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# Genomic Instability in *Rhizobium phaseoli*

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**Experience from different laboratories indicates that *Rhizobium* strains can generate variability in regard to some phenotypic characteristics such as colony morphology or symbiotic properties. On the other hand, several reports suggest that under certain stress conditions or genetic manipulations *Rhizobium* cells can present genomic rearrangements. In search of frequent genomic rearrangements, we analyzed three *Rhizobium* strains under laboratory conditions that are not considered to cause stress in bacterial populations. DNAs from direct descendants of a single cell were analyzed in regard to the hybridization patterns obtained, using as probes different recombinant plasmids or cosmids; while most of the probes utilized did not show differences in the hybridization patterns, some of them revealed the occurrence of frequent genomic rearrangements. The implications and possible biological significance of these observations are discussed.**

Bacteria of the genus *Rhizobium* interact with the roots of leguminous plants, eliciting a symbiotic process. During symbiosis, a new organ is formed, the nodule, product of the differentiation of both bacteria and plant cells. In the nodule atmospheric nitrogen is reduced to ammonia, which in turn is assimilated by the plant.

It is a common observation that *Rhizobium* strains can generate variability in colony morphology and symbiotic properties. Such observations have led to certain practices that diminish the possibilities of losing some important characteristics. Preservation of strains and its use for inoculation are frequently performed without the isolation of single colonies. Strains that are supposed to be homogeneous are commonly passed through symbiotic cycles to select clones that present the desired properties.

Several reports indicate that, when exposed to certain stress conditions or genetic manipulations, *Rhizobium* cells can undergo genomic rearrangements (1–4, 6, 9, 20, 23, 25, 26). In some cases, such rearrangements alter the symbiotic properties of the strains.

We have performed experiments to analyze genomic rearrangements that occur at high frequency under commonly used laboratory conditions that are not considered to cause stress in bacterial populations. The experimental approach was similar to that reported by Sapienza et al. (21), which demonstrated the presence of frequent genomic rearrangements in the archaebacteria *Halobacterium halobium* and *Halobacterium volcanii*. Such an approach consisted of analyzing direct descendants of a single bacterial cell in regard to the DNA hybridization patterns obtained against different recombinant plasmids. We have analyzed two *Rhizobium* phaseoli (*Rhizobium leguminosarum* biovar *phaseoli*) strains and one *Rhizobium* strain originally isolated from *Dalea leporina*. We detected genomic rearrangements that occur at high frequency in the three strains.

Our data support the hypothesis that in *Rhizobium* spp. genomic rearrangements occur frequently under laboratory conditions and that strains should be considered as heterogeneous collections of similar but not identical organisms. The possible biological significance of this phenomenon is discussed.

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## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Two strains of *R. phaseoli*, CFN-42 (15) and CFN-285 (10), and one strain of *Rhizobium* sp. originally isolated from *D. leporina*, CFN-249 (10), were used. *Escherichia coli* S17-1(pSUP5011) was used to introduce Tn*5-mob* into *R. phaseoli* (22). Recombinant plasmids pMF9 and pMF18 were obtained from a gene library of strain CFN-42; pMF101 and pMF122 were from a gene library of strain CFN-285. Both libraries were made in the *Eco*RI site of pBR329. They contain single *Eco*RI fragments that detect repeated DNA families in *R. phaseoli* (7). The sizes of the inserts were 5, 0.8, 2.0, and 3.0 kilobases for pMF9, pMF18, pMF101, and pMF102, respectively. Other recombinant plasmids utilized were pCQ15 (15, 16), which carries a 4.7-kilobase *Eco*RI insert with nitrogenase structural genes, and pSUP5011 (22), which detects Tn*5* sequences. Cosmids cMAP-16 and cMAP-30 are pSUP205 plasmids (22) harboring 40-kilobase inserts from the symbiotic plasmid of CFN-249 (10). *Rhizobium* bacteria were grown at 30°C in PY medium (12), and *E. coli* strains were grown at 37°C in LB medium (11).

**DNA fingerprints, filter blot hybridization, and plasmid profiles.** Total DNA digested with *Eco*RI restriction endonuclease was subjected to electrophoresis in 1% agarose gels and either stained with ethidium bromide (DNA fingerprint) or blotted onto nitrocellulose according to Southern (24). Recombinant plasmids or cosmids were labeled with $^{32}P$ by nick translation (17) at high specific activity ($10^8$ cpm/μg of DNA) and used as probes for hybridization. For each lane, 2 × $10^6$ cpm of probe DNA were used. The blots were prehybridized for 2 h at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.2% Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.1 M phosphates (pH 6.7), and 0.05 mg of salmon sperm DNA per ml. They were then hybridized for 16 h at 65°C in the same solution with the labeled probe. The filters were washed once with 2× SSC–0.1% sodium dodecyl sulfate for 10 min at room temperature and three times with 0.1× SSC–0.1% sodium dodecyl sulfate for 30 min at 50°C.

Autoradiography in the presence of intensifying screens was carried out at −70°C for 24 h. Analysis of plasmid profiles was performed by the Eckhardt procedure (5).

**Tn*5* labeling of *Rhizobium* spp.** A kanamycin-resistant mutant of CFN-285 was obtained by the conjugative transfer of Tn*5*-*mob* from S17-1(pSUP5011) to CFN-285 as described by Simon (22).

**Isolation of individual *Rhizobium* cells by micromanipulation.** A diluted liquid culture of *Rhizobium* spp. was extended on a Neubauer chamber covered with a thin layer of agar. With the aid of the microscope, isolated bacteria were localized and then picked with a needle covered with soft agar and placed in solid PY medium for growth. Bacterial colonies were obtained in 70% of the cases. Manipulation was carried out under sterile conditions.

## RESULTS

The rationale of the experiments performed can be summarized as follows: if direct descendants of a single cell show differences in the DNA hybridization patterns obtained when certain cloned DNA fragments are used as probes, it follows that a genomic rearrangement occurred at a certain moment, from the time the culture was a single cell to the time of harvesting individual colonies derived from such a cell.

In the experiment shown in Fig. 1, a purified colony of *R. phaseoli* CFN-285 was randomly labeled with Tn*5*. From the $Km^r$ cells an individual bacterial clone was purified by repeatedly isolating a single colony. The selected colony represented a culture of approximately 18 generations after being a single cell. The colony was grown in liquid medium for another four generations (16 h), and samples of the culture were used for genomic analysis and for plating to isolate individual clones derived from the culture. Twenty single colonies were grown in liquid for four generations more, and each culture was harvested for genomic analysis.

Genomic analysis consisted of plasmid profiles, fingerprints of totally digested DNA, and genome Southern hybridization of totally digested DNA, using as probes a fragment detecting the Tn*5* sequences as well as different recombinant plasmids. Such recombinant plasmids were derived from gene libraries of certain *Rhizobium* strains (see Materials and Methods) and were previously shown to detect repeated DNA sequences in *R. phaseoli* (7). The results of the genomic analysis obtained with five different bacterial clones are presented in Fig. 1. All 20 bacterial clones analyzed had identical plasmid profiles (Fig. 1A) and DNA fingerprints (Fig. 1B), and hybridization with Tn*5* sequences revealed a band of the same mobility in all of them (Fig. 1C). Recombinant plasmid pCQ15 (Fig. 1D) revealed the same pattern in all of the bacterial clones. Recombinant plasmid pMF101 (Fig. 1E) gave the same pattern in all clones except clone 3, in which the absence of a band (see arrow) is evident. Recombinant plasmid pMF122 (Fig. 1F) detected a difference in mobility in a band in clone 4. Genomic analysis of the other 15 clones analyzed in this experiment as well as the original culture from which such clones were derived gave results identical to those with clones 1, 2, and 5 (not shown). Three other recombinant plasmids utilized did not reveal any differences among the 20 clones (not shown).

Nine experiments similar to that presented in Fig. 1 were performed with strain CFN-285 (Fig. 2; Table 1). All experiments were derived from descendants of the single cell used as starting material in the experiment presented in Fig. 1. All bacterial clones analyzed (a total of 541, including 9 different

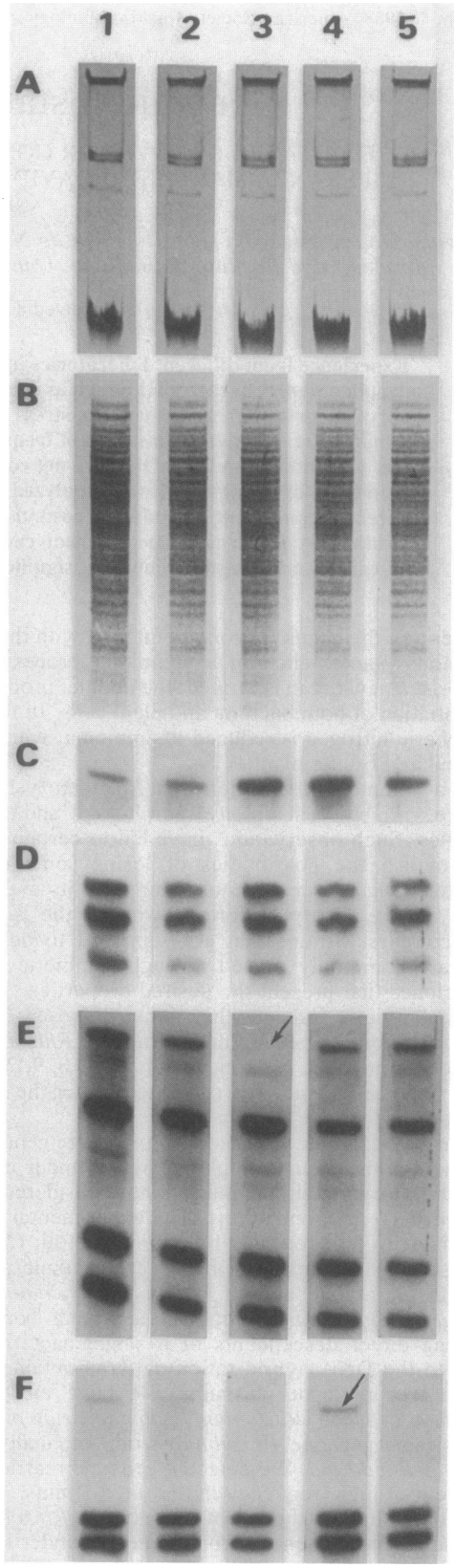



FIG. 1. Detection of genomic rearrangements in strain CFN-285. Independent clones of CFN-285 were isolated as indicated in the text. (A) Plasmid profiles stained with ethidium bromide. (B) DNA fingerprints stained with ethidium bromide. (C–F) Autoradiographs of filter blot hybridization, using pSup 5011 (C), pCQ15 (D), pMF101 (E), or PMF122 (F) as probe.

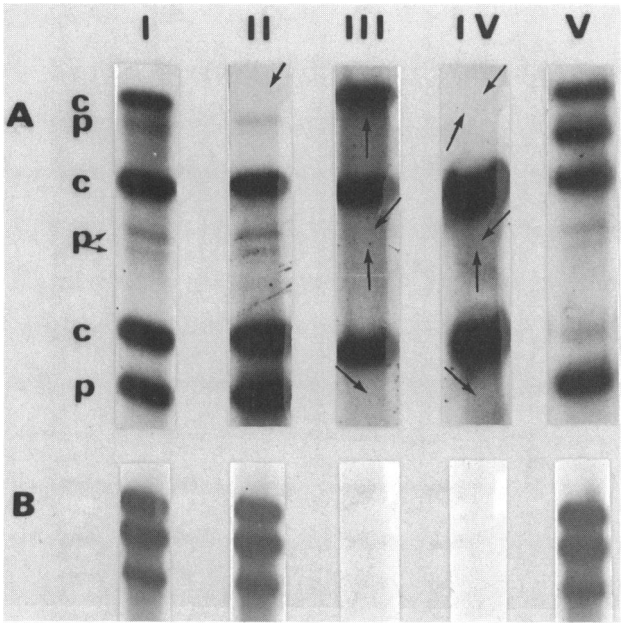



FIG. 2. Different genomic rearrangements detected in strain CFN-285. Shown are autoradiographs of filter blot hybridization from five typical clones of CFN-285, using pMF101 (A) or pCQ15 (B) as probe. I to V indicate the different types of clones described in the text, which also defines the "c" and "p" labels.

experiments) showed the same plasmid profile and DNA fingerprint, and the Tn*5* sequence revealed a band of similar mobility in all of them (not shown). According to the patterns detected with pMF101, four different types of bacterial clones were obtained (Fig. 2A). Type I, the original and most frequent, showed seven bands. Three such bands (labeled c) are present in the chromosome or in a plasmid not detected by the Eckhardt procedure, while the others (labeled p) correspond to the symbiotic plasmid (plasmid b of strain CFN-285). Evidence for the genomic localization was obtained as follows: each of the four plasmids of strain CFN-285 was independently transferred to an *Agrobacterium tumefaciens* derivative cured of all of its native plasmids (18), and Southern blots from each transconjugant were hybridized with labeled pMF101. The only transconjugant that hybridized was that containing the symbiotic plasmid. Bands labeled p hybridized with this transconjugant, while those labeled c did not. At the same time the plasmid profile transferred to nitrocellulose only showed hybridization with the symbiotic plasmid region (not shown).

Figure 2 shows all patterns obtained. Type II showed the absence of one chromosomal band, the same alteration presented by clone 3 in Fig. 1E. Type III clones revealed the absence of all four bands present in the symbiotic plasmid. Type IV clones presented a pattern that seems to be a combination of the alterations in types II and III. A correlation existed between the patterns obtained with pMF101 and *nif* gene hybridization. Figure 2B shows that types I and II hybridized with *nif* genes, revealing the three reiterated *nif* regions present in the symbiotic plasmids of *R. phaseoli* (10, 15), while types III and IV did not hybridize at all. Type V was found in only one bacterial clone in an experiment described below. In this type, hybridization shows the absence or large decrease of one chromosomal band concomitant with the appearance of a new band, while hybridization with the *nif* probe showed a normal pattern.

Table 1 presents a summary of the different experiments performed. When experiments were initiated with a clone representing type I, descendants presented pattern I (95%), II (1%), or III (4%). Experiments started with type II clones presented a type II (86%) or IV (14%) pattern. All descendants of type III tested kept the same pattern. Type IV seems to be a combination of the two genomic rearrangements that give rise to types II and III. That type IV was not obtained when starting with type I cells is consistent since the additive frequency of both events ($0.01 \times 0.04$) should give only about 1 type IV in 2,500 descendants of type I. We analyzed only about one-fifth of this amount. That in the sample analyzed type II clones gave rise to type IV while type III did not is also consistent with the higher frequency of the rearrangement that gives rise to type III versus that which originated type II.

Ten bacterial clones corresponding to each of the four different types were hybridized with three other recombinant plasmids that reveal reiterated DNA families present in the chromosome. No differences in hybridization patterns were observed (data not shown).

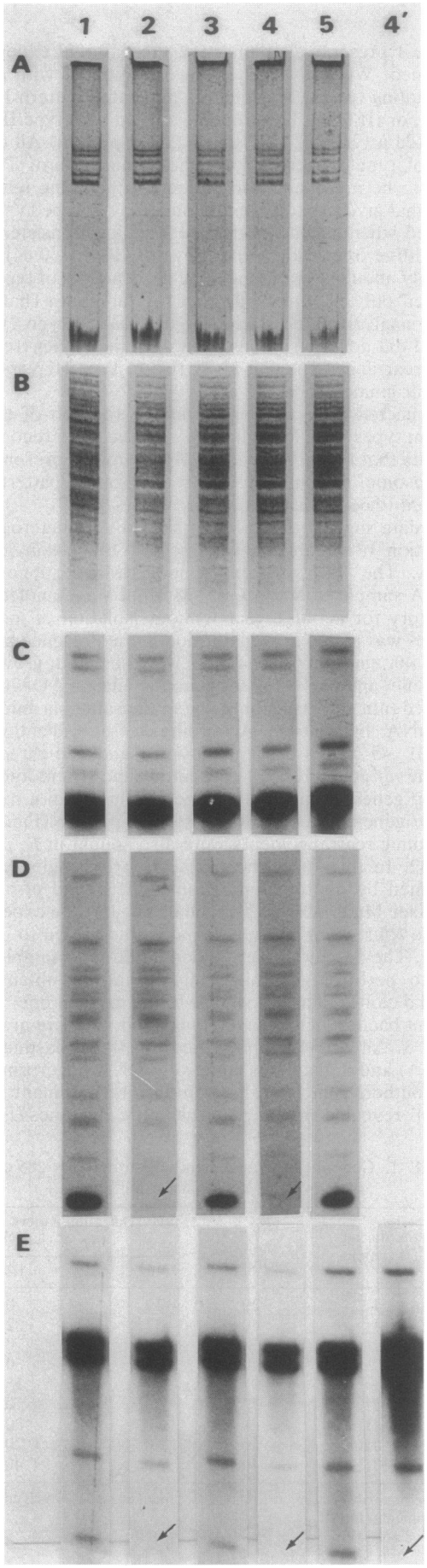
Our data suggest that a strain should be a heterogeneous population of organisms with regard to certain genomic regions. The following experiment further supports this view. A sample of strain CFN-285 that was handled in the laboratory for about 1 year without isolation of individual colonies was plated at high dilution in the presence of 0.01% Tween 40, and individual clones were selected, grown for a short time, and harvested for genomic analysis. Of 70 clones analyzed (not shown), all presented the same plasmid profile and DNA fingerprint. According to hybridization with pMF101, 45 clones presented type I and had three bands with the *nif* gene probe, 1 presented type II and hybridized with *nif* genes, 23 presented type III and did not hybridize with *nif* genes, and 1 showed a type V pattern (Fig. 2).

Genomic rearrangements were also sought in *R. phaseoli* CFN-42. In this case, the purification of single cells was performed by micromanipulation with the aid of a microscope (see Materials and Methods). To start the experiment, a single *Rhizobium* cell was isolated and grown to obtain a colony. The colony was grown on liquid, and samples were used to perform genomic analysis and to obtain single bacterial clones in a way similar to the original one. Twenty different bacterial clones were analyzed, and five are shown in Fig. 3. All 20 clones presented the same plasmid profile (Fig. 3A) and indistinguishable total DNA fingerprints (Fig. 3B). Southern blot hybridization with recombinant plasmid pMF101 revealed similar patterns in the 20 clones (Fig. 3C).

TABLE 1. Genomic rearrangements in strain CFN-285 detected with pMF101 as probe[a]

| Expt | Starting pattern | No. of clones analyzed | Hybridization pattern obtained (no. of clones) | | | |
|---|---|---|---|---|---|---|
| | | | I | II | III | IV |
| 1 | I | 20 | 19 | 1 | 0 | 0 |
| 2 | I | 40 | 37 | 1 | 2 | 0 |
| 3 | I | 20 | 20 | 0 | 0 | 0 |
| 4 | I | 20 | 20 | 0 | 0 | 0 |
| 5 | I | 291 | 274 | 2 | 15 | 0 |
| 6 | II | 70 | 0 | 60 | 0 | 10 |
| 7 | III | 20 | 0 | 0 | 20 | 0 |
| 8 | III | 20 | 0 | 0 | 20 | 0 |
| 9 | III | 40 | 0 | 0 | 40 | 0 |

[a] Experiments were similar to those presented in Fig. 1, which corresponds to experiment 1 (see text).



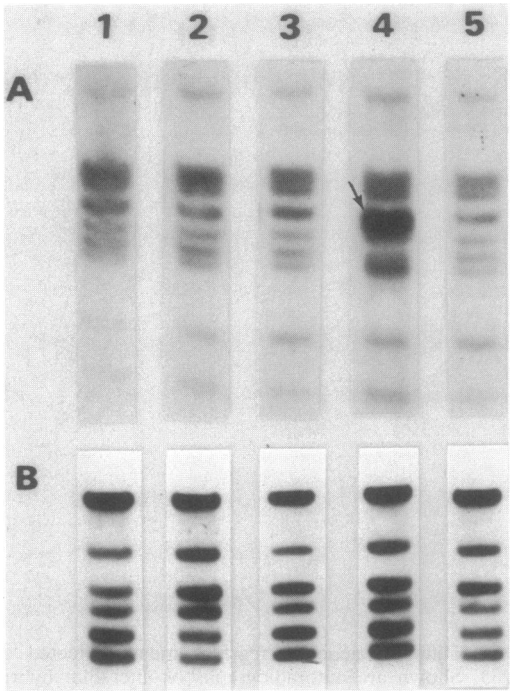




FIG. 4. Detection of genomic rearrangements in strain CFN-249. Independent clones of CFN-249 were isolated as indicated in the text. Shown are autoradiographs of filter blot hybridization from five clones using cMAP-30 (A) or cMAP-16 (B) as probe.

Plasmid pMF18 revealed an interesting difference among the clones. One band (indicated by arrows) showed marked differences in intensity, while the other bands were relatively similar as corroborated by densitometry (not shown). Plasmid pMF9 (Fig. 3E) also revealed differences among the clones, since the band with lower molecular weight (see arrows) is absent in some of them. Two additional probes that reveal reiterated DNA elements did not show any difference among the clones.

*Rhizobium* sp. strain CFN-249 originally isolated from the tropical legume *D. leporina* was also studied (Fig. 4). Purification of the starting cell as well as the derived individual clones was performed as for strain CFN-285 (see above) but without labeling the initial sample with Tn*5*. Twenty different individual bacterial clones derived from a single cell were analyzed. Southern blots were hybridized with five different randomly selected recombinant cosmids, each one harboring an average of 40 kilobases of DNA from the symbiotic plasmid of CFN-249. Genomic analysis showed no differences in plasmid profiles and DNA fingerprints (not shown). Four of the five cosmids used as probes did not reveal differences in any of the 20 bacterial clones analyzed. Only one cosmid (cMAP30) detected a difference in the pattern of one of the bacterial clones. Results of hybridization of five bacterial clones with two of the cosmids are

FIG. 3. Detection of genomic rearrangements in strain CFN-42. Individual clones from CFN-42 were isolated as indicated in Materials and Methods. (A) Plasmid profiles stained with ethidium bromide. (B) DNA fingerprints stained with ethidium bromide. (C–E) Autoradiographs of filter blot hybridization, using pMF101 (C), pMF18 (D), or pMF9 (E) as probe. Lane 4′ is an overexposure of the autoradiograph from clone 4.

presented in Fig. 4. The arrow indicates the detected rearrangement in clone 4 hybridized with cosmid cMAP30.

## DISCUSSION

By analyzing descendants of a single *Rhizobium* cell, the experiments presented gave direct evidence of the occurrence of genomic rearrangements under laboratory conditions.

For these experiments to be valid, it is imperative to rule out artifacts due to strain contamination or partial restriction endonuclease digestions. Before any experiment was started, a single colony was isolated through several cycles of plating at high dilution in the presence of detergent. Genomic analysis of the different bacterial clones always included a plasmid profile and DNA fingerprint. These characteristics remained unaltered in all cases presented in which genomic rearrangements were shown with some hybridization probes. We have analyzed >50 different native isolates of *R. phaseoli*, and we have not found any two strains with identical fingerprints and plasmid profiles. In addition, with strain CFN-285, an internal Tn*5* control was included. In all cases analyzed, the Tn*5* was detected in the same genomic position. Moreover, for strain CFN-42, cells were isolated directly by micromanipulation under a microscope. In regard to the possibility of partial DNA digestions, clones representative of the different patterns obtained were challenged by increasing the time or enzyme concentration during digestion, and in all cases the pattern remained the same (not shown).

Most experiments were performed with *R. phaseoli* CFN-285. However, the screening performed in *R. phaseoli* CFN-42 and *Rhizobium* sp. CFN-249 also revealed genomic variability, indicating that this phenomenon is of a more general nature.

Mechanisms responsible for the genomic rearrangements observed are not known at the present time. Homologous recombination between different elements of reiterated DNA families could be involved as well as transposition of insertion sequences. We have found that the *Rhizobium* genome contains a large amount of repeated DNA sequences (7), and different laboratories have reported the presence of insertion sequences in *Rhizobium* and *Bradyrhizobium* species (9, 14, 19).

The analysis performed in this study would only detect frequent events. It must be emphasized that changes were detected only with some hybridization probes and that several others did not reveal any difference in the genome. On the other hand, at least in the best analyzed case, hybridization of clones of strain CFN-285 with pMF101 generated similar patterns in different experiments. These observations suggest that frequent genomic rearrangements are probably confined to certain regions of the genome. Soberon-Chavez et al. (23) reported rearrangements that alter the symbiotic plasmid of another *R. phaseoli* strain with loss of *nif* genes as well as nodulation ability. In the case of some *Archaebacteria*, which have many repeated DNA sequences and present frequent genomic rearrangements (21), these appear to be circumscribed to only some zones of the genome, having a high A+T content (8, 13).

Our data support the hypothesis that *Rhizobium* strains can generate subpopulations. Such a situation is exemplified by the experiment performed with strain CFN-285 in which, after 1 year of handling under laboratory conditions, about 35% of the cells showed differences compared with the starting organism.

Variability in growth and symbiotic properties is a common characteristic in *Rhizobium* strains. We propose that frequent genomic rearrangements are the molecular basis of such variability. In some cases this variability could be an important problem in handling and preserving the properties of *Rhizobium* strains, in particular when they are used as inoculants. Further experiments are certainly needed to clarify the mechanisms and extent of genomic variability to find ways to stabilize relevant genetic information.

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