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Reiterated DNA Sequences in *Rhizobium* and *Agrobacterium* spp.

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Repeated DNA sequences are a general characteristic of eucaryotic genomes. Although several examples of DNA reiteration have been found in procaryotic organisms, only in the case of the archaebacteria *Halobacterium halobium* and *Halobacterium volcanii* [C. Sapienza and W. F. Doolittle, Nature (London) 295:384–389, 1982], has DNA reiteration been reported as a common genomic feature. The genomes of two *Rhizobium phaseoli* strains, one *Rhizobium meliloti* strain, and one *Agrobacterium tumefaciens* strain were analyzed for the presence of repetitive DNA. *Rhizobium* and *Agrobacterium* spp. are closely related soil bacteria that interact with plants and that belong to the taxonomical family *Rhizobiaceae*. *Rhizobium* species establish a nitrogen-fixing symbiosis in the roots of legumes, whereas *Agrobacterium* species is a pathogen in different plants. The four strains revealed a large number of repeated DNA sequences. The family size was usually small, from 2 to 5 elements, but some presented more than 10 elements. *Rhizobium* and *Agrobacterium* spp. contain large plasmids in addition to the chromosomes. Analysis of the two *Rhizobium* strains indicated that DNA reiteration is not confined to the chromosome or to some plasmids but is a property of the whole genome.

Reiterated DNA sequences have been considered a general characteristic of eucaryotic genomes. In procaryotic genomes, several reports indicate the existence of repeated DNA sequences (1, 13, 14, 17, 23, 24, 30, 39, 49), but it has been demonstrated as a common genomic feature only in a few cases. For instance, Sapienza and Doolittle (42) showed that DNA reiteration is a common feature in the genome of the archaebacteria *Halobacterium halobium* and *Halobacterium volcanii*.

We have previously reported that nitrogen fixation genes (*nif* genes) are reiterated in *Rhizobium phaseoli* (33, 34), the symbiont of the common bean plant *Phaseolus vulgaris*. Reports from several laboratories have shown that *nif* gene sequences are reiterated in different organisms, including *Rhodopseudomonas* spp. (44), *Anabaena* spp. (35), *Calothrix* spp. (21), *Clostridium* spp. (6), *Azotobacter* spp. (20), *Frankia* sp. (46), and some strains of *Rhizobium* spp. (10, 26, 28, 29, 31).

Besides nitrogen fixation gene sequences, different repeated DNA sequences have been found in *Rhizobium* strains belonging to different cross-inoculation groups. These sequences include regulatory regions (3, 40), an early nodulation gene (2, 15, 19, 25), and insertion sequences (22, 41). Masterson and Atherly (27) have recently reported that the symbiotic plasmid of a *Rhizobium fredii* strain contains several repeated DNA sequences.

Altogether, these reports suggest that repeated DNA sequences might be a general characteristic of the *Rhizobium* genome. Accordingly, we analyzed the genome of some bacterial strains, including *R. phaseoli*, *Rhizobium meliloti*, the symbiont of alfalfa, and the plant pathogen *Agrobacterium tumefaciens*. We found that the *Rhizobium* genome contains an unusual number of reiterated DNA sequences and that this characteristic is shared by the taxonomically related *A. tumefaciens*.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Molecular cloning. To prepare bacterial gene libraries, total DNA from each strain was digested with *Eco*RI restriction endonuclease and the fragments were cloned in the *Eco*RI site of pBR329 by using *Escherichia coli* MC1061 as a recipient for transformation.

Filter blot hybridization. Total DNA digested with *Eco*RI restriction endonuclease was subjected to electrophoresis in a 1% agarose gel and blotted onto nitrocellulose by the method of Southern (48). Recombinant plasmids were labeled with ^{32}P by nick translation (36) 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 was 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 (Pharmacia Fine Chemicals, Piscataway, N.J.), 0.2% bovine serum albumin, 0.2% polyvinylpirrolidone, 0.1 M phosphates (pH 6.7), and 0.05 mg of salmon sperm DNA per ml. The blots 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 containing 0.01% sodium dodecyl sulfate for 10 min at room temperature and three times with 0.1× SSC containing 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.

Transfer of *R. phaseoli* plasmids to *A. tumefaciens*. *R. phaseoli* CFN-42 and CFN-285 were randomly mutagenized with Tn*5-mob* (45). Different Km^r derivatives of each strain were mated with the plasmidless *A. tumefaciens* GMI9023 in triparental matings by using pJB3JI as a helper plasmid for mobilization. *Agrobacterium* transconjugants were analyzed for plasmid profiles by the Eckhardt procedure (12), and strains carrying each different plasmid were selected. A

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Source or reference
R. phaseoli	
CFN-1	CFN collection[a]
CFN-23	CFN collection
CFN-42	CFN collection
CFN-226	CFN collection
CFN-275	CFN collection
CFN-285	CFN collection
CFN-307	CFN collection
CIAT 281	Peter Graham[b]
Nitragin USA 8251	Nitragin[c]
R. meliloti 2011	J. Denarié[d]
A. tumefaciens	
C58	16
GMI9023	38
E. coli MC1061	5
Plasmids	
pBR329	7
pJB3JI	4

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further screening for Tc^S colonies was performed to obtain pJB3JI-free *Agrobacterium* derivatives.

RESULTS

Identification of reiterated DNA sequences. The presence of an unusually large number of repetitive DNA sequences in procaryotic organisms has been well documented by Sapienza and Doolittle (42) for the archaebacteria *H. halobium* and *H. volcanii*. The approach they followed consisted in analyzing the patterns obtained by hybridizing Southern blots of total DNA against randomly isolated cloned fragments of the same DNA. We followed a similar approach to search for repeated DNA in two strains of *R. phaseoli*, one strain of *R. meliloti*, and one strain of *A. tumefaciens*.

To ascertain that the experiments were initiated with a single cell, the strains used were purified by repeatedly suspending single colonies in the presence of detergent (0.01% Tween 40) and plating at high dilution. Total DNA from each strain was extracted and totally digested with the restriction endonuclease *Eco*RI. A sample of each digest was cloned in the *Eco*RI site of pBR329, whereas other samples were subjected to agarose gel electrophoresis and blotted onto nitrocellulose by the method of Southern (48). Forty recombinant plasmids containing single inserts were purified from randomly selected clones of each genomic library. Each plasmid was labeled with ^{32}P by nick translation and used as probe to hybridize, under high stringency conditions, against a Southern blot of the corresponding strain. In these experiments, homologous bands in addition to the insert are evidence of DNA reiteration.

The results obtained with *R. phaseoli* CFN-42 and CFN-285, *R. meliloti* 2011, and *A. tumefaciens* C58 are presented in Fig. 1. The number of bands that showed hybridization with each recombinant plasmid was scored and is plotted in Fig. 1A. For each strain, several recombinant plasmids hybridized to other bands in addition to that of the insert: 55, 78, 38, and 43% in *R. phaseoli* CFN-42 and CFN-285, *R. meliloti* 2011, and *A. tumefaciens* C58, respectively. Since partial DNA digestions could alter the interpretation of the results, several recombinant plasmids detecting repeated sequences were hybridized against DNA digests obtained in different conditions. In all cases tested, the increase in enzyme concentration or time in DNA digestion compared with that for the conditions used in the experiments presented here did not alter the number of hybridizing bands (not shown). The level of reiteration was usually low, in the order of 2 to 5 bands; however, some clones hybridized with more than 10 bands. All the clones detecting reiterated sequences gave different hybridization patterns, indicating that different repeated elements were being screened. Examples of Southern blot hybridization that reveal different degrees of reiteration from each strain tested are presented in Fig. 1B. Some reiterated bands show a low intensity; since the hybridization was performed under high-stringency conditions, we infer that in these cases the DNA repetition corresponds to only a small portion of the insert. In the other cases, repeated bands show a high intensity which suggests that long stretches of DNA are reiterated or that several copies of the repeated sequences are present in such bands.

Genomic localization of reiterated DNA sequences. Fast-growing *Rhizobium* strains such as those used in this work, as well as *A. tumefaciens*, contain large plasmids in addition to the chromosome. In *Rhizobium* spp. several symbiotic factors, including nitrogenase structural genes and early nodulation genes, are located in a single plasmid in each strain (*sym* plasmid) (18, 32, 37). Also, tumor-inducing factors have been localized in an *Agrobacterium* plasmid (Ti plasmid) (50). We analyzed the locations of some repeated elements in *R. phaseoli* CFN-42. This strain contains six plasmids of about 190 kilobases (kb) (plasmids a and b), 220 kb (plasmid c), 280 kb (plasmid d, *sym* plasmid) 400 kb (plasmid e), and 500 kb (plasmid f). In an initial screening (data not shown), 20 recombinant plasmids that detected reiterated families were hybridized against plasmid profiles of the same strain transferred to nitrocellulose filters. Six clones showed intense hybridization with one or two plasmids. The rest did not show significant hybridization with the plasmid profile, suggesting a chromosomal location for those repeated elements.

To determine the locations of individual members of selected DNA families, plasmids a, b, c, d, and f from strain CFN-42 were transferred to *A. tumefaciens* GMI9023, a derivative cured of its native plasmids (38). We have not been able to either transfer plasmid e to *Agrobacterium* sp. or to cure it from CFN-42. Plasmid profiles and *Eco*RI digests of the original *Rhizobium* and *Agrobacterium* strains as well as those of the *Agrobacterium* transconjugants were hybridized with labeled recombinant plasmids representing some repeated families. Examples were found of repeated families located in one plasmid only, in more than one plasmid, in the chromosome and some plasmids, and in the chromosome only. Several recombinant plasmids revealed bands in *Agrobacterium* sp., indicating homology between both organisms.

Results obtained with some of these repeated DNA families are presented in Fig. 2. The plasmid profiles of the original *Rhizobium* strain and the different *Agrobacterium* transconjugants are shown in Fig. 2A. Recombinant plasmid pMF9 hybridized with plasmids a and d in the plasmid

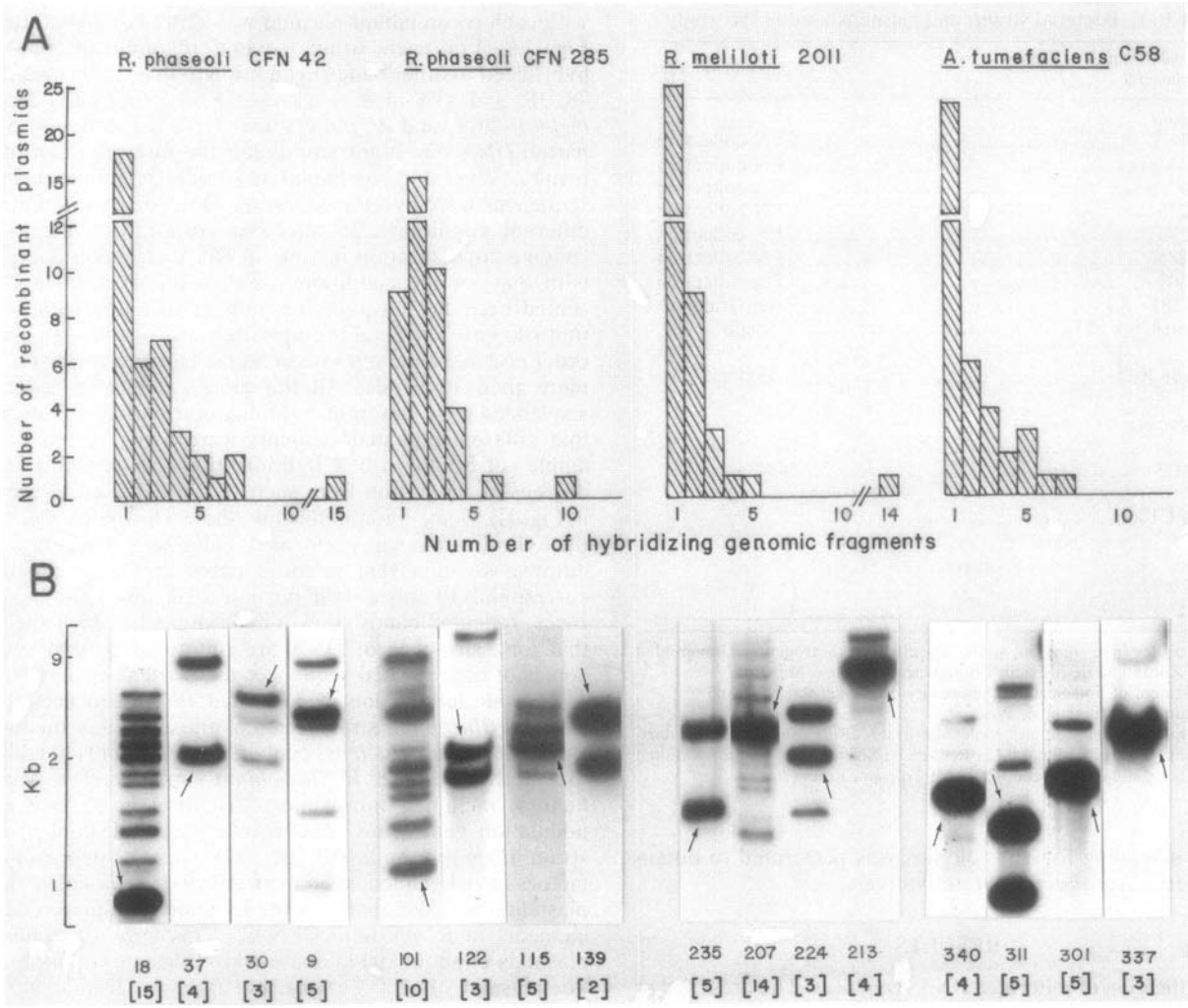

FIG. 1. Reiteration of DNA sequences in *R. phaseoli* CFN-42 and CFN-285, *R. meliloti* 2011, and *A. tumefaciens* C58. Total DNA from each strain was purified and digested with *Eco*RI restriction endonuclease. One sample of the digest was cloned in the *Eco*RI site of pBR329. Other samples were subjected to agarose gel electrophoresis and blotted onto nitrocellulose. From each genomic library, 40 recombinant plasmids containing single inserts from randomly selected clones were isolated and labeled with ^{32}P by the nick translation procedure. Each plasmid was used as probe to hybridize with a Southern blot of its corresponding DNA digest. (A) The number of bands probed by each recombinant plasmid is plotted for the different strains. (B) Four selected examples of autoradiography from each strain, showing different degrees of reiteration. The recombinant plasmids used as probes are all named pMF plus the number that is indicated under each lane. The number of scored bands is indicated in brackets at the bottom. The arrows indicate the bands corresponding to the actual probes. The indicated length in kilobases (kb) was calculated by using λ bacteriophage DNA digested with *Hin*dIII as a marker.

profiles (Fig. 2B) and showed five hybridization bands in the Southern blots of the *Rhizobium* strain. Hybridization against the Southern blots of *Agrobacterium* transconjugants indicated that three of such probed bands are present in plasmid d, whereas the other two are present in plasmid a (Fig. 2C). Recombinant plasmid pMF30 did not show hybridization to the plasmid profile (not shown). In Southern blots, plasmid pMF30 revealed three bands in the original *Rhizobium* strain and a different band in all the *Agrobacterium* strains (Fig. 2D). We infer that the bands detected in the *Rhizobium* strain are present in the chromosome, whereas the other band reveals sequence homology among the original *Rhizobium* and *Agrobacterium* strains.

Similar experiments were performed with strain CFN-285. This strain contains four plasmids (Fig. 2E) of 130 (plasmid a), 400 (plasmid b, *sym* plasmid), and about 500 kb (plasmids c and d). As in strain CFN-42, repeated DNA families were not confined to particular replicons but showed different patterns of distribution in the genome. One example is presented in Fig. 2E through G. Clone pMF101 hybridized with plasmid b (Fig. 2F) and with 10 bands in the Southern blot of the original *Rhizobium* strain (Fig. 2G). Five of these bands correspond to plasmid b, whereas the other five bands did not hybridize with any plasmid, and we infer that they are present in the chromosome. One band detected in all *Agrobacterium* transconjugants, but not in the *Rhizobium* strain, represents homology with the *Agrobacterium* chromosome. Although more comprehensive experiments are necessary to obtain a general view on the location of repeated elements, these experiments do show that DNA reiteration is not confined to specific replicons but is a property of the whole genome.

Presence of reiterated DNA families in different *R. phaseoli* strains. To find out whether DNA sequences that show

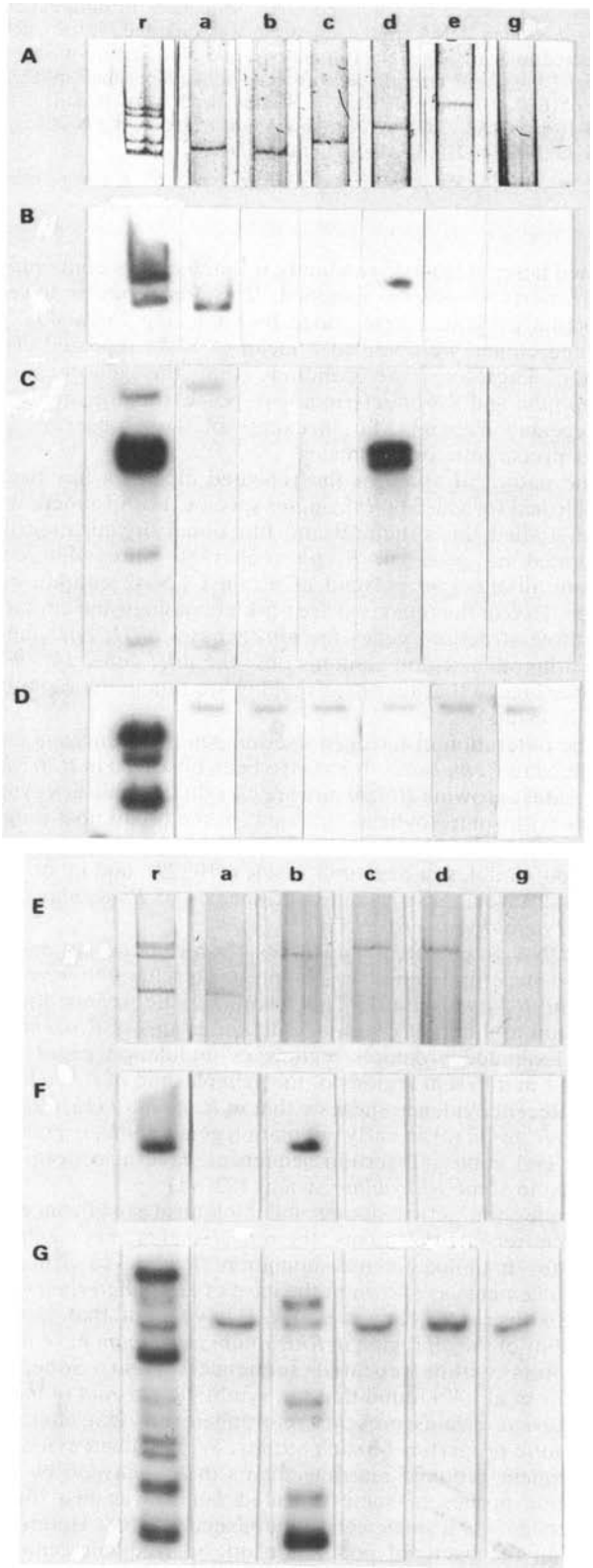

FIG. 2. Genomic localization of reiterated DNA sequences in *R. phaseoli* CFN-42 and CFN-285. The donor *Rhizobium* strains CFN-42 or CFN-285, the recipient *Agrobacterium* strain GMI9023, and the *Agrobacterium* transconjugants bearing different *Rhizobium* plasmids were grown in liquid medium and portions were used to obtain plasmid profiles (12) and for total DNA isolation followed by digestion with *Eco*RI and agarose gel electrophoresis. The gels from both plasmid profiles and digests were blotted onto nitrocellulose and hybridized under high-stringency conditions against selected recombinant plasmids that probed repeated DNA families. (A through D) Experiments with strain CFN-42. Original *Rhizobium* strain (lane r) and *Agrobacterium* strain (lane g). (A) Ethidium bromide strain of plasmid profiles from the different strains; (B) autoradiography of plasmid profiles hybridized against recombinant plasmid pMF9; (C) autoradiography of DNA digests hybridized against recombinant plasmid pMF9; (D) autoradiography of DNA digests hybridized against recombinant plasmid pMF30. (E through G) Experiments with strain CFN-285. Donor *Rhizobium* strain (lane r), *Agrobacterium* transconjugants containing plasmids a, b, c, d, and recipient *Agrobacterium* strain (lane g). (E) Ethidium bromide stain of plasmid profiles from the different strains; (F) autoradiography of plasmid profiles hybridized against recombinant plasmid pMF101; (G) autoradiography of DNA digests hybridized against recombinant plasmid pMF101.

reiteration in one strain constitute a repeated family in other strains, several inserts identifying repeated DNA in either strain CFN-42 or CFN-285 were used as probes to hybridize with DNA digests from several strains obtained from different geographical origins. We analyzed eight different DNA families. All of them showed repeated DNA sequences in the different strains screened. Three examples are presented in Fig. 3. Although the band patterns are different in each strain, all of them show repeated DNA with each of the probes used. This is also the case of *nif* genes. We analyzed about 50 strains of *R. phaseoli*, and all but 2 showed reiterated nitrogen fixation gene sequences (26).

DISCUSSION

This report shows that a substantial percentage of randomly cloned fragments from *R. phaseoli*, *R. meliloti*, and *A. tumefaciens* hybridize to additional fragments in their respective genomes. Since all the clones detecting repeated sequences showed different hybridization patterns, it might be assumed that they represent different DNA families. The genome size of *Rhizobium* spp. has been estimated as 5,300 kb (8), and that of *Agrobacterium* sp. has been estimated as 5,500 kb (9). In the small portion of the genome that we have screened (about 3% in each strain), an average of 22 DNA families per strain was detected, ranging from 15 in *R. meliloti* to 33 in *R. phaseoli* CFN-285, containing an average of 3.5 elements per family. If each collection of clones screened represents a random sample of the whole genome, one could expect to find an average of about 200 repeated DNA families per genome. This is far in excess of the amount of repeated elements than can be accounted for by known bacterial repeated sequences, including insertion elements, rDNA, repeated genes, or extended regulatory sequences.

No direct experiments to randomly screen repeated DNA elements have been reported for well-studied bacterial species such as *E. coli*. In *E. coli* numerous genetic and recombinant DNA studies have shown that most genes are present in single copies, with rDNA and insertion sequences being the best-known repeated elements. From calculations reported by Dykhuizen et al. (11) and assuming a random distribution of insertion sequence elements in the *E. coli* genome, there must be one element every 100 kb. From this it can be derived that to detect any of the known *E. coli* insertion sequences in experiments analogous to those per-

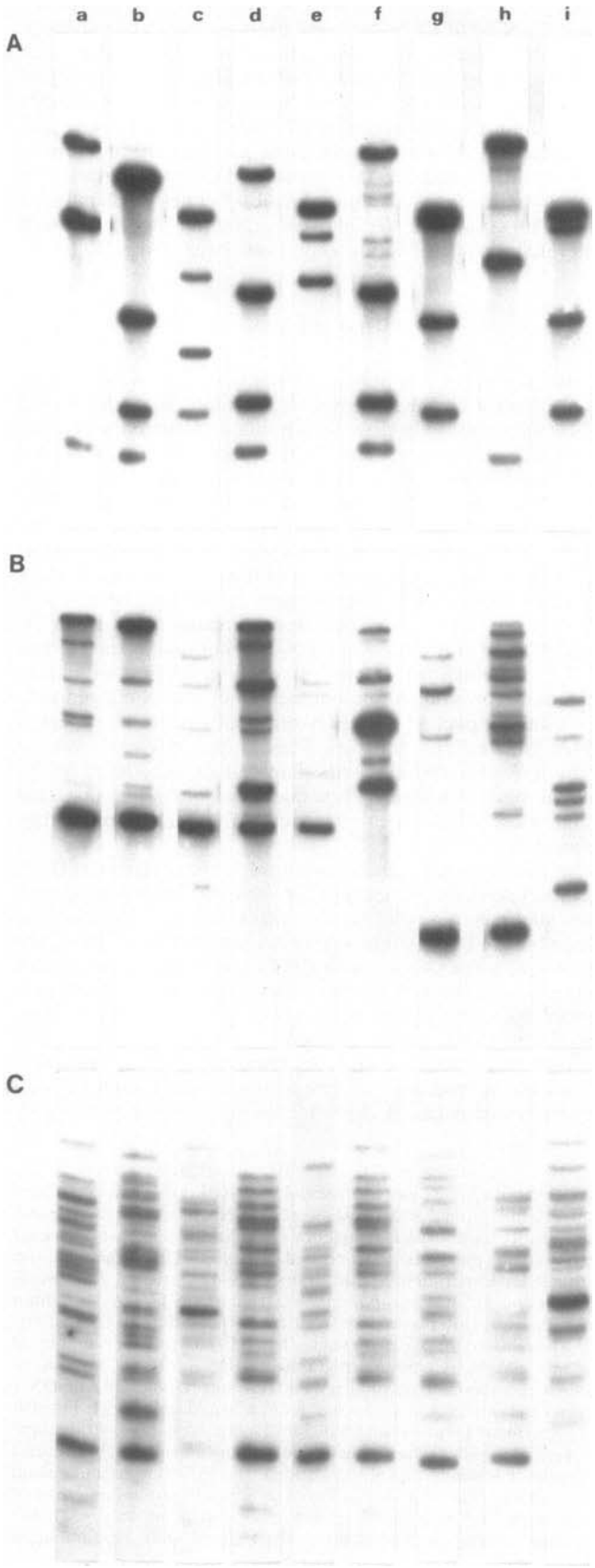

FIG. 3. Presence of reiterated DNA sequences in different *R. phaseoli* strains. DNA from each strain was digested with *Eco*RI, subjected to agarose gel electrophoresis, and blotted onto nitrocellulose. Blots were hybridized with recombinant plasmids pMF30 (A), pMF20 (B), or pMF18 (C). Strains used were in lanes: a, CFN-1; b, CIAT 281; c, Nitragin USA 8251; d, CFN-285; e, CFN-42; f, CFN-226; h, CFN-307; i, CFN-275.

formed here, at least 25 randomly isolated clones containing 4-kb inserts should be screened. This frequency is lower than that presented here, since by analyzing 25 randomly isolated clones we obtained a mean of 13.75 repeated elements. Therefore, we conclude that the genomes of *Rhizobium* and *Agrobacterium* spp. possess a large number of repeated elements, far in excess of those observed in other procaryotic organisms.

The nature of some of the repeated elements has been established for various *Rhizobium* species. For instance, we have studied the structural and functional organization of reiterated *nif* genes in *R. phaseoli* (34). Three different regions of the *sym* plasmid of strain CFN-42 contain *nif* genes. Two of the regions share a 5-kb homology and contain the three structural genes for nitrogenase, *nifH*, *nifD*, and *nifK*; the other region contains only the *nifH* gene (34). We have evidence that the three regions are functional (unpublished results).

The reiteration of nitrogen fixation gene sequences is not confined to *R. phaseoli*. It has also been observed in *R. fredii* (31), a fast-growing *Rhizobium* species that establishes symbiosis with some soybean cultivars, in the broad host-range strain ANU240 (28), in strain ORS571 that produces stem and root nodules in *Sesbania* species (10, 29), and in strains originally isolated from different species of *Phaseolus* and from *Pachyrhyzus erosus* (26).

A DNA fragment that includes the promoter for nitrogenase structural genes was found to be reiterated severalfold in *R. meliloti* (3). This characteristic seems to be common to different cross-inoculation groups of *Rhizobium* spp. Extended promoter regions of nodulation genes are present in different regions of the *sym* plasmid of *R. meliloti* (40). Recent evidence suggests that in *R. meliloti* (15, 19, 25) and in *R. fredii* (2) an early nodulation gene, *nodD*, is present in several copies. Insertion sequences have also been described in some *Rhizobium* strains (22, 41).

Besides the actual nature and biological significance of different repeated elements, the repeated elements might be sites for homologous recombination, leading to genomic rearrangements as shown in the case of *Halobacterium* spp. (43). In this regard Kaluza et al. (22) have found that several deletions of the *nif* region in *Rhizobium japonicum* have their endpoints within repeated sequences. Also, Soberón-Chávez et al. (47) found that the symbiotic plasmid of many *R. phaseoli* strains presents rearrangements that alter the symbiotic properties of such strains. We obtained evidence of frequent genomic rearrangements in *R. phaseoli* by hybridizing probes of some repeated families against direct descendents of a single cell (unpublished results). Heterogeneity in the bacterial population due to frequent genomic rearrangements would reveal additional hybridization bands in the types of experiments that we used to score DNA reiteration. In our experiments, some probed bands could actually be due to genomic rearrangements. Experiments are now in progress to analyze the dynamics of the *Rhizobium* genome.

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