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Indigenous plasmids and cultural characteristics of rhizobia nodulating chickpeas (*Cicer arietinum* L.)

E. Cadahía, A. Leyva, and T. Ruiz-Argüeso

Departamento de Microbiología, E. T. S. de Ingenieros Agrónomos, Universidad Politécnica, E-28040 Madrid, Spain

Abstract. We examined 27 strains of chickpea rhizobia from different geographic origins for indigenous plasmids, location and organization of nitrogen fixation (*nif*) genes, and cultural properties currently used to separate fast- and slow-growing groups of rhizobia. By using an in-well lysis and electrophoresis procedure one to three plasmids of molecular weights ranging from 35 to higher than 380 Mdal were demonstrated in each of 19 strains, whereas no plasmids were detected in the eight remaining strains. Nitrogenase structural genes homologous to *Rhizobium meliloti nif*HD, were not detected in plasmids of 26 out of the 27 strains tested. Hybridization of *Eco*RI digested total DNA from these 26 strains to the *nif* probe from *R. meliloti* indicated that the organization of *nif*HD genes was highly conserved in chickpea rhizobia. The only exception was strain IC-72 M which harboured a plasmid of 140 Mdal with homology to the *R. meliloti nif* DNA and exhibited also a unique organization of *nif*HD genes. The chickpea rhizobia strains showed a wide variation of growth rates (generation times ranged from 4.0 to 14.5 h) in yeast extract-mannitol medium but appear to be relatively homogeneous in terms of acid production in this medium and acid reaction in litmus milk. Although strains with fast and slow growth rates were identified, DNA/DNA hybridization experiments using a *nif*HD-specific probe, and the cultural properties examined so far do not support the separation of chickpea rhizobia into two distinct groups of the classical fast- and slow-growing types of rhizobia.

Key words: *Cicer arietinum* – *nif* Genes – Plasmids – Rhizobia – DNA/DNA hybridization – Nitrogen fixation – Chickpeas

Cicer arietinum (chickpeas, garbanzos) is the third most widely grown grain legume in the world (van der Maesen 1972). Since the work of Raju (1936), rhizobia nodulating chickpeas are thought to be highly host-specific. Based on cross-inoculation studies, Gaur and Sen (1979) suggested that *C. arietinum* and its nodule bacteria should be considered in a separate cross-inoculation group.

Bacteria involved in the nitrogen-fixing symbiosis with leguminous plants are currently classified into two genera, *Rhizobium* (fast growers) and *Bradyrhizobium* (slow growers), based mainly upon the cross-inoculation group concept and physiological properties in yeast extract-mannitol medium (Jordan 1984). The information available regarding physiological and genetic properties of chickpea rhizobia is scarce. Strains with clearly distinct growth rates have been identified (Raju 1936; Okon et al. 1972) and this fact suggests the existence of two types of strains, each with properties characteristic of fast- or slow-growing groups of rhizobia. However, no further characterization has been carried out and the taxonomic position of chickpea rhizobia is uncertain (Vincent et al. 1979). They have been assigned to both *Rhizobium* and *Bradyrhizobium* genera (Jordan 1982, 1984). DNA homology studies have related three strains of chickpea nodule bacteria to rhizobia nodulating *Lotus* spp (Crow et al. 1981).

The presence of large plasmids is a common feature in both fast- and slow-growing groups of rhizobia (Prakash et al. 1980; Dénarié et al. 1981). However, nitrogenase structural genes (*nif*) are linked to large plasmids in fast-growing *Rhizobium* (Nuti et al. 1979; Hombrecker et al. 1981; Banfalvi et al. 1981; Rosenberg et al. 1981) but no evidence has been found for a plasmid location of *nif* genes in slow-growing *Bradyrhizobium* (Masterson et al. 1982; Haugland and Verma 1981). To our knowledge no specific reports have been published concerning the plasmid content and the location of *nif* genes in rhizobia nodulating chickpeas.

In this study, we have examined 27 strains of chickpea rhizobia from worldwide collections, for cultural characteristics currently used to separate fast- and slow-growing types of rhizobia, plasmid content and location and organization of *nif* genes, in order to obtain insights on its taxonomic position and to provide a foundation for molecular studies in these agriculturally important endosymbiotic bacteria.

Materials and methods

Bacterial strains. Twenty seven strains of rhizobia nodulating chickpeas (*Cicer arietinum* L.) were used. Strains 27a2 and 27a9 were obtained from J. C. Burton, Nitragin Company, Milwaukee, Wis, USA; strains Ca7, USAB-67, 3889-big, 3HOa1, 3HOa5, 3HOa9 and 3HOa10 from H. H. Keyser, USDA, Beltsville, Md, USA; strains 43b and 50b from R. Lobel, Volcani Center, Bet Dagan, Israel; strains CB1189 and CC1192 from J. Brockwell, SCIRO, Division of Plant Industry, Canberra, Australia; strains IC-2002, IC-2008, IC-6, IC-41, IC-60 and IC-76 from O. P. Rupela, ICRISAT, Patancheru, India; strain 522 from O.A.Berestetsky, All-Union Research Institute for Agriculture Microbiology, Leningrad, USSR; strains IC-72M and

Offprint requests to: T. Ruiz-Argüeso

IC-94M from J. E. Ruiz Sainz, University of Sevilla, Sevilla, Spain; strains UPM-Ca7, UPM-Ca36, UPM-CA116, UPM-Ca126 and UPM-Ca142 were isolated in this laboratory from nodulated chickpeas grown in Spain. Strains of *Rhizobium meliloti* Rme 41 and L5.30, *Agrobacterium tumefaciens* C58 and *Escherichia coli* 1230 (pR68-45) were supplied by J. Olivares, CSIC, Granada, Spain.

Cultural characteristics. Generation times were calculated from the exponential growth phase of cultures grown in the yeast extract-mannitol broth (YMB) medium described by Vincent (1970). Side-arm 150 ml Erlenmeyer flasks containing 30 ml of the YMB medium were inoculated with 1 ml of a culture pregrown in the same medium and incubated at 28° C in a rotary platform shaker (200 rpm). Optical density readings were measured at 550 nm with a Bausch and Lomb Spectronic 21 Spectrophotometer at intervals of 2, 4 or 6 h depending on the growth rate of the strain.

For growth reactions in litmus milk, tubes containing 10 ml of litmus milk medium (Skerman 1967) were inoculated with 0.1 ml of a exponentially growing culture in YMB, incubated for 2 weeks at 28° C and examined for pH changes, peptonization ("serum zone" formation) and reduction of litmus.

Plasmid profile analysis. Plasmids were identified by using the in-well cell lysis and electrophoresis procedure of Eckhardt (1978) as modified by Rosenberg et al. (1982), except that horizontal 0.6% agarose gels were used instead of the vertical gels, the bacteria-lysozyme and dodecyl sulfate layers were not mixed in the gel wells, and the agarose sealing step was omitted.

Plasmid molecular weights were determined from their relative mobilities in agarose gel by using pR68-45 (mol. wt. 39 Mdal, Riess et al. 1980) pRme-L5.30 (mol. wt. 91 Mdal, Casse et al. 1979), pRme-41 (mol. wt. 140 Mdal, Casse et al. 1979) and pAT-C58 (mol. wt. 273 Mdal, Rosenberg et al. 1982) as molecular weight standards. A linear relationship was found between relative mobilities and the log of molecular weights from 40 to 273 Mdal. The sizes of plasmids higher than 273 Mdal were calculated extrapolating the straight line outside the lineality interval and estimated as higher than the calculated value.

Isolation and restriction of genomic DNA. Total DNA was isolated from 10 ml of late-log-phase cultures in TY medium (Beringer 1974) as described by Corbin et al. (1982). Digestions of DNA with restriction endonuclease *Eco*RI (Boehringer Mannheim, FRG) were carried out as specified by the manufacturer. The resulting DNA fragments were separated by electrophoresis in 1% agarose gels.

Hybridization experiments. DNA from plasmid pID1 containing *nif*HD genes of *R. meliloti* (Banfalvi et al. 1981) was used for the preparation of *nif* probe. The plasmid DNA was isolated by the procedure of Birnboim and Doly (1979) and labeled with α^{32} P dATP (410 Ci/mmol, Amersham International) by nick translation (Rigby et al. 1977).

Eckhardt agarose gels used for visualization of plasmids, were dried for 1 h in a slab gel dryer (Biorad, Calif, USA), soaked for 30 min first in denaturation buffer (500 mM NaOH, 150 mM NaCl) and then in a neutralization buffer (pH 8, 500 mM Tris, 150 mM NaCl). Finally the gels were dried again for 30 min. Genomic DNA restriction fragments, electrophoresed in agarose gels were depurinated by treatment with 0.25 M HCl for 15 min and transferred onto nitrocellulose filters (Millipore, France) by the method of Southern (1975).

Hybridization of nick-translated DNA to Eckhardt dried gels and filters was done in 50% deionized formamide, 5 × SSC (1 × SSC is 0.15 M NaCl plus 0.15 M sodium citrate, pH 7.0), 1 × Denhardt solution (Denhardt 1966), 0.2% sodium dodecyl sulfate (SDS), 0.02% denatured herring sperm DNA, at 42° C for 48 h. After hybridization, gels and filters were washed four times for 30 min at 37° C in 2 × SSC, 0.1% SDS, followed by an additional wash at room temperature in 0.1 × SSC, 0.1% SDS. The gels and filters were finally dried and exposed to MAFE, RPX-ray film at − 70° C with CRONEX intensifying screens.

Table 1. Cultural characteristics of various strains of chickpea rhizobia. Abreviations and symbols: Acid, acidification; + c, clear upper serum zone; + t, turbid upper serum zone

Strains	Generation[a] time (h)	pH in YMB[b]	Changes in litmus milk	
			Reaction	Serum zone
3HOa9	4.0 ± 0.1	6.5 ± 0.1	Acid	+ c
522	4.0 ± 0.2	4.7 ± 0.2	Acid	+ c
27a9	4.1 ± 0.3	6.5 ± 0.1	Acid	+ c
27a2	4.7 ± 0.2	6.4 ± 0.1	Acid	+ c
3HOa5	4.7 ± 0.3	6.2 ± 0.2	Acid	+ c
CB1189	4.7 ± 0.1	6.4 ± 0.2	Acid	+ c
3HOa1	5.0 ± 0.2	6.3 ± 0.1	Acid	+ c
3HOa10	5.0 ± 0.2	6.5 ± 0.1	Acid	+ c
CC1192	5.0 ± 0.4	6.4 ± 0.1	Acid	+ c
IC-2002	5.1 ± 0.1	6.1 ± 0.1	Acid	+ c
3889-big	5.3 ± 0.4	6.2 ± 0.3	Acid	+ c
Ca-7	5.4 ± 0.2	6.3 ± 0.2	No change	+ t
USAB-67	5.7 ± 0.2	6.2 ± 0.3	No change	+ t
UPM-Ca7	5.7 ± 0.3	5.0 ± 0.4	Acid	+ c
IC-76	5.8 ± 0.1	4.9 ± 0.4	Acid	+ c
IC-6	5.9 ± 0.2	6.3 ± 0.2	No change	No change
IC-60	6.0 ± 0.4	5.1 ± 0.1	Acid	+ c
IC-41	6.5 ± 0.3	6.2 ± 0.1	No change	No change
UPM-Ca142	6.7 ± 0.2	4.6 ± 0.1	No change	+ t
UPM-Ca116	7.5 ± 0.4	5.1 ± 0.4	Acid	+ c
IC-2008	7.8 ± 0.1	5.9 ± 0.3	No change	No change
UPM-Ca126	7.9 ± 0.6	5.3 ± 0.2	Acid	+ c
UPM-Ca36	8.0 ± 0.2	4.7 ± 0.2	No change	No change
50b	8.0 ± 0.5	6.5 ± 0.1	No change	+ t
IC-94M	8.5 ± 0.8	6.6 ± 0.1	Acid	+ t
IC-72M	8.6 ± 0.6	6.6 ± 0.1	Acid	+ t
43b	14.5 ± 0.6	6.3 ± 0.1	No change	+ t

[a] Values are means of three replicate cultures ± SEM

[b] pH Measurements were made after 5 days of growth in yeast extract-mannitol broth (YMB) medium (pH 6.8). Values are means of at least three determinations ± SEM

Results

Cultural properties. Generation times and pH changes in YMB medium and in litmus milk by 27 strains of chickpea rhizobia are shown in Table 1. The generation times ranged from 4.0 h to 14.5 h. Although 16 strains had generation times lower than 6 h, and 5 strains exhibited clearly slow growth rates (8 h or longer), the chickpea rhizobia examined can not be divided into two exclusive groups of fast- and

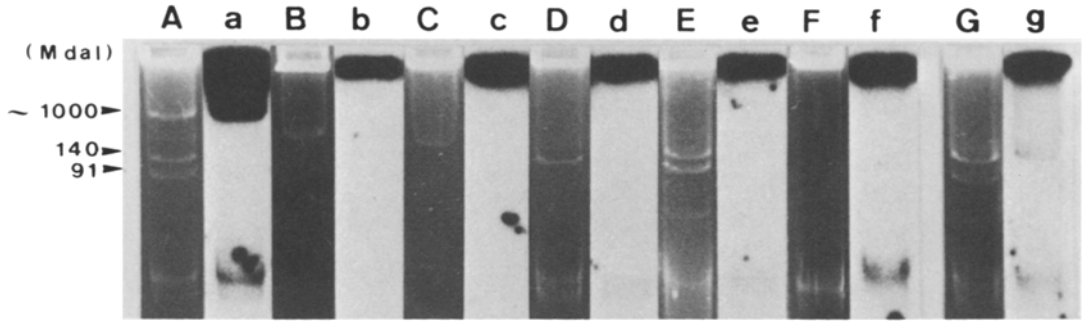

Fig. 1. Composite photograph showing hybridization of α^{32} P-labeled pID1 DNA (containing *Rhizobium meliloti nif*HD) to DNA from one strain of *R. meliloti* and six strains of chickpea rhizobia. *Capital letters* indicate photographs of ethidium bromide-stained Eckhardt gels; *small letters* indicate the corresponding autoradiograms of the dried Eckhardt gels after hybridization. *Lanes Aa*, mixture (1:1) of *R. meliloti* strains Rme-41 and L5.30 containing marker plasmids of molecular masses shown in margin; *lanes Bb to Gg* correspond to chickpea rhizobia: strain 3HOa5 (Bb), strain UPM-Ca7 (Cc), strain 43b (Dd), strain UPM-Ca36 (Ee), strain UPM- Ca116 (Ff), strain IC-72M (Gg)

slow-growers since there are strains exhibiting intermediate growth rates. All strains acidified the YMB medium after 5 days of incubation, although most of them produced slight acidification and only 9 strains lowered the pH below 6.0. No correlation was found between the magnitude of the pH changes and the growth rates. For instance, the most active acid-producing strains, 522 and UPM-Ca36, were clearly fast- and slow-growing strains, respectively. The most common pattern of changes in litmus milk was an acid reaction, production of a clear serum zone, and absence of reduction of litmus milk. Some strains did not cause observable changes in litmus milk medium.

Plasmid identification. Plasmids were consistently resolved in 19 of the 27 strains of *Cicer* rhizobia examined by the modified in-well cell lysis procedure of Eckhardt. Representative strains are shown on Fig. 1 (lanes B, C, D, E, G). The molecular weight of plasmids was estimated by their relative mobilities on agarose gels using plasmids of known molecular weights as standard. Eleven different groups of strains were identified on the basis of the number and size of the plasmids (Table 2). The sizes ranged from 36 to 233 Mdal, except for strains included in group 5 that contained a megaplasmid of molecular weight > 380 Mdal. Only one plasmid was resolved in each of the 13 strains belonging to groups 2, 3, 4, and 5. Groups from 6 to 11 were represented by only one strain containing 2 or 3 plasmids.

No plasmids were detected in the 8 strains (30%) included in group 1. Strain UPM-Ca126 (Fig. 1, lane F) is a representative example of this group. The absence of detectable plasmids was repeatedly observed, although the method used for plasmid isolation resolved cccDNA molecules of molecular weight as high as the megaplasmid of *R. meliloti* (Fig. 1, lane A) of ca. 1000 Mdal (Burkardt and Burkardt 1984). Moreover, when each of these strains showing no detectable plasmids were mixed (ratio 1:1) to a plasmid-containing strain, immediately before lysing the cells, no interference was observed with the resolution of the plasmid DNA bands of the last strain. This suggests that no DNAses or other factors were preventing the detection of cccDNA in strains of group 1.

Location of nitrogenase structural genes. In order to test whether *nif* genes of chickpea rhizobia were carried on plasmids, we separated the plasmid DNA of each strain in Eckhardt gels and directly hybridized the dried gels with a radioactive probe from pID1 DNA carrying the *nif*HD genes of *R. meliloti* (Banfalvi et al. 1981). No hybridization to pID1 DNA was obtained for plasmids of any but one of the 27 strains of chickpea rhizobia examined. Lanes Bb to Ff in Fig. 1 are representative examples of negative results.

Table 2. Number and molecular weight of indigenous plasmids in strains of rhizobia nodulating chickpeas

Strain group	Strains[a]	Number of plasmids	Molecular[b] weight ($\times 10^6$)
1	UPM-Ca116, UPM-Ca126, 3HOa9, USAB-67, 50b, IC-41, IC-60, 522	0	
2	43b	1	132 ± 4
3	UPM-Ca142, IC-2008	1	119 ± 11
4	UPM-Ca7, 3HOa10	1	233 ± 7
5	27a2, 27a9, 3HOa1, 3HOa5, CB1189, CC1192, 3889-big, IC-2002	1	> 380
6	Ca7	2	195 ± 14 83 ± 5
7	IC-94M	2	128 ± 4 47 ± 2
8	IC-72M	2	139 ± 6 91 ± 3
9	IC-76	2	94 ± 4 47 ± 4
10	UPM-Ca36	3	155 ± 5 123 ± 7 47 ± 3
11	IC-6	3	140 ± 8 60 ± 4 36 ± 3

[a] Strains within each group contained plasmids that comigrate on agarose gels

[b] Molecular weights were calculated from the relative mobilities in three separate gels using the standard plasmids described in Material and methods

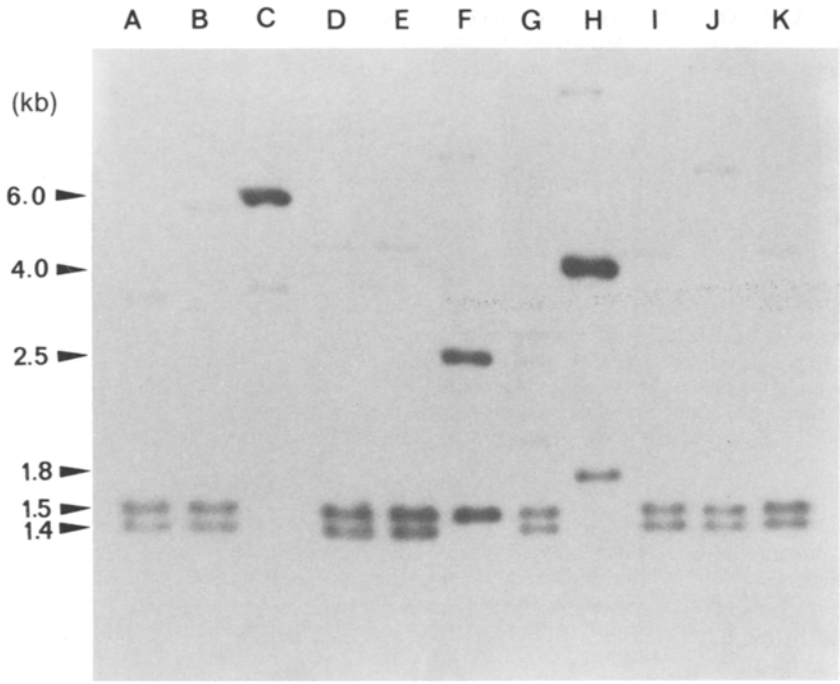

Fig. 2
Southern blots of *Eco*RI digested total DNA from one strain of *Rhizobium meliloti* and ten strains of chickpea rhizobia hybridized to α^{32} P-labeled pID1 DNA (containing *R. meliloti nif*HD). Chickpea rhizobia are on *lanes A* (strain 43b), *B* (strain IC-6), *C* (strain IC-72M), *D* (strain 3HOa5), *E* (strain UPM-Ca7), *F* (strain 3 HOa9), *G* (strain 522), *I* (strain CC1192), *J* (strain Ca 7) and *K* (strain 27a2). *Lane H: R. meliloti* strain Rme-41. The sizes (kb) of the *Eco*RI fragments calculated by using *Hind*III restricted lambda DNA as a marker are shown

Repeated hybridization attemps yielded identical results. A strong hybridization signal was always obtained to the megaplasmid of *R. meliloti* included as control (Fig. 1, lanes A, a). Autoradiograms from all strains showed specific hybridization to the chromosomal DNA retained in the well of the electrophoresis gel.

The exception was strain IC-72 M which contains a plasmid of ca. 140 Mdal (pRci72Ma) weakly hybridizing to the *R. meliloti nif* genes (Fig. 1, lanes G, g). Since this was a rare result, efforts were made to confirm the purity of the culture. Chickpea plants were inoculated with the strain IC-72M and 4 isolates obtained from nodules were analysed for plasmid profiles and location of *nif* genes. All the isolates exhibited the same plasmid profile and showed the *nif* specific signal in the same plasmid band as the inoculum culture.

The purified total DNAs from the 27 strains of chickpea rhizobia were digested with *Eco*RI and DNA fragments were separated by electrophoresis and transferred to nitrocellulose paper by the method of Southern. When Southern blots of *Eco*RI-digested total DNA were hybridized to radioactively labeled DNA from pID1, 25 out of the 27 strains exhibited identical hybridization patterns with relatively strong homology to the 1.5 and 1.4 kb fragments (Fig. 2, lanes A, B, D, E, G, I, J, K). Strain 3HOa9 showed hybridization to only one of the common fragments (1.5 kb) and to an additional fragment of 2.6 kb (Fig. 2, lane F). Strain IC-72 M, that exhibited homology to the *nif* probe on a plasmid, had a completely different hybridization pattern (Fig. 2, lane C) showing strong homology to only one *Eco*RI fragment of 6.0 kb. Weak hybridization signals were also observed to one or more *Eco*RI fragments of different sizes depending on the strain.

Discussion

Growth rates and acid production in YMB medium, as well as reaction in litmus milk, have been used to separate fast- and slow-growing groups of rhizobia (Vincent et al. 1979; Kennedy and Greenwood 1982; Jordan 1984). Fast-growing rhizobia are reported to have generation times ranging from 2 to 6 h, to produce an acid reaction in YMB and to form in litmus milk a clear upper serum zone, usually accompanied by alkalinization, although an acid reaction is characteristic of many *Rhizobium meliloti* cultures (Graham and Parker 1964). The slow-growing group exhibits generation times of 8 h or longer, does not acidify the YMB medium, and causes an alkaline reaction in litmus milk without production of serum zone. The strains of chickpea rhizobia studied by us do not match any of these two groups. Although strains with growth rates characteristic of typical fast- and slow-growing rhizobia were identified, no correlation was found between growth rates and pH changes in YMB (all strains acidified) or changes in litmus milk (none of the strains alkalinized). With regard to these two last properties chickpea rhizobia resemble fast growers. A similar lack of correlation between growth rates and pH changes has been reported by Hernández and Focht (1984) in cowpea rhizobia.

Plasmids were reproducibly resolved in approximately 70% of the examined strains of *Cicer* rhizobia. The most representative plasmid profile included strains harbouring only one plasmid of molecular weight higher than 380 Mdal. This pattern seems to correspond to that reported by Broughton et al. (1984) for three strains of *Cicer* rhizobia. In contrast, no plasmids were consistently found in the remaining 30% of strains, which included both fast- and slow-growers. The absence of discernible plasmids has also been shown in slow-growing strains of *R. japonicum* (Cantrell et al. 1982) and cowpea rhizobia (Rolfe and Shine 1984).

By using *nif*HD DNA of *R. meliloti* as hybridization probe we have obtained no evidence for a plasmid location of these *nif* genes in any of the strains of chickpea rhizobia examined, except in strain IC-72 M. Although we can not discard that *nif* genes are located in a very large plasmid that we were unable to resolve, these results suggest a chromo-

somal location of *nif*HD genes in chickpea rhizobia. A chromosomal location of structural *nif* genes has also been reported in the slow-growing *Bradyrhizobium japonicum* (Haugland and Verma 1981; Masterson et al. 1982). Unlike plasmids of fast-growing soybean rhizobia (Masterson et al. 1982; Sadowsky and Bohlool 1983) and other fast-growing strains nodulating legumes characteristically infected by slow-growing rhizobia strains (Broughton et al. 1984), plasmids from fast-growing strains of chickpea rhizobia have not shown detectable *nif* structural genes. Strain IC-72M exhibited a weak, but consistent homology to *R. meliloti nif* genes in a medium-sized plasmid. This indicates that at least some *nif* structural genes are plasmid linked in this strain. However, whether the hybridization signal is due in fact to authentic *nif* genes is currently under investigation.

Genomic Southern blots, hybridized to *R. meliloti nif*HD genes, showed that the pattern of *Eco*RI restriction fragments with homology to the *nif* probe was almost identical in 26 out of the 27 strains examined. These results indicate that in chickpea rhizobia the organization of *nif*HD genes is highly conserved regardless of the growth rates. This conservation is more remarkable in the light of the fact that the group of strains examined includes isolates from very different geographical origins like India, Mexico, Spain, Israel or USSR.

The data presented here suggest that on the basis of culture characteristics, location and *Eco*RI restriction sites conservation of *nif*HD DNA, chickpea rhizobia can not be split into two distinct groups of strains corresponding to the classical fast- and slow-growing rhizobial groups. Since, in addition, chickpea rhizobia are very highly host specific (Raju 1936; Gaur and Sen 1979), these endosymbiotic bacteria should probably be maintained in a single species.

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