APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1996, p. 2029–2036 Vol. 62, No. 6
0099-2240/96/$04.00+0

Typing of Rhizobia by PCR DNA Fingerprinting and PCR-Restriction Fragment Length Polymorphism Analysis of Chromosomal and Symbiotic Gene Regions: Application to *Rhizobium leguminosarum* and Its Different Biovars

GISÈLE LAGUERRE,* PATRICK MAVINGUI,† MARIE-REINE ALLARD, MARIE-PAULE CHARNAY,‡ PHILIPPE LOUVRIER, SYLVIE-ISABELLE MAZURIER, LIONEL RIGOTTIER-GOIS, AND NOËLLE AMARGER

Laboratoire de Microbiologie des Sols, Institut National de la Recherche Agronomique, 21034 Dijon Cedex, France

Received 6 November 1995/Accepted 5 April 1996

Characterization of 43 strains of *Rhizobium leguminosarum* biovars viciae, trifolii, and phaseoli was performed by two methodologies based on PCR amplification, i.e., PCR DNA fingerprinting of interrepeat sequences and restriction fragment length polymorphism (RFLP) analysis of PCR-amplified chromosomal and symbiotic gene regions. Groupings generated by PCR DNA fingerprinting with either extragenic palindromic repetitive primers or two different single random primers were correlated with similar levels of resolution. Although less discriminating, PCR-RFLP analysis of intergenic spacer between genes coding for 16S and 23S rRNA (16S and 23S rDNA) yielded intraspecific polymorphisms. The classification of strains was independent of the biovar status and was in agreement with those obtained by PCR DNA fingerprinting. Intrabiovar variation within symbiotic gene regions was detected by PCR-RFLP analysis of *nifDK* and *nodD* gene regions, but the strains were grouped according to their biovar. The rDNA intergenic spacer and *nif* primers were verified to be universal for rhizobial species by testing of various reference strains, whereas the *nod* primers designed in this study were biovar or species specific for *R. leguminosarum* and *Rhizobium etli*. Classifications of *R. leguminosarum* strains by the PCR-based methods were correlated with those previously obtained by conventional total DNA restriction profile comparisons and RFLP analysis using chromosomal and symbiotic gene probes. Ranges of discriminating powers were also equivalent between the two approaches. However, the PCR-based methods are much less time-consuming and are therefore more convenient.

Rhizobium is a genus of soil bacteria whose members are able to establish a nitrogen-fixing symbiosis with legumes. In most *Rhizobium* species, genes essential for symbiosis, and in particular genes that determine the plant host specificity, are carried on a symbiotic plasmid. *Rhizobium* strains contain in addition other phenotypically cryptic plasmids, and plasmid DNA may represent up to 50% of the genome (35).

Restriction fragment length polymorphism (RFLP) analysis of Southern-blotted genomic DNA with various chromosome and symbiotic plasmid probes has been used to type strains in different species of *Rhizobium* (5, 10, 12, 14, 20, 24, 26, 36, 53). Distinct symbiotic gene hybridization patterns could be found in identical chromosomal types, and correlations were not always found between chromosomal and symbiotic plasmid types, suggesting interstrain transfer and recombination of symbiotic sequences in the course of evolution (12, 20, 24, 26, 36, 53). Therefore, for a proper characterization of *Rhizobium* strains, chromosomal and symbiotic gene markers should be concomitantly used.

Recently, various methods based on the PCR have been proposed to characterize *Rhizobium* strains and to examine genetic relationships in *Rhizobium* groups. Direct sequencing of genes coding for 16S rRNA (16S rDNA) amplified by PCR (8, 23, 33, 38, 48, 50, 51) and RFLP analysis of these PCR-amplified sequences (PCR-RFLP analysis) (22) have been used to establish genetic relationships and to characterize *Rhizobium* strains at the species and higher levels. Sequences of 16S rDNA are known to be highly conserved among eubacteria (49), and analysis of genetic variations in this region is not appropriate to differentiate strains within species. Greater variability can be obtained by analysis of intergenic spacer (IGS) sequences between 16S and 23S rDNA (16S-23S rDNA IGS sequences) in order to examine chromosomally encoded genetic variations at the intraspecies level (1). Both sequencing and RFLP analysis of 16S-23S rDNA IGS sequences have been recently used for differentiation of strains within various bacterial species, including chickpea rhizobia (11, 28, 29, 32).

Other PCR methods based on direct analysis by gel electrophoresis of multiple DNA fragments amplified simultaneously have been used to type rhizobia (PCR DNA fingerprinting). Random primers have been proposed to fingerprint *Rhizobium* strains (2, 7, 13). Dooley et al. (7) proposed this approach for identification and phylogenetic grouping of *Rhizobium* isolates. DNA fingerprints can also be generated by using pairs of primers derived from repetitive extragenic palindromic (REP)

* Corresponding author. Mailing address: Laboratoire de Microbiologie des Sols, INRA, 17, rue Sully, B.V. 1540, 21034 Dijon Cedex, France. Phone: 33 80 63 30 93. Fax: 33 80 63 32 24. Electronic mail address: laguerre@dijon.inra.fr.

† Present address: Centro de Investigacion sobre Fijacion de Nitrogeno, Universidad National Autonoma de Mexico, Cuernavaca, Morelos, Mexico.

‡ Present address: Institut National Agronomique-Paris-Grignon, Laboratoire de Recherche de Science des Sols, 78850 Thiverval-Grignon, France.

and enterobacterial repetitive intergenic consensus (ERIC) sequences (47). de Bruijn (4) demonstrated the usefulness of DNA fingerprinting by PCR using REP and ERIC primers (REP- and ERIC-PCR) for the identification and classification of members of several *Rhizobium* species. These methods have been used to type several rhizobial strains (19, 27, 30).

The PCR DNA fingerprint methods reflect variations on DNA sequences located throughout the whole genome, but they do not permit the investigation of the diversity of symbiotic plasmids among chromosomally closely related strains. Enough variability was found in the IGS region between *nifD* and *nifK* genes, which are nitrogenase structural genes located on the symbiotic plasmid in most *Rhizobium* species, for typing, by PCR-RFLP analysis, of nitrogen-fixing strains of the genus *Frankia* (16).

While various PCR-based approaches have been (or could be) used to type rhizobial strains, we lack comparative data to evaluate their respective levels of resolution and limitations. This is necessary in order to make a decision among the methods and to compare results generated by them. In the present study, we used and compared PCR-RFLP analyses of chromosomal DNA sequences (16S-23S rDNA IGS sequences) and of the symbiotic *nifDK* gene region to type and classify strains of the different biovars of *Rhizobium leguminosarum*. We have also developed a PCR-RFLP method to analyze variations in nodulation (*nod*) genes by using the region of the *nodD* gene, a gene essential in regulation of *nod* gene expression, as a target. In addition, the strains were examined by PCR DNA fingerprinting using random and REP primers. The sample of strains was chosen to represent various combinations of chromosomal types and symbiotic (*nod* and *nif*) gene hybridization patterns previously characterized on the basis of conventional RFLP analysis (12, 24, 26). In addition, members of other rhizobial species were included. We have thereby aimed to evaluate the usefulness and limitations of different PCR-based methods to characterize *Rhizobium* strains.

MATERIALS AND METHODS

Bacterial strains. Forty-three *R. leguminosarum* strains representing the three biovars, viciae, trifolii, and phaseoli, of the species were used in this study. They were chosen from among those previously characterized by total DNA restriction fingerprinting and RFLP analysis of total DNA using various chromosomal and plasmid-encoded symbiotic gene probes (12, 24, 26). In addition, we analyzed a collection of 19 reference strains representing most of the various rhizobial species, including the type strains of *R. leguminosarum*, *Rhizobium tropici*, *Rhizobium etli*, *Rhizobium loti*, *Rhizobium galegae*, *Rhizobium huakuii*, *Sinorhizobium meliloti*, and *Sinorhizobium fredii*. The additional strains were *R. leguminosarum* bv. trifolii USDA 2011, *R. leguminosarum* bv. phaseoli 8002, *S. meliloti* RCR2011, *R. tropici* CFN299, *Rhizobium* (*Cicer*) sp. strain USDA 3233, *Rhizobium* (*Phaseolus*) sp. strains R602 and H152, *Rhizobium* (*Leucaena*) sp. strain USDA 3497, *Sinorhizobium teranga* ORS 1007, *Bradyrhizobium* (*Lupinus*) sp. strain MSDJ 718, and *Bradyrhizobium japonicum* USDA 110. Sources of the reference strains have been previously reported (22), except for *R. leguminosarum* bv. phaseoli 8002, which was kindly provided by J. E. Beringer.

Oligonucleotide primers. The sequences of the oligonucleotide primers used for PCR are shown in Table 1. Primers SPH1 (13) and PucFor (2) were used for randomly primed PCR fingerprinting. The REP1R-I and REP2-I primer pair (47) was used for REP-PCR fingerprinting. Primers FGPS1490 and FGPL132′ were chosen to amplify the 16S-23S rDNA IGS regions. FGPS1490 derives from conserved sequences in the 3′ part of 16S rDNA genes (29), and reverse primer FGPL132′ corresponds to the 5′ part of the 23S rDNA gene right next to the IGS (34). For amplification of *nifD* through *nifK* fragments, primer FGPD807, corresponding to the *nifD* sequence conserved among nitrogen-fixing bacteria, was used (16). Reverse primer FGPK492′, kindly provided by P. Normand (University of Lyon), was defined by comparison of *nifK* sequences of *B. japonicum*, *Klebsiella pneumoniae*, and *Frankia* strains. The primers used to amplify the *nod* gene regions were chosen by comparison of sequences of *nod* gene regions of *R. leguminosarum* bv. viciae, trifolii, and phaseoli available in the EMBL and GenBank Data Libraries. Primer NBA12 and reverse primer NBF12′ were designed from the conserved *nod* box sequences of promoter regions of genes *nodA* and *nodF* (41), respectively, flanking the *nodD* genes of *R. leguminosarum* bv. viciae and trifolii (Fig. 1A). This pair of primers was used to amplify the *nodD* through *nodF* gene region in *R. leguminosarum* bv. viciae and trifolii. Primer NODD2PH678 corresponds to an oligonucleotide sequence in the 5′ part of the *nodD2* gene of *R. leguminosarum* bv. phaseoli (3), and the reverse primer NODD3PH2152′ corresponds to a sequence in the 5′ part of the published sequence of the *R. leguminosarum* bv. phaseoli gene *nodD3* (3) (Fig. 1B). This primer pair allowed the amplification of the *nodD2* through *nodD3* gene region in *R. leguminosarum* bv. phaseoli. Reverse primer NODDRL2′ corresponds to a consensus oligonucleotide sequence in the 3′ region of the *nodD* genes of *R. leguminosarum* bv. viciae (42) and trifolii (37) and the *nodD2* gene of *R. leguminosarum* bv. phaseoli (3) (Fig. 1). Primer pairs NBA12-NODDRL2′ and NODD2PH678-NODDRL2′ were used to amplify the *nodD* gene of *R. leguminosarum* bv. viciae and trifolii and the *nodD2* gene of *R. leguminosarum* bv. phaseoli, respectively. Oligonucleotides FGPS1490 and FGPL132′ were synthesized by Appligene (Strasbourg, France), and all the others were synthesized by Eurogentec (Seraing, Belgium).

TABLE 1. Oligonucleotides used as PCR primers

Primer	5′-3′ nucleotide sequence[a]	Reference
FGPS1490	TGCGGCTGGATCACCTCCTT	29
FGPS132′	CCGGGTTTCCCCATTCGG	34
FGPD807	CACTGCTACCGGTCGATGAA	16
FGPK492′	GATGACCTCGGCCAT	31
NBA12	GGATSGCAATCATCTAYRGMRTGG	This study
NBF12′	GGATCRAAAGCATCCRCASTATGG	This study
NODDRL2′	CTCSCSSAWCCAKATGYTYCC	This study
NODD2PH678	TGAGTTGCAAGGGCCTTGATC	This study
NODD3PH2152′	AGATGACTGCGCCCCCGATAG	This study
PucFor	GTAAAACGACGGCCAGT	2
REP1R-I	IIIICGICGICATCIGGC	47
REP2-I	ICGICTTATCIGGCCTAC	47
SPH1	GACGACGACGACGAC	13

[a] Abbreviations: S = C or G; Y = C or T; M = A or C; R = A or G; W = A or T; K = G or T; and I = inosine.

PCR amplification. Total DNA extraction and bacterial cell preparation for PCR amplification were done as previously described (22). The PCRs were carried out with 50 to 100 ng of pure genomic DNA or with 5 to 15 μl of bacterial cell suspension as template DNA. PCRs with random primers SPH1 and PucFor and REP primers were performed as described by Harrison et al. (13), Coutinho et al. (2), and de Bruijn (4), respectively, except that 1 U of *Taq* polymerase (Appligene) was used in a 25-μl reaction volume. The other PCRs were carried out in 100-μl reaction volume by mixing template DNA with the polymerase reaction buffer (10 mM Tris-Cl [pH 9; 25°C], 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$); 20 μM (each) dATP, dCTP, dTTP, and dGTP; 0.05 μM (each) primers; and 1 U of *Taq* polymerase, except for amplification of *nodD* through *nodF* gene regions, for which reactions were made with 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, and 0.1 μM each primer. All reaction mixtures were sealed with a thin layer of paraffin oil. PCR amplifications were performed by using a thermal cycler (TRIO-Thermoblock TB1; Biometra, Göttingen, Germany) with the following standard 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 final extension at 72°C for 3 min. The temperature profile was modified for amplification of the *nodD* through *nodF* and *nifD* through *nifK* gene regions by increasing the annealing temperature to 57°C and 63°C, respectively. For randomly primed PCRs and REP-PCRs, aliquots of 3 to 8 μl of PCR products were analyzed by horizontal electrophoresis in 1.5% agarose gels in TAE buffer (40 mM Tris-HCl, 4 mM sodium acetate, 1 mM EDTA [pH 7.9]). Electrophoresis was carried out at 100 V for 1 h with standard gels (6 by 8 cm) (Bethesda Research Laboratories Horizon 58 apparatus). For the other PCRs, amplified DNA was examined by electrophoresis in 0.9% agarose gels with 4-μl aliquots of PCR products. Gels

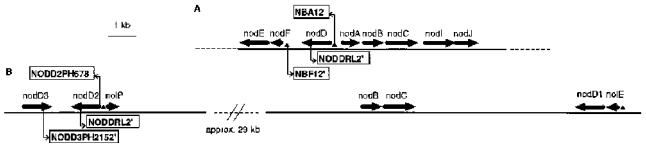

FIG. 1. Locations of oligonucleotide sequences used as primers for PCR amplification of *nod* gene regions in *R. leguminosarum* bv. viciae and trifolii (updated from reference 46) (A) and in *R. leguminosarum* bv. phaseoli (3) (B). Priming directions are indicated by arrows. Black triangles indicate the positions of *nod* boxes. approx., approximately.

were stained with ethidium bromide and photographed under UV illumination with Polaroid type 665 positive-negative film.

Restriction fragment analysis. Aliquots (8 μl) of PCR products were digested with 10 U of restriction endonuclease in 10-μl reaction volumes by using the manufacturer's recommended buffer and temperature. The following restriction endonucleases were used: *Alu*I (Gibco BRL, Cergy Pontoise, France), *Cfo*I (Boehringer Mannheim, Meylan, France), *Dde*I, *Hae*III, *Hin*fI, *Msp*I, *Nde*II, and *Taq*I (Appligene). Restricted DNA was analyzed by horizontal electrophoresis in 3 to 4% agarose (NuSieve 3:1 or MetaPhor; FMC, Rockland, Maine) gels. Electrophoreses were carried out at 80 V for 4 h with standard gels (11 by 14 cm) (Bethesda Research Laboratories Horizon 11-14 apparatus), and the gels were stained and photographed as described above. Similarities between each pair of strains were estimated from the proportion of shared restriction fragments to the total number of fragments identified for all the strains examined or from the proportion of shared restriction sites for cluster analysis of the *nodD* gene by using a simple matching coefficient (40). Dendrograms were constructed from the similarity matrix by the unweighted pair group method with arithmetic mean (UPGMA) (39). In order to test the goodness of fit of cluster analysis, cophenetic value matrices were calculated and compared with the original similarity matrices that were UPGMA clustered by using the NTSYS-pc analysis package (version 1.8; Exeter Software, Setauket, N.Y.). Relationships between strain groupings made by the various methods were assessed by comparison of the data matrices with NTSYS-pc. Matrix correlation was estimated by the correlation coefficient *r*, which is the normalized Mantel test statistic *Z*. Statistical significance tests were performed by comparing the observed *Z* value with its permutational distribution (1000 random permutations) and computing the probability, *P*, that the random *Z* values were superior or equal to the observed *Z* value.

RESULTS

PCR DNA fingerprinting. Forty-three strains of *R. leguminosarum* bv. viciae, trifolii, and phaseoli were initially analyzed by REP-PCR DNA fingerprinting. The results are shown in Table 2 and Fig. 2A. The strains which exhibited identical fingerprints were grouped in the same REP-PCR fingerprint type. Since some types shared most of their bands and thus appeared very similar, they were sorted into the same REP group (identified by a single letter). Thirty-nine types, distributed into 11 groups, were recorded. The strains were further examined by PCR DNA fingerprinting using two different oligonucleotides as a single random primer, SPH1 and PucFor. The classification of strains and the levels of resolution were similar to those achieved by REP-PCR DNA fingerprinting (Fig. 2B and C).

However, we observed that the banding patterns obtained by PCR could markedly vary for the same strain depending on the supplier of primers and sometimes for the same supplier depending on batches of primers; the various sources of *Taq* DNA polymerase; and, for randomly amplified polymorphic DNA primer PucFor, the type of thermal cycler used (data not shown).

PCR-RFLP analysis of 16S-23S rDNA IGS regions. 16S-23S rDNA IGS regions of the 43 *R. leguminosarum* strains were amplified with primers FGPS1490 and FGPL132′. Most strains produced single bands ranging from 1,160 to 1,400 bp as estimated by summing the sizes of the restriction fragments after digestion with restriction enzymes (Table 3). Some strains, of which the purity was carefully checked, reproducibly exhibited an additional band in the same range of size (Fig. 3, lanes 9, 10, and 13). The 19 additional rhizobial strains all produced single bands in the range of sizes described above with the exception of the *R. loti*, *R. huakuii*, and *B. japonicum* strains, which produced single amplified fragments that were smaller in size, i.e., about 900 to 1,000 bp (data not shown).

Seven 4-base-cutting restriction endonucleases were used to digest the 16S-23S rDNA IGS fragments amplified from the 43 *R. leguminosarum* strains. Twelve to 13 distinct restriction patterns, with two to nine restricted fragments per pattern, were detected with each restriction enzyme (Table 3; Fig. 4A). Fourteen different combinations of restriction patterns representing 13 main rDNA IGS types were recorded among the 43 strains (types V and V^+ were regrouped, since type V shared all its restriction fragments with type V^+, which harbored additional fragments due to the PCR amplification of an extra band). By using only two restriction enzymes, e.g., *Hae*III and *Taq*I, the same level of resolution could be obtained.

TABLE 2. Comparison of results of REP-PCR DNA fingerprinting, PCR-RFLP analysis of 16S-23S rDNA IGS regions, and RFLP analysis of Southern-blotted DNA using a chromosomal DNA probe with *R. leguminosarum* strains

REP-PCR DNA fingerprinting pattern[a]	PCR-RFLP rDNA IGS type[b]	Chromosomal hybridization pattern type[a,c]	Biovar[d]	No. of strains
a1	I	a1	V	1
a2	I	a1	V	2
a3	I	a1	V	1
a4	I	a1	V	1
a5	I	a1	P	1
a6	I	a1	T	1
a7	I	a1	P	1
a8	I	a1	T	2
a9	I	a1	P	2
b1	I	a1	V	2
c1	II	a2	V	1
c2	II	a2	T	1
c3	II	a2	T	1
c4	II	a2	T	1
c5	II	a2	P	1
c6	III	a2	T	1
d1	I	$a3^-$	T	1
d2	I	$a3^-$	T	1
e1	VI	b	V	1
e2	VI	b	V	1
f1	IV	c1	V	1
f2	IV	c2	V	1
f3	IV	$c1^+$	T	1
f4	IV	$c1^+$	T	1
g1	IX	d	V	1
g2	X	d^-	V	1
g3	XI	d	V	1
h1	VII	e	V	1
h2	VII	e	V	1
i1	VIII	f	V	1
i2	VIII	f	V	1
i3	VIII	f	V	1
j1	XII	h	V	1
k1	V	i1	V	1
k6	V^+	i1	P	1
k2	XIII	i1	P	1
k3	XIII	i1	P	1
k4	XIII	i1	P	1
k5	XIII	i1	P	1

[a] Closely related patterns were designated by the same letter.
[b] The rDNA IGS types are defined in Table 3.
[c] The chromosomal hybridization pattern types were previously determined by Southern blotting *Eco*RI-restricted total DNA and probing it with the chromosomal DNA probe plac12 (12, 24, 25).
[d] V, viciae; T, trifolii; P, phaseoli.

Comparison of PCR and conventional DNA fingerprinting and RFLP analysis of chromosomal DNA regions. To estimate relationships between the 13 rDNA IGS types, a similarity matrix was calculated by analyzing a total of 151 restriction fragments. The matrix was used to construct a dendrogram based on the UPGMA algorithm (Fig. 5A). The results were in agreement with the classification of the strains into 11 REP groups, although 3 of them (c, g, and k) were subdivided into two or three rDNA IGS types and, conversely, rDNA IGS type I included REP groups a, b, and d (Table 2). Generally, strains classified into the same REP group were clustered at levels of

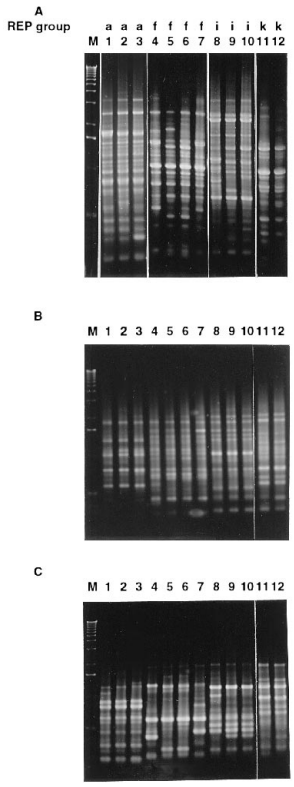

FIG. 2. Examples of PCR fingerprints of *R. leguminosarum* bv. viciae, trifolii, and phaseoli strains produced by using primer pair REP1R-1-REP2-I (A), single random primer SPH1 (B), and single random primer PucFor (C). Lanes 1 to 3, REP group a; lanes 4 to 7, REP group f; lanes 8 to 10, REP group i; lanes 11 and 12, REP group k; lanes M, molecular weight marker (1-kb ladder; Bethesda Research Laboratories).

similarity of at least 85% by RFLP analysis of rDNA IGS regions. Exceptions were strains of REP group k, which were classified in rDNA IGS types V and XIII. These IGS types did not cluster, but the uncut PCR products showed a length difference of more than 100 bp (Table 3; Fig. 3).

All strains were previously typed by total DNA restriction fingerprinting and RFLP analysis of Southern-blotted total DNA by hybridization with a chromosomal DNA region as a probe, plac12 (12, 24–26). At the higher levels of resolution, classification of the 43 strains into 39 types obtained by total DNA restriction fingerprinting was identical to grouping in 39 types by REP-PCR DNA fingerprinting (data not shown). PCR DNA fingerprinting was therefore found to be as sensitive as conventional DNA fingerprinting. The 11 REP groups and the 13 rDNA IGS types were also consistent with the 13 chromosomal hybridization pattern types (Table 2), with some differences in discrimination of particular groups or types as described above for correspondence between REP groups and rDNA IGS types. On the whole, similar levels of resolution were thus obtained by the conventional and the PCR methods.

PCR-RFLP analysis of symbiotic gene regions. Primers FGP807 and FGP492′ were used to amplify IGS regions between *nifD* and *nifH* genes and parts of these genes, which are located in most *Rhizobium* strains on symbiotic plasmids. The 43 *R. leguminosarum* strains and the 19 additional reference rhizobial strains were examined. The majority of the strains produced a single band of about 1,250 bp (data not shown). The size of the amplified fragment was smaller for *B. japonicum* USDA 110 (about 700 bp). Four *R. leguminosarum* bv. phaseoli strains and *R. tropici* CIAT 899 produced an additional band of about 550 bp. DNA from *Rhizobium* (*Phaseolus*) sp. strain H152 was not amplified; however, this was expected, since it had not shown homology when its total DNA was probed with a *nifKDH* DNA probe (12, 23). DNA of the *R. huakuii* strain could not be amplified under the PCR conditions used.

TABLE 3. rDNA IGS types and restriction patterns of *R. leguminosarum* strains revealed by RFLP analysis of PCR-amplified 16S-23S rDNA IGS regions

rDNA IGS type[a,b]	Size (bp)[b,c]	Restriction pattern type[b,d] of 16S-23S rDNA IGS regions digested with:						
		*Alu*I	*Cfo*I	*Dde*I	*Hae*III	*Msp*I	*Nde*II	*Taq*I
I	1,350	A	A	A	A	A	A	A
II	1,280	B	B	B	B	B	B	B
III	1,280	C	B	B	B	B	B	C
IV	1,280	D	C	C	C	C	C	D
V	1,280	E	D	D	D	D	D	E
V$^+$	1,280$^+$	E$^+$	D$^+$	D$^+$	D$^+$	D$^+$	D$^+$	E$^+$
VI	1,210	F	E	E	E	E	E	F
VII	1,350	G	F	F	F	F	F	G
VIII	1,350	H	G	G	G	G	G	H
IX	1,400$^+$	I	H	H	H	H	H	I
X	1,350$^+$	I	I	H+I	I	H	H$^+$	I
XI	1,350	J	J	I	J	I	I	J
XII	1,210	K	K	J	K	J	J	K
XIII	1,160	L	L	K	L	K	K	L

[a] The rDNA IGS types represent the combination of restriction patterns obtained with seven restriction endonucleases.
[b] A superscript plus sign indicates that an extra band which yielded additional restricted fragments for some restriction patterns (Fig. 4A) was detected in the PCR product (Fig. 3).
[c] Mean size of the undigested PCR products estimated by summing the sizes of the restricted fragments.
[d] The different pattern types detected with each endonuclease among the 43 strains analyzed (Fig. 4A) are shown.

Results of RFLP analysis using eight 4-base-cutting restriction endonucleases of amplified *nifD* through *nifK* fragments from the 43 *R. leguminosarum* strains are shown in Table 4. Five to 11 distinct restriction patterns were obtained with each restriction enzyme. Fifteen *nif* types, defined by the different combinations of restriction patterns, were found within the sample of strains. *Hae*III was the most discriminating restriction enzyme, resolving the strains into 11 restriction pattern types (Fig. 4B). Three additional restriction enzymes, i.e., *Cfo*I, *Hin*fI, and *Msp*I, were necessary to obtain the same level of resolution as was obtained with the combination of the eight restriction enzymes.

Then *nod* regions of the symbiotic plasmids were examined.

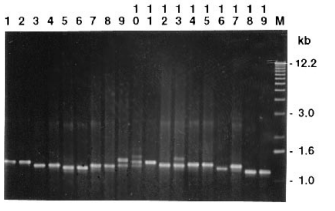

FIG. 3. Gel electrophoresis showing size variability of PCR-amplified 16S-23S rDNA IGS regions from *R. leguminosarum* strains grouped into rDNA IGS types I (lanes 1 and 2), II (lanes 3 and 4), VI (lanes 5 and 6), IV (lanes 7 and 8), IX (lane 9), X (lane 10), XI (lane 11), VII (lanes 12 and 13), VIII (lanes 14 and 15), XII (lane 16), V (lane 17), and XIII (lanes 18 and 19). Lane M, molecular weight marker (1 kb ladder, Bethesda Research Laboratories).

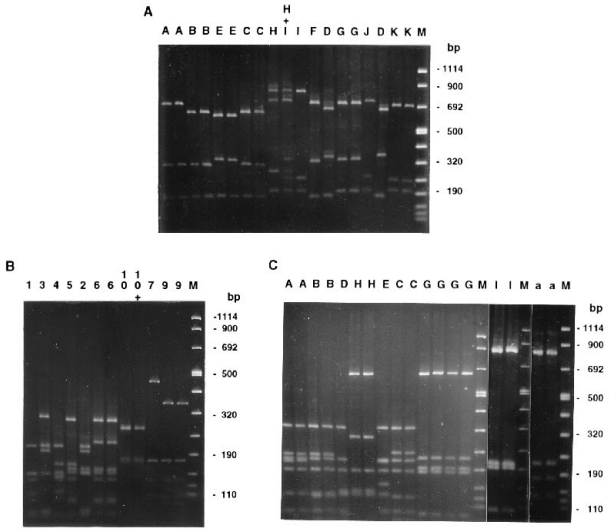

FIG. 4. Restriction patterns of PCR-amplified 16S-23S rDNA IGS regions digested with *Dde*I (A), *nifD* through *nifK* fragments digested with *Hae*III (B), and *nodD* through *nodF* or *nodD2* through *nodD3* fragments digested with *Hae*III (C). The lane assignments correspond to restriction pattern types which are given in Tables 3 and 4. Lanes M, molecular weight marker VIII (Boehringer Mannheim).

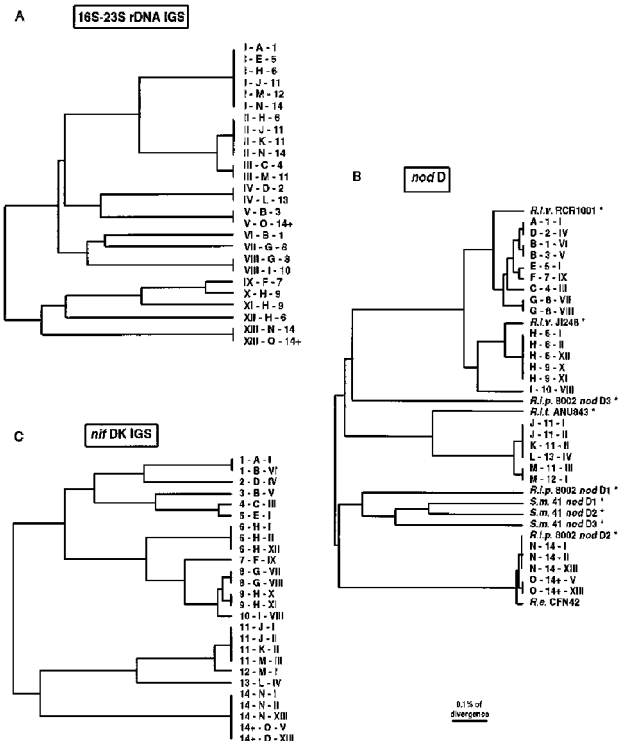

FIG. 5. Dendrograms (UPGMA) of similarities between rDNA IGS types (A), between *nod* types (B), and between *nif* types (C) revealed by PCR-RFLP analysis. The rDNA IGS types are numbered from I to XIII (Table 3), the *nod* types are designated by letters (Table 4), and the *nif* types are numbered from 1 to 14 (Table 4). In dendrogram B, *nod* types of *R. leguminosarum* (*R.l.*) bv. viciae (*v.*), trifolii (*t.*), and phaseoli (*p.*) and of *S. meliloti* (*S.m.*) strains that are designated by an asterisk were included by inferring their restriction site polymorphism from the *nodD* sequences available in GenBank (accession numbers Y00548, J03671, X03721, X54214, X54215, X04473, X04474, and X53820). *R.e.*, *R. etli*. Cophenetic correlations were as follows: $r = 0.91$ (A), $r = 0.99$ (B), and $r = 0.98$ (C).

Primer NODD2PH678 was associated with primer NOD D3PH2152′ to amplify fragments which included *nodD2*, the IGS sequence between *nodD2* and *nodD3*, and about 120 bp of *nodD3* in *R. leguminosarum* bv. phaseoli (Fig. 1). All *R. leguminosarum* bv. phaseoli strains produced a single band of about 1,490 bp, which corresponded to the expected size according to the published sequence of *R. leguminosarum* bv. phaseoli nodulation genes (3). The primer pair NBA12-NBF12′ was used to amplify DNA fragments including *nodD* and the IGS sequence between *nodD* and *nodF* in *R. leguminosarum* bv. viciae and trifolii (Fig. 1). *R. leguminosarum* bv. trifolii strains produced a single band of about 1,450 bp, which was about 130 bp larger than the expected size, 1,317 bp, according to the published sequence of *R. leguminosarum* bv. trifolii nodulation genes (37). The sizes of the single amplified bands produced by the *R. leguminosarum* bv. viciae strains ranged from about 1,360 to 1,490 bp (Table 4).

Results of RFLP analysis of *nodD* through *nodF* or *nodD2* through *nodD3* regions are given in Table 4. Examples of restriction patterns are shown in Fig. 4C. Fifteen *nod* types were discriminated with the eight restriction endonucleases used, but a combination of only three restriction enzymes, e.g., *Cfo*I, *Hae*III, and *Taq*I, was necessary to resolve the strains into the 15 *nod* types. Similar levels of resolution were thus obtained by analysis of either *nod* or *nifD* through *nifK* regions. However, some strains undifferentiated by one method were subtyped by the other. Eighteen composite genotypes which we called *nod-nif* types were recorded (Table 4).

Primer pair NBA12-NBF12′ did not permit amplification of DNA from rhizobia other than *R. leguminosarum* bv. viciae and trifolii. Likewise, primer pair NODD2PH678-NODD3PH 2152′ permitted amplification only of DNA from *R. leguminosarum* bv. phaseoli and *R. etli* bv. phaseoli. These results were expected because of the differences in organization of *nod* gene clusters among the rhizobial species or biovars. In order to compare homologous DNA sequences between the different biovars of *R. leguminosarum*, DNA fragments restricted to the *nodD* genes were analyzed. By use of primer pair NBA12-NODDRL2′, a single fragment, including 876 of 969 bp (42) of the *nodD* gene, was amplified from each *R. leguminosarum* bv. viciae or trifolii strain, without detectable variations in size among the PCR products. This result indicated that the *nodD* through *nodF* IGS regions and not the *nodD* gene are variable in length in *R. leguminosarum* bv. viciae and bv. trifolii. Likewise, primer pair NODD2PH678-NODDRL2′ permitted amplification of 875 of 954 bp (3) of the *nodD2* gene for each *R. leguminosarum* bv. phaseoli strain. NODD2PH678-NOD DRL2′ also permitted amplification of *nodD* gene fragments from *R. leguminosarum* bv. viciae and trifolii and from *R. etli* bv. phaseoli, but they did not allow amplification of fragments from strains of other rhizobial species under the PCR conditions used.

RFLP analysis of *nodD* or *nodD2* gene fragments amplified from *R. leguminosarum* and *R. etli* was performed and found to be almost as discriminating as the analysis of about 1,350 to 1,500 bp of *nodD* through *nodF* and *nodD2* through *nodD3* regions. The map locations of the restriction sites were inferred from the known *nodD* gene sequences of *R. leguminosarum* bv. viciae, trifolii, and phaseoli strains. Additional *nod* types of *R. leguminosarum* bv. viciae, trifolii, and phaseoli and of *S. meliloti* strains were included in this analysis on the basis of their restriction site polymorphism directly inferred from the *nodD* gene sequences available in GenBank. A total of 153 restriction sites were examined. UPGMA cluster analysis of

TABLE 4. RFLP analysis of PCR-amplified *nodD* through *nodF*, *nodD2* through *nodD3*, and *nifD* through *nifK* gene regions and comparison with RFLP analysis of Southern-blotted total DNA probed with symbiotic gene regions from *R. leguminosarum* strains

nod-nif types[a,b]	Size (bp)[c]	Restriction pattern types[a,d] of *nod* and *nif* gene regions digested with:								*nod-nif* hybridization pattern type(s)[e]	Biovar[f]	No. of strains
		*Alu*I	*Cfo*I	*Dde*I	*Hae*III	*Hin*fI	*Msp*I	*Nde*II	*Taq*I			
A-1	1,490	A-1	A-1	A-1	A-1	A-1	A-1	A-1	A-1	A1	V	1
B-1	1,490	A-1	A-1	A-1	A-1	A-1	A-1	A-1	B-1	A1	V	2
B-3	1,490	A-3	A-3	A-1	A-2	A-1	A-3	A-3	B-3	A2	V	1
C-4	1,490	A-3	B-4	A-1	B-3	A-1	B-3	A-4	A-4	B	V	2
D-2	1,360	B-2	C-2	B-1	C-1	B-1	C-2	B-2	C-2	G1, G2	V	2
E-5	1,490	A-3	D-4	C-1	D-3	C-2	D-3	C-4	D-4	D	V	1
F-7	1,390	C-4	E-1	D-1	E-5	D-1	E-4	D-5	E-3	F	V	1
G-8	1,440	D-4	F-5	E-1	F-5	E-1	F-4	E-3	F-3	J	V	3
H-6	1,490	E-4	G-5	F-1	G-4	F-1	G-4	F-5	G-5	I1	V	5
H-9	1,490	E-4	G-5	F-1	G-5	F-1	G-5	F-3	G-3	I2	V	2
I-10	1,490	F-4	H-5	G-1	H-6	G-1	H-4	G-3	H-3	E	V	2
J-11	1,450	G-5	I-6	H-2	I-7	H-3	I-6	H-6	I-6	α1, α2, α3, β1, β2	T	6
K-11	1,450	G-5	J-6	H-2	I-7	H-3	I-6	H-6	I-6	ε1	T	1
L-13	1,450	G-5	J-8	H-3	I-9	H-4	J-7	H-6	I-6	ε2	T	2
M-11	1,450	H-5	J-6	H-2	I-7	H-3	K-6	H-6	I-6	χ	T	1
M-12	1,450	H-5	J-7	H-2	I-8	H-3	K-6	H-6	I-6	η	T	1
N-14	1,490	a-6	a-9	a-4	a-10	a-5	a-8	a-7	a-7	a	P	6
O-14$^+$	1,490	a-6$^+$	b-9$^+$	a-4$^+$	a-10$^+$	a-5$^+$	a-8$^+$	a-7$^+$	a-7$^+$	b	P	4

[a] A superscript plus sign indicates that an extra band which yielded additional restricted fragments for some restriction patterns (Fig. 4B) was detected in the PCR product.

[b] The *nod* types (A to O) represent the combination of restriction patterns obtained with eight restriction endonucleases from RFLP analysis of *nodD* through *nodF* fragments (types A to M) and *nodD2* through *nodD3* fragments (types N and O). The *nif* types (1 to 14$^+$) resulted from RFLP analysis of *nifD* through *nifK* fragments.

[c] Mean size of the undigested PCR products of *nodD* through *nodF* or *nodD2* through *nodD3* fragments estimated by summing the sizes of the restricted fragments.

[d] The different pattern types detected with each endonuclease among the 43 strains analyzed are designated by uppercase letters for *nodD* through *nodF* fragments (*R. leguminosarum* bv. viciae and trifolii), lowercase letters for *nodD2* through *nodD3* fragments (*R. leguminosarum* bv. phaseoli), and numbers for *nifD* through *nifK* fragments (Fig. 4).

[e] *nod-nif* hybridization pattern types were previously determined by Southern blotting of restricted total DNA and probing with various *nod* gene and *nif* gene probes (12, 21, 24). The *nod-nif* hybridization pattern types identified by the same letter correspond to identical patterns obtained by probing with the *nod* gene probes, but some of them, e.g., A1 and A2, were further subtyped when the *nif* gene probes were used.

[f] V, viciae; T, trifolii; P, phaseoli.

nod types was performed with the fraction of shared restriction sites (Fig. 5B). Numerical analysis of 143 restriction fragments resulting from RFLP data for the *nifD* through *nifK* gene region was also performed (Fig. 5C).

In both analyses, the 43 *R. leguminosarum* strains formed three main clusters which corresponded to the three biovars of *R. leguminosarum*. The correlation between both classifications was good according to the Mantel test ($r = 0.81$; $P = 0.001$). Two subclusters were delineated within the sample of *R. leguminosarum* bv. viciae strains by analysis of both *nifD* through *nifK* and *nodD* gene regions, but *nod-nif* types F-7 and G-8 were grouped with *nod-nif* types A-1 to E-5 according to *nodD* analysis and with *nod-nif* types H-6, H-9, and I-10 according to *nifD* through *nifK* region analysis. The comparison of the *nif* and *nod* type similarity matrices was performed with the data restricted to the sample of *R. leguminosarum* bv. viciae strains, and the correlation coefficient was found to be low ($r = 0.19$; $P = 0.05$).

Among the strains of *R. leguminosarum* bv. trifolii and bv. phaseoli examined, the symbiotic gene regions appeared closely related within biovars and less polymorphic than those of *R. leguminosarum* bv. viciae. However, a level of divergence similar to that observed in biovar viciae strains was revealed within biovar trifolii when the reference strain ANU843 was included in the analysis of *nodD* genes (Fig. 5B). Thirty-three and 26 polymorphic restriction sites were detected among the *R. leguminosarum* bv. viciae and bv. trifolii strains, respectively. We have verified that this diversity remains limited to the intrabiovar level by including *nodD* gene sequences from another rhizobial species, *S. meliloti*, in our analysis. In contrast, the *nodD2* genes of the biovar phaseoli strains examined were closely related. For the *nodD2* through *nodD3* amplified fragments from the *R. leguminosarum* bv. phaseoli strains examined, only one restriction site localized in the *nodD2* gene was found to be polymorphic. The *nodD* gene region amplified from *R. etli* CFN42 clustered with *nodD2* genes of *R. leguminosarum* bv. phaseoli strains and differed from them by only one to two restriction sites. As expected from previous phylogenetic studies based on sequence comparisons (6, 52), the three different copies of *R. leguminosarum* bv. phaseoli *nodD* genes were as distantly related to each other as are *nodD* genes from the different biovars or species, and the three different copies of the *S. meliloti nodD* gene were clustered at the species level.

The results of PCR-RFLP analysis were consistent with those previously obtained by RFLP analysis of total DNA probed with symbiotic gene probes (Table 4). For *R. leguminosarum* bv. viciae and bv. phaseoli strains, the levels of resolution were similar with the two techniques. Within the sample of *R. leguminosarum* bv. viciae strains, 11 different *nod-nif* hybridization pattern types were discriminated by conventional RFLP analysis. By PCR-RFLP analysis of the *nod* and *nif* regions, only one restriction enzyme (*Hae*III or *Msp*I) was required to detect 10 *nod-nif* types. For *R. leguminosarum* bv. trifolii strains, less polymorphism was detected by PCR-RFLP analysis (four *nod-nif* types) than by conventional RFLP analysis (eight *nod-nif* hybridization pattern types).

Comparison of RFLP analyses of symbiotic gene and chromosomal DNA regions. By contrast to RFLP analysis of symbiotic gene regions, RFLP analysis of chromosomal rDNA IGS regions grouped the *R. leguminosarum* strains independently of their biovars, as expected from a previous study based on conventional RFLP analysis (24). The correlations between

the *nod* and the rDNA IGS similarity matrices and between the *nif* and the rDNA IGS matrices were relatively low, although significant ($P = 0.001$), with r values of 0.66 and 0.52, respectively. Likewise, within biovars, the groupings into *nod* or *nif* types did not correspond to the grouping into chromosomal types (Fig. 5). When the matrix comparisons were performed within each biovar, the correlations between symbiotic gene regions and rDNA IGS matrices were very low (r values ranging from 0.31 to 0.13) and generally not significant ($P > 0.05$).

DISCUSSION

Groupings of *R. leguminosarum* strains obtained by the PCR-based methods investigated in this study were in good agreement with the previous genotypic classification of these strains obtained by total DNA restriction fingerprinting and RFLP analysis of total DNA using chromosomal and symbiotic gene sequences as probes.

The most rapid and discriminating methods are based on PCR DNA fingerprinting. Our results showed that they reflected the variability of chromosomal DNA regions but did not reflect the variability of symbiotic gene regions in the *R. leguminosarum* species and biovars. Therefore, PCR DNA fingerprinting does not provide information about the possibility that different genotypes have the same symbiotic plasmid or, conversely, that the same chromosomal backgrounds harbor different symbiotic plasmids.

The patterns generated by the REP primers and the random primers were rather complex, with multiple bands of variable intensity; this limits a quantitative estimation of relatedness between strains. In addition, because of the lack of reproducibility that we occasionally observed, results should not be easily comparable between different laboratories; this limits the use of this method for identification of strains. Nevertheless, despite these limitations, these simple methods provide an efficient means for rapidly typing a large number of strains under well-standardized experimental conditions.

By PCR-RFLP analysis of 16S-23S rDNA IGS regions, the discriminating power was sufficient to group chromosomally closely related strains on the basis of the simple, reproducible, and, hence, easily analyzable patterns of restriction fragments. Analysis of combined data from various restriction enzymes enables estimation of relatedness between 16S-23S rDNA IGS regions. However, variability in length of the PCR products not only between rhizobial species but also between genotypes distantly or closely related within *R. leguminosarum* was recorded (for example, the strains that were distributed in 16S-23S rDNA types V and XIII showed very similar PCR DNA fingerprints and had identical chromosomal hybridization patterns). Length variability of rDNA IGS regions both between and within bacterial species has been reported (1, 18). In bacteria, the rRNA operon is present in multiple copies. At least three copies occur in *Rhizobium* species (12, 15). The multiple PCR products observed for some *R. leguminosarum* strains suggest variations in the length of IGS regions between the different copies, which could be partly explained by insertion of various tRNA genes in IGS regions (18). The amplification of multiple PCR products from a single individual could also be due to the formation of heteroduplex DNA structures and of single-stranded DNA (17). Therefore, length polymorphism of restriction fragments between 16S-23S rDNA IGS types does not necessarily correspond to differences in restriction sites, and distances between genotypes could be overestimated. Map location of restriction sites could not be performed, because sequence data for 16S-23S rDNA IGS regions were not available for *Rhizobium* species.

PCR-RFLP analysis of symbiotic gene regions grouped the strains within the *R. leguminosarum* species and within biovars independently of their chromosomal types. Within biovar viciae, the low correlation between groupings resulting from PCR-RFLP analysis of *nifD* through *nifK* and *nodD* gene regions could reflect recombination between different symbiotic genes. Similar levels of polymorphism were obtained for the two symbiotic gene regions. Our data indicated that the *nodD2* gene sequence is conserved between *R. leguminosarum* bv. phaseoli and *R. etli* and that it is weakly polymorphic within biovar phaseoli. This result contrasts with the diversity detected in *R. leguminosarum* bv. viciae and bv. trifolii. Collectively, our results are consistent with those of previous studies showing that the phylogeny of *nod* genes was well correlated with the systematics of host legumes but was not correlated with the taxonomy of the bacteria (6, 43, 52). Enough information was obtained from the present PCR-RFLP analysis of *nodD* genes to reveal their phylogenetic relationships in accordance with data based on nucleotide sequence comparison (6, 45, 52).

The DNA sequences used as PCR primers for REP (or ERIC) DNA fingerprinting and PCR-RFLP analysis of 16S-23S rDNA IGS regions are conserved among a wide range of bacterial species. REP and/or ERIC primers even enabled PCR amplification within fungal genera (9, 44). The *nif* primers used in this study were designed to be universal for the nitrogen-fixing bacteria (16, 31). We have indeed verified that rDNA IGS and *nif* gene regions could be amplified for various rhizobial species. The random primers SPH1 and PucFor have also been used successfully to type strains of diverse rhizobial species (2, 7). On the other hand, the *nod* primers described in this study are biovar- or species-specific for *R. leguminosarum* and *R. etli*. However, it should be possible to design universal primers to amplify *nod* gene regions of other rhizobial species from the numerous available DNA sequences.

Thus, the PCR-based methods that we have evaluated offer a convenient alternative to conventional RFLP analyses with the same range of levels of resolution and the same possibility of typing either the whole genome or specific DNA regions. As they are much less time-consuming, avoiding fastidious DNA extraction and hybridization, they are more suitable for large-scale identification and classification of bacterial collections and for study of large populations at the intraspecies level.

ACKNOWLEDGMENTS

We are grateful to P. Normand for helpful discussion and for supplying us with *nif* primer sequences. We also thank S. M. Nour for helping with the English text, F. Dessaint for discussion of statistic tests, and N. Darphin for technical assistance.

This work was partly funded by the Conseil Régional de Bourgogne.

REFERENCES

1. **Barry, T., G. Colleran, M. Glennon, L. K. Dunican, and F. Gannon.** 1991. The 16s/23s ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods Applic. **1:**51–56.
2. **Coutinho, H. L. C., B. A. Handley, H. E. Kay, L. Stevenson, and J. E. Beringer.** 1993. The effect of colony age on PCR fingerprinting. Lett. Appl. Microbiol. **17:**282–284.
3. **Davis, E. O., and A. W. B. Johnston.** 1990. Analysis of three *nod*D genes in *Rhizobium leguminosarum* biovar *phaseoli*; *nod*D1 is preceded by *nol*E, a gene whose product is secreted from the cytoplasm. Mol. Microbiol. **4:**921–932.
4. **de Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58:**2180–2187.
5. **Demezas, D. H., T. B. Reardon, J. M. Watson, and A. H. Gibson.** 1991. Genetic diversity among *Rhizobium leguminosarum* bv. trifolii strains revealed by allozyme and restriction fragment length polymorphism analyses.

Appl. Environ. Microbiol. **57:**3489–3495.
6. **Dobert, R. C., B. T. Breil, and E. W. Triplett.** 1994. DNA sequence of the common nodulation genes of *Bradyrhizobium elkanii* and their phylogenetic relationships to those of other nodulating bacteria. Mol. Plant-Microbe Interact. **7:**564–572.
7. **Dooley, J. J., S. P. Harrison, L. R. Mytton, M. Dye, A. Cresswell, L. Skot, and J. R. Beeching.** 1993. Phylogenetic grouping and identification of *Rhizobium* isolates on the basis of random amplified polymorphic DNA profiles. Can. J. Microbiol. **39:**665–673.
8. **Eardly, B. D., J. P. W. Young, and R. K. Selander.** 1992. Phylogenetic position of *Rhizobium* sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *nifH* genes. Appl. Environ. Microbiol. **58:**1809–1815.
9. **Edel, V., C. Steinberg, I. Avelange, G. Laguerre, and C. Alabouvette.** 1995. Comparison of three molecular methods for the characterization of *Fusarium oxysporum* strains. Phytopathology **85:**579–585.
10. **Engvild, K. C., E. S. Jensen, and L. Skot.** 1990. Parallel variation in isoenzyme and nitrogen fixation markers in a *Rhizobium* population. Plant Soil **128:**283–286.
11. **Frothingham, R., and K. H. Wilson.** 1993. Sequence-based differentiation of strains in the *Mycobacterium avium* complex. J. Bacteriol. **175:**2818–2825.
12. **Geniaux, E., G. Laguerre, and N. Amarger.** 1993. Comparison of geographically distant populations of *Rhizobium* isolated from root nodules of *Phaseolus vulgaris*. Mol. Ecol. **2:**295–302.
13. **Harrison, S. P., L. R. Mytton, L. Skot, M. Dye, and A. Cresswell.** 1992. Characterization of *Rhizobium* isolates by amplification of DNA polymorphisms using random primers. Can. J. Microbiol. **38:**1009–1015.
14. **Hartmann, A., and N. Amarger.** 1991. Genotypic diversity of an indigenous *Rhizobium meliloti* field population assessed by plasmid profiles, DNA fingerprint, and insertion sequence typing. Can. J. Microbiol. **37:**600–608.
15. **Huber, I., and S. Selenska-Pobell.** 1994. Pulsed-field electrophoresis-fingerprinting, genome size estimation and *rrn* loci number of *Rhizobium galegae*. J. Appl. Bacteriol. **77:**528–533.
16. **Jamann, S., M. P. Fernandez, and P. Normand.** 1993. Typing method for N_2-fixing bacteria based on PCR-RFLP—application to the characterization of *Frankia* strains. Mol. Ecol. **2:**17–26.
17. **Jensen, M. A., and N. Straus.** 1993. Effects of PCR conditions on the formation of heteroduplex and single-stranded DNA products in the amplification of bacterial ribosomal DNA spacer regions. PCR Methods Applic. **3:**186–194.
18. **Jensen, M. A., J. A. Webster, and N. Straus.** 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. **59:**945–952.
19. **Judd, A. K., M. Schneider, M. J. Sadowsky, and F. J. de Bruijn.** 1993. Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl. Environ. Microbiol. **59:**1702–1708.
20. **Kajalainen, S., and K. Lindström.** 1989. Restriction fragment length polymorphism analysis of *Rhizobium galegae* strains. J. Bacteriol. **171:**5561–5566.
21. **Laguerre, G.** Unpublished data.
22. **Laguerre, G., M. R. Allard, F. Revoy, and N. Amarger.** 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. **60:**56–63.
23. **Laguerre, G., M. P. Fernandez, V. Edel, P. Normand, and N. Amarger.** 1993. Genomic heterogeneity among French *Rhizobium* strains isolated from *Phaseolus vulgaris* L. Int. J. Syst. Bacteriol. **43:**761–767.
24. **Laguerre, G., E. Geniaux, S. I. Mazurier, R. Rodríguez-Casartelli, and N. Amarger.** 1993. Conformity and diversity among field isolates of *R. leguminosarum* bv. *viciae*, bv. *trifolii*, and bv. *phaseoli* revealed by DNA hybridization using chromosome and plasmid probes. Can. J. Microbiol. **39:**412–419.
25. **Laguerre, G., S. I. Mazurier, and N. Amarger.** 1990. Field isolates belonging to different biovars of *R. leguminosarum* can share identical chromosomal background, p. 550. *In* P. M. Gresshoff, L. E. Roth, G. Stacey, and W. E. Newton (ed.), Nitrogen fixation: achievements and objectives. Chapman and Hall, New York.
26. **Laguerre, G., S. I. Mazurier, and N. Amarger.** 1992. Plasmid profiles and restriction fragment length polymorphism of *Rhizobium leguminosarum* bv. *viciae* in field populations. FEMS Microbiol. Ecol. **101:**17–26.
27. **Leung, K., S. R. Strain, F. J. de Bruijn, and P. J. Bottomley.** 1994. Genotypic and phenotypic comparisons of chromosomal types within an indigenous soil population of *Rhizobium leguminosarum* bv. trifolii. Appl. Environ. Microbiol. **60:**416–426.
28. **Matar, G. M., B. Swaminathan, S. B. Hunter, L. N. Slater, and D. F. Welch.** 1993. Polymerase chain reaction-based restriction fragment length polymorphism analysis of a fragment of the ribosomal operon from *Rochalimaea* species for subtyping. J. Clin. Microbiol. **31:**1730–1734.
29. **Navarro, E., P. Simonet, P. Normand, and R. Bardin.** 1992. Characterization of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. Arch. Microbiol. **157:**107–115.
30. **Nick, G., and K. Lindström.** 1994. Use of repetitive sequences and the polymerase chain reaction to fingerprint the genomic DNA of *Rhizobium galegae* strains and to identify the DNA obtained by sonicating the liquid cultures and root nodules. Syst. Appl. Microbiol. **17:**265–273.
31. **Normand, P. (University of Lyon, Villeurbanne, France).** Personal communication.
32. **Nour, S. M., J. C. Cleyet-Marel, D. Beck, A. Effosse, and M. P. Fernandez.** 1994. Genotypic and phenotypic diversity of *Rhizobium* isolated from chickpea (*Cicer arietinum* L.). Can. J. Microbiol. **40:**345–354.
33. **Oyaizu, H., N. Naruhashi, and T. Gamou.** 1992. Molecular methods of analysing bacterial diversity: the case of rhizobia. Biodivers. Conserv. **1:**237–249.
34. **Ponsonnet, C., and X. Nesme.** 1994. Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions. Arch. Microbiol. **161:**300–309.
35. **Prakash, R. K., and A. G. Atherly.** 1986. Plasmids of *Rhizobium* and their role in symbiotic nitrogen fixation. Int. Rev. Cytol. **104:**1–24.
36. **Schofield, P. R., A. H. Gibson, W. F. Dudman, and J. M. Watson.** 1987. Evidence for genetic exchange and recombination of *Rhizobium* symbiotic plasmids in a soil population. Appl. Environ. Microbiol. **53:**2942–2947.
37. **Schofield, P. R., and J. M. Watson.** 1986. DNA sequence of *Rhizobium trifolii* nodulation genes reveals a reiterated and potentially regulatory sequence preceding *nodABC* and *nodFE*. Nucleic Acids Res. **14:**2891–2903.
38. **Segovia, L., J. P. W. Young, and E. Martinez-Romero.** 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli sp. nov.* Int. J. Syst. Bacteriol. **43:**374–377.
39. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical taxonomy. W. H. Freeman & Co., San Francisco.
40. **Sokal, R. R., and C. D. Michener.** 1958. A statistical method for evaluating systematic relationships. Univ. Kans. Sci. Bull. **38:**1409–1438.
41. **Spaink, H. P., R. J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg.** 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. Plant Mol. Biol. **9:**27–39.
42. **Squartini, A., R. J. M. van Veen, T. Regensburg-Tuink, P. J. J. Hooykaas, and M. P. Nuti.** 1988. Identification and characterization of the *nod*D gene in *Rhizobium leguminosarum* strain 1001. Mol. Plant-Microbe Interact. **1:**145–149.
43. **Ueda, T., Y. Suga, N. Yahiro, and T. Matsuguchi.** 1995. Phylogeny of Sym plasmids of rhizobia by PCR-based sequencing of a *nodC* segment. J. Bacteriol. **177:**468–472.
44. **van Belkum, A., W. G. V. Quint, B. E. de Pauw, W. J. G. Melchers, and J. F. Meis.** 1993. Typing of *Aspergillus* species and *Aspergillus fumigatus* isolates by interrepeat polymerase chain reaction. J. Clin. Microbiol. **31:**2502–2505.
45. **van Rhijn, P. J. S., B. Feys, C. Verreth, and J. Vanderleyden.** 1993. Multiple copies of *nodD* in *Rhizobium tropici* CIAT899 and BR816. J. Bacteriol. **175:**438–447.
46. **van Rhijn, P. J. S., and J. Vanderleyden.** 1995. The *Rhizobium*-plant symbiosis. Microbiol. Rev. **59:**124–142.
47. **Versalovic, J., T. Koeuth, and J. R. Lupski.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:**6823–6831.
48. **Willems, A., and D. Collins.** 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequence. Int. J. Syst. Bacteriol. **43:**305–313.
49. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
50. **Yanagi, M., and K. Yamasato.** 1993. Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. **107:**115–120.
51. **Young, J. P. W., H. L. Downer, and B. D. Eardly.** 1991. Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J. Bacteriol. **173:**2271–2277.
52. **Young, J. P. W., and A. W. B. Johnston.** 1989. The evolution of specificity in the legume-*Rhizobium* symbiosis. Trends Ecol. Evol. **4:**341–349.
53. **Young, J. P. W., and M. Wexler.** 1988. Sym plasmid and chromosomal genotypes are correlated in field populations of *Rhizobium leguminosarum*. J. Gen. Microbiol. **134:**2731–2739.