L. leucocephala* and in having only a single copy of *nifH*).

by Lange (1961), but there were no data on the nitrogen fixation activity. Our results show that *Phaseolus vulgaris* may be nodulated effectively by non-specific *Rhizobium* strains from tropical legumes. These strains do not show *nif* reiterations. We propose that those strains isolated primarily as *R. phaseoli* and that do not have *nif* reiterations may be representatives of fast-growing rhizobia from tropical legumes that are capable of nodulating *Phaseolus vulgaris*. Indeed, we have found *Leucaena Rhizobium* strains forming nodules on bean roots in soils where *Leucaena* trees grow.

*Rhizobium* strains from *Dalea leporina*, *Crotalaria pumila* and *Macroptilium gibbosifolium*, besides nodulating their original host, also nodulate *Phaseolus* and *Leucaena* effectively. *R. phaseoli* strains CIAT 899, UMR 1020 and UMR 1026 also nodulate *Leucaena*. In contrast, *R. phaseoli* '*nifH*-reiterated' strains are more specific, nodulating effectively only *Phaseolus vulgaris* (cultivar and wild-type) and *Phaseolus coccineus* (data not shown; Graham & Halliday, 1977). If the primitive *Rhizobium* had a broad host range and evolved to become more specific for certain legumes, then the *nifH* gene reiterations may be considered a marker of this specialization in one of the evolutionary trends of *R. phaseoli*.

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