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Specific Detection of *Bradyrhizobium* and *Rhizobium* Strains Colonizing Rice (*Oryza sativa*) Roots by 16S-23S Ribosomal DNA Intergenic Spacer-Targeted PCR

ZHIYUAN TAN,[1,2] THOMAS HUREK,[3] PABLO VINUESA,[4] PETER MÜLLER,[4] JAGDISH K. LADHA,[5] AND BARBARA REINHOLD-HUREK[1,2]*

Group Symbiosis Research, Max Planck Institute for Terrestrial Microbiology, D-35043 Marburg,[1] Laboratory of General Microbiology[2] and Cooperation Laboratory of the Max Planck Institute of Marine Microbiology,[3] Faculty of Biology and Chemistry, University of Bremen, D-28334 Bremen, and Department of Cellular Biology and Applied Botany, Philipps-Universität Marburg, D-35032 Marburg,[4] Germany, and Soil and Water Science Division, International Rice Research Institute, Los Banos, Philippines[5]

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In addition to forming symbiotic nodules on legumes, rhizobial strains are members of soil or rhizosphere communities or occur as endophytes, e.g., in rice. Two rhizobial strains which have been isolated from root nodules of the aquatic legumes *Aeschynomene fluminensis* (IRBG271) and *Sesbania aculeata* (IRBG74) were previously found to promote rice growth. In addition to analyzing their phylogenetic positions, we assessed the suitability of the 16S-23S ribosomal DNA (rDNA) intergenic spacer (IGS) sequences for the differentiation of closely related rhizobial taxa and for the development of PCR protocols allowing the specific detection of strains in the environment. 16S rDNA sequence analysis (sequence identity, 99%) and phylogenetic analysis of IGS sequences showed that strain IRBG271 was related to but distinct from *Bradyrhizobium elkanii*. *Rhizobium* sp. (*Sesbania*) strain IRBG74 was located in the *Rhizobium-Agrobacterium* cluster as a novel lineage according to phylogenetic 16S rDNA analysis (96.8 to 98.9% sequence identity with *Agrobacterium tumefaciens;* emended name, *Rhizobium radiobacter*). Strain IRBG74 harbored four copies of rRNA operons whose IGS sequences varied only slightly (2 to 9 nucleotides). The IGS sequence analyses allowed intraspecies differentiation, especially in the genus *Bradyrhizobium,* as illustrated here for strains of *Bradyrhizobium japonicum, B. elkanii, Bradyrhizobium liaoningense,* and *Bradyrhizobium* sp. (*Chamaecytisus*) strain BTA-1. It also clearly differentiated fast-growing rhizobial species and strains, albeit with lower statistical significance. Moreover, the high sequence variability allowed the development of highly specific IGS-targeted nested-PCR assays. Strains IRBG74 and IRBG271 were specifically detected in complex DNA mixtures of numerous related bacteria and in the DNA of roots of gnotobiotically cultured or even of soil-grown rice plants after inoculation. Thus, IGS sequence analysis is an attractive technique for both microbial ecology and systematics.

Rhizobia are classically defined as symbiotic bacteria capable of eliciting and invading root or stem nodules on leguminous plants, where they differentiate into N_2-fixing bacteroids. Based on their 16S ribosomal DNA (rDNA) sequences, these nodule endosymbionts constitute a polyphyletic assemblage of bacteria grouped into four major phylogenetic branches of the α-2 subclass of the class *Proteobacteria*. Rhizobial strains are currently placed in the following genera: *Allorhizobium* (emended genus, *Rhizobium*), *Mesorhizobium, Rhizobium,* and *Sinorhizobium* constitute one of the rhizobial clades, whereas *Azorhizobium, Bradyrhizobium,* and *Methylobacterium* are each located on a different and well-resolved phylogenetic branch (45, 57). These legume symbionts are phylogenetically intertwined with several nonsymbiotic bacterial genera, including pathogenic, phototrophic, and denitrifying strains (for a review, see reference 48).

A remarkable ecological feature of rhizobia is their ability to thrive in very different environments. Many soils contain a rather large population of nonsymbiotic rhizobia that are found both in the bulk soil and in the rhizospheres of legumes and other plants (39, 40, 43). Some of these saprophytic or rhizospheric bacteria may become symbiotic by the horizontal acquisition of a symbiotic plasmid or a chromosomal symbiotic island (44), allowing them to synthesize and secrete strain-specific lipochitin-oligosaccharides for host nodulation and intracellular invasion. Rhizobia are also found as viable cells in water, where they are able to infect and nodulate aquatic legumes, such as *Aeschynomene* spp. and *Sesbania* spp. (8).

More recently, it has been recognized that these legume symbionts may also occur as endophytes in the roots of cereals, such as rice (*Oryza breviligulata* and *Oryza sativa*) (8, 14, 47, 55), wheat, and maize (39). These findings have stimulated research on rice growth promotion by rhizospheric and endophytic rhizobia (22). The isolation of plant growth-promoting rhizobia (PGPR) capable of enhancing rice yield under greenhouse and field conditions (55) is remarkable, since rice is the most important food crop produced in the world.

This study focuses on two rhizobial PGPR strains that were previously shown to promote rice growth (6, 7). These strains were isolated from root nodules of the aquatic legumes *Aeschynomene fluminensis* (IRBG271) (29, 41) and *Sesbania*

* Corresponding author. Mailing address: Institute of General Microbiology, Faculty of Biology and Chemistry, University of Bremen, P.O. Box 33 04 40, D-28334 Bremen, Germany. Phone: (49) 421-218-2370. Fax: (49) 421-218-4042. E-mail: breinhold@uni-bremen.de.

aculeata (IRBG74) (J. K. Ladha, unpublished data), respectively, and had an uncertain taxonomic status. Therefore, one goal of this study was to determine their phylogenetic positions both by 16S rDNA and intergenic spacer (IGS) sequence analysis.

An important requirement for an agronomically useful rhizobium-graminea interaction is that the inoculated bacteria are able to establish a significant population on or in the host roots, particularly under competitive conditions in nonsterile substrates. Since the majority of bacteria cannot be easily cultured from their natural environments (35), a second objective of this study was to develop a culture-independent method for the rapid and easy detection of these rhizobial strains on rice roots. In microbial ecology, a wide range of methods for detection and identification of specific microorganisms in environmental samples has been developed. These include classical and molecular genetic methods. Traditional methods are mainly fluorescent-antibody and selective plating techniques, each of which is useful but limited in some respects (34, 39). Molecular biological techniques, such as phylogenetic probes or PCR-based approaches, allow the detection of particular DNA sequences and therefore can be used to trace their presence in target organisms directly in the environment (4, 9). The rDNA operon (*rrn*) is a particularly useful target for the development of nucleic acid hybridization- and PCR-based assays. In prokaryotes, the rDNA operon encodes the 16S (*rrs*), 23S (*rrl*), and 5S (*rrf*) rRNA genes. Although the 16S rRNA gene has been most widely used, the 16S-23S rDNA IGS region has received increased attention as a target in molecular detection and identification schemes (5, 30). In contrast to rRNA genes, which are remarkably well conserved throughout most bacterial species, the IGS regions exhibit a large degree of sequence diversity and length variation (24). Even within species, the IGS sequence variation may be very high, thus allowing intraspecies strain differentiation, as recently also shown for rhizobial strains (11, 30, 49, 51, 53).

Here we show that the presence of highly variable sequence stretches within IGS regions allows the development of a rapid, easy-to-perform, and sensitive PCR protocol for the specific detection of PGPR strains in the rhizospheres of rice plants cultivated in a gnotobiotic system as well as in nonsterile rice field soil. Phylogenetic sequence analysis of the 16S and IGS rDNA sequences of these PGPR strains consistently showed that IRBG271 is phylogenetically related to *Bradyrhizobium elkanii,* while strain IRBG74 is related to *Agrobacterium tumefaciens* bv. 1 (emended name, *Rhizobium radiobacter* [57]). Potential advantages and limitations of IGS sequence analyses for phylogenetic inference in rhizobia are discussed.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. The strains tested in this study are listed in Table 1. All the strains were routinely grown on YM medium, which consisted of SM medium (36) with the carbon source, potassium malate, replaced by 1% mannitol. All strains were grown at 28°C unless otherwise stated.

Extraction of DNA and techniques for DNA manipulation. Three milliliters of 3-day-old cultures (fast-growing bacteria) or 6-day-old cultures (slow-growing strains) was collected by centrifugation, and DNA was isolated after cell lysis with *N*-laurylsarcosin (5% [wt/vol]) and phenol-chloroform extraction as described previously (19). Rice roots were collected and vigorously washed by vortexing them in sterile distilled water with seven changes. The washed roots were frozen in liquid nitrogen and powdered in a mortar before being resuspended in 400 μl of DNA extraction buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 100 mM NaCl, 1.0% sodium dodecyl sulfate) and incubated at 60°C for 30 min. Cell debris was removed by centrifugation, and the supernatant was used for DNA extraction as described above. General techniques for DNA manipulations were carried out according to standard protocols (1).

Sequencing of 16S rRNA gene and 16S-23S rRNA IGS regions. The methods for directly sequencing the PCR products of 16S rRNA genes were as described before (21), using an ALFexpress automated sequencer (Amersham Pharmacia Biotech). For the general IGS PCR, a forward primer, 926f (5′-GGT TAA AAC T[C/T]A AA[G/T] GAA TTG ACG G-3′, corresponding to a conserved region of the 3′ end of bacterial 16S rDNA, *Escherichia coli* sequence positions 901 to 926), and a reverse primer, 115r/23S (5′-CCG GGT T[T/G/C]C CCC ATT CGG-3′, corresponding to a conserved region of the 5′ end of 23S rDNA, *E. coli* sequence positions 97 to 115), were used to amplify the 16S-23S rDNA IGS region. The amplification was carried out by the following steps: initial denaturation at 94°C for 3 min; 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 90 s; and then extension at 72°C for 10 min. The PCR products of the IGS were dialyzed and sequenced directly by using the Cy-5-end-labeled primer 1492fc (5′-AAG TCG TAA CAA GGT A[A/G]C CGT-3′, corresponding to *E. coli* 16S rDNA sequence positions 1471 to 1492) and the reverse primer 85rc/23S (5′-CCC CAC GGC TT[A/T] TCG CA[A/G] CGT ATC AC-3′, corresponding to *E. coli* 23S rDNA sequence positions 59 to 85). Sequencing reactions of 600 to 700 nucleotides (nt) yielded an almost-complete overlap only for short IGS sequences, which were thus sequenced from both strands. For large IGS fragments, the overlap was accordingly shorter.

Determination of the *rrn* operon copy number in rhizobial genomes. To detect different copies of rRNA genes, Southern blots of chromosomal DNA (3 μg per lane digested with different restriction endonucleases) were hybridized with 16S rDNA-targeted gene probes or an oligonucleotide probe, respectively. Digoxigenin-labeled fragments of 16S rDNA were generated by PCR using the Dig-DNA labeling and detection kit (Roche) with the forward primer 342f (5′-CTC CTA CGG GAG GCA G-3′) and the reverse primer 926r (5′-YYC CGT CAA TTC CTT TAA GTT T-3′). The template for PCR was chromosomal DNA of strain IRBG74 or IRBG271, respectively. High-stringency hybridization was carried out at 65°C. Hybridization with a digoxigenin-labeled oligonucleotide (926f; 5′-AAA CTY AAA KGA ATT GA-3′) was carried out at 45°C (19).

For sequence analysis of different IGS copies in strain IRBG74, genomic DNA was digested with *Pst*I, and fragments were separated by agarose gel electrophoresis in two different lanes. One lane was used for Southern blot hybridization with oligonucleotide 926f, and the other lane was used for excision of the corresponding fragments from the agarose gel. The gel slices were washed separately in Tris-EDTA buffer for 15 min and then disrupted by the freeze-thaw method in 50 μl of Tris-EDTA buffer. Five microliters of supernatant was used for PCR amplification of 16S rDNA with adjacent IGS sequences, using primers 25f (5′-AAC TKA AGA GTT TGA TCC TGG CTC-3′) and 115r/23S. Amplification products were cloned into the Topo TA vector (Invitrogen). Positive clones were used for plasmid sequencing.

Phylogenetic sequence analysis and design of strain-specific IGS-targeted primers. The determined rDNA sequences together with reference sequences retrieved from GenBank were aligned by using the Ribosomal Database Project (31). The distances of aligned sequences (corresponding to *E. coli* 16S rDNA positions 57 to 1440) were calculated by the Jukes-Cantor method (25). The tree topology was inferred by the neighbor-joining method (38), and the phylogenetic tree was constructed with the Treecon software package (50). The sequences of IGS regions and known closely related sequences obtained were aligned by using CLUSTAL W, version 1.8 (46), and the tree was constructed as described above. Two sets of IGS-targeted primers were designed as follows: R2ssf (5′-CCT GGA TCA ACG CGG TAT-3′) and R2ssr (5′-CCA TAG CCG CTC CAA AGG A-3′) for strain IRBG74 and R3ssf (5′-GAG CGC TGT GCG ATG CAT CG-3′) and R3ssr (5′-GCT CAT CTT GCG ATG AAC GAG-3′) for strain IRBG271.

PCR conditions for specific IGS PCRs. Strains from the rhizobia, sinorhizobia, mesorhizobia, bradyrhizobia, and agrobacteria were divided into 11 genomic DNA pools, 6 of which (A, B, C, D, E, and F) contained samples from slow-growing strains, while the other 5 (G, H, I, J, and K) contained samples from fast-growing strains. Each DNA pool consisted of 5 to 10 strains (Table 1). Pure genomic DNA of strain IRBG74 or IRBG271 was used as a positive control. The final concentration of each genomic DNA was 10 ng/μl, and 0.5 μl of DNA solution was used as a PCR template. PCRs were carried out in 25-μl volumes using Ready-To-Go PCR beads (Amersham Pharmacia Biotech) with 0.5 μl of each primer at 50 μM. Cycle conditions for direct and nested specific IGS amplifications were identical: initial denaturation at 94°C for 3 min; 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 90 s; and then extension at 72°C for 10 min, with primers R2ssf-R2ssr or R3ssf-R3ssr, respectively. Five microliters of the amplification product was used for agarose (1.1%) gel electrophoresis. For nested PCR, the amplification products of general IGS PCRs (see above) were

TABLE 1. Isolates and reference strains used

DNA pool	Strain or isolate[a]	Host plant	Geographic origin	Source or reference[b]
	Bradyrhizobium sp. strain IRBG271	*Aeschynomene fluminensis*	Philippines	IRRI
	Rhizobium sp. strain IRBG74	*S. aculeata*	Philippines	IRRI
A	*Azospira oryzae* 6a3^T = LMG 9096^T	*Leptochloa fusca*	Pakistan	37
	B. elkanii USDA76^T	*Glycine max*	United States	USDA
	Bradyrhizobium sp. strain TAL 209*	*Vigna radiata*	Thailand	41
	Bradyrhizobium sp. strain TAL 289*	*Indigofera endecaphylla*	Mexico	41
	Bradyrhizobium sp. strain TAL 1037*	*Indigofera brevicalix*	Kenya	41
	Bradyrhizobium sp. strain TAL 1521*	*Acacia manginum*	Hawaii	41
B	*Bradyrhizobium* sp. strain CIAT 109*	*Desmodium intortum*	Zaire	CIAT
	Bradyrhizobium sp. strain CIAT 1502*	*Desmodium incanum*	Hawaii	CIAT
	Bradyrhizobium sp. strain CIAT 2335*	*Desmodium ovalifolium*	Brazil	CIAT
	B. elkanii USDA31, USDA46	*G. max*	United States	USDA
C	*B. japonicum* B 15	*G. max*	China	CCBAU
	B. japonicum USDA6^T	*G. max*	Japan	USDA
	B. japonicum X1-3, X6-9	*G. max*	China	51
	B. japonicum USDA62, USDA110, USDA123	*G. max*	United States	USDA
	B. japonicum DSM 30131	*G. max*	Japan	DSMZ
D	*Bradyrhizobium* sp. strain BC-C1, BC-C2	*Chamaecytisus proliferus*	Gran Canaria, Canary Islands	51
	Bradyrhizobium sp. strain BC-P7, BTA-1	*C. proliferus*	La Palma, Canary Islands	51
	Bradyrhizobium sp. strain TAL1000	*Arachis hypogaea*	Hawaii	HAMBI
	Bradyrhizobium sp. strain FN13, CICS70	*Lupinus montanus*	Mexico	3
	Bradyrhizobium sp. strain Spr7-9	*A. hypogaea*	China	58
E	*Mesorhizobium loti* NZP 2213^T	*Lotus corniculatus*	New Zealand	NZP
	M. loti NZP 2227, NZP 2234	*Lotus* sp.		NZP
	Mesorhizobium amorphae ACCC 19665^T	*Amorpha fruticosa*	China	CCBAU
	Mesorhizobium sp. strain HL56	*A. fruticosa*	China	CCBAU
	Mesorhizobium ciceri USDA 3378^T (UPM-Ca7)^T	*Cicer arietinum*	Spain	USDA
F	*Mesorhizobium huakuii* A106, PL-52	*Astragalus sinicus*	China	CCBAU
	M. huakuii CCBAU 2609^T	*A. sinicus*	China	CCBAU
	Mesorhizobium mediterraneum USDA3392^T	*Cicer arietinum*	Spain	USDA
	Mesorhizobium plurifarium LMG 1892^T	*Acacia senegal*	Senegal	LGM
	Mesorhizobium plurifarium USDA4413^T	*A. senegal*	Senegal	USDA
	Mesorhizobium tianshanense A-1BS^T	*Glycyrrhiza uralensis*	China	CCBAU
G	*Rhizobium etli* CFN42^T	*Phaseolus vulgaris*	Mexico	CFN
	R. galegae HAMBI 1185	*Galega* sp.	United Kingdom	HAMBI
	R. galegae HAMBI 503	*Galega officinalis*	United States	HAMBI
	R. galegae HAMBI 540^T	*Galega orientalis*	Finland	HAMBI
	R. galegae 59A2		United States	USDA
H	*Rhizobium giardinii* USDA2914^T (H152^T)	*P. vulgaris*	France	USDA
	R. hainanense I12	*Centrosema pubescens*	China	CCBAU
	R. hainanense 166^T	*Desmodium smuatum*	China	CCBAU
	R. hainanense H14	*Desmodium heterophyllum*	China	CCBAU
	Rhizobium huautlense S02^T	*Sesbania herbacea*	Mexico	CIFN
	Rhizobium gallicum USDA2918^T (R602sp^T)	*P. vulgaris*	France	USDA
I	*Rhizobium leguminosarum* 162X68	*Trifolium* sp.	United States	USDA
	R. leguminosarum USDA2370^T		United States	USDA
	Rhizobium mongolense USDA1844^T	*Medicago ruthenica*	China	USDA
	R. tropici type A CFN299	*P. vulgaris*	Mexico	CFN
	R. tropici type A C-05-I	*P. vulgaris*	Brazil	CFN
	R. tropici type B BR853	*Leucaena leucocephala*	Brazil	CFN
	R. tropici type B CIAT 899^T	*P. vulgaris*	Columbia	CFN
J	*Sinorhizobium fredii* 2048	*Glycine soja*	China	CCBAU
	S. fredii USDA194, USDA205^T	*G. soja*	China	USDA
	Sinorhizobium meliloti USDA1002^T		United States	USDA
	S. meliloti 102F28	*Medicago sativa*		
	S. meliloti H1	*Melilotus albus*	China	CCBAU
	Sinorhizobium terangae LMG 7834^T (ORS1009^T)	*Acacia laeta*	Senegal	LGM
	Sinorhizobium xinjiangense CCBAU 110^T	*G. max*	China	CCBAU
	S. xinjiangense CCBAU 108, Rx22	*G. max*	China	CCBAU

Continued on following page

TABLE 1—*Continued*

DNA pool	Strain or isolate[a]	Host plant	Geographic origin	Source or reference[b]
K	*A. oryzae* 6a3^T = LMG 9096^T	*Leptochloa fusca*	Pakistan	37
	R. rubi HAMBI 1187^T			HAMBI
	R. radiobacter IAM 12048^T			IAM
	R. radiobacter IAM 13129			IAM
	R. rhizogenes IAM 13570^T			IAM

[a] ∗, Phylogenetically close to the *B. elkanii rrn* branch (P. Vinuesa, unpublished data).
[b] CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China; ACCC, Agricultural Center Culture Collection, Chinese Academy of Agriculture, Beijing, China; CIFN, Centro de Investigación sobre Fijación de Nitrógeno, Cuernavaca, Mexico; CIAT, Centro Internacional de Agricultura Tropical, Cali, Columbia; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; NZP, Culture Collection of the Department for Scientific and Industrial Research, Biochemistry Division, Palmerston North, New Zealand; ORS, ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement et Coopération, Dakar, Senegal; USDA, *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, U.S. Department of Agriculture, Beltsville, Md.; IRRI, International Rice Research Institute, Los Banos Philippines; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

diluted 50-fold in sterile distilled water, and 1 μl was used for IGS-targeted specific PCR. In plant inoculation experiments, 70 ng of DNA extracted from rice roots per reaction mixture was used as a PCR template.

Rice germination and cultivation. Seeds of *O. sativa* IR36 were dehusked, surface sterilized, and germinated on agar plates as previously described (20). The seedlings were aseptically transferred to glass tubes containing 5 ml of sterile plant medium (13) supplemented with 100 mg of glucose (instead of malate) per liter and 0.2% agar instead of quartz sand. Alternatively, the seedlings were transferred to 5 g of unsterilized dried rice field soil (unfertilized), which had been collected from the Camargue region of France and was saturated with distilled water prior to transplanting. Bacterial cells grown aerobically on liquid YM medium were washed twice in plant medium and inoculated into the rice test tubes at 2×10^7 cells of each strain per tube, with eight replicates per experiment. The rice plants were grown in a greenhouse for 15 days (at 30°C; 2,200 lx of light added for 14 h; 80% humidity); the lower parts of the tubes were immersed in ink-stained water to shade the roots.

Nucleotide sequence accession numbers. The 16S rDNA sequence of *Rhizobium* sp. (*Sesbania*) strain IRBG74 and *Bradyrhizobium* sp. (*Aeschynomene*) strain IRBG271 have been deposited in GenBank under accession numbers AF364836 to AF364839 and AF271638, respectively. The accession numbers of IGS sequences were as follows: AF271639, *Bradyrhizobium japonicum* USDA123; AF271640, *Bradyrhizobium.* sp. (*Arachis*) strain TAL 1000; AF271641, *B. japonicum* X1-3; AF271642, *B. japonicum* USDA62; AF271643, *B. japonicum* DSM 30131; AF324182, *B. elkanii* USDA46; AF324181, *Bradyrhizobium* sp. strain BTA-1; AF271644, *A. tumefaciens* IAM 13129; AF271645, *Rhizobium tropici* CIAT 899; AF271646, *Rhizobium galegae* HAMBI 540; AF271647, *Bradyrhizobium* sp. strain IRBG271; AF271648, *Rhizobium* sp. strain IRBG74.

RESULTS

Phylogenetic position of rice growth-promoting rhizobial strains according to 16S rDNA sequence analysis. Almost-complete 16S rDNA sequences of isolates IRBG74 and IRBG271 were obtained and used for phylogenetic analysis together with published rhizobial reference sequences. The phylogenetic tree derived from the sequence distance values is shown in Fig. 1. The sequences of both PGPR strains appeared to be distinct from those of known species of rhizobia. That of strain IRBG271 was related, but not identical, to the sequence of *B. elkanii* USDA76^T, the type strain of the species (28). The identity level of the 16S rDNA sequences of isolates IRBG271 and *B. elkanii* USDA76^T or *B. japonicum* LMG 6138 was 99 or 98.2%, respectively. Isolate IRBG74 clustered in the *Rhizobium-Agrobacterium* branch, being more closely related to the former *A. tumefaciens* (emended name, *R. radiobacter*). The levels of 16S rDNA sequence identity between strain IRBG74 and *R. radiobacter* LMG 196, *Rhizobium rubi* LMG 156, and *R. galegae* IAM 13631 were 98.4, 97, and 95.4%, respectively. However, isolate IRBG74 differed from former *Agrobacterium* spp. in two major respects: it was isolated from nodules of *S. aculeata,* and it harbored a *nifH* gene (as detected by PCR and by Southern hybridization [not shown]). Thus, we refer to it as *Rhizobium* sp. (*Sesbania*).

Comparison of rhizobial 16S-23S rDNA IGS regions. Since only a few published sequences were available when the project was started, the 16S-23S rDNA IGS regions of several reference strains belonging to the *Rhizobium-Agrobacterium* cluster as well as to the *Bradyrhizobium* cluster were sequenced (for sequences and alignment, see http://www.institute.uni-bremen.de/~reinhol/IGS.html). It has been reported that most 16S-23S IGSs lack tRNA sequences in *Bacillus subtilis* (16); however, in *Rhizobium,* former *Agrobacterium,* and *Bradyrhizobium* species, the presence of tandem tRNA^{Ile} and tRNA^{Ala} genes was observed, as previously (27) and more recently (49, 53) reported for *Bradyrhizobium* strains.

In fast-growing rhizobia and agrobacteria, the tRNA genes showed some sequence variability, while they were highly conserved among the slow-growing bradyrhizobia. In both groups, the IGS sequences read from the *rrs* to the *rrl* gene, contained a conserved region that was followed first by a highly variable region (region I), after which the genes for tRNA^{Ile} and tRNA^{Ala} were found, interrupted by a variable region (II). Downstream of the tRNA genes, a third highly variable region (III) was located preceding a conserved region.

Copy numbers of *rrn* operons in rhizobial isolates. Since some bacterial species may have multiple rDNA operons, the operon numbers in the strains under question were determined by Southern hybridization using a digoxigenin-labeled 16S rDNA fragment (Fig. 2A) or oligonucleotide (Fig. 2B). In *Bradyrhizobium* sp. strain IRBG271, only one hybridizing fragment was detected in chromosomal DNA when it was digested with five different restriction endonucleases (Fig. 2A), indicating that the genome contains only one copy. In contrast, in *Rhizobium* sp. strain IRBG74, three to four different hybridizing fragments were found with four restriction endonucleases (not shown). In order to exclude false positives due to putative internal restriction sites, hybridizations were repeated with an oligonucleotide probe targeted to a conserved site of the 16S rDNA gene (926f). Again, four hybridizing fragments were detected (Fig. 2B), indicating that this strain contains four different *rrn* operon copies. To detect putative sequence polymorphisms in these copies, the four different copies of 16S

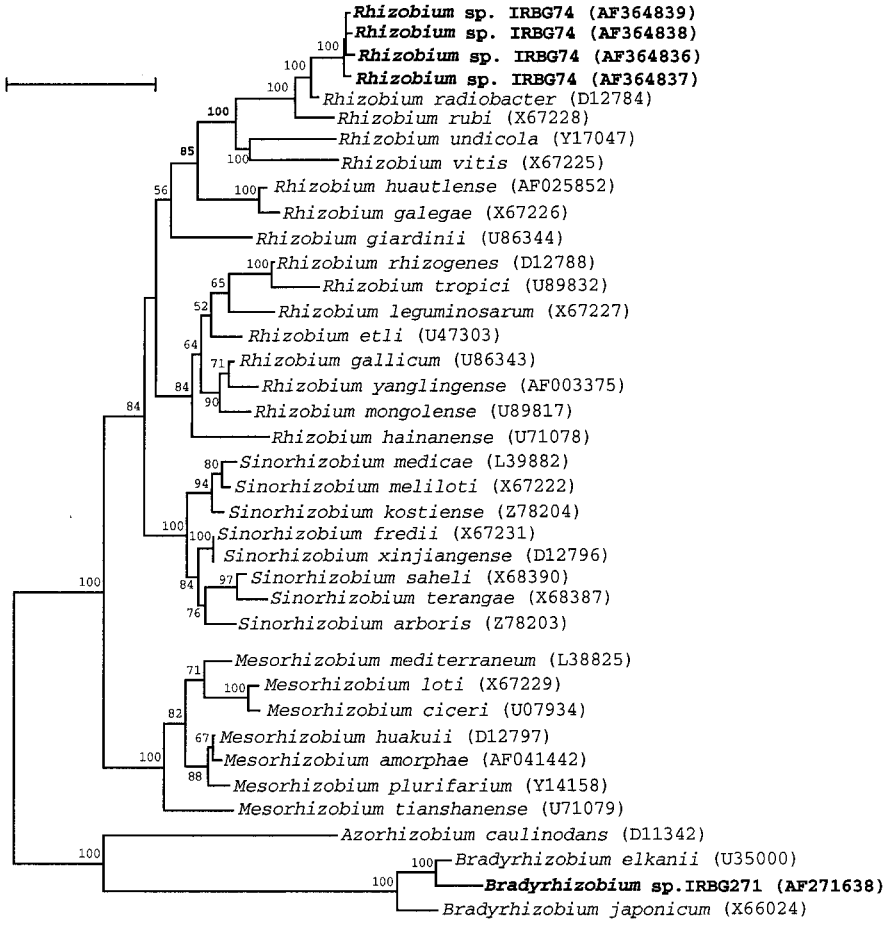

FIG. 1. Neighbor-joining dendrogram derived from a 16S rDNA sequence distance matrix (Jukes-Cantor) of the root nodule isolates IRBG74 and IRBG271 and known related *Rhizobium* (including former *Agrobacterium*), *Sinorhizobium, Mesorhizobium,* and *Bradyrhizobium* species. Bootstrap confidence levels greater than 50% are indicated at the internodes. GenBank accession numbers are shown in parentheses. Bar = 5% nucleotide divergence.

rDNA with the adjacent IGS region were amplified by PCR, cloned, and sequenced. The 16S rDNA genes diverged only slightly (4 to 6 nt [Fig. 1]), and sequence variation within the IGS was small (2 to 9 nt [see alignment]).

Phylogenetic analysis of rhizobial IGS sequences. Aligned sequences checked carefully by hand using GeneDoc software (http://www.cris.com/~ketchup/genedoc.shtml) were used to construct a phylogenetic tree (Fig. 3). The reference strains of the three validly described *Bradyrhizobium* species were well resolved, clustering with other representatives of each species. Strain IRBG271 was found to be most closely related to the *B. elkanii* cluster (sequence identity to *B. elkanii* USDA76^T, 91.6%), as deduced also from the 16S rDNA analyses. The *Chamaecytisus* isolate BAT-1 was more closely related to but distinct from the *B. japonicum* lineage (sequence identity, 90.6 to 93.7%), which is consistent with previously published partial 16S rDNA sequence and IGS PCR-restriction fragment length polymorphism (RFLP) analyses (51, 52). Among the fast-growing strains of rhizobia and agrobacteria, strains of different species were clearly differentiated from each other. However, not all nodes were statistically significant (Fig. 3), and the tree topology partially differed from 16S rDNA analyses (Fig. 1). This is most likely due to the high IGS sequence variability in these strains, which was significantly greater than for the slow-growing rhizobia. *Rhizobium* sp. strain IRBG74 clustered again with *R. radiobacter* (formerly *A. tumefaciens*), displaying 86% sequence identity (Fig. 3).

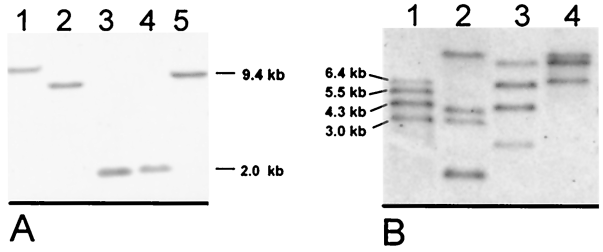

FIG. 2. Southern blot hybridization of genomic DNA of *Bradyrhizobium* sp. strain IRBG271 (A) and *Rhizobium* sp. strain IRBG74 (B) for detection of the 16S rDNA copy number. Hybridization was performed with a homologous 16S rDNA gene probe (A) or a universal oligonucleotide probe (926f) (B). Each lane was loaded with 3 μg of DNA digested with *Bam*HI (A, lane 1), *Eco*RI (A, lane 2), *Sma*I (A, lane 3; B, lane 2), *Asp*718 (A, lane 4), *Pst*I (A, lane 5, and B, lane 1), *Pvu*II (B, lane 3), or *Rsr*II (B, lane 4).

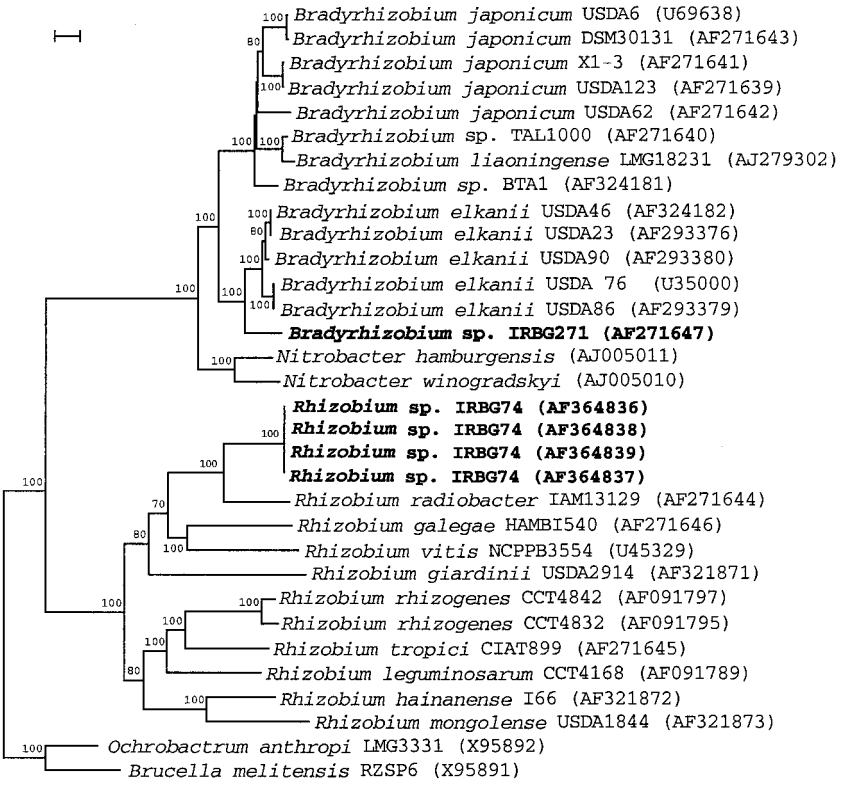

FIG. 3. Neighbor-joining dendrogram derived from a 16S-23S rDNA IGS sequence distance matrix (Jukes-Cantor) of *Bradyrhizobium* sp. and *Rhizobium* sp. clusters. Bootstrap confidence levels greater than 50% are indicated above the nodes. GenBank accession numbers are shown in parentheses. Bar = 5% nucleotide divergence.

Specificity of the IGS-targeted primers. Strain-specific primers were developed from the highly variable regions I and III: R3ssf and R3ssr, amplifying a 451-bp fragment, for *Bradyrhizobium* sp. strain IRBG271 and R2ssf and R2ssr, yielding a 988-bp product, for *Rhizobium* sp. IRBG74. In order to test a wide range of strains for an assessment of primer specificity, pools of DNA samples from different strains were used. Eleven genomic DNA pools containing DNA from rhizobia, sinorhizobia, mesorhizobia, bradyrhizobia, and agrobacteria (shown in Table 1) were used as PCR templates. To assess whether the mixture of DNAs conferred any inhibitory effects on PCR, reactions were also carried out with an internal positive control, the DNA of the rhizobial isolates being added. *Bradyrhizobium* sp. strain IRBG271 was compared with slow-growing strains (mesorhizobia and bradyrhizobia) (Fig. 4A), while *Rhizobium* sp. strain IRBG74 was separately compared with fast-growing strains (rhizobia, sinorhizobia, and agrobacteria) (Fig. 4B). For the nested PCR, the IGS region was first amplified from the mixtures by using general primers, and the diluted PCR product was subsequently used as a template for the strain-specific PCR. The amplification products from DNAs of pure cultures were of the expected size, approximately 450 bp for strain IRBG271 and 1,000 bp for strain IRBG74 (Fig. 4, lanes 1). When DNA pools were used, a product of this size was amplified only when DNA of the specific strain, *Bradyrhizobium* sp. strain IRBG271 (Fig. 4A) or *Rhizobium* sp. strain IRBG74 (Fig. 4B), was added as a template. Thus, the PCR protocol was considered to be sufficiently specific to differentiate the rhizobial strains, even in DNA mixtures of closely related species.

Application of specific IGS-targeted PCR primers to detect rhizobial colonization of rice roots. In order to assess whether the PCR protocol developed here was sufficiently sensitive and specific to detect colonization of roots by the rhizobial isolates, rice seedlings were inoculated either under gnotobiotic conditions or in complex soil. The total DNA isolated from the roots was subjected to PCR. In the nested IGS PCR using the specific primers R2ssf-R2ssr or R3ssf-R3ssr, single amplification products of the expected size (450 or 1,000 bp) were obtained (Fig. 5 and 6). In uninoculated control plants (Fig. 5 and 6, lanes 6), no amplification product was obtained, indicating that rice DNA (Fig. 5) or DNA of roots colonized by soil bacteria (Fig. 6) was not giving rise to false amplification products. The lack of an amplification product from soil-grown roots also indicated that the Asian rhizobial strains were not present in the rice field soil from France, or if present, they were not efficiently colonizing the rice cultivar.

Seedlings inoculated with *Rhizobium* sp. strain IRBG74 or *Bradyrhizobium* sp. strain IRBG271 separately (Fig. 5 and 6, lanes 2) or in a mixture of both (Fig. 5 and 6, lanes 3), gave rise to amplification products of the appropriate size, indicating that the bacteria were colonizing the roots sufficiently well to be detected by our PCR assay. This was especially interesting for soil-grown roots (Fig. 6), where the rhizobial isolates had to compete with the microflora present in the rice field soil. As an additional competitive constraint, a grass-endophytic diazo-

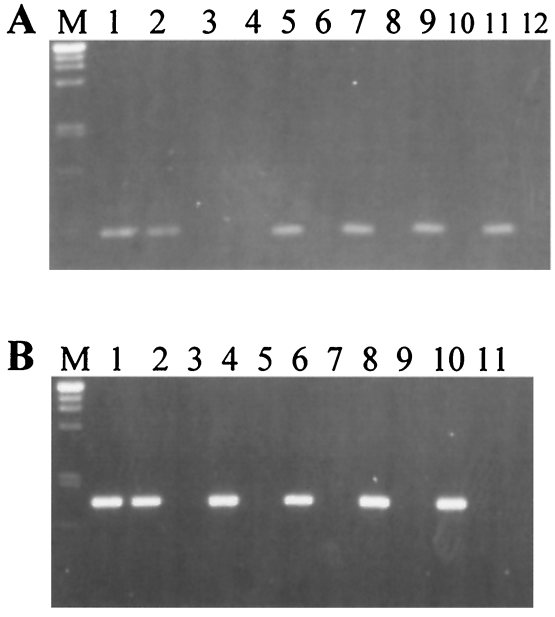

FIG. 4. Amplification products of nested PCR using IGS-targeted primers specific for *Bradyrhizobium* sp. strain IRBG271 (A) or *Rhizobium* sp. strain IRBG74 (B) in pure culture (lanes 1) or in the presence of a mixture of related bacteria (lanes 2 through 12). DNA pools of numerous reference strains (Table 1) of *Bradyrhizobium* spp. (A) or *Rhizobium, Mesorhizobium,* and *Sinorhizobium* (B) were used as templates. The specific IGS-targeted primers R3ssf-R3ssr (A) and R2ssf-R2ssr (B) were used in the second reaction mixture; 5 μl of the amplification product was loaded on a 1.1% agarose gel. The products were approximately 450 (A) or 1,000 (B) bp in size. M, size marker (λ DNA digested with *Pst*I). (A) Lanes: 1, strain IRBG271; 2, strain pool A plus IRBG271; 3, strain pool A; 4, strain pool B; 5, strain pool C plus IRBG271; 6, strain pool C; 7, strain pool D plus IRBG271; 8, strain pool D; 9, strain pool E plus IRBG271; 10, strain pool E; 11, strain pool F plus IRBG271; 12, strain pool F. (B) Lanes: 1, IRBG74; 2, strain pool G plus IRBG74; 3, strain pool G; 4, strain pool H plus IRBG74; 5, strain pool H; 6, strain pool I plus IRBG74; 7, strain pool I; 8, strain pool J plus IRBG74; 9, strain pool J; 10, strain pool K plus IRBG74; 11, strain pool K.

troph *Azospira oryzae* 6a3, originally isolated from Kallar grass (37), was coinoculated (Fig. 5 and 6, lanes 4 and 5). In both experimental settings, the gnotobiotic agar system and soil, the rhizobia were still detectable in root DNA. An unnested PCR approach applying specific IGS PCR directly, without a prior general amplification of bacterial IGS fragments, led to a loss of specificity: DNA fragments of aberrant size were amplified from uninoculated roots and inoculated samples, especially when soil-grown plants were analyzed. The direct PCR protocol was specific, however, when applied to DNA of pure cultures (not shown). The protocol developed for nested IGS-targeted PCR, however, was sufficiently sensitive and specific to detect and identify the rhizobial isolates on roots.

DISCUSSION

Strains of *Azorhizobium, Bradyrhizobium,* and *Rhizobium* have been identified as endophytes of different rice cultivars and species growing naturally or under cultivation in disjunct geographical regions around the world (8, 14, 47, 55). One objective of this study, therefore, was to determine the phylogenetic placement of the rice growth-promotig rhizobial strains IRBG74 and IRBG271, which are fast- and slow-growing isolates, respectively.

Our 16S rDNA phylogenetic analysis of rhizobial strains revealed the same overall topologies described by others (10, 57). The new *S. aculeata* isolate IRBG74 is placed in the former *Agrobacterium-Allorhizobium* clade, the closest phylogenetic neighbors being strains of *A. tumefaciens* bv. 1 and *Agrobacterium rubi* (now *R. radiobacter* and *R. rubi*, respectively). The high 16S rDNA sequence identity of strain IRBG74 to these strains (96.8 to 98.9 and 97%, respectively) strongly suggests its taxonomic placement in the genus *Rhizobium* according to Young (57). To our knowledge, this is the first report of a rhizobial strain from the *R. radiobacter-Rhizobium undicola* clade exhibiting a rice growth-promoting effect.

The *A. fluminensis* isolate IRBG271 was placed in the *Bradyrhizobium* clade in our analysis, being most closely related to *B. elkanii* USDA76^T (99% sequence identity). This high similarity level is not sufficient, however, to classify isolate IRBG271 as a *B. elkanii* strain, since it is well documented for many bacterial groups, including rhizobia, that full-length rDNA sequence analysis provides insufficient taxonomic resolution to distinguish closely related (geno)species (3, 15, 42, 49).

Recently, several reports have shown the suitability of IGS PCR-RFLP analysis for the rapid genotypic characterization, identification, and grouping of large collections of *Bradyrhizobium* strains at much higher taxonomic resolution than *rrs* sequence or PCR-RFLP analysis (12, 49, 51). Therefore, sequencing of the IGS region would allow the full exploitation of

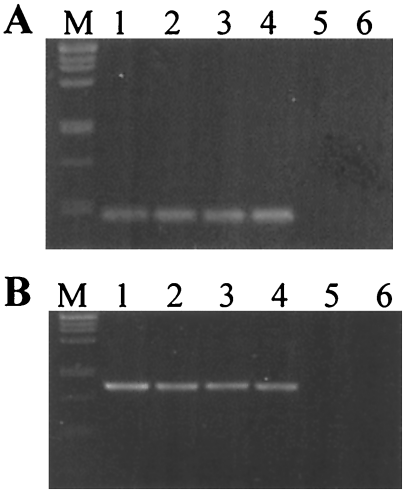

FIG. 5. Detection of *Bradyrhizobium* sp. strain IRBG271 (A) or *Rhizobium* sp. strain IRBG74 (B) in association with roots of inoculated rice plants by nested IGS-targeted PCR, without and with the presence of other bacteria. The DNA of roots of rice seedlings grown in gnotobiotic culture in agar medium was used for amplification, with the specific IGS-targeted primers R3ssf-R3ssr (A) and R2ssf-R2ssr (B) used in the second reaction mixture; 5 μl of the amplification product was loaded on a 1.1% agarose gel. The products were approximately 450 (A) or 1,000 (B) bp in size. M, size marker (λ DNA digested with *Pst*I). Lanes 1, pure culture of IRBG271 (A) or IRBG74 (B); lanes 2 to 4, rice inoculated with strain IRBG271 (A) or strain IRBG74 (B); lanes 3, rice inoculated with IRBG271 and IRBG74; lanes 4, rice inoculated with IRBG271, IRBG74, and *A. oryzae* 6a3; lanes 5, rice inoculated with *A. oryza* 6a3 only; 6, uninoculated rice.

this variable region for phylogenetic analysis of rhizobia, as frequently used in plant systematics and recently reported for several microbial groups (2, 17, 56). Two key studies that used IGS sequence analysis for inter- and intraspecific phylogenetic analysis of *Bradyrhizobium* strains were published during the review phase of this manuscript (49, 53). These studies revealed a very good correlation between groupings obtained by IGS sequence analysis, total DNA-DNA hybridization, and AFLP fingerprinting data. Willems et al. (53) reported that all the *Bradyrhizobium* genospecies identified by DNA-DNA hybridization were also resolved by IGS sequence analysis. However, IGS sequence similarity levels within the different genospecies were found to vary considerably, and therefore these authors found it impossible to use one particular level of IGS sequence similarity to delineate genospecies. A relatively low IGS sequence variation (94 to 100% sequence identity) was found within most of their AFLP clusters, whereas related genospecies displayed 81 to 89% IGS sequence similarity (53). The notable taxonomic resolution achieved by IGS sequencing, and the phylogenetic consistency of the delineated groups is also clearly illustrated in our analysis. The *B. japonicum* strains USDA6^T, USDA123, and USDA62 have *rrs* sequences that diverge <1% and belong to the distinct serogroups denoted by their respective strain numbering. The first two strains belong to DNA homology group I of Hollis et al. (18), whereas USDA62 belongs to homology group Ia. This is reflected by the placement of USDA62 as a separate branch of the clade formed by strains USDA6^T and USDA123 on our IGS dendrogram. The 16S rDNA sequence of *B. liaoningense* LMG18231 differs by only 3 nt from that of *B. japonicum* USDA6^T (49, 54) but is well resolved on the IGS dendrogram as a distinct strain that is closely related to *B. japonicum*. The same applies for *Bradyrhizobium* sp. (*Chamaecytisus*) strain BTA-1, which is consistent with previous reports using 16S and IGS rDNA PCR-RFLP analyses and stable low-molecular-weight RNA fingerprints (23, 52). All these strains form a clade that is well resolved at highly significant bootstrap values from a cluster containing the *B. elkanii* reference strains, in good agreement with other recent reports (49, 53). Therefore, we conclude that IGS sequence analysis is a powerful tool to delineate inter- and intraspecific *Bradyrhizobium* groups at statistically significant levels. As discussed by Willems et al. (53), most of these clades will correspond to different genomic species. However, due to the the highly variable nature of the IGS region, in some instances this genetic marker might reveal infraspecific genotypic differences that are not apparent when studying overall genomic similarities.

The phylogenetic placement of strain IRBG271 on the IGS dendrogram as a sister branch to the cluster formed by *B. elkanii* strains (91.65% similarity with USDA76^T) suggests, therefore, that it may correspond to a new genomic species. Strains IRBG271 and USDA76^T were found to be related but distinguishable by their cellular fatty acid compositions (41), indicating that strain IRBG271 may eventually be classified as a new species related to *B. elkanii*. We refrain here from making a formal species proposal, since more isolates related to IRBG271 should be characterized using a polyphasic taxonomic approach.

A potential limitation of 16S-23S rDNA IGS sequences for phylogenetic analysis in prokaryotes is the fact that many bacterial species possess several copies of the *rrn* operon, which may differ from one another, as demonstrated unambiguously by several genome-sequencing projects. This applies also to rhizobia, particularly to the fast-growing strains like IRBG74, whereas most *Bradyrhizobium* sp. strains, like IRBG271, typically contain a single *rrn* operon (27, 53). Although strain IRBG74 was found to harbor four copies of rRNA genes, the sequence divergence among different copies was very low (2 to 9 nt) and therefore did not blur our analysis. Strain IRBG74, which was found to be related to *R. radiobacter* based on *rrs* sequence analysis, had a significantly different IGS region (only 86% identity), suggesting that it is genomically quite distinct from *R. radiobacter* and therefore may also represent a new rhizobial species. However, as discussed for strain IRBG271, the exact taxonomic affiliation of strain IRBG74 can only be ascertained by using a polyphasic taxonomic approach.

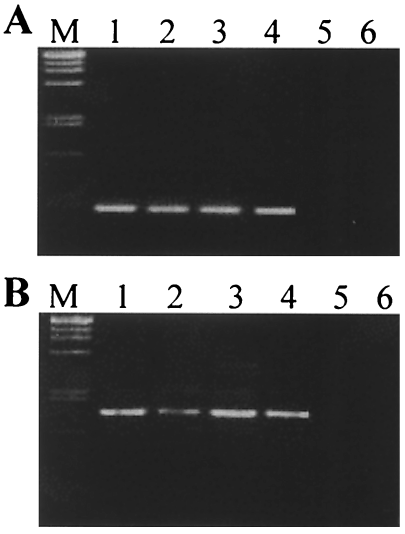

FIG. 6. Nested IGS-targeted PCR for detection of *Bradyrhizobium* sp. strain IRBG271 (A) or *Rhizobium* sp. strain IRBG74 (B) in association with inoculated rice roots grown in unsterilized soil. The plants were grown in unsterilized rice field soil from France inoculated with different bacterial strains, and total DNA was extracted after 15 days. For the PCR conditions and sample loading, see the legend to Fig. 5.

As a general conclusion, IGS sequences appeared to be more suitable for phylogenetic analyses in *Bradyrhizobium* than in the *Rhizobium* group. In the latter, at many nodes, the tree topology was statistically not well supported and differed from the 16S rDNA-based data. An illustrative example of this point is the clustering of *R. tropici* CIAT899 with *Rhizobium hainanense* I66 rather than with *Rhizobium rhizogenes* strains, as would be expected from the *rrs* sequence analysis. These inconsistencies are certainly a consequence of the high variability found in IGS sequences, including several gaps and insertions. However, this intrinsic sequence variability was very valuable for strain differentiation and identification in both bradyrhizobia and rhizobia, making IGS sequence analysis a very attractive technique for phylogenetic studies of these bacteria.

The second objective of this work was to develop a culture-independent, easy-to-perform, and sensitive technique to allow the specific detection of our rice growth-promoting strains in the rhizospheres of rice plants. At present, it is not known which mechanisms are responsible for the observed growth promotion that some rhizobia exert on rice. Since the reported

N_2-fixing activities measured in the rice rhizospheres is generally too low to account for the observed growth effect (8, 55), other factors, like phytohormone production, may be also involved, as is the case for other endophytic and rhizospheric associations (33). However, growth promotion on the field scale will only take place if the inoculated strains are able to establish themselves in the rhizosphere, on or inside the host roots. Therefore, it is of critical importance to be able to assess the colonizing abilities of potential inoculant strains, particularly under competitive conditions in nonsterile substrates. The high variability of the IGS region allowed the design of specific primers for the identification of streptococcal species or strains (5). Here we show the broad applicability of this strategy by using it to specifically detect our rice growth-promoting rhizobia colonizing rice roots.

To test the specificity of the PCR protocol, a large number of rhizobial and agrobacterial strains from different geographic locations and genera were used, including those found to be most closely related to IRBG74 and IRBG271 based on IGS sequence analyses. In complex mixtures of bacterial DNAs, as well as with the even more complex DNA mixtures extracted from roots grown in soil, the assay was remarkably specific. It is noteworthy that our PCR tests showed that the strains were even able to compete for colonization sites at rice roots when coinoculated with other grass endophytes like *A. oryzae* (37) or when rice seedlings were grown in unsterilized rice field soil, which should harbor plenty of other competing bacteria. This is in good agreement with the finding that these isolates promote plant growth by causing an enhanced seedling vigor in soil under greenhouse conditions (6, 7). However, 6 weeks after inoculation, the inoculated strains could not be isolated any longer from the rice roots (6). It is known that the majority of bacteria in a given ecosystem cannot be cultivated by conventional microbiological methods (35), and this has also been shown for diazotrophic endophytic bacteria occurring naturally in rice roots (14). Nonsporulating bacteria released into an environment might reach a physiological state in which they are viable but difficult to cultivate (26). Since the detection method that we have developed is solely based on the presence of bacterial DNA, it overcomes problems of cultivation, allowing us to prove the presence of rhizobia even in an unculturable state. Since IGS sequence variability can also be exploited to design strain-specific oligonucleotides for in situ hybridization, this *rrn* operon region is clearly of great relevance for both microbial systematics and ecology.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
2. **Baldwin, B. G., and S. Markos.** 1998. Phylogenetic utility of the external transcribed spacer (ETS) of 18S-26S rDNA: congruence of ETS and ITS trees of *Calycadenia* (*Compositae*). Mol. Phylogenet. Evol. **10:**449–463.
3. **Barrera, L. L., M. E. Trujillo, M. Goodfellow, F. J. Garcia, I. Hernandez-Lucas, G. Davila, P. van Berkum, and E. Martinez-Romero.** 1997. Biodiversity of bradyrhizobia nodulating *Lupinus* spp. Int. J. Syst. Bacteriol. **47:**1086–1091.
4. **Bej, A. K., M. H. Mahbubani, J. L. Dicesare, and R. M. Atlas.** 1991. Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. Appl. Environ. Microbiol. **57:**3529–3534.
5. **Berridge, B. R., J. D. Fuller, J. De Azavedo, D. E. Low, H. Bercovier, and P. F. Frelier.** 1998. Development of specific nested oligonucleotide PCR primers for the *Streptococcus iniae* 16S-23S ribosomal DNA intergenic spacer. J. Clin. Microbiol. **36:**2778–2781.
6. **Biswas, J. C., J. K. Ladha, and F. B. Dazzo.** 2000. Rhizobial inoculation improves nutrient uptake and growth of lowland rice. Soil Sci. Soc. Am. J. **64:**1644–1650.
7. **Biswas, J. C., J. K. Ladha, F. B. Dazzo, Y. G. Yanni, and B. G. Rolfe.** 2000. Rhizobial inoculation influences seedling vigor and yield of rice. Agron. J. **92:**880–886.
8. **Chaintreuil, C., E. Giraud, Y. Prin, J. Lorquin, A. Ba, M. Gillis, P. de Lajudie, and B. Dreyfus.** 2000. Photosynthetic bradyrhizobia are natural endophytes of the African wild rice *Oryza breviligulata*. Appl. Environ. Microbiol. **66:**5437–5447.
9. **Chaudhry, G. R., G. A. Toranzos, and A. R. Bhatti.** 1989. Novel method for monitoring genetically engineered microorganisms in the environment. Appl. Environ. Microbiol. **55:**1301–1304.
10. **de Lajudie, P., E. Laurent-Fulele, A. Willems, U. Torck, R. Coopman, M. D. Collins, K. Kersters, B. Dreyfus, and M. Gillis.** 1998. *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. Int. J. Syst. Bacteriol. **48:**1277–1290.
11. **de Oliveira, V. M., H. L. C. Coutinho, B. W. S. Sobral, C. T. Gumaraes, J. D. Van Elsas, and G. P. Manfio.** 1999. Discrimination of *Rhizobium tropici* and *R. leguminosarum* strains by PCR-specific amplification of 16S-23S rDNA spacer region fragments and denaturing gradient gel electrophoresis (DGGE). Lett. Appl. Microbiol. **28:**137–141.
12. **Doignon-Bourcier, F., A. Willems, R. Coopman, G. Laguerre, M. Gillis, and P. de Lajudie.** 2000. Genotypic characterization of *Bradyrhizobium* strains nodulating small Senegalese legumes by 16S-23S rRNA intergenic gene spacers and amplified fragment length polymorphism fingerprint analyses. Appl. Environ. Microbiol. **66:**3987–3997.
13. **Egener, T., T. Hurek, and B. Reinhold-Hurek.** 1999. Endophytic expression of *nif* genes of *Azoarcus* sp. strain BH72 in rice roots. Mol. Plant-Microbe Interact. **12:**813–819.
14. **Engelhard, M., T. Hurek, and B. Reinhold-Hurek.** 2000. Preferential occurrence of diazotrophic endophytes, *Azoarcus* spp., in wild rice species and land races of *Oryza sativa* in comparison with modern races. Environ. Microbiol. **2:**131–141.
15. **Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk, Jr.** 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. **42:**166–170.
16. **Fox, K. F., A. Fox, M. Nagpal, P. Steinberg, and K. Heroux.** 1998. Identification of *Brucella* by ribosomal-spacer-region PCR and differentiation of *Brucella canis* from other *Brucella* spp. pathogenic for humans by carbohydrate profiles. J. Clin. Microbiol. **36:**3217–3222.
17. **Grundmann, G. L., M. Neyra, and P. Normand.** 2000. High-resolution phylogenetic analysis of NO_2^--oxidizing *Nitrobacter* species using the *rrs-rrl* IGS sequence and *rrl* genes. Int. J. Syst. Evol. Microbiol. **50:**1893–1898.
18. **Hollis, A. B., W. E. Kloos, and G. E. Elkan.** 1981. DNA:DNA hybridization studies of *Rhizobium japonicum* and related *Rhizobiaceae*. J. Gen. Microbiol. **123:**215–222.
19. **Hurek, T., S. Burggraf, C. R. Woese, and B. Reinhold-Hurek.** 1993. 16S rRNA-targeted polymerase chain reaction and oligonucleotide hybridization to screen for *Azoarcus* spp., grass-associated diazotrophs. Appl. Environ. Microbiol. **59:**3816–3824.
20. **Hurek, T., B. Reinhold-Hurek, M. Van Montagu, and E. Kellenberger.** 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. J. Bacteriol. **176:**1913–1923.
21. **Hurek, T., B. Wagner, and B. Reinhold-Hurek.** 1997. Identification of N_2-fixing plant- and fungus-associated *Azoarcus* species by PCR-based genomic fingerprints. Appl. Environ. Microbiol. **63:**4331–4339.
22. **James, E. K., P. Gyaneshwar, W. L. Barraquio, N. Mathan, and J. K. Ladha.** 2000. Endophytic diazotrophs associated with rice, p. 119–140. *In* J. K. Ladha and P. M. Reddy (ed.), The quest for nitrogen fixation in rice. IRRI, Manila, Philippines.
23. **Jarabo-Lorenzo, A., E. Velázquez, R. Pérez-Galdona, M. C. Vega-Hernández, E. Martínez-Molina, P. F. Mateos, P. Vinuesa, E. Martínez-Romero, and M. León-Barrios.** 2000. Restriction fragment length polymorphism analysis of PCR-amplified 16S rDNA and low molecular weight RNA profiling in the characterisation of rhizobial isolates from shrubby legumes endemic to the Canary Islands. Syst. Appl. Microbiol. **23:**418–425.
24. **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.
25. **Jukes, T. H., and C. R. Cantor (ed.).** 1969. Evolution of protein molecules. Academic Press, New York, N.Y.
26. **Kaprelyants, A., J. C. Gottschal, and D. K. Kell.** 1993. Dormancy in nonsporulating bacteria. FEMS Microbiol. Rev. **104:**271–286.
27. **Kuendig, C., C. Beck, H. Hennecke, and M. Goettfert.** 1995. A single rRNA gene region in *Bradyrhizobium japonicum*. J. Bacteriol. **177:**5151–5154.
28. **Kuykendall, L. D., B. Saxena, T. E. Devine, and S. E. Udell.** 1992. Genetic

diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. **38:**501–505.
29. **Ladha, J. K., and R. B. So.** 1994. Numerical taxonomy of photosynthetic rhizobia nodulating *Aeschynomene* species. Int. J. Syst. Bacteriol. **44:**62–73.
30. **Laguerre, G., P. Mavingui, M. R. Allard, M. P. Charnay, P. Louvrier, S. I. Mazurier, L. Rigottiergois, and N. Amarger.** 1996. 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. Appl. Environ. Microbiol. **62:**2029–2036.
31. **Maidak, B. L., J. R. Cole, C. T. Parker, G. M. Garrity, N. Larsen, B. Li, T. G. Lilburn, M. J. McCaughey, G. J. Olsen, R. Overbeek, S. Pramanik, T. M. Schmidt, J. M. Tiedje, and C. R. Woese.** 1999. A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res. **27:**171–173.
32. **Nick, G., P. de Lajudie, B. D. Eardly, S. Suomalainen, L. Paulin, X. Zhang, M. Gillis, and K. Lindström.** 1999. *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya. Int. J. Syst. Bacteriol. **49:**1359–1368.
33. **Okon, Y., and C. A. Labandera-Gonzalez.** 1994. Agronomic applications of *Azospirillum*—an evaluation of 20 years worldwide field inoculation. Soil Biol. Biochem. **26:**1591–1601.
34. **Olsen, P. E., and W. A. Rice.** 1989. *Rhizobium* strain identification and quantification in commercial inoculants by immunoblot analysis. Appl. Environ. Microbiol. **55:**520–522.
35. **Pace, N. R.** 1997. A molecular view of the microbial diversity and the biosphere. Science **276:**734–740.
36. **Reinhold, B., T. Hurek, and I. Fendrik.** 1985. Strain-specific chemotaxis of *Azospirillum* spp. J. Bacteriol. **162:**190–195.
37. **Reinhold-Hurek, B., and T. Hurek.** 2000. Reassessment of the taxonomic structure of the diazotrophic genus *Azoarcus sensu lato* and description of three new genera and new species, *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov. and *Azonexus fungiphilus* gen. nov., sp. nov. Int. J. Syst. Evol. Microbiol. **50:**649–659.
38. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
39. **Schloter, M., W. Wiehe, B. Assmus, H. Steindl, H. Becke, G. Hoeflich, and A. Hartmann.** 1997. Root colonization of different plants by plant-growth-promoting *Rhizobium leguminosarum* bv. trifolii R39 studied with monospecific polyclonal antisera. Appl. Environ. Microbiol. **63:**2038–2046.
40. **Segovia, L., D. Pinero, R. Palacios, and E. Martinez Romero.** 1991. Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum*. Appl. Environ. Microbiol. **57:**426–433.
41. **So, R. B., J. K. Ladha, and J. P. W. Young.** 1994. Photosynthetic symbionts of the *Aeschynomene* species form a cluster with *Bradyrhizobium* on the basis of fatty acid and rRNA analyses. Int. J. Syst. Bacteriol. **44:**392–403.
42. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
43. **Sullivan, J. T., B. D. Eardly, P. Van Berkum, and C. W. Ronson.** 1996. Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. Appl. Environ. Microbiol. **62:**2818–2825.
44. **Sullivan, J. T., and C. W. Ronson.** 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc. Natl. Acad. Sci. USA **95:**5145–5149.
45. **Sy, A., E. Giraud, P. Jourand, N. Garcia, A. Willems, P. de Lajudie, Y. Prin, M. Neyra, M. Gillis, C. Boivin-Masson, and B. Dreyfus.** 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J. Bacteriol. **183:**214–220.
46. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
47. **Ueda, T., Y. Suga, N. Yahiro, and T. Matsuguchi.** 1995. Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. J. Bacteriol. **177:**1414–1417.
48. **van Berkum, P., and B. D. Eardly.** 1998. Molecular evolutionary systematics of the *Rhizobiaceae*, p. 1–24. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The Rhizobiaceae. Kluwer Academic Publishers, Dordrecht, The Netherlands.
49. **van Berkum, P., and J. J. Fuhrmann.** 2000. Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence. Int. J. Syst. Evol. Microbiol. **50:**2165–2172.
50. **Van de Peer, Y., and R. de Wachter.** 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Appl. Biosci. **10:**569–570.
51. **Vinuesa, P., J. L. W. Rademaker, F. J. de Bruijn, and D. Werner.** 1999. Characterization of *Bradyrhizobium* spp. strains by RFLP analysis of amplified 16S rDNA and rDNA intergenic spacer regions, p. 275–279. *In* E. Martinez and M. Hernandez (ed.), Highlights of nitrogen fixation research. Kluwer Academic/Plenum Publishers, New York, N.Y.
52. **Vinuesa, P., J. L. W. Rademaker, F. J. Debruijn, and D. Werner.** 1998. Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. Appl. Environ. Microbiol. **64:**2096–2104.
53. **Willems, A., R. Coopman, and M. Gillis.** 2001. Comparison of sequence analysis of 16S-23S rDNA spacer regions, AFLP analysis and DNA-DNA hybridizations in *Bradyrhizobium*. Int. J. Syst. Evol. Microbiol. **51:**623–632.
54. **Xu, L. M., C. Ge, Z. Cui, J. Li, and H. Fan.** 1995. *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. Int. J. Syst. Bacteriol. **45:**706–711.
55. **Yanni, Y. G., R. Y. Rizk, V. Corich, A. Squartini, K. Ninke, S. Philip-Hollingsworth, G. Orgambide, F. De Bruijn, J. Stoltzfus, D. Buckley, T. M. Schmidt, P. F. Mateos, J. K. Ladha, and F. B. Dazzo.** 1997. Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots and assessment of its potential to promote rice growth. Plant Soil **194:**99–114.
56. **Yoon, J. H., S. T. Lee, S. B. Kim, M. Goodfellow, and Y. H. Park.** 1997. Inter- and intraspecific genetic analysis of the genus *Saccharomonospora* with 16S to 23S ribosomal DNA (rDNA) and 23S to 5S rDNA internally transcribed spacer sequences. Int. J. Syst. Bacteriol. **47:**661–669.
57. **Young, J. M., L. D. Kuykendall, E. Martinez-Romero, A. Kerr, and H. Sawada.** 2001. A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. Int. J. Syst. Evol. Microbiol. **51:**89–103.
58. **Zhang, X., G. Nick, S. Kaijalainen, Z. Terefework, L. Paulin, S. W. Tighe, P. H. Graham, and K. Lindstrom.** 1999. Phylogeny and diversity of *Bradyrhizobium* strains isolated from the root nodules of peanut (*Arachis hypogaea*) in Sichuan, China. Syst. Appl. Microbiol. **22:**378–386.