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Evidence of Horizontal Transfer of Symbiotic Genes from a *Bradyrhizobium japonicum* Inoculant Strain to Indigenous Diazotrophs *Sinorhizobium* (*Ensifer*) *fredii* and *Bradyrhizobium elkanii* in a Brazilian Savannah Soil[▽]

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The importance of horizontal gene transfer (HGT) in the evolution and speciation of bacteria has been emphasized; however, most studies have focused on genes clustered in pathogenesis and very few on symbiosis islands. Both soybean (*Glycine max* [L.] Merrill) and compatible *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains are exotic to Brazil and have been massively introduced in the country since the early 1960s, occupying today about 45% of the cropped land. For the past 10 years, our group has obtained several isolates showing high diversity in morphological, physiological, genetic, and symbiotic properties in relation to the putative parental inoculant strains. In this study, parental strains and putative natural variants isolated from field-grown soybean nodules were genetically characterized in relation to conserved genes (by repetitive extragenic palindromic PCR using REP and BOX A1R primers, PCR-restriction fragment length polymorphism, and sequencing of the 16SrRNA genes), nodulation, and N_2-fixation genes (PCR-RFLP and sequencing of *nodY-nodA*, *nodC*, and *nifH* genes). Both genetic variability due to adaptation to the stressful environmental conditions of the Brazilian Cerrados and HGT events were confirmed. One strain (S 127) was identified as an indigenous *B. elkanii* strain that acquired a *nodC* gene from the inoculant *B. japonicum*. Another one (CPAC 402) was identified as an indigenous *Sinorhizobium* (*Ensifer*) *fredii* strain that received the whole symbiotic island from the *B. japonicum* inoculant strain and maintained an extra copy of the original *nifH* gene. The results highlight the strategies that bacteria may commonly use to obtain ecological advantages, such as the acquisition of genes to establish effective symbioses with an exotic host legume.

Genome sequencing has demonstrated that a high percentage of the genes of prokaryotes results from horizontal gene transfer (HGT), which has stimulated broad discussion about basic concepts of bacterial evolution and speciation (4, 5, 12, 32, 39). Many genes horizontally transferred are organized in clusters called "genomic islands" (GEIs) in DNA regions frequently inserted in the vicinity of tRNA genes and flanked by repeat sequences (5). The GEIs may confer to the recipient bacterium an increased adaptive capacity and also improved fitness to environmental changes. One major example, and probably the most studied case, of horizontal transfer of GEIs is that of the prokaryotic genes required for pathogenicity in eukaryotic hosts (5, 16).

Several species of the family *Leguminosae* are capable of establishing symbiotic associations with a variety of diazotrophic bacteria belonging to the genera *Allorhizobium*, *Bradyrhizobium*, *Burkholderia*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium*, and *Sinorhizobium* (now *Ensifer*) and probably others to be described, and the group is usually collectively termed "rhizobia." The rhizobial symbiotic genes analyzed so far are organized in clusters localized either on symbiotic plasmids or in GEIs on the chromosome (5, 10, 13, 25, 26). Not much is known about HGT of the symbiotic GEIs. A pioneer study was performed in a New Zealand soil devoid of rhizobia capable of nodulating *Lotus corniculatus*, in which a strain of *Mesorhizobium loti* (ICMP3153) was introduced along with the host plant (48). After 7 years, genotypically diverse strains of *M. loti* were isolated from the root nodules, and as both the inoculant and the isolated strains contained the same symbiotic DNA region, Sullivan et al. (48) suggested that nonsymbiotic indigenous strains of *M. loti* acquired the genes through HGT. After that, Sullivan and Ronson (47) characterized and verified that the 500-kb symbiotic element of *M. loti* was transmissible to at least three genomic species of nonsymbiotic mesorhizobia under laboratory conditions. This element was thus termed a "symbiosis island," analogous to pathogenicity islands. Later, a putative 611-kb symbiosis island showing properties similar to those of strain ICMP3153 was described in the genome of *M. loti* strain MAFF303099 (49).

In *Bradyrhizobium japonicum* strain USDA 110, Kündig et al. (30) first reported that all known genes related to nodulation and N_2 fixation were clustered in a chromosomal region of about 400 kb. The symbiotic region was characterized by a G+C content different from that of the genome as a whole,

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TABLE 1. Soybean rhizobial strains used in this study

Serogroup	Strain(s)	Description
566	SEMIA 566	*B. japonicum* strain isolated from a North American inoculant in 1966 and used in Brazilian commercial inoculants from 1966 to 1978.
	CPAC 15 (SEMIA 5079)	*B. japonicum* natural variant of SEMIA 566 used in commercial inoculants since 1992.
	S 127, S 340, S 370, S 372, S 478, S 490, S 516	Natural variants of SEMIA 566 adapted to the Brazilan Cerrados soils and showing higher capacity of N_2 fixation than the putative parental strain.
586	SEMIA 586 (CB 1809)	*B. japonicum* strain originally isolated in the United States but received from Australia in 1966; used in Brazilian commercial inoculants in 1977.
	CPAC 7 (SEMIA 5080)	*B. japonicum* natural variant of SEMIA 586 used in commercial inoculants since 1992.
	CPAC 390, CPAC 392, CPAC 394, CPAC 402, CPAC 403, CPAC 404	Natural variants of SEMIA 586 adapted to the Brazilian Cerrados and showing higher competitiveness than the putative parental strain.

suggesting integration into the chromosome after HGT from a different strain (30, 41). Later, the complete genome sequence of strain USDA 110 revealed the presence of a 680-kb DNA region that included the 400-kb symbiosis island previously described (26). In experiments performed in soils and microcosms, Minamisawa et al. (35) described HGT of nodulation genes from a highly reiterated sequence-possessing (HRS) strain of *B. japonicum* to a *Bradyrhizobium elkanii* lacking common *nod* genes. However, after successive transfers in vitro, the acquired genes were lost. As the highly reiterated sequence-possessing strain harbors high copy numbers of the insertion sequences (ISs) RSα and RSβ, Minamisawa et al. (35) proposed that the ISs elements might be involved in the HGT process.

Both soybean (*Glycine max* [L.] Merrill) and compatible *B. japonicum* and *B. elkanii* strains are exotic to Brazil and have been massively introduced in the country mainly since the earlier 1960s (9, 20, 23). Soybean is now the most important grain crop in the country, occupying almost 45% of the cropped land. The nitrogen (N) required for plant growth is acquired via biological N_2 fixation, with no need to supply N fertilizers. The great majority of the 22 million hectares cropped with the legume today show established populations of inoculant strains, estimated at 10^3 to 10^6 soybean rhizobia per g of soil, but massive reinoculation with selected strains of *B. japonicum* and *B. elkanii* is still a common practice among farmers to maximize the effectiveness of the biological process and to optimize yields (20, 21, 23).

Since the late 1990s, our group has characterized several rhizobial strains reisolated from field-grown soybean nodules in areas of the Brazilian Cerrados originally devoid of soybean. In these areas, exotic strains of *B. japonicum* and *B. elkanii* were introduced a decade ago. Compared to the putative parental strains, several of these isolates have shown differences in morphological, serological, physiological, genetic, and symbiotic properties (3, 19, 22, 33, 38, 44). Variability might be caused by the stressful environmental conditions of the Cerrados, where long periods of high temperature and drought often prevail (18). However, increasing evidence of HGT events is accumulating (2, 11). In this study, a detailed genetic characterization of some strains previously obtained by our group (3, 44) has confirmed the occurrence of HGT of symbiotic genes among inoculant and indigenous rhizobia in the Brazilian Cerrados.

MATERIALS AND METHODS

Rhizobial strains. The strains were obtained from the Brazilian Cerrados, a savannah-like zone characterized by acid soils, with high temperatures year-round and with a 4- to 9-month period of drought annually (1). The soils at the experimental area were initially devoid of rhizobia capable of effectively nodulating soybean; exotic *B. japonicum* strains SEMIA 566 and SEMIA 586 (identical to CB 1809) were introduced together with the host plant as the first crop, after which annual cropping of soybean took place. Strains SEMIA 566 and SEMIA 586 belong to different serogroups, and their characteristics have been described elsewhere (3, 23, 44).

Variant strains of both the SEMIA 566 (3) and SEMIA 586 (44) serogroups used in this study were obtained from nodules of field-grown soybean plants some years after their initial introduction and were classified according to their serological reactions. In comparison to the putative parental genotypes, variant strains were different in several morphological, physiological, genetic, and symbiotic properties (3, 22, 44). We selected seven variant strains of SEMIA 566 and six of SEMIA 586. Two other strains used were CPAC 7 (identical to SEMIA 5080) and CPAC 15 (identical to SEMIA 5079), variants of SEMIA 586 and SEMIA 566, respectively, also obtained after adaptation to the Cerrados soils (3, 18, 19, 38, 40, 44); both have been used in Brazilian commercial inoculants since 1992 (23, 40). SEMIA 566 and SEMIA 586 were used as controls. Table 1 summarizes the main characteristics of the strains used in this study.

Morphological, serological, physiological, and symbiotic characterization. The main morphological (colony morphology and mucus production on yeast-mannitol agar [YMA] [51]), serological (immuno-agglutination reaction with polyclonal antisera prepared against the somatic thermo-stable antigens of SEMIA 566 and SEMIA 586), and physiological (acid or alkaline reaction on YMA) properties were determined as described before (9).

N_2-fixation capacity was evaluated in a greenhouse experiment performed in a completely randomized block design with three replicates. Soybean plants of cultivar BR 16 were individually inoculated with each strain and grown in Leonard jars containing N-free nutrient solution, as described before (9). At 30 days after emergence plants were evaluated in terms of nodule number and dry weight, shoot dry weight, and total N content of shoots, as described before (22).

Nodule occupancy was evaluated in a greenhouse experiment with seeds co-inoculated with a combination of each strain and of *B. elkanii* SEMIA 587 (1:1; 10^8 cells ml^{-1} of each), which is serologically distinct. Plants were harvested at 30 days after emergence, and nodule occupancy was evaluated by immuno-agglutination as described before (3).

Amplification of DNA with primers for conserved and symbiotic genomic regions. Total genomic DNA from the strains was obtained (27), and the amplification of conserved regions was achieved by repetitive extragenic palindromic PCR (rep-PCR) with REP (44) and BOX A1R (27) primers. The amplification of the DNA region coding for the 16 rRNA gene was carried out with fD1 and rD1 primers, as described before (34).

Symbiotic genes chosen for this study included *nifH*, a DNA region encoding the *nodY-nodA* region, and the *nodC* gene. Primers nifHF and nifHI (31) were

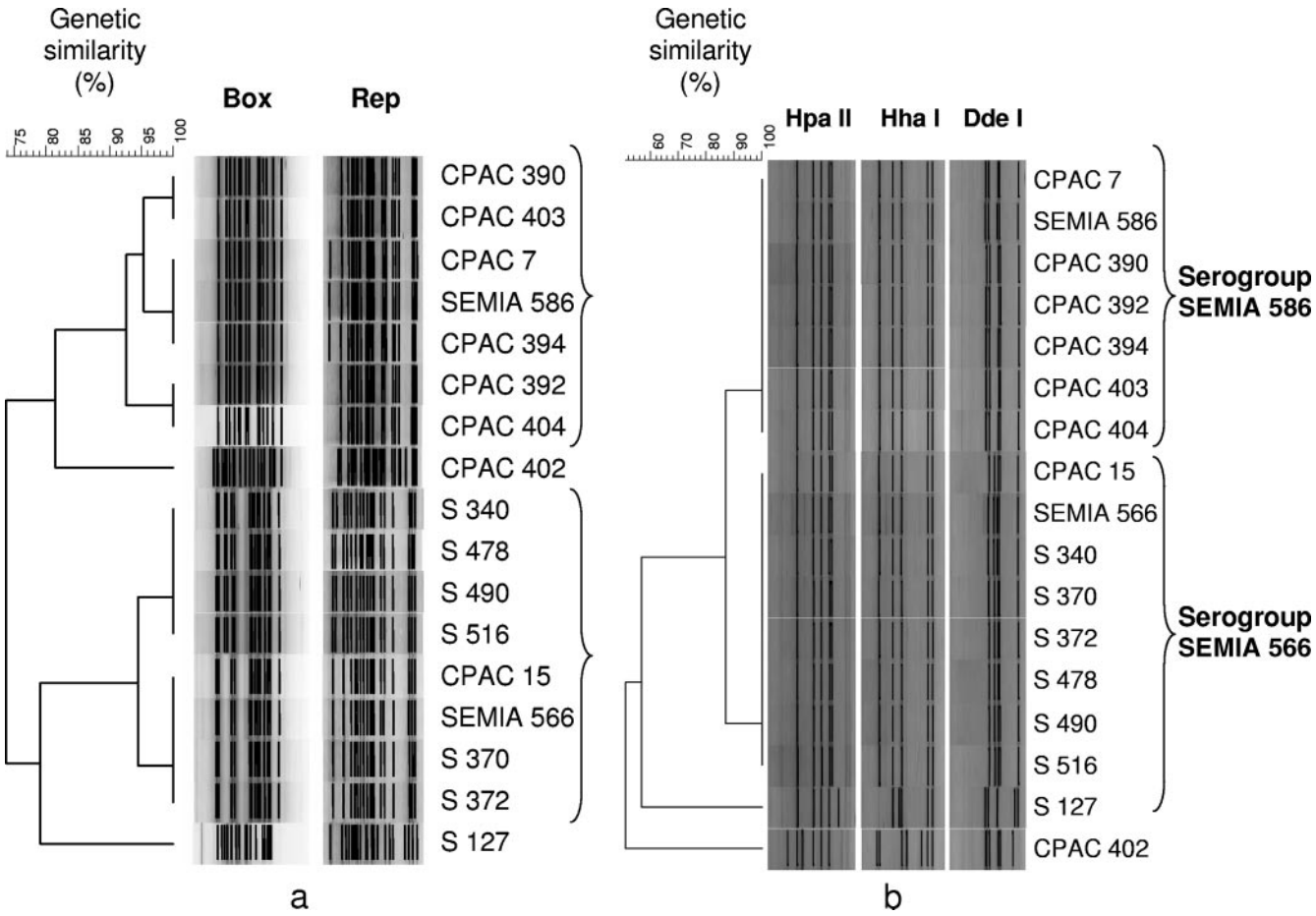

FIG. 1. Cluster analysis (UPGMA with the coefficient of Jaccard) of the DNA amplification products obtained in the analyses of: rep-PCR (primers BOX A1R and REP) (a) and PCR-RFLP of the 16S rRNA genes (b) of 17 soybean rhizobia. Strains belonged to two serogroups, one comprised of the parental *B. japonicum* strain SEMIA 586 (also CB1809) and the commercial variant CPAC 7 and the other comprised of the *B. japonicum* parental strain SEMIA 566 and the commercial variant CPAC 15.

used for the amplification of about 800 bp of the 885-bp *nifH* gene. A 2,000-bp DNA region including the *nodY* and *nodA* genes was amplified with primers TsnodDI-1a, TsnodB2, and TsnodB3 (37). Amplification conditions have been described elsewhere (31, 37).

For the PCR amplification of about 1,200 bp of the 1,400-bp *nodC* gene, we designed the primers nodCBjF (5′-GATCAACTCATCGCTGGA-3′) and nodCBjR (5′-GCCTTACGAATAGGAGCA-3′), based on the genome of *B. japonicum* strain USDA 110. The PCR mixture included, in a 50-μl final volume: template DNA (20 ng); PCR buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl); 0.2 mM (each) dATP, dCTP, dTTP, and dGTP; 3 mM $MgCl_2$; 15 pmol of each *nodC* primer; and 1 U of *Taq* polymerase. Temperature cycles were the following: 2 min at 95°C and 35 cycles consisting of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, with a final cycle at 72°C for 2 min.

PCR-RFLP of the DNA regions coding for the 16S rRNA, *nifH*, *nodY-nodA*, and *nodC* genes. The PCR products obtained for each gene region were individually digested with restriction endonucleases, as follows: HhaI, DdeI, and HpaII for the 16S rDNA; HhaI, HpaII, and MboI for the *nodC* region; DdeI, HaeIII, HpaII, and HindIII for the *nifH* region; and HpaII, DdeI, HhaI, and MboI for the *nodY-nodA* region. Digestion conditions were as described by the manufacturer. PCR-restriction fragment length polymorphism (RFLP) products were analyzed by horizontal electrophoresis in 3% agarose, as described before (36).

Analysis of rep-PCR and PCR-RFLP products. The analyses of rep-PCR and PCR-RFLP products were performed using Bionumerics software (version 1.50; Applied Mathematics, Kortrijk, Belgium). The sizes of the fragments in each analysis were normalized according to the molecular weight of the DNA size markers (included in the right, left, and central lanes of each gel). The PCR products were submitted to similarity analyses using the unweighted-pair group method using average linkages (UPGMA) algorithm (45) and the coefficient of Jaccard (24), and a tolerance of 3% was established in the Bionumerics software.

Sequencing of the whole 16S rRNA gene and partial sequencing of the *nifH*, *nodY-nodA*, and *nodC* genes. The 16S rRNA and *nodY-nodA* genes were sequenced straight from PCR products. To obtain the complete 16S rRNA sequences, primers fD1, Y2, 362f, 786f, and 1203f were used, as described by Menna et al. (34). A DNA region spanning 397 bp of the *nodY* gene and 54 bp of the *nodA* gene was sequenced using the nodKup and nodAp4 primers (46). For the partial sequencing of the *nifH* and *nodC* genes, the PCR products obtained were first cloned into the TOPO TA vector (Invitrogen), following the procedure described by the manufacturer. The PCR products were purified as described before (34), and sequencing was performed with the use of the DYEnamic ET terminator reagent (Amersham Biosciences) and analyzed in a MegaBace 1000 DNA Analysis System (Amersham Biosciences), according to parameters described previously (34).

Sequencing analysis and phylogeny of the 16S rRNA and symbiotic genes. The high-quality sequences obtained in both 3′ and 5′ directions were assembled using the programs phred (6, 7), phrap (http://www. phrap.org), and Consed (14) and were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov /BLAST) to seek significant alignments. The sequenced 16S rRNA genes were also submitted for taxonomical identification to the Ribosomal Database Project II (http://rdp.cme.msu.edu/) using the Naive Bayesian rRNA Classifier, version 1.0. Phylogenetic trees were generated using default parameters (29), K2P distance model (28), and the neighbor-joining algorithm (43). Statistical support for tree nodes was evaluated by bootstrap analyses (8) with 2,000 samplings (17).

Phylogenetic analyses of the whole sequences of the 16S rRNA and of the partial sequences of *nifH*, *nodY-nodA*, and *nodC* genes were conducted using

MEGA software, version 3.1 (29). The sequences obtained were aligned and compared to those of the type or reference strains (accession numbers of the GenBank Database in parentheses), as follows. For the 16S rRNA gene, the following strains were considered: *B. elkanii* USDA 76^T (U35000); *B. japonicum* USDA 6^T (U69638), SEMIA 566 (AF236086), and SEMIA 586 (AF236087); *Sinorhizobium* (*Ensifer*) *fredii* USDA 205^T (X6723) (*Sinorhizobium* and *Ensifer* should be now combined as the genus *Ensifer* [52]; however, in this paper we will refer to the symbiotic bacteria as *Sinorhizobium*) and the variant strains S 127 (DQ485704), CPAC 402 (DQ485718), CPAC 7 (AF234889), and CPAC 15 (AF234888). For the *nifH* gene, the following strains were used: *B. japonicum* USDA 110 (AF322012); *B. elkanii* USDA 76^T (AB094963); *S. fredii* USDA 191 (Z95229) and the variant strains S 127 (DQ485701) and CPAC 402 (DQ485714 and DQ485715). For the *nodY-nodA* region, alignment was performed with the following strains: *B. japonicum* USDA 110 (AF322013); *B. elkani* USDA 94 (U04609) and the variant strains CPAC 15 (DQ485694); CPAC 7 (DQ485696); SEMIA 586 (DQ485698); SEMIA 566 (DQ485700); S 127 (DQ485703); S 340 (DQ485705); S 370 (DQ485706); S 372 (DQ485707); S 478 (DQ485708); S 490 (DQ485709); S 516 (DQ485710); CPAC 390 (DQ485711); CPAC 392 (DQ485712); CPAC 394 (DQ485713); CPAC 402 (DQ485717); CPAC 403 (DQ485719) and CPAC 404 (DQ485720). Finally, for the comparison of *nodC* genes, the following strains were used: *B. japonicum* USDA 110 (AF322013); *B. elkanii* CCBAU 23174 (DQ010032); *S. fredii* HH 103 (DQ060002) and the variant strains CPAC 15 (DQ485693); CPAC 7 (DQ485695); SEMIA 586 (DQ485697); SEMIA 566 (DQ485699); S 127 (DQ485702) and CPAC 402 (DQ485716).

Nucleotide sequence accession numbers. GenBank accession numbers DQ485693 to DQ485720 were given to the nucleotide sequences determined in this study.

RESULTS

Morphological, physiological, and symbiotic characterization. In general, variant strains of both serogroups showed a higher production of mucus on YMA medium than the putative parental inoculant strains. The *B. japonicum* variant strain CPAC 402 (serogroup of SEMIA 586) showed more abundant mucus production than the parental strain SEMIA 586 (data not shown). Strain CPAC 402 also showed a faster growth rate and a more acidic reaction on YMA medium than the parental strain SEMIA 586 (data not shown).

Evaluation of nodulation (nodule number and dry weight), plant growth (shoot dry weight), N_2 fixation (total shoot N), and nodule occupancy parameters under greenhouse and N-free conditions (data not shown) confirmed the ranking of the previously reported strains (3, 22). Variants of serogroup SEMIA 566 were in general statistically superior in N_2 fixation performance (nodulation and shoot N) in comparison to the parental strain, while the majority of the variants of SEMIA 586 were statistically superior in the nodule occupancy capacity (data not shown).

Comparative analysis of variant and parental strains based on the profiles obtained by rep-PCR and PCR-RFLP of the 16S rRNA gene. Except for S 127 and CPAC 402, the strains belonging to the same serogroup were clustered at a level of similarity higher than 85% in the rep-PCR analysis (Fig. 1a).

The 17 *B. japonicum* strains were also clustered based on the PCR-RFLP of the whole 16S rRNA gene (Fig. 1b). Strains belonging to the same serogroup, except again for CPAC 402 and S 127, showed complete similarity of restriction patterns, and the two clusters were joined at a similarity level of 88%, differing only in the profiles obtained with the restriction endonuclease DdeI (Fig. 1b). The similarity of the 16S rRNA genes of CPAC 402 and S 127 with the other strains was lower than 60%, suggesting that they belong to a different species.

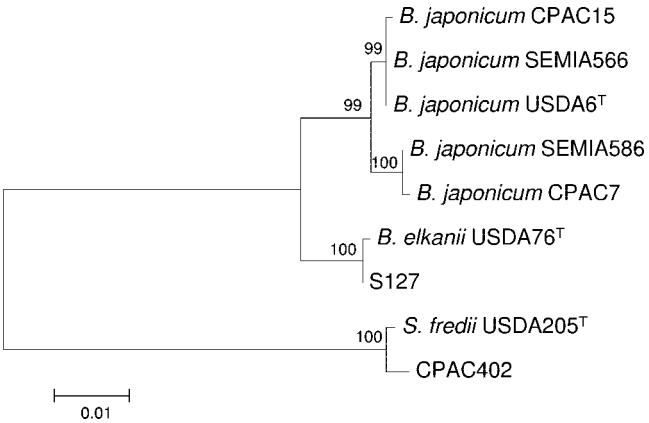

FIG. 2. Phylogenetic tree based on the 16S rRNA sequences of *B. japonicum* strains USDA 6^T, SEMIA 566, SEMIA 586, CPAC 7, and CPAC 15; *S. fredii* USDA 205^T; *B. elkanii* USDA 76^T; and the variant strains S 127 and CPAC 402. The numbers in the main branches indicate bootstrap values obtained with 2,000 replicates.

Sequencing of the 16S rRNA gene. The complete sequencing of the 16S rRNA gene indicated that neither S 127 nor CPAC 402 is *B. japonicum*; S 127 showed greater similarity with *B. elkanii* and CPAC 402 showed greater similarity to *S. fredii* (Fig. 2).

PCR-RFLP and sequencing analyses of the *nodC*, *nodY-nodA*, and *nifH* genes. Approximately 1,200 bp of the 1,400-bp *nodC* gene were obtained and analyzed by PCR-RFLP, and identical profiles were obtained for all 17 strains (Fig. 3a).

The PCR products of the *nodC* genes from strains CPAC 15, CPAC 7, SEMIA 586, SEMIA 566, S 127, and CPAC 402 were sequenced, resulting in about 1,200 bp. In the phylogenetic analysis, three main branches were observed, one clustering the *B. japonicum* strains (USDA 110, SEMIA 566, SEMIA 586, CPAC 15, and CPAC 7) and the S 127 (*B. elkanii*) and CPAC 402 (*S. fredii*) strains (Fig. 4). The second branch is represented by the *B. elkanii* strain CCBAU 23174, and the third branch is represented by the *S. fredii* HH 103 strain. It is noteworthy that the *nodC* nucleotide sequences of *B. elkanii* strain S 127 and of *S. fredii* strain CPAC 402 were identical to those of *B. japonicum* strains (Fig. 4), indicating that they might have been acquired by HGT from the inoculant *B. japonicum*.

When the 2,000-bp DNA fragments including the *nodY* and *nodA* genes of the strains were submitted to PCR-RFLP analysis, all strains were grouped at a similarity level of 99%, except for S 478 and S 127 (*B. elkanii*), which were joined to the others at 78% and 44% levels of similarity, respectively (Fig. 3b). The S 478 strain showed the same pattern of fragments as the *B. japonicum* strains, with the exception of the use of HhaI restriction endonuclease. Furthermore, the use of all four restriction endonucleases indicated extra fragments not detected in *B. japonicum* strains, suggesting the existence of an extra copy of the gene. A 451-bp DNA fragment spanning 397 bp of the *nodY* gene and 54 bp of the *nodA* gene was sequenced, and complete similarity of bases was obtained for all but strain S 127 (Fig. 5). The sequenced *nodY-nodA* DNA fragment of the S 127 (*B. elkanii*) strain showed high similarity (99%) with *B. elkanii* USDA 94 *nodK* (homolog gene to the *nodY* of *B.*

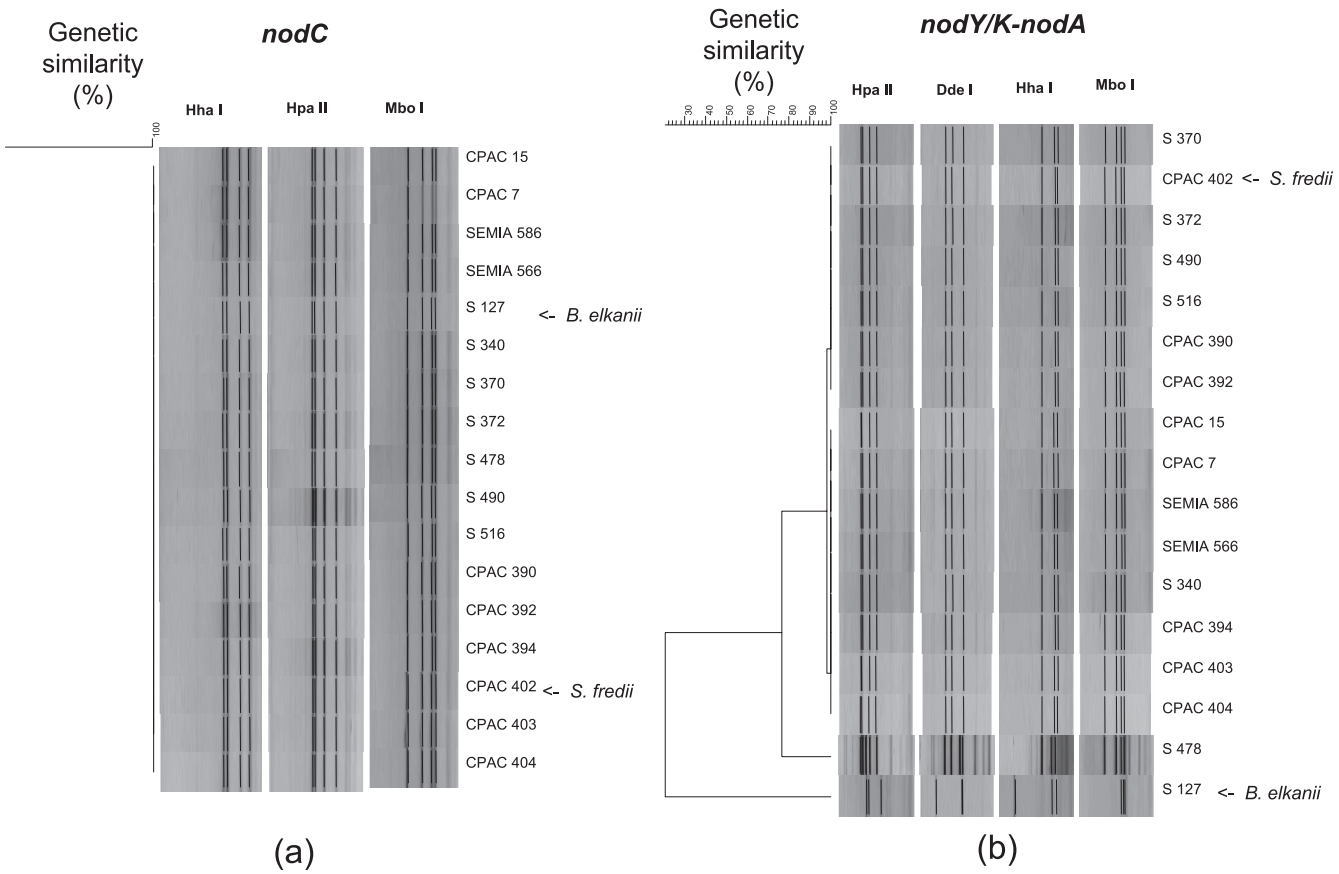

FIG. 3. Cluster analysis (UPGMA with the coefficient of Jaccard) of the DNA fragments obtained in the analysis of PCR-RFLP of the *nodC* (a) and *nodY-nodA* (b) regions of 17 strains of soybean rhizobia. Strains belonged to two serogroups, one comprised the parental *B. japonicum* SEMIA 586 (also CB 1809) and the commercial variant CPAC 7 and the other comprised of the parental *B. japonicum* SEMIA 566 and the commercial variant CPAC 15.

japonicum) and *nodA* genes. The *nodY-nodA* DNA fragment from CPAC 402 (*S. fredii*) was 100% similar to *B. japonicum* *nodY-nodA* sequences, thus indicating that it has also been acquired by HGT from the *B. japonicum* inoculant strains (Fig. 5).

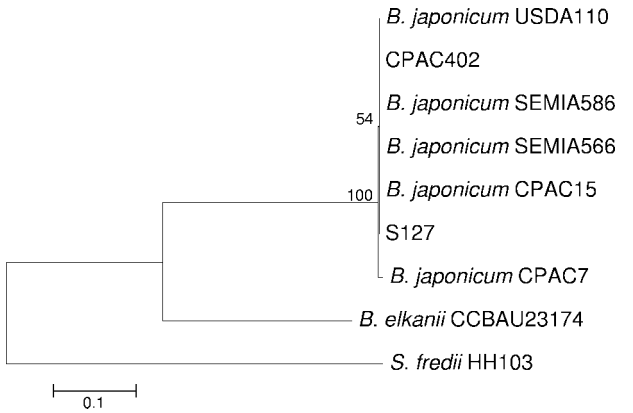

FIG. 4. Phylogenetic tree based on the *nodC* partial sequences of *B. japonicum* strains USDA 110, SEMIA 586, SEMIA 566, CPAC 7, and CPAC 15; *B. elkanii* CCBAU23174; and *S. fredii* HH103. The numbers in the main branches indicate bootstrap values obtained with 2,000 replicates.

Finally, when fragments of about 800 bp of the *nifH* gene were analyzed by PCR-RFLP, similar profiles were again obtained for all strains except for S 127 (*B. elkanii*) and CPAC 402 (*S. fredii*) (Fig. 6). However, while strain S 127 showed different restriction patterns with all four enzymes, strain CPAC 402 showed bands common to those of *B. japonicum* and also extra bands, suggesting the existence of two *nifH* gene copies. In addition, CPAC 402 was the only strain in which the sum of fragments obtained in the *nifH* PCR-RFLP analysis was greater than the length of the PCR-amplified gene (800 bp), more evidence of an extra copy of the gene (Fig. 6).

Confirming the presence of two copies of *nifH* gene in strain CPAC 402. Based on the sequences deposited in the GenBank, we have determined that restriction endonuclease HindIII cuts only the *B. japonicum nifH*, while AvaII cuts only the *S. fredii* *nifH*. The PCR-amplified *nifH* fragments of strain CPAC 402 (*S. fredii*) were digested with HindIII and AvaII, respectively. The uncut fragments obtained (about 800 bp) were isolated, purified, sequenced, and submitted to a search for similarities in the GenBank database (Table 2). The 800-bp *nifH* gene sequences from the CPAC 402 and S 127 strains were also submitted to phylogenetic analysis along with the *B. japonicum*, *B. elkanii*, and *S. fredii* reference strains (Fig. 7). The *nifH* gene of strain S 127 was highly related to *B. elkanii* strain

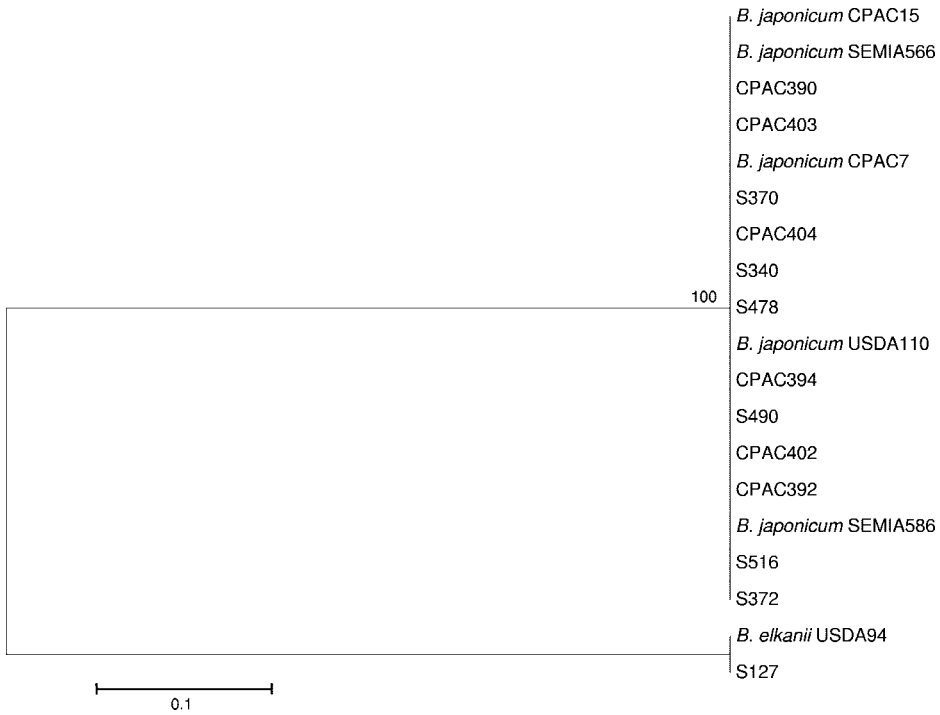

FIG. 5. Phylogenetic tree based on the *nodY-nodA* partial sequences of *B. japonicum* strains USDA 110, SEMIA 566, SEMIA 586, CPAC 7, and CPAC 15; *B. elkanii* USDA 94; and the variant strains CPAC 390, CPAC 403, S 370, CPAC 404, S 340, S 478, CPAC 394, CPAC 402, CPAC 392, S 516, S 372, and S 127. The numbers in the main branches indicate bootstrap values obtained with 2,000 replicates.

USDA 76. Additionally, the results confirmed the existence of two *nifH* copies in CPAC 402, one endogenous to *S. fredii* and another obtained probably through HGT from the *B. japonicum* inoculant strain. Finally, four plasmids were identified in strain CPAC 402 (data not shown).

Summary of sequencing data. Information about the *nifH*, *nodY*, *nodA* and *nodC* sequences of both S 127 (*B. elkanii*) and CPAC 402 (*S. fredii*) strains is summarized in Table 2.

DISCUSSION

The detection of a high percentage of genes arising from HGT in the genomes of prokaryotes, including the conserved 16S rRNA, has raised debates about evolutionary concepts, such as the recognition of a universal tree of life and the definition of the boundaries between species. HGT of whole genomic islands has also been reported, resulting in profound

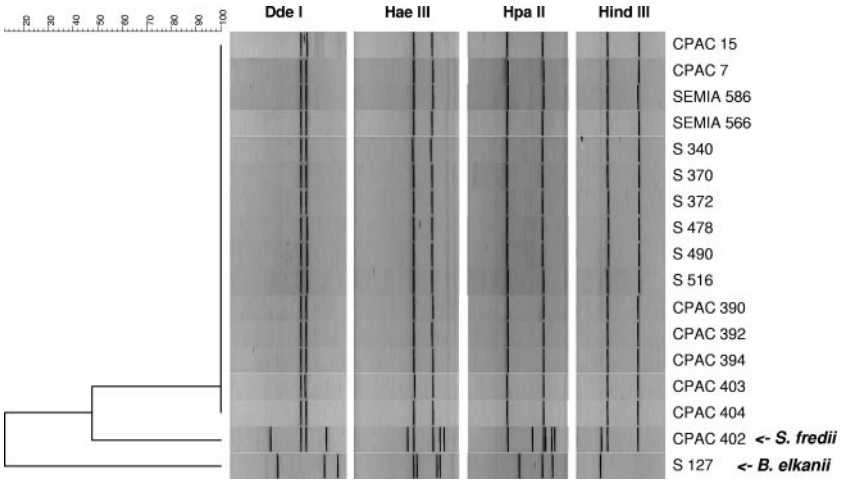

FIG. 6. Cluster analysis (UPGMA with the coefficient of Jaccard) of the DNA fragments obtained in the analysis of PCR-RFLP of the *nifH* genes of 17 soybean rhizobia. Strains belonged to two serogroups, one comprised of the parental *B. japonicum* SEMIA 586 (also CB 1809) and the commercial variant CPAC 7 and the other comprised of the parental *B. japonicum* SEMIA 566 and the commercial variant CPAC 15.

TABLE 2. Summary of nucleotide identity of the conserved and symbiotic genes of origin of strains CPAC 402 and S 127

Variant strain	Species showing nucleotide identity for indicated genes			
	16S rRNA	*nodC*	*nodY-nodA* and *nodK-nodA*	*nifH*
S 127	*B. elkanii*	*B. japonicum*	*B. elkanii*	*B. elkanii*
CPAC 402	*S. fredii*	*B. japonicum*	*B. japonicum*	*B. japonicum*, *S. fredii*

modifications in physiological functions, and a major example is represented by pathogenicity islands (4, 5, 12, 16, 32, 39, 50). In general, less attention has been paid to genetic studies with nonpathogenic prokaryotes including the symbiotic diazotrophic bacteria. However, increasing concerns over global pollution and the depletion of soil nutrients are revitalizing the importance of the symbiotic N_2 fixation, revealing the need for better understanding the ecological aspects of the symbioses under field conditions.

Over the last decade our group has recovered dozens of soybean rhizobial isolates from fields in which exotic inoculant strains of *B. japonicum* and *B. elkanii* were introduced several years ago. Differences in morphological, physiological, genetic, and symbiotic properties in comparison to the putative parental strain were described (3, 11, 22, 33, 44) and first attributed to adaptation to the prevailing stressful tropical conditions (3, 18, 22, 44); however, evidence of HGT events is accumulating (2, 11).

To confirm that HGT of nodulation and N_2 fixation genes was occurring under field conditions, we elected first to characterize conserved genes (using rep-PCR and 16S rRNA genes). Subsequently, we used two sets of symbiotic genes because of their distant localization in the symbiosis island of *B. japonicum*: the *nod* (*nodY*, *nodA*, and *nodC*) and *nifH* genes (15). The analysis of 13 variant strains belonging to two different serogroups has confirmed variability due to the adaptation to the Cerrados, which is especially evident from the rep-PCR products. However, we have identified two rhizobial strains, S 127 and CPAC 402, showing mixed properties of indigenous rhizobia and exotic *B. japonicum* inoculant strains.

Sequencing analysis of the 16S rRNA gene has classified S 127 as *B. elkanii* and CPAC 402 as *S. fredii*. The analysis of symbiotic genes of *B. elkanii* S 127 has identified partial transference of the symbiotic island, with the *nodC* gene showing similarity to the *B. japonicum* inoculant strain and *nodK-nodA* and *nifH* genes highly similar to the indigenous *B. elkanii* strain. However, strong evidence of HGT of the whole symbiosis island of *B. japonicum* was verified in *S. fredii* strain CPAC 402, as *nodC*, *nodY-nodA*, and *nifH* genes were similar to those of the inoculant strain; furthermore, CPAC 402 has maintained an extra copy of the indigenous *nifH* gene. Evidence of other extra copies of nodulation and N_2 fixation genes in CPAC 402 was also obtained in study. Finally, it should be mentioned that both indigenous *B. elkanii* and *Sinorhizobium* spp. have been isolated as microsymbionts of leguminous indigenous trees in Brazil (34).

The plasticity of the *B. japonicum* genome and particularly of the symbiosis island has been highlighted (15, 26), and 19% of the symbiosis island of strain USDA 110 might be represented by genes involved in integration and recombination events (15). Of all ISs identified in the genome of USDA 110, 60% were located in the symbiosis island (26) and might be involved in the transfer of functional genes (35). In addition, under controlled conditions, Rogel et al. (42) also obtained transconjugants of *Ensifer adhaerens* strain ATCC 33499 containing the symbiotic plasmid of *Rhizobium tropici* strain CFN 299. *E. adhaerens* is a soil bacterium species that multiplies by budding and is predatory on other bacteria, but after receiving the symbiotic plasmid of *R. tropici*, strain ATCC 33499 was capable of forming N_2-fixing nodules in both *Phaseolus vulgaris* and *Leucena leucocephala*. In our study, the predatory properties of *Ensifer* may have facilitated the acquisition of foreign genes that would give an ecological advantage to the bacterium.

The detection of *B. japonicum* genes in the *B. elkanii* and *S. fredii* strains strongly suggests that HGT events from the inoculant strain massively introduced in these soils occur under field conditions. It is important to emphasize that this is the first report of HGT of symbiosis islands between different genera of symbiotic bacteria (*Bradyrhizobium* and *Sinorhizobium* [*Ensifer*]) in a natural environment. The acquisition of *B. japonicum* genes may have enabled indigenous rhizobia to effectively nodulate the new, exotic host legume.

The results obtained in this study highlight the strategies that bacteria may use under field conditions to obtain ecological advantage. The detection of HGT was enabled by the use of molecular biology tools, but gene transference probably occurred long ago, since the first introduction of exotic inoculant strains in the soil. Apparently, the HGT events did not result in any symbiotic disadvantage, as today higher soybean grain yields are obtained in the Cerrados based exclusively on N_2 fixation (23). However, since we have determined that HGT of symbiotic genes occurs in nature even among distantly related bacteria, a more careful monitoring of the rhizobial community should be considered. Concerns are now being raised about the possibility that pathogenic bacteria may acquire N_2 fixation genes and also about the ecological impact on the rhizosphere of a well-adapted indigenous population of rhizobia with a homogenous symbiosis island.

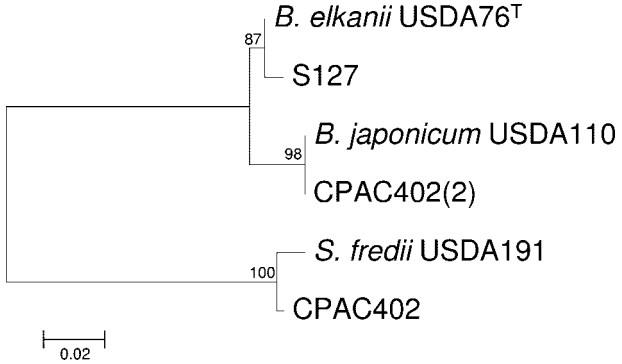

FIG. 7. Phylogenetic tree based on the *nifH* partial sequences of *B. japonicum* strain USDA 110, *B. elkanii* USDA 76, *S. fredii* USDA 191, and the variant strains S 127 and CPAC 402. The two *nifH* sequences from CPAC 402 are discriminated. The numbers in the main branches indicate bootstrap values obtained with 2,000 replicates.

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