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Characterization of *Rhizobium etli* and other *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. in an Austrian soil

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Abstract

***Phaseolus vulgaris L.* (common bean) is nodulated by rhizobia present in the fields around the Seibersdorf laboratory despite the fact that common bean has not been grown for a long time. Using PCR analysis with repetitive primers, plasmid profiles, *nifH* profiles, PCR-RFLP analysis of the 16S rRNA gene and of the 16S rRNA–23S rRNA intergenic spacer and the nodulation phenotype, two well-differentiating groups could be distinguished. One group showed high similarity to *Rhizobium* sp. R602sp, isolated from common bean in France, while the other showed the same characteristics as *R. etli*. We detected little variation in the symbiotic regions but found higher diversity when using approaches targeting the whole genome. Many isolates obtained in this study might have diverged from a limited number of strains, therefore the Austrian isolates showed high saprophytic and nodulation competence in that particular soil.**

Keywords: Phaseolus vulgaris, Rhizobium etli, rhizobial diversity

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Introduction

Phaseolus vulgaris L. (common bean) has two centres of origin in the Americas, in Mesoamerica and in the southern Andes, which developed independently (Gebts 1990). Seeds of common bean were imported to Europe after the discovery of America in 1492 and were grown extensively already 60 years later (Gebts & Bliss 1988). Rhizobia that can form nitrogen-fixing nodules on *Phaseolus vulgaris* were found to be genetically distant and phylogenetically diverse (Piñero *et al.* 1988; Eardly *et al.* 1995). Traditionally, common-bean-nodulating rhizobia have been classified as *R. leguminosarum* bv. *phaseoli* (Jordan 1984) according to the host plant they nodulate, but later two other species, *R. etli* and *R. tropici* (Martínez-Romero *et al.* 1991; Segovia *et al.* 1993), have been defined. Both, *R. leguminosarum* bv. *phaseoli* and *R. etli* maintain multiple copies of the nitrogenase reductase gene (*nifH*) on the symbiotic plasmid but they possess different 16S rRNA gene sequences (Quinto *et al.* 1982; Martínez *et al.* 1985; Segovia *et al.* 1993). *R. tropici* has only one *nifH* gene copy on the symbiotic plasmid (Martínez-Romero *et al.* 1991). *R. tropici* and *R. etli* nodulate a large number of hosts but their host ranges are different (Martínez-Romero *et al.* 1991; Hernandez-Lucas *et al.* 1995). Phylogenetic analysis based on partial 16S rRNA sequences revealed that *R. tropici* is related to *R. leguminosarum*, while *R. etli* was very close to *Rhizobium* sp. Or191 isolated from ineffective alfalfa nodules in Oregon, USA (Eardly *et al.* 1985) and to strain FL27 isolated from common bean in Mexico (Piñero *et al.* 1988). In general, strains of *Rhizobium* nodulating common bean are of American origin, similar to their host plant. For a long time, *R. leguminosarum* bv. *phaseoli* was believed to be the only microsymbiont of common bean in Europe, but recently *R. tropici* and two new genomic species, *Rhizobium* sp. H152 and *Rhizobium* sp. R602sp, were also found in French soils (Laguerre *et al.* 1993; Amarger *et al.* 1994).

In the fields around the Seibersdorf laboratory common bean is well nodulated, although this crop has not been cultivated for several decades. In this study different approaches were used to classify rhizobia nodulating common bean, targeting the nodulation phenotype as well as symbiotic and chromosomal regions of the genome.

Materials and methods

Bacterial strains

Surface-sterilized seeds of common bean (*Phaseolus vulgaris* L. cv. Extender), faba bean (*Vicia faba* cv.

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Weiselburger), pea (*Pisum sativum* cv. Rheinperle), red clover (*Trifolium repens* cv. Reichersberger) and alfalfa (*Medicago sativa* cv. Saranac) were grown in Leonard jars using soil from fields around the Seibersdorf laboratory. Soil characteristics have been reported by Zapata *et al.* (1987). Common bean has not been cultivated in the laboratory fields for at least 30 years and is presently a rarely grown plant in this region. Rhizobial strains were isolated from surface-sterilized nodules (Somasegaran & Hoben 1985) on yeast–mannitol agar plates (Danso & Alexander 1974). Twenty-six isolates of common bean nodules and 18 isolates, each of faba bean, pea, clover and alfalfa, were used for further characterization. As a control, *Phaseolus vulgaris* L. cv. Extender seeds, with and without surface sterilization, were grown in sterile sand. Reference strains were obtained either from G. Laguerre, France, or from the culture collection at the Seibersdorf Laboratory.

Sample preparation for DNA amplification from cell cultures

Isolates were grown on YM agar plates for 16 h at 28 °C. Cells were resuspended in 100 μL TE (Ausubel *et al.* 1994) and the OD at 600 nm of all samples was adjusted to 2.6. Then, the samples were deep frozen for 4 min at – 70 °C. Afterwards, the cells were set on ice for 1 min, boiled for 2 min, again left on ice for 1 min and boiled again for 2 min. Finally, the cells were centrifuged for 2 min at 14000 r.p.m. and the supernatant was used for the PCR assay.

PCR using repetitive primers

A slightly modified protocol to that described previously (de Bruijn 1992) using PCR with repetitive extragenic palindromic (REP) primers was applied to fingerprint the strains of *Rhizobium* isolated from common bean, faba bean, pea, clover and alfalfa. This protocol yields smaller DNA fragments resulting in more consistent patterns. PCR amplifications were performed in a total reaction volume of 25 μL containing 1 × PCR reaction buffer (50 mM KCl; 20 mM Tris HCl, pH 8.0), 200 μM each of dATP, dCTP, dGTP and dTTP (Pharmacia-LKB), 3 mM $MgCl_2$, 2 μM of each primer, 3 μL of cell extract and 2 U *Taq* DNA polymerase (Gibco, BRL). All amplifications were performed with a Perkin-Elmer thermocycler (GeneAmp PCR System 9600). The temperature cycle for PCR with REP primers consisted of an initial denaturation step at 95 °C for 1 min, followed by 35 cycles of 50 s denaturation at 94 °C, 1 min annealing at 40 °C and 2 min extension at 72 °C and a final extension step for 4 min at 72 °C. The total reaction volumes were examined on 1.5% agarose gels.

RPO1-PCR was also used to differentiate rhizobial strains isolated from common bean. This method employs a single primer, RPO1 (Richardson *et al.* 1995; Schofield & Watson 1985), that works like a random primer. The PCR reaction was carried out as described above in a 25-μL reaction volume using 2.5 μL cell extract and 0.4 μM primer RPO1. The temperature cycle for primer RPO1 was: five cycles of 30 s denaturation at 94 °C, 2 min annealing at 50 °C and 90 s extension at 72 °C; followed by 35 cycles of 10 s at 94 °C, 50 s at 55 °C and 90 s at 72 °C; followed by a final cycle of 20 s at 94 °C, 40 s at 55 °C and 5 min at 72 °C. Amplification products were examined on 1.5% agarose gels.

For data analysis all fingerprints used for comparison were run on the same gel and all bands were scored. The similarity values were calculated by using the analysis program RFLPscan™ (Scanalytics) and they represent the ratio of shared bands over total bands within two lanes being compared during a matching operation. Dendrograms were generated by using the SAHN (Sequential Agglomerative Hierarchical and Nested; Sneath & Sokal 1973) analysis of the program NTSYS-pc (Applied Biostatistics, Inc.).

PCR-RFLP analysis of the 16S rRNA gene and of the 16S–23S rRNA intergenic spacer

PCR amplification of the 16S rRNA gene followed by RFLP analysis (Laguerre *et al.* 1994) was performed with the rhizobial isolates obtained from common bean and with reference strains. PCR conditions were as described above using a 100-μL reaction volume with 8-μL cell extract and 0.1 μM primers rD1 and fD1 (Weisburg *et al.* 1991). The following temperature cycle was used: an initial denaturation step of 1 min at 95 °C followed by 30 cycles of 50 s denaturation at 94 °C, 1 min annealing at 48 °C and 2 min extension at 72 °C and a final extension step of 4 min at 72 °C. Aliquots (17–19 μL) of PCR products were digested with the following restriction enzymes: *Dde*I, *Msp*I, *Nde*II and *Taq*I (Pharmacia-LKB). The resulting DNA fragments were analysed by horizontal agarose gel electrophoresis in 3% agarose gels.

The PCR-RFLP analysis was carried out with the rhizobial isolates obtained from common bean as described above for the 16S rRNA gene using the primers pHr and p23SROI (Massol-Deya *et al.* 1995). Aliquots of PCR products (17–19 μL) were digested with the restriction enzymes *Alu*I, *Hae*III, *Hha*I, *Msp*I and *Pal*I (Pharmacia-LKB).

Analysis of plasmids

For the Southern hybridization analysis with a *R. etli nifH* gene probe, genomic DNAs from the isolates nodulating common bean and the reference strains *R. etli* CFN42 and *Rhizobium* sp. R602sp were digested with *Bam*HI and the resulting fragments were separated by electrophoresis using 0.8% agarose gels. The gels were

blotted on to Hybond-N membranes (Amersham). For preparation of the *nifH* gene probe, plasmid pKW112 containing a *nifH* gene of *R. etli* strain CFN42 (Wilson *et al.*1995) was digested with *Sal*I. After isolating the 300 bp *nifH* internal fragment from a 1.3% agarose gel utilizing the eneclean II Kit (Bio 101), the probe was labelled with α-[^{32}P]dATP to high specific activity (10^8 counts/min/μg NA) by using the Multiprime DNA Labelling System (Amersham) according to the manufacturer´s protocol. The membranes were hybridized with the *nifH* gene probe in Rapid-hyb buffer (Amersham) at 65 °C for 2 h. Washing and autoradiography was carried out as described previously (Sessitsch *et al.* in press).

Plasmid profiles of all isolates nodulating common bean were carried out as described by Hynes *et al.* (1985).

Nodulation host range

Surface-sterilized seeds of *Phaseolus vulgaris* cv. Extender, *Vicia faba* cv. Weiselburger, *Pisum sativum* cv. Rheinperle, *Trifolium repens* cv. Reichersberger, *Medicago sativa* cv. Saranac, *Glycine max* cv. Clay, *Vigna unguiculata* cv. Red Caloona, *Leucaena leucocephala* cv. Cunningham, *Gliricidia sepium* and *Acacia albida* were germinated on 1.5% (w/v) water-agar plates. The seedlings were transplanted into sterile modified Leonard jars (Vincent 1970) containing sand and N-free nutrient solution (Somasegaran & Hoben 1985). Each seedling was inoculated with 10^7 cells of selected common bean isolates and of *R.* sp. R602sp. Plants were grown in the greenhouse at 20–25 °C and harvested 28 days after planting.

Control experiments were carried out in order to ensure that the broad host range detected was not due to contamination. Two nodule isolates were prepared from the different nodulated host plants followed by inoculation of sterile *Phaseolus vulgaris*, *Vigna unguiculata*, *Leuceana* and *Gliricidia* seedlings with those isolates. In addition, nodule isolates of the various host plants were compared by RPO1-PCR fingerprinting.

Results

PCR using repetitive primers

Fingerprints of 26 rhizobial isolates of common bean and of 18 rhizobial isolates, each of faba bean, pea, clover and alfalfa, were obtained using REP primers. When using REP-PCR, the common bean strains could be divided into two well-differentiated groups A and B with, respectively, four and three subdivisions and one strain with an unique pattern (CbS-21). Group A represented about 40% and group B around 60% of all common bean nodule isolates (Table 1). The dendrogram showed high similarity between group A isolates and *R.* sp. R602sp, while group B rhizobia did not show high similarity to any of the reference strains used (Fig. 2). Nodule isolates from faba bean, pea, alfalfa and clover showed higher diversity; seven different profiles were found among 18 faba bean isolates, 10 profiles among 18 pea isolates and 13 profiles among 18 clover and among 18 alfalfa isolates. One identical REP profile was found among isolates from faba bean and pea. Fingerprints of common bean isolates obtained by PCR using the RPO1 primer resulted in a similar grouping, but the discrimination level was slightly lower, group A comprised two subdivisions, group B could be divided in three and again CbS-21 had an unique pattern (Table 1). In this analysis, all group A isolates except CbS-18 showed the same fingerprint as *R.* sp. R602sp, but CbS-18 showed high similarity to *R.* sp. R602sp. Group B isolates showed low similarity to the common bean nodulating reference strains used. Although CbS-21 showed quite distinct profiles in both analyses, it was included in group B as it showed highest similarity to those isolates (Fig. 2). In addition, other, less discriminative, analyses (see below) revealed high similarity to group B isolates.

PCR-RFLP of the 16S rRNA gene and the 16S rRNA–23S rRNA intergenic spacer

Analysis of the 16S rRNA gene was used to classify the isolated common bean rhizobia into species. The DNA fragment patterns obtained by digesting the 16S rRNA

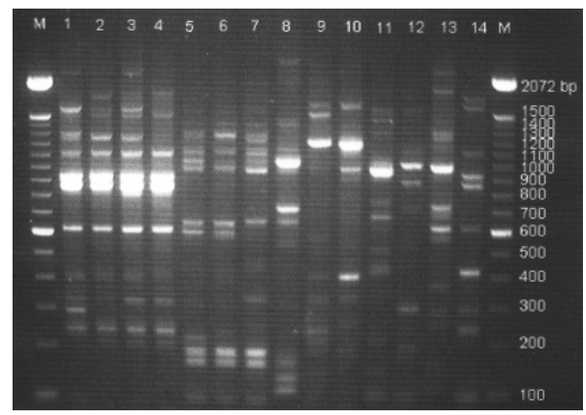

Fig. 1 REP-PCR fingerprint patterns of common bean nodulating isolates and of reference strains. Lanes showing DNA molecular weight standards are labelled with 'M'. Lanes 1–8 show the REP-PCR products obtained from representative group isolates of common bean CbS-1 (lane 1), CbS-3 (lane 2), CbS-17 (lane 3), CbS-18 (lane 4), CbS-2 (lane 5), CbS-4 (lane 6), CbS-7 (lane 7) and CbS-21 (lane 8). Lanes 9–14 show the REP PCR products of the common bean reference *Rhizobium* strains *R. tropici* type CIAT899 (lane 9), *R. tropici* type CFN299 (lane 10), *R. etli* CFN42 (lane 11), *R. leguminosarum* bv. *phaseoli* H131 (lane 12), *R.* sp. FL27 (lane 13) and *R.* sp. R602 (lane 14).

Isolate	REP-PCR	RP01-PCR	PCR-RFLP OF THE 16S-23S rRNA intergenic spacer				
			*Alu*I	*Hae*III	*Hha*I	*Msp*I	*Pal*I
Group A isolates							
CbS-1*	AI	AI	AI	AI	AI	AI	AI
CbS-3	AII	AI	AI	AII	AII	AI	AII
CbS-5	AII	AI	AI	AII	AII	AI	AII
CbS-8	AII	AI	AI	AII	AII	AI	AII
CbS-12	AII	AI	AI	AII	AII	AI	AII
CbS-13	AII	AI	AI	AII	AII	AI	AII
CbS-15	AII	AI	AI	AIII	AI	AI	AIII
CbS-17	AIII	AI	AI	AIII	AI	AI	AIII
CbS-18	AIV	AII	AI	AIV	AIII	AI	AIII
CbS-22	AII	AI	AI	AII	AII	AI	AII
CbS-23	AIII	AI	AI	AIII	AI	AI	AIII
Group B isolates							
CbS-2	BI	BI	BI	BI	BI	BI	BI
CbS-4	BII	BII	BI	BI	BI	BI	BI
CbS-6	BI	BI	BI	BI	BI	BI	BI
CbS-7	BIII	BIII	BII	BII	BII	BII	BII
CbS-9	BII	BI	BI	BI	BI	BI	BI
CbS-10	BII	BII	BI	BI	BI	BI	BI
CbS-11	BI	BI	BI	BI	BI	BI	BI
CbS-14	BII	BI	BI	BI	BI	BI	BI
CbS-16	BI	BI	BI	BI	BI	BI	BI
CbS-19	BI	BI	BI	BI	BI	BI	BI
CbS-20	BI	BI	BI	BI	BI	BI	BI
CbS-24	BII	BI	BI	BI	BI	BI	BI
CbS-25	BII	BII	BI	BI	BI	BI	BI
CbS-26	BI	BI	BI	BI	BI	BI	BI
CbS-21	BIV	BIV	BIII	BIII	BIII	BIII	BIII

*Underlined isolates indicate representative group isolates.

Table 1 Groupings of Austrian *Phaseolus vulgaris* nodule isolates derived from PCR patterns using repetitive primers and PCR-RFLP analysis of the 16S–23S rRNA intergenic spacer

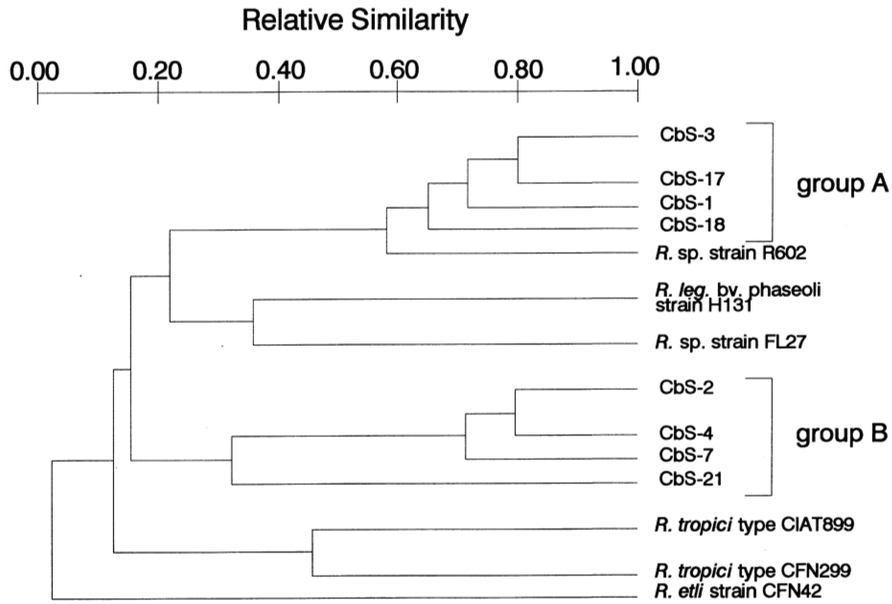

Fig. 2 Dendrogram of common bean nodulating isolates derived from REP-PCR fingerprints.

Table 2 PCR-RFLP analysis of the 16S rRNA gene of common bean nodule isolates

Strain, Isolate	Restriction pattern of the amplified 16S rRNA gene			
	*Dde*I	*Msp*I	*Nde*II	*Taq*I
R. l. bv. *viciae* VF39	a	a	a	a
R. l. bv. *phaseoli* H131	a	a	a	a
R. meliloti 2001	a	a	b	a
R. galegae 625	c	f	f	e
R. loti NZP2234	a	j	e	d
R. haukii CCBAU2609T	a	j	e	e
R. fredii MSDJ1536	a	d	d	c
R. spp. (Phaseolus) FL27	a	a	h	e
R. spp. (Phaseolus) R602sp	a	a	h	e
R. spp. (Phaseolus) H152	d	b	g	f
R. etli bv. *phaseoli* CFN42	a	a	b	a
R. tropici type IIA CFN299	b	c	c	b
R. tropici type IIB CIAT899	a	b	a	a
CbS-1, -3, -5, -8, -12, -13, -15, -17, -18, -22, -23	a	a	h	e
CbS-2, -4, -6, -7, -9, -10, -11, -14, -16, -19, -20, -21, -24 -25, 26	a	a	b	a

gene with different restriction enzymes were compared with those from reference strains (Table 2). Again, the common bean nodule isolates formed two groups, corresponding to the groups A and B described above. Group A rhizobia were found to be different from *R. leguminosarum*. bv. *phaseoli*, *R. tropici* or *R. etli*, but had the same profiles as *R.* sp. strain FL27, which was isolated in Mexico (Piñero *et al.* 1988) and *R.* sp. R602sp, a French isolate (Laguerre *et al*. 1993; Amarger *et al.* 1994). Group B isolates showed the same patterns as *R. etli* and the presence of three copies of *nifH*, revealed by Southern hybridization analysis, confirmed their close relationship to *R. etli.*

Higher diversity was found by PCR-RFLP analysis of the 16S rRNA–23S rRNA intergenic spacer region. Group A and B rhizobia isolated from common bean could be divided into several subgroups which correlated to a certain extent with the results obtained from other comparisons (Table 1).

Analysis of plasmids

We determined the *nifH* gene hybridization patterns of the common bean isolates. With all group B isolates, except isolate CbS-21, the same pattern was found showing three bands, about 9.8, 6.5 and 5.1 kb in size. They shared the 9.8-kb and 6.5-kb bands with *R. etli* type strain CFN42. (Martínez *et al.* 1985). Isolate CbS-21 showed three bands of 13.0, 11.3 and 2.9 kb. Group A rhizobia and *R.* sp. R602sp showed a single band of 8.3 kb. Figure 3 shows the *nifH* profiles of representative group isolates of the common bean nodulating rhizobia.

Group A and group B strains were also distinguishable by their plasmid profiles. Three plasmids showing the same profile were visualized in group A strains and five plasmids in group B strains, with one exception, CbS-7, that had a different pattern with six plasmids.

Nodulation host range

Different host ranges were found among isolates of group A and B. The representative isolates of each subgroup were all able to nodulate common bean from which they were isolated initially. They did not nodulate plants commonly grown in that soil as faba bean, pea, alfalfa and clover. Isolates of group A (CbS-1, CbS-3, CbS-17 and CbS-18) and *R.* sp. R602sp showed a broader host range than isolates of group B as they formed nodules on *Vigna unguiculata*, *Leucaena* and *Gliricidia*. Nodule isolates from these plants showed the same host range after re-inoculation and the same RPO1 fingerprints as the original inoculant strain. Isolates of group B (CbS-2, CbS-4, CbS-7 and CbS-21) nodulated only common bean. All nodules formed on the different hosts were red, so we assumed that they contained leghaemoglobin.

In the control experiment, where *P. vulgaris* cv. Extender seeds were grown in sterile sand, no nodules were formed.

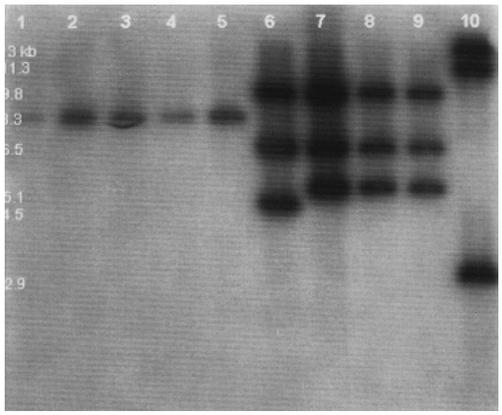

Fig. 3 Autoradiogram of a Southern blot of *Bam*HI-digested genomic DNA hybridized with a 300 bp *nifH* fragment of strain CFN42. Strains tested were *Rhizobium* sp. R602 (lane 1), CbS-1 (lane 2), CbS-3 (lane 3), CbS-17 (lane 4), CbS-18 (lane 5), *R. etli* CFN42 (lane 6), CbS-2 (lane 7), CbS-4 (lane 8), CbS-7 (lane 9) and CbS-21 (lane 10).

Discussion

Because common bean had not been cultivated previously in the fields around the Seibersdorf laboratory but was nodulated well without inoculation, the main goal of this study was to characterize *Rhizobium* strains nodulating this crop. Nodulation of common bean was reported to be quite promiscuous (Eardly *et al.* 1995; van Berkum *et al.* 1996), and even strains of *R. meliloti* and *R. fredii* have been found to nodulate common bean (Bromfield & Barran 1990; Eardly *et al.* 1985, 1992; Sadowsky *et al.* 1988). In order to detect any cross-inoculation, rhizobia were also isolated from other legumes such as faba bean, pea, red clover and alfalfa, all of which nodulate with indigenous strains. Isolates from those legumes showed high diversity when analysed by REP-PCR. Our analysis did not reveal that bean belonged to the same cross-inoculation group as the other legume plants analysed. Cross-inoculation was found as expected among rhizobia nodulating pea and faba bean as both are nodulated by *R. leguminosarum* bv. *viciae*. Using a polyphasic approach, two well-differentiated groups could be distinguished among the isolates from common bean, which were assigned in this paper as group A and B.

Results obtained from PCR using REP primers have been demonstrated to be in agreement with those obtained from multilocus enzyme electrophoresis (de Bruijn 1992) and RFLPs (Judd *et al.* 1993). These and the present findings confirm that this method is an appropriate tool to analyse bacterial communities. However, it has been previously reported that REP-PCR fingerprints reflect the variability of chromosomal DNA regions of *Rhizobium* but not the variability of symbiotic DNA regions (Laguerre *et al.* 1996). Richardson *et al.* (1995) showed that *Rhizobium* can be differentiated by their RPO1-PCR amplification pattern at the strain level. In the present study, the classification obtained using this method correlated in general well with that obtained by other methods, although less variation within group A could be detected. As PCR methods using repetitive primers reflect differences in the whole genome (chromosome), we also applied other methods targeting specific parts of the genome.

Classification of bacteria has been based to a great extent on the 16S rRNA gene (Willems & Collins 1993). Laguerre *et al.* (1994) showed in a recent study that the RFLP analysis of the PCR-amplified 16S rRNA gene was in full agreement with data based on DNA-rRNA hybridizations and sequence analysis of the 16S rRNA gene. Isolates belonging to group A could not be assigned to any of the recognized species nodulating *P. vulgaris*, i.e. *R. leguminosarum* bv. *phaseoli*, *R. etli* or *R. tropici*. However, the RFLP-patterns obtained were the same as from *Rhizobium* sp. R602sp, which was collected in France and classified as a new species (Laguerre *et al.* 1993, 1994). The partial 16S rRNA gene sequence of R602sp was found to be identical to strain FL27 (Laguerre *et al.* 1993), a Mexican isolate from common bean which is poor in nitrogen fixation (Piñero *et al.* 1988). Isolates from group B showed the same 16S rRNA-RFLP pattern as *R. etli* type strain CFN42 (Segovia *et al.* 1993) that was distinct from other patterns. Southern hybridization with a *nifH* gene probe of strain CFN42 revealed the presence of three *nifH* gene copies in all isolates within family B. This finding also indicated the presence of *R. etli* in this particular soil in Austria as only *R. etli* and *R. leguminosarum* bv. *phaseoli* maintain multiple *nifH* genes. The fact that the fingerprints generated by PCR with repetitive primers did not show high similarity to the *R. etli* reference strain CFN42 could be due to the high genomic instability reported in *R. etli* (Flores *et al.* 1988; Brom *et al.* 1991). Furthermore, it has been argued that repetitive elements change faster than the genome as a whole (Martínez-Romero 1994) as they might be involved in recombination and amplification events (Flores *et al.* 1988). The intergenic spacer between the 16S rRNA and the 23S rRNA genes is not well conserved and thus exhibits a large degree of variation (Massol-Deya *et al.* 1995), that can be used for differentiation at the strain level. Nour *et al.* (1994) found that RFLP analysis of the 16S rDNA plus intergenic spacer is in accordance with results obtained by multilocus enzyme electrophoresis. The different patterns found among isolates belonging to group A and B were in general in good correlation with PCR analysis using repetitive primers. Using plasmid profiles and *nifH* patterns, little variation among the common bean isolates was detected. More discriminative methods would be needed in order to determine the variation of symbiotic genes, which are located on plasmids in most fast-growing strains of *Rhizobium*.

From the results obtained we suggest that the *Phaseolus vulgaris*-nodulating strains found in Austria were derived from rhizobia originating in Mesoamerica which might have been imported as seed contaminants. Two analyses indicate that isolates of group B are strains of *R. etli*, a species that has its centre of origin in Mesoamerica. Diversity is not very high among the Austrian isolates as the host plant is not native in the area and is not extensively cultivated in Austria. However, it seems that some strains persist well even in the absence of the host plant and at least some of the strains were shown to be very competitive (A. Sessitsch *et al.* unpublished data). It is striking that no differences could be detected among isolates of group A using methods that target symbiotic regions in the genome. Nevertheless they differ in the intergenic region between 16S rRNA and 23S rRNA and variances were detected by PCR with repetitive primers. These isolates might have evolved from one strain and the members of group A might represent divergent lineages selected by this particular environment. In the case of group B, two isolates, CbS-7 and CbS-21, were very distinct from the majority of the members of this group according to most of the analyses. We presume that these strains carried these differences already when introduced into this soil. Among the other isolates of group B, small differences could be detected by using PCR with repetitive primers and also these isolates might represent better adapted descendants derived from one strain.

Sequence analysis of the 16S rRNA gene of strains of group A will help to assign the species of this group and to understand phylogenetic relationships to other *Rhizobium* species nodulating common bean. As the isolates obtained in this study possess high saprophytic competence and as some of them appear to be very competitive when nodulating the host plant, studying rhizobial diversity in a particular soil might help to select competitive and adapted inoculant strains.

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