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Rhizobium tropici nodulates field-grown *Phaseolus vulgaris* in France

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Abstract

Two hundred and eighty seven isolates of *Rhizobium* nodulating *Phaseolus vulgaris* L. were sampled in France from four geographically distant field populations. They were characterized by their colony morphology and by plasmid profiles. A representative sample was further characterized: a) by the ability of each isolate to nodulate a potential alternative host *Leucaena leucocephala* and to grow on specific media, and b) by RFLP analysis of PCR amplified 16S rRNA genes. On the basis of their phenotypic and genetic characteristics the isolates could be assigned either to *Rhizobium leguminosarum* bv *phaseoli*, or to *R. tropici*. The two species co-occurred at three sites. *R. leguminosarum* bv *phaseoli* represented 2%, 4%, 72% and 100% of the population at the four different sites. Eighteen and 22 different plasmid profiles were identified within *R. tropici* and *R. leguminosarum* bv *phaseoli*, respectively. Some of them were conserved between distant geographical regions. The fact that *R. tropici* was found in France shows that this species is not limited to tropical regions and gives additional evidence of the multi-specific nature of the *Phaseolus* microsymbiont, even over a geographically limited area.

Introduction

The common bean, *Phaseolus vulgaris* L., is native to the Americas. Independent domestications of wild populations took place at least 10,000 years ago in Mesoamerica and in the Southern Andes. Soon after European discovery of central America in 1492, it was imported to Western Europe where it was being grown extensively less than 60 years later (Gepts and Bliss,1988). Field-grown common beans are usually nodulated but they are generally considered weak in nitrogen fixation (Vincent, 1974). Their response to inoculation has proved extremely variable and inoculation failure is common (Graham,1981). This could be related to the promiscuity observed in *P. vulgaris*. Under laboratory conditions beans nodulate with a wide range of rhizobia (Bromfield and Barran 1990; Eardly et al., 1985; Graham and Parker, 1964 ; Sadowsky et al., 1988) which in many cases elicit ineffective nodules. However, although the level of genetic diversity among *Rhizobium* isolated from bean nodules in different geographical regions appears to be very high (Pinero et al.,1988), the extent of this genetic diversity in one field and its potential implications for the crop are not well documented.

To date, three species of *Rhizobium*, *R. leguminosarum* bv *phaseoli* (Jordan, 1984), *R. tropici* (Martinez Romero et al., 1991) and *R. etli* bv *phaseoli* (Segovia et al., 1993) have been recognized as microsymbionts of *P. vulgaris*. The

latter species was created recently to reclassify American *Rhizobium leguminosarum* bv *phaseoli* Type I strains. All the strains described so far as belonging to *R. tropici*, are also of American origin but only from tropical areas. A high level of diversity exists within each of these two species (Martinez-Romero et al., 1991; Pinero et al, 1988). Until recently, *R. leguminosarum* bv *phaseoli* was believed to be the only species of *Rhizobium* nodulating beans in Western Europe. However in France, Laguerre et al. (1993b) observed the co-occurence, among the bean isolates from a single field population, of *R. leguminosarum* bv *phaseoli* and of a group of strains which was distinct from the three recognized *Rhizobium* species nodulating beans. On the basis of DNA-DNA hybridization data and of sequence analysis of partial 16S ribosomal DNA (rDNA), this group has been characterized as a new genomic species: *Rhizobium* sp. H152 (Laguerre et al., 1993a). Another group of bean isolates collected from various regions in France (Geniaux et al., 1993) was also characterized as a new genomic species: *Rhizobium* sp. R602. The sequence of the analyzed fragment of the 16S rRNA gene from *Rhizobium* sp R602 (Laguerre et al., 1993a) was not different from that of *Rhizobium* sp FL27 (Eardly et al., 1992) collected in the Americas (Pinero et al., 1988). Beans grown in Western Europe can thus be nodulated by at least three different taxons, one of them being common to Europe and the Americas. However, we do not know whether the occurrence of each species, singly or in association, within a population of nodulating rhizobia is limited to or correlated with a plant genotype, a soil type, or other environmental characteristics. As one of the genomic species described, *Rhizobium* sp. H152, is composed of ineffective isolates, its dominance in the nodules of a crop could have agronomic consequences. It seems important, therefore, to gain more information on the occurrence of the different species of *Rhizobium* that nodulate beans in different field environments. This is the objective of the present study.

We chose three sites with acidic soils and one site with a neutral soil. Two varieties of bean were grown in this latter site in order to detect a possible host-plant effect. Growth characteristics and plasmid profiles were used as a primary rapid screening of the isolates. A representative sample was then further characterized by using symbiotic characteristics and RFLP analysis of PCR amplified 16S rRNA genes, a method which allows differentiation between *Rhizobium* species (Laguerre et al., 1994).

Material and methods

Sampling and isolation of indigenous rhizobia

Nodules were collected from bean plants grown in the uninoculated plots of inoculation trials. Field trials were carried out in 1992 at three sites (A, B, C) located approximately 50 to 120 km apart in the southwest of France, a green bean producing region, and at one site (D) located on the INRA Experimental Farm close to Dijon in the central east of France. There was no known history of bean inoculation at any of the sites. Some characteristics of the different sites are given in Table 1. At each location, prior to sowing, five soil samples of approximately 500 g were collected to a depth of 20 cm, mixed and used to estimate the numbers of indigenous rhizobia nodulating beans by the most probable number method (Vincent, 1970). The experimental design was four randomized blocks of 120 m^2, each split into four (3 × 10 m) treatments, one uninoculated and three inoculated with different strains. There were 6 rows per plot. Nodules were collected from the four central rows of each uninoculated plot. Three samples of two to three plants were taken at random in each plot and the roots were placed in plastic bags for subsequent isolation, 2-4 days later. At site D, two bean varieties were grown and a single sampling of 5 plants in one row was made per plot.

From each of the plants sampled, one to three nodules (depending on the site and the abundance of nodules) were excised at random. Isolation of rhizobia was performed as described by Vincent (1970).

Table 1. Characteristics of the sampling sites

Location	Soil type	Soil pH[a]	Rhizobia per g soil	Preceding crop	Bean variety	Number of isolates
A Ste Livrade (33)	sandy loam	5.5	23009	Tomato	Capitole	56
B Cestas (47)	sand	5.4	620	Potato	Sonore	111
C Luxey (40)	sand	5.7	<100	Tobacco	Tavera	60
D. Breteniéres (21)	clay	7.1	3900	Oats	FDC10	34
					S102	38

[a] in water

Reference strains and growth conditions

R. leguminosarum bv *viciae*, strain 10004^T was obtained from the American Type Culture Collection. *R. etli* bv *phaseoli* CFN42^T and *R. tropici* CFN299 were obtained from E. Martinez, CIFN, Universidad Nacional Autonoma de Mexico, Cuernavaca, Mexico. *R. tropici* CIAT 899^T was donated by P. Graham, Department of Soil Science, University of Minnesota, St Paul, Minnesota, U.S.A.. *Rhizobium* sp. H152 and R602 were from our laboratory collection. All the strains were maintained on agar slopes of Bergersen's medium (1961) supplemented with 0.02 % yeast extract (MGY).

Plasmid profile determination

The number of isolates analyzed are given in Table 1. Bacterial isolates were grown in TY medium (Beringer, 1974) for 24 h at 28°C. Plasmid profiles were obtained by using the procedure of Eckhardt (1978) as modified by Wheatcroft et al., (1990).

Cultural characteristics

Citrate, glycine, lactate and dulcitol were tested for utilization as sole source of carbon at a concentration of 1 g per liter of minimal medium (MM) containing per liter : K_2HPO_4, 1 g; KH_2PO_4, 1 g; $FeCl_3$, $6H_2O$, 0.01 g; $MgSO_4$, $7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1 g; agar,15 g; pH was adjusted to 6.8. Tolerance to kanamycin, nalidixic acid, and streptomycin were tested by growing bacteria on TY media containing 10 μg mL^{-1} of kanamycin sulphate, 40 μg L^{-1} mL of nalidixic acid or 80 μg mL^{-1} of streptomycin sulphate. The ability to grow on LB medium (Miller, 1972) was also tested. Growth on the different media was tested in Petri dishes by applying approximately 1×10^5 bacteria with a multipoint inoculator to the surface of the agar.

Nodulation and effectiveness tests

The ability to nodulate *Leucaena* spp. in addition to *P. vulgaris* is a useful criterion to distinguish *R. leguminosarum* bv *phaseoli* and *R. etli* bv *phaseoli* from the three other taxons, *R. tropici, Rhizobium* sp H152 and *Rhizobium* sp R602. Isolates were tested for their ability to nodulate *Phaseolus vulgaris* cv Vernandon and *Leucaena leucocephala.* Seeds were surface sterilized with saturated Ca hypochlorite, rinsed and germinated in sterile perlite. Prior to surface sterilization, seeds of *L. leucocephala* were treated for 20 min in concentrated sulphuric acid and rinsed with water. Seedlings of bean were grown according to the method of Gibson (1963) using liquid Jensen's medium (Vincent, 1970) and filter paper wicks instead of agar slopes. *L. leucocephala* was grown on slopes of Jensen's agar medium. Inoculation was performed two days after planting with approximately 1×10^9 bacteria per plant. Three replicates were inoculated with each isolate.

Effectiveness tests were done on plants grown in 5 L plastic pots filled with perlite placed on a 5 cm layer of heat sterilized clay beads. Eight

seeds of *P. vulgaris* cv Vernandon were surface sterilized, planted in pots and later thinned to 4. Inoculation was performed with 10 mL, of a suspension containing approximately 1×10^9 bacteria per mL. There were four replicates per treatment. Plants were grown in a greenhouse under natural light with a daily minimum-maximum temperature of 18-24°C. A nitrogen free solution (Amarger, 1981) was supplied when needed through an automatic watering system. Plants were harvested seven weeks after planting. Aerial parts were dried and weighed.

PCR amplification

Cells were grown on agar slopes of TY medium for 24 h at 28°C and washed in sterile distilled water. The cells were pelleted by centrifugation and resuspended in sterile distilled water. The cell suspension was adjusted to an OD_{620} of 0.5 by dilution in water. The primers fDl and rDl described by Weisburg et al. (1991) were used to amplify nearly full-length 16S rRNA genes. They were synthesised by Bioprobe Systems, France. PCR amplification was carried out in a 100-μL, reaction volume. DNA was amplified by mixing 5 μL of cell suspension as template in the polymerase reaction buffer (10 m*M* Tris-Cl, pH 9.0 at 25°C, 50 m*M* KCl, 1 % Triton X 500, 1.5 m*M* $MgCl_2$) with 20 m*M* each of dATP, dCTP, dTTP and dGTP (Pharmacia-LKB), 0.1 m*M* each of the primers and 2.5 U of Taq DNA polymerase (Promega, Madison, USA). DNA amplification was done in a Biometra TRIO-Thermoblock TBl (Göttingen, FRG) using the following temperature profile: an initial denaturation at 95°C for 3 min, 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C) and extension (2 min at 72°C), and a final extension at 72°C for 3 min. Amplified DNA was checked by horizontal electrophoresis in 0.9 % agarose with 5 μL aliquots of PCR product.

Restriction fragment analysis

Aliquots (5 to 10 μL) of PCR products were digested with restriction endonucleases (Boeringer, Mannheim, France) according to the manufacturer's instructions but using an excess of enzyme (5 U / reaction). Two enzymes, *Msp*I and *Nde*II, were used. Restricted DNA was analysed by horizontal electrophoresis in 3 % agarose (NuSieve 3:1, FMC, Rockland, USA). Electrophoreses were carried out at 80 V for 3 h with 11 × 14 cm gels (Bethesda Research Laboratories Horizon 11-14 apparatus). Gels were photographed under UV illumination with Polaroid type 665 positive/negative film.

Results

Colony morphology

Upon isolation and purification of the isolates on MGY medium, two types of isolates could be differentiated on the basis of the time of appearance and morphology of the colonies. One group of isolates, group I, formed colonies appearing between 36-40 h which turned white opaque and measured 3-5 mm after 3 days. They were not observed in site D but respectively 55/56, 107/111 and 17/60 isolates of sites A, B and C were of this type. The other group of isolates, group II, formed wet and semitranslucent colonies which appeared after 40-48 h and were 2-4 mm in diameter within 3 days.

Plasmid profiles

Plasmid profiles consisted of 2 to 6 bands. Examples are shown in Figure 1. The profiles were reproducible with the exception of a high molecular weight band (> 1000 kb) which could be seen or not depending on the gel. Its presence was not taken into account. Forty different profiles were observed among the 287 isolates analysed. A schematic representation of these profiles is given in Figure 2. Two groups of plasmid profiles could be distinguished. In plasmid profiles numbered 1 to 18, the size of the highest plasmid band was equivalent to that of *R. tropici* CFN 299, i.e. 400 kb. In the other group, profiles numbered 19 to 40, at least one band with a size greater than 400 kb was present in each profile. The distribution

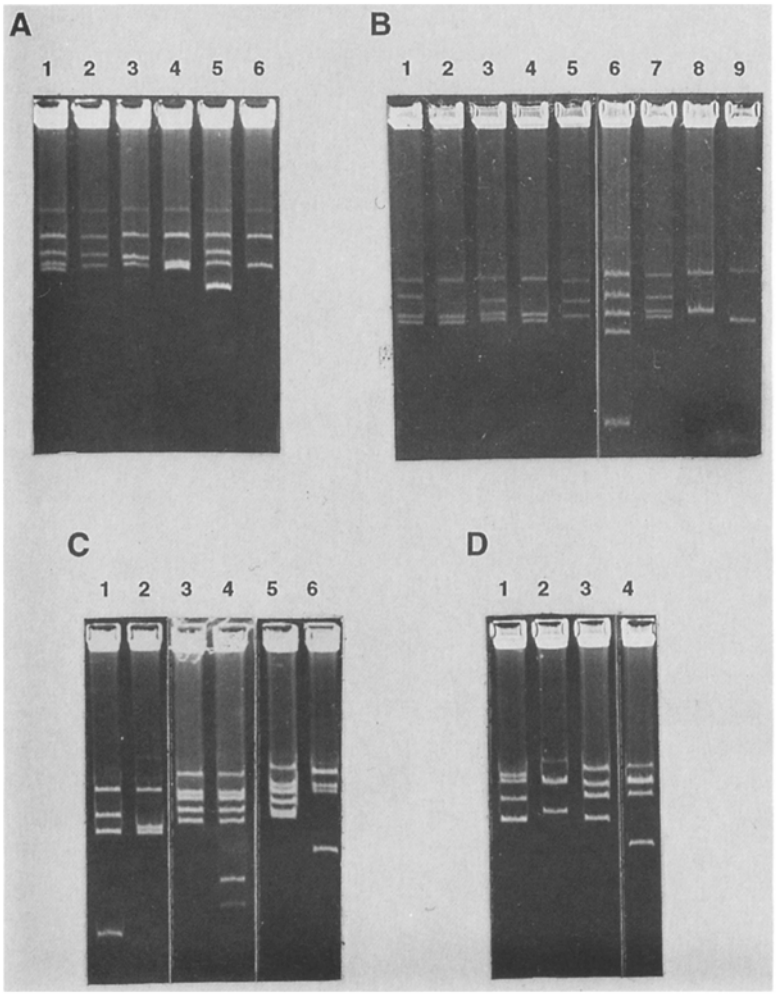

Fig. 1. Examples of agarose gel electrophoresis of plasmids of bean isolates from the four sites. (A) isolates from site A with plasmid profile types: 1 (lane 1), 4 (lane 2), 6 (lane 3), 2 (lane 4), 5 (lane 5), 3 (lane 6). (B) isolates from site B with plasmid profile types: 10 (lane 1), 2 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), 16 (lane 6), 1 (lane 7), 3 (lane 8), 13 (lane 9). (C), isolates from site C with plamid profile types: 17 (lane 1), 2 (lane 2), 19, (lane 3), 24 (lane 4), 23 (lane 5), 25, (lane g). (D) isolates from site D with plasmid profile types: 34 (lane 1), 33 (lane 2), 35 (lane 3), 32 (lane 4).

of isolates of the different locations within each plasmid profile is given in Figure 2. The plasmid profile of every isolate that formed white opaque colonies on MGY medium (group I) corresponded to one profile of type 1 to 18. This kind of isolate was largely dominant among isolates from site A and B. It represented all but one of the isolates in site A, and all but four in site B. It was also represented in 28 % of the isolates of site C but was not found in site D. The plasmid profiles of the isolates growing more slowly (group II) were of type 19 to 40. These isolates were dominant in site C and occupied all the nodules from site D. Some plasmid types were conserved between sites. For example, isolates of plasmid types 1 and 2 were the most abundant in site A and B and isolates of plasmid type 2 were also present in site C. Isolates of plasmid type 19, were present in sites A, B and C. At each site more than 50 % of the isolates were grouped in two plasmid types. Twelve isolates (4 %) had unique plasmid profiles.

Isolates representative of the different plasmid types within each site were sampled for further analysis. For each site and each plasmid type, two

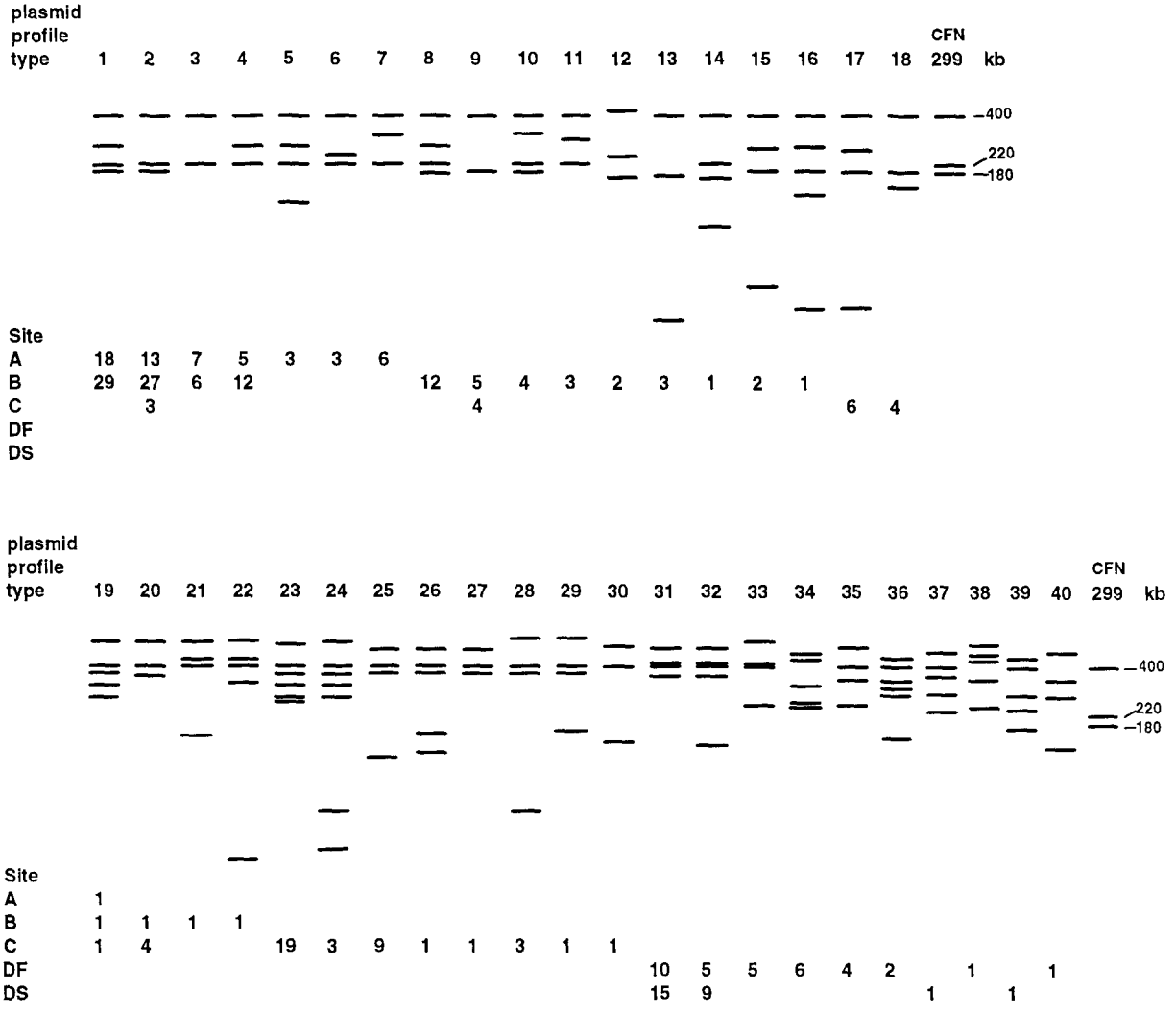

Fig. 2. Schematic representation of plasmid profile types found among the bean isolates and distribution of isolates from each site within each plasmid profile type

isolates were sampled when the number of isolates was more than ten and one when the number was between two and ten. Plasmid types 19, 20, 21, 29, and 35 were also sampled.

Nodulation phenotype

The 46 isolates we sampled nodulated *P. vulgaris* cv Vernandon. Twenty nine of these isolates also nodulated *L. leucocephala*. These isolates which nodulated *Leucaena* all belonged to the group I defined above based on colony morphology and plasmid profile.

Cultural characteristics

The ability to grow on different media, which were chosen because they allow differential growth of the type strains of *Rhizobium* species that nodulate beans (Table 2), was tested for the same sample of isolates. Again, isolates could be grouped in two different clusters which coincided with the two groups already defined. Isolates from group I showed the cultural characteristics of *R. tropici* CFN299 (Table 2). Isolates from group II had the characteristics of *R. leguminosarum* ATCC 10004^T.

Table 2. Phenotypic characteristics of reference strains of bean nodulating *Rhizobium* species and of two groups of isolates described in the text

Characteristics	*R. leguminosarum* ATCC 10004^T	*R. etli* CFN42^T	*R. tropici* CIAT899^T	*R. tropici* CFN299	*Rhizobium* sp H152	*Rhizobium* sp R602	Group I	Group II
Colony morphology on MGY medium	wet st[b]	wet st	wet st	white opaque	wet st	wet st	white opaque	wet st
Growth on :								
MM[a] + citrate	–	–	+	–	–	–	–	–
MM + glycine	–	–	+	–	–	–	–	–
MM + lactate	–	–	–	–	+	–	–	–
MM + dulcitol	+	–	–	–	–	–	–	+
TY + kanamycin	–	–	–	–	–	+	–	–
TY + nalidixic acid	–	+	+	+	+	+	+	–
TY + streptomycin	–	+	+	–	–	+	–	–
LB	–	–	+	–	–	–	–	–
Nodulation of *Leucaena*	–	–	+	+	+	+	+	–

[a] The media used are described in Materials and Methods

[b] st: semitranslucent.

RFLP analysis of PCR-amplified 16S rRNA genes

A subsample representing every plasmid profile type which contained at least two isolates was characterized by RFLP analysis of PCR-amplified 16S rRNA genes. One fragment of amplified DNA could be visualized for each isolate tested (results not shown). Isolates of group I produced a fragment whose size was about 100 bp greater than that of group II isolates from (1600 bp vs 1500 bp). This higher size of the 16S rRNA genes is a characteristic of the *R. tropici* strains CFN299 and C-05-35 (Laguerre et al., 1994) and is due to the presence of an insert in the 16S rRNA genes (Willems and Collins, 1993). Two distinct restriction patterns were observed for each of the two endonucleases tested, *Msp*I and *Ndel*II. The grouping of isolates according to restriction patterns corresponded, for both enzymes, to the groups defined using other criteria. Restriction patterns of one isolate representative of each group and of strains CFN299 and ATCC 10004^T are shown in Figure 3. Restriction patterns of group I were identical to those of *R. tropici* CFN299, and patterns of group II to those of *R. leguminosarum* ATCC 10004^T.

Effectiveness test

Effectiveness was estimated on a sample of isolates from the three sites. Three classes of effectiveness based on the dry matter of the aerial parts after seven weeks of growth were differentiated. Isolates which did not allow the plants to grow more than the uninoculated treatment were considered ineffective. Those that allowed an accumulation of dry matter equivalent to at most 50 % of that accumulated by the reference strain, CIAT899^T, were considered moderately effective. The remaining isolates that allowed a growth of more than 50 % of that of CIAT899^T were considered effective. The results are shown in Table 3. Ineffective isolates were only found in group I.

Discussion

On the basis of symbiotic, cultural and morphological traits and of polymorphism of 16S rRNA

Table 3. Effectiveness of some isolates from sites, A, B, C and D

Site	Group[a]	Number of isolates			
		Total	Effective	Moderately effective	Ineffective
A	I	8	4	3	1
B	I	11	6	3	2
C	I	5	1	2	2
	II	5	5		
D	II	6	4	2	

[a] Groups are based on the phenotypic and genotypic characteristics described in the test

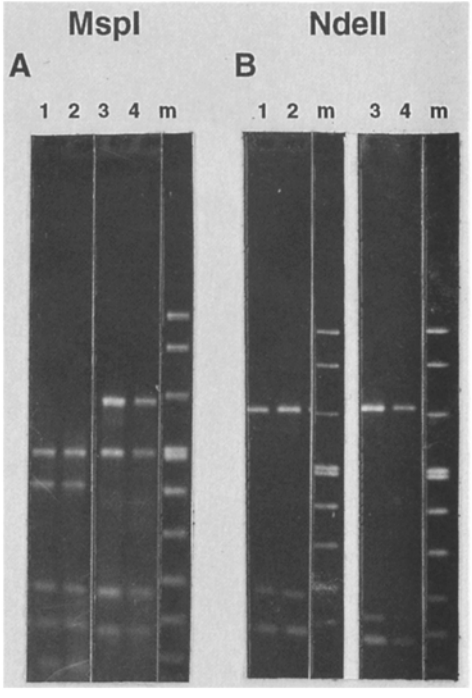

Fig. 3. Agarose gel electrophoresis of amplified 16S rDNA digested with *Msp*I (A) and *Nde*II (B) from reference strains and bean isolates: *R leguminosarum* ATCC 10004^T (lane 1), isolate of group II (lane 2), *R. tropici* IIA CFN 299 (lane 3), isolate of group I (lane 4). Lane m:, molecular weight marker VIII (Boeringer Mannheim, France): 1114 bp, 906 bp, 692 bp, 501 bp, 489 bp, 404 bp, 320 bp, 242 bp, 190 bp, 147 bp.

genes, two well differentiated groups of *Rhizobium* were distinguished among the rhizobia nodulating *P. vulgaris* at four sites. The similarity of phenotypic and genotypic characteristics of each group to those of reference strains led us to assign these groups of isolates to two species, *R. leguminosarum* bv *phaseoli* and *R. tropici*. Within *R. tropici* two subgroups have been identified by Martinez-Romero et al. (1991), type IIA represented by strain CFN299 and type IIB represented by strain CIAT899^T. This subdivision was justified by a rather low level of DNA-DNA hybridization and different phenotypic characteristics. Group I isolates described in the present study and present at 3 of the 4 sites had the same phenotypic characteristics as strain CFN299. Moreover, the presence of an insertion in the 16S rRNA genes, which has been revealed here by the size of the amplified 16S RNA genes, so far has only been observed in CFN299 and one other *R. tropici* type IIA strain (Willems and Collins, 1993). These isolates can therefore be logically considered as *R. tropici* type IIA. Isolates of group II shared symbiotic characteristics with *R. leguminosarum* bv *phaseoli* and *R. etli* bv *phaseoli*. These two latter species have been distinguished from each other by whole DNA hybridization tests, their partial 16S rRNA gene sequences and their multilocus enzyme electrophoretic profiles (Segovia et al., 1993), but the only known phenotypic trait that allows easy differentiation is the resistance of *R. etli* to nalidixic acid. On the basis of their nalidixic sensitivity and their 16S rDNA restriction patterns typical of *R. leguminosarum*, the isolates of group II were classified as *R. leguminosarum* bv *phaseoli*.

Up until now, strains of *Rhizobium* assigned to the species *R. tropici* have been isolated exclu-

sively from tropical regions of South and Central America (Martinez-Romero et al., 1991). The identification of *R. tropici* in a temperate region of Western Europe is a new finding. *R. tropici* was found in the three sites in southwest France. This area is characterized by acidic sandy soils. The better tolerance to acidity of *R. tropici* compared with *R. leguminosarum* could be a factor determining their presence in these soils.

R. leguminosarum bv *phaseoli* was isolated from every site. It was the only species isolated from the nodules of the two bean varieties sown at site D. At the sites located in the southwest, its representation was only 2 and 4% at sites A and B but reached 72% at site C where the total population of rhizobia which nodulates bean was the lowest. It should be noted that five different varieties of bean were used in the field experiments. Whether the differences observed between the sites in the distribution of the two *Rhizobium* species in the bean nodules is due to the selectivity exerted by the plant genotype or whether these differences reflect differential competitiveness or abundance of *Rhizobium* genotypes is not known. Simultaneous characterisation of soil populations would be necessary to answer this question. The absence of *Rhizobium* spp. H152 and R602 in the nodule isolates analyzed was surprising as *Rhizobium* sp. R602 has been found at several locations in France and *Rhizobium* sp. H152 formed one-third of the bean nodules six years before at a site located 500 m away from site D (Geniaux et al., 1993; Laguerre et al., 1993b).

The diversity of plasmid profiles within each species was noteworthy. It was comparable to the level found in other studies (Brockman and Bezdicek, 1989; Laguerre et al., 1992; Mozo et al., 1988). Two plasmid profile types (1 and 2) of *R. tropici* were dominant at sites A and B and one of them, type 2, was also present at site C. The conservation of plasmid profiles over large distances has already been observed for *R. leguminosarum* bv *phaseoli*, *Rhizobium* sp. H152 and *Rhizobium* sp. R602 (Geniaux et al., 1993). Although the number of isolates analysed was different between sites A and B, it can be seen that the population structure was conserved between these two sites which are separated by a distance of about 120 km. This phenomenon has already been noted by Geniaux et al. (1993). However, no generalization can be made as the populations from site C and D were different from each other, from those of sites A and B and from those described by Geniaux et al. (1993).

The estimation of the effectiveness, although based on a limited number of isolates, shows that isolates differed in their effectiveness. More than half of the *R. tropici type* IIA isolates tested were moderately effective or ineffective while 9/11 of the *R. leguminosarum* bv *phaseoli* isolates were effective. Were these results confirmed over a larger sample of isolates, it could mean than nitrogen fixation might be limited at the sites where *R. tropici* type IIA is dominant.

Previous studies have shown that three different rhizobial taxons nodulated beans grown in France (Geniaux et al., 1993; Laguerre et al., 1993a). The results presented here show that a fourth group identified as *R. tropici* is also present in the nodules of beans grown in the country. There was considerable diversity of plasmid profiles within *Rhizobium tropici* type IIA. Although *P. vulgaris* is not indigenous to Western Europe, with a history of less than 500 years, the microsymbionts are very diverse, representing at least four different species which are themselves polymorphic. This would suggest that *Rhizobium* spp. able to nodulate beans pre-existed in European soils when bean was first introduced. This could explain the rapid extension of the crop across the continent.

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