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Evaluation of the Diversity of Cyclodextrin-Producing *Paenibacillus graminis* Strains Isolated from Roots and Rhizospheres of Different Plants by Molecular Methods

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To address the diversity of cyclodextrin-producing *P. graminis* strains isolated from wheat roots and rhizospheres of maize and sorghum sown in Australia, Brazil, and France, restriction fragment length polymorphism analysis of part of genes encoding RNA polymerase (*rpoB*-RFLP) and DNA gyrase subunit B (*gyrB*-RFLP) was used to produce genetic fingerprints. A phylogenetic tree based on *rpoB* gene sequences was also constructed. The isolates originated from Brazil could be separated from those from Australia and France, when data from the *rpoB*-based phylogenetic tree or *gyrB*-RFLP were considered. These analyses also allowed the separation of all *P. graminis* strains studied here into four clusters; one group formed by the strains GJK201 and RSA19^T, second group formed by the strains MC22.02 and MC04.21, third group formed by the strains TOD61, TOD 221, TOD302, and TOD111, and forth group formed by all strains isolated from plants sown in Cerrado soil, Brazil. As this last group was formed by strains isolated from sorghum and maize sown in the same soil (Cerrado) in Brazil, our results suggest that the diversity of these *P. graminis* strains is more affected by the soil type than the plant from where they have been isolated.

Keywords: *Paenibacillus graminis*, cyclodextrin-producing strains, plant rhizospheres, *rpoB*-RFLP, *gyrB*-RFLP

Different bacteria use starch as a carbon and energy source for growth and can produce cyclodextrin glucanotransferase (CGTase). This enzyme catalyzes the cleavage of the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety giving rise to the cyclodextrins, which are cyclic (α-1,4)-linked oligosaccharides consisting of 6, 7, 8 glycosyl units that possess a unique ability to act as molecular containers by entrapping guest molecules in their internal cavity. Cyclodextrins have been mainly used as complexing agents to increase the aqueous solubility of poorly water-soluble drugs, to reduce or prevent gastrointestinal or ocular irritation, to reduce or eliminate unpleasant smells or tastes, to prevent drug-drug or drug-additive interactions, or even to convert oils and liquid drugs into microcrystalline or amorphous powders (Qi and Zimmermann, 2005).

Paenibacillus is a widely distributed bacterial genus in the environment. Some of the species belonging to this genus have been isolated from soil (Axelrood *et al.*, 2002; Garbeva *et al.*, 2003), water (Ross *et al.*, 2001) and different plant rhizospheres (Seldin *et al.*, 1983; von der Weid *et al.*, 2000; Berge *et al.*, 2002). Different studies have already shown the industrial and agricultural importance of the different species of *Paenibacillus*. Among the 72 recognized species of *Paenibacillus* (NCBI home page, July 2006), *P. macerans* (Gordon *et al.*, 1973; Takano *et al.*, 1986), *P. campinasensis* (Yoon *et al.*, 1998), *P. stellifer* (Suominen *et al.*, 2003), and *P. illinoisensis* (Doukyu *et al.*, 2003) are the only species studied so far concerning the isolation and/or characterization of CGTases. However, studies on the CGTase gene (*cgt*) were performed exclusively in *P. macerans* (Jeang *et al.*, 2005).

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During a bioprospecting study, searching for new cyclodextrin-producing strains, 15 *Paenibacillus* strains were isolated from the rhizosphere of maize and sorghum sown in Cerrado soil, Sete Lagoas, MG, Brazil. These strains were phenotypically very homogeneous. They were nitrogen-fixers and were identified as *P.*

Table 1. Strains used in this study and their *rpoB*, *gyrB*, and *nifKDH* RFLP patterns

		rpoB			***gyrB***			***nifKDH***
Strains	Origin/reference	*Eco*RI	*Hin*dIII	*Eco*RV	*Eco*RI	*Hin*dIII	*Eco*RV	*Eco*RI
RSA19^T	Rhizosphere of maize, Ramonville-Saint-Agne, France; *Berge et al.*, 2002	B	B	B	A	B	C	A
TOD61	Roots of wheat, Dieulouard, France; *Berge et al.*, 2002	A	A	A	A	A	C	ND
TOD111	Roots of wheat, Dieulouard, France; *Berge et al.*, 2002	A	A	A	A	A	C	ND
TOD221	Roots of wheat, Dieulouard, France; *Berge et al.*, 2002	A	A	A	A	A	C	ND
TOD302	Roots of wheat, Dieulouard, France; *Berge et al.*, 2002	A	A	A	A	B	B	ND
GJK201	Roots of wheat, Kapunda, Australia; *Berge et al.*, 2002	B	B	B	A	B	C	ND
MC04.01	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	B	B	B	A
MC04.06	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	A	C	A
MC04.11	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	B	B	B	A
MC04.16	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	A	C	A
MC04.21	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	C	C	A	C	B	B	B
MC22.02	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	C	C	A	C	B	A	B
MC22.12	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	B	A	ND
MC22.13	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	A	C	A
MC22.19	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	A	C	A
MC11.09	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	A	C	A
MC36.04	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	B	B	A
MC36.22	Rhizosphere of maize sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	B	B	A
BR601.02	Rhizosphere of sorghum sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	A	B	A
BR601.06	Rhizosphere of sorghum sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	A	C	A
BR304.07	Rhizosphere of sorghum sown in Cerrado soil, Sete Lagoas, Minas Gerais, Brazil; this study	A	A	A	D	A	B	A

The RFLP patterns correspond to those presented in Table 2.

graminis on the basis of phenotypic and molecular methods. The species *P. graminis* was described by Berge *et al.* (2002) and the strains studied were isolated from the rhizosphere of maize and wheat roots, sown in France or Australia. Although starch utilization has been observed in these strains, no information was available concerning cyclodextrin production.

Therefore, the aim of this study was first to determine the presence of cyclodextrin among the six *P. graminis* strains described by Berge *et al.* (2002) and then to study the diversity of all cyclodextrin-producing strains isolated from France, Australia and Brazil. Determining their diversity will help to understand whether there is a correlation between strains and the soil type, plant or country of isolation. In order to achieve that, restriction fragment length polymorphism analysis of part of genes encoding RNA polymerase (*rpoB*-RFLP) and DNA gyrase subunit B (*gyrB*-RFLP) was chosen, because both genes are present as single copies in bacterial genomes. Using those methods, the limitations of 16S rRNA-based phylogenetic analysis due to the presence of up to 10 copies of the 16S rRNA gene in *Paenibacillus* species are avoided (Nübel *et al.*, 1996; Berge *et al.*, 2002; Silva *et al.*, 2003). Furthermore, a comparative analysis of the sequences of the *rpoB* gene was performed. Although the genes *rpoB* and *gyrB* have already been used for taxonomic and phylogenetic studies in different bacterial species (Renesto *et al.*, 2001; Drancourt and Raoult, 2002; La Duc *et al.*, 2004; Mota *et al.*, 2004) and for the determination of the intraspecific diversity of *Bacillus licheniformis* (De Clerck and De Vos, 2004) and *P. durus* strains (Albuquerque *et al.*, 2006), it is the first time they have been used within *P. graminis* strains.

Materials and Methods

Bacterial strains and growth conditions

Six strains of *P. graminis* isolated from rhizosphere of maize and roots of wheat in France and Australia and described by Berge *et al.* (2002), together with 15 strains previously isolated from the rhizosphere of different cultivars of maize and sorghum sown in Minas Gerais, Brazil and described in this study are listed in Table 1. Their sources and their RFLP groups based on the *rpoB*, *gyrB*, and *nifKDH* genes are also presented in this table. All strains isolated in this study were identified as *P. graminis* by using the cultural and biochemical tests proposed by Gordon *et al.* (1973) and the API 50CH and API 20E kits (Appareils et Procédés d'Identification, bioMérieux sa, France) as described in Seldin and Penido (1986). All strains were stored aerobically at room temperature on GB agar slants (Seldin *et al.*, 1983) supplemented with $CaCO_3$ (w/v). For growth of *P. graminis* cells, cultures were inoculated still in TBN medium (Seldin *et al.*, 1984) and incubated at 32°C.

Crystalline dextrins production

The test for crystalline dextrins was applied to all strains in test tubes containing a combination of rolled oats (0.5 g), $CaCO_3$ (0.2 g) and distilled water (10 ml). After 24 h, 2 and 5 days of incubation at 34°C, a drop of the supernatant liquid of each culture was mixed on a microscope slide with a drop of Gram's iodine and dried. If present, dextrins could be seen microscopically at the edge of the dried film as dark-brown or blue hexagonal crystals.

Preparation of genomic and plasmid DNAs

Isolation and purification of total DNA from cultures of *P. graminis* were performed as described by Seldin and Dubnau (1985). DNAs were quantified spectrophotometrically (GeneQuant apparatus, Pharmacia). DNAs were digested for 16 h at 37°C with 10 to 20 U of *Eco*RI, *Eco*RV, or *Hin*dIII per μg of DNA. Agarose gel electrophoresis of restricted DNA was performed with 0.8% agarose in Tris-borate-EDTA buffer (Sambrook *et al.*, 1989) at 2 V/cm for 16 h at room temperature. Bacteriophage lambda DNA digested with *Hin*dIII and labeled with digoxigenin-11-dUTP (DIG; Boehringer Mannheim Biochemicals-BMB) was routinely used as the molecular weight marker. Plasmid pSA30 (*Klebsiella pneumoniae nifKDH* gene cluster cloned into pACYC184; Cannon *et al.*, 1979) was isolated by the alkaline lysis method of Birnboim and Doly (1979). The plasmid preparation was purified in CsCl-ethidium bromide gradients as described by Sambrook *et al.* (1989).

Amplified ribosomal DNA (rDNA) restriction analysis (ARDRA)

For amplification of *Paenibacillus* 16S rDNA fragments, a primer set consisting of the specific forward primer PAEN515F and the universal reverse primer 1377R (Shida *et al.*, 1997) was used. The amplification conditions were: 25 cycles of 94°C (1 min), 58°C (1 min, 30 s) and 72°C (1 min, 30 s). An initial procedure consisting of 5 min at 94°C was applied to avoid initial mispriming and enhance the specificity. A final extension step was run for 5 min at 72°C and the reaction tubes were then cooled to 4°C. All reaction mixtures (50 μl) contained: 1 μl of template DNA (50 to 100 ng), 10 mM Tris·HCl, pH 8.3, 10 mM KCl, 0.2 mM of each deoxynucleoside triphosphate, 3 mM $MgCl_2$, 1 μM of each primer, and 2 U of *Taq* DNA polymerase (Promega). Samples (10 μl) of the 16S rRNA amplified products were then digested with the endonucleases *Hin*fI and *Hae*III

(Promega), for 16 h, according to the manufacture's specifications. Agarose (2%) gel electrophoresis of restricted DNA was performed at 80 V for 4 h at room temperature.

Preparation of probes, blotting, and hybridization conditions

Probes for the *P. graminis rpoB* and *gyrB* genes were generated by PCR. For *gyrB*-RFLP analysis, a *gyrB* gene fragment (1.2 kb) of *P. graminis* RSA19^T was amplified using the primers UP-1 and UP-2r as described by Yamamoto and Harayama (1995). The amplification conditions were: a hot start at 94°C for 5 min, 30 cycles of 94°C (1 min), 60°C (1 min) and 72°C (2 min), followed by a final extension at 72 °C for 7 min. All reaction mixtures (50 μl) contained: 1 μl of template DNA (100 ng), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 μM of each primer, and 2.5 U of *Taq* DNA polymerase. For amplification of *rpoB* gene fragment (340 bp), a primer set consisting of a forward primer (*rpoB*1698f) and a reverse primer (*rpoB*2041r) was used as described by Dahllöf *et al.* (2000). The amplification conditions were: 1 cycle (5 min, 95°C), 6 cycles (30 s, 94°C; 1.5 min, 40°C; 1.5 min, 72°C), 19 cycles (30 s, 94°C; 1.5 min, 50°C; 1.5 min, 72°C), with a final 10 min extension period at 72°C. The reaction mix (50 μl) contained 1 μl of template DNA (100 ng), 10 mM Tris-HCl, pH 8.3, 10 mM KCl, 25 pmol of each primer, 2.5 mM of each deoxynucleoside triphosphate, 20 μg of BSA, 2.6 mM $MgCl_2$, and 5 U of *Taq* polymerase. Negative controls (without DNA) were run in all amplifications and PCR products were visualized by 1.4% agarose gel electrophoresis followed by staining with ethidium bromide.

The PCR products obtained after amplification of *P. graminis* RSA19^T DNA with primers for the *rpoB* and *gyrB* genes and the plasmid pSA30 digested with *Eco*RI were then DIG (digoxigenin-11-dUTP) labeled using the protocol provided by Roche Molecular Biochemicals (Germany). Digested genomic DNAs of all *P. graminis* strains (Table 1) were loaded onto positively-charged nylon membranes as described before (Sambrook *et al.*, 1989). Prehybridization and hybridization conditions using the DIG-labeled probe were those described by the manual for DIG Nucleic Acid detection kit (Roche). The nylon membrane was subjected to stringent washing steps after hybridization at 65°C for 16 h. Signals were detected using a CSPD-based chemiluminescence detection kit (Roche), as recommended by the manufacturer.

Statistical analyses

The banding profiles obtained in the hybridization experiments with different probes were used to generate a matrix indicating the presence or absence (scored as 1 or 0, respectively) of bands in the different hybridization patterns, using the DICE coefficient. Dendrograms were obtained on the basis of these data by the unweighted pair group method with arithmetic averages (UPGMA) algorithm using the NTSYS software package (version 2.02J, Exeter Software, USA).

Cloning and sequencing of PCR products

rpoB PCR products of the expected size of about 340 bp generated with DNA extracted from all *P. graminis* strains were cloned using the pGEM T-easy vector according to the instructions of the manufacturer (Promega, USA). After transformation of *E. coli* JM109 competent cells, selected clones were then sequenced using the M13f and M13r primers and an ABI Prism 3100 automatic sequencer (Applied Biosystems, USA).

DNA sequence analyses

Sequences generated in this study, as well as *rpoB* sequences from *Paenibacillus* strains recovered from the database, were aligned by using Clustal X (Thompson *et al.*, 1997). The phylogenetic trees were calculated by the neighbour-joining method (Saitou and Nei, 1987), using p-distance and 1000 replicates of bootstrap. Tree construction was performed with the software MEGA 3 (Kumar *et al.*, 2004).

Results and Discussion

Six strains isolated from the rhizosphere of maize and roots of wheat were described as a new *Paenibacillus* species, named *P. graminis*, by Berge *et al.* (2002). In the same year, 15 isolates (Table 1) were obtained from TBN plates previously inoculated with pasteurized samples of rhizosphere soil of maize (n = 12) and sorghum (n = 3) in our laboratory. All these strains were tested for crystalline dextrin production and showed positive results after 24 h of incubation. This property used to be considered a reliable characteristic for the separation of *P. macerans* (Gordon *et al.*, 1973). However, more recently new species of *Paenibacillus* were described forming crystalline dextrins in rolled oats, such as *P. campinasensis* (Yoon *et al.*, 1998), *P. stellifer* (Suominen *et al.*, 2003) and *P. illinoisensis* (Doukyu *et al.*, 2003). Therefore, the 15 strains were tested using the methods and media described by Gordon *et al.* (1973) for their correct identification. All strains showed to be very homogeneous phenotypically and presented the same results as described in Berge *et al.* (2002) for *P. graminis*. Different tests (for example: reduction of nitrate, utilization of mannitol and lactose as carbon sources) differentiated the isolates from the other cyclodextrin-producing *Paenibacillus* species (Yoon *et al.*, 1998;

Doukyu *et al.*, 2003; Suominen *et al.*, 2003). To confirm their identity, the 15 strains were tested using the API 50CH and API 20E kits and all strains were able to produce acid from glycerol, L-arabinose, D-xylose, β methyl-xyloside, galactose, D-glucose, fructose, mannose, mannitol, α-methyl-D-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, starch, glycogen, gentibiose, and D-turanose, as did the strains of *P. graminis* (Berge *et al.*, 2002; this study). Furthermore, they showed positive result for 2-nitrophenyl-β-D-galactopyranoside (ONPG) and negative for citrate, H_2S production, urease, indol, gelatine, lysine and ornithine decarboxilases when the kit API 20E was used.

Different approaches (PCR-RFLP and MLEE assays) have been already used as a rapid tool for the characterization and the establishment of the taxonomic position of isolates belonging to the nitrogen-fixing species of *Paenibacillus* species (Berge *et al.*, 2002; Coelho *et al.*, 2003). In ARDRA experiments, strains of *P. graminis* could be separated from all other species studied using *Hin*fI (Coelho *et al.*, 2003). Therefore, in this study, DNAs from the type strain of *P. graminis* (RSA19^T) and from two representatives of the new isolates (MC36.04 and MC04.16) were amplified with the specific primer for the genus *Paenibacillus* (PAEN515F) and the universal primer 1377R (Shida *et al.*, 1997) and all strains produced a single band of about 860 bp (data not shown). PCR products were then digested with *Hin*fI and *Hae*III, and the same pattern was observed in all strains tested: three bands were observed when the DNAs were digested with *Hin*fI, while two bands were obtained with *Hae*III. Using all phenotypic and genetic (ARDRA) features, the identification of our isolates as *P. graminis* was unambiguous.

The production of crystalline dextrins was then tested in this study using the six strains of *P. graminis* described by Berge *et al.* (2002). In all strains, dark-brown or blue hexagonal crystals could be observed microscopically at the edge of the dried film (data not shown). With this observation, *P. graminis* can be included in the list of *Paenibacillus* species able to produce cyclodextrins.

Furthermore, as the strains of *P. graminis* was described by Berge *et al.* (2002) as nitrogen fixers, all strains were tested for the presence of homology to the *K. pneumoniae nifKDH* gene cluster (nitrogenase structural genes cloned in pACYC184-originating plasmid pSA30) by Southern hybridization. Recombinant DNA probes containing *Klebsiella* structural *nif* genes have been used to identify DNA fragments with related sequences from several nitrogen-fixing organisms, including strains of *P. durus* (Seldin *et al.*, 1998) and *P. polymyxa* (von der Weid *et al.*, 2000). Total DNA (digested with *Eco*RI) from all strains studied here showed homology to pSA30 and two hybridization patterns could be observed (Table 1). These patterns allowed the separation of strains MC22.02 and MC04.21 from the remaining strains, as these strains showed two bands of 8.8 and 1.2 kb homologous to *nifKDH*, while the first band observed in the other DNAs was smaller (8.0 kb, Table 2).

The aim of the further hybridization analyses was to address the heterogeneity among cyclodextrin-producing *P. graminis* strains isolated from wheat roots and different rhizospheres of maize and sorghum sown in Australia, Brazil, and France in order to understand whether there is a correlation between strains and the soil type, plant or country of isolation. Also, grouping the strains can help further studies on the determination of physicochemical properties and the genetics of the cyclodextrin production in *P. graminis*. Previous studies already demonstrated the usefulness of *rpoB* gene to identify isolates of different bacteria as an alternative to 16S rRNA gene (Renesto *et al.*, 2001; Drancourt and Raoult, 2002; De Clerk and De

Table 2. Size of the bands used to group strains based on the hybridization patterns obtained using *rpoB*, *gyrB* genes from *P. graminis* RSA19^T and *nifKDH* (plasmid pSA30) as probes

Gene/ Endonucleases	Size of the fragments (kb)		RFLP patterns (Table 1)
rpoB			
*Hin*dIII	3.8		A
	3.8	6.5	B
	3.8	8.2	C
*Eco*RI	10		A
	11.3		B
	10	8	C
*Eco*RV	1.1		A
	1.1	1.9	B
gyrB			
*Hin*dIII	6.5	3.6	A
	6.5		B
*Eco*RI	8.5	6.4	A
	8.0	2.8	B
	6.4	4.4	C
	4.4	2.8	D
*Eco*RV	6.2	3.6	A
	3.6	0.9	B
	3.6		C
nifKDH			
*Eco*RI	8.8	1.2	A
	8.0	1.2	B

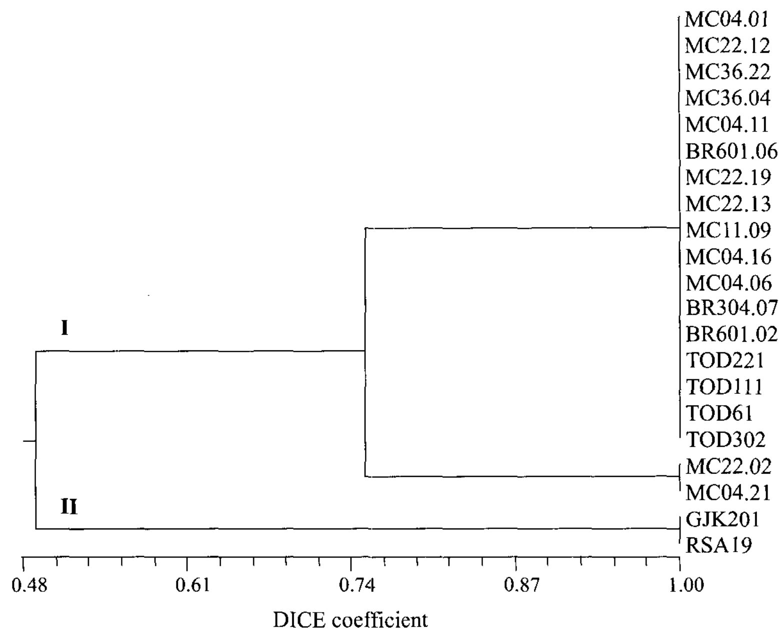

Fig. 1. Dendrogram (UPGMA) of genetic relationships among strains of *P. graminis* based on data from hybridization studies using the *rpoB* gene as a probe.

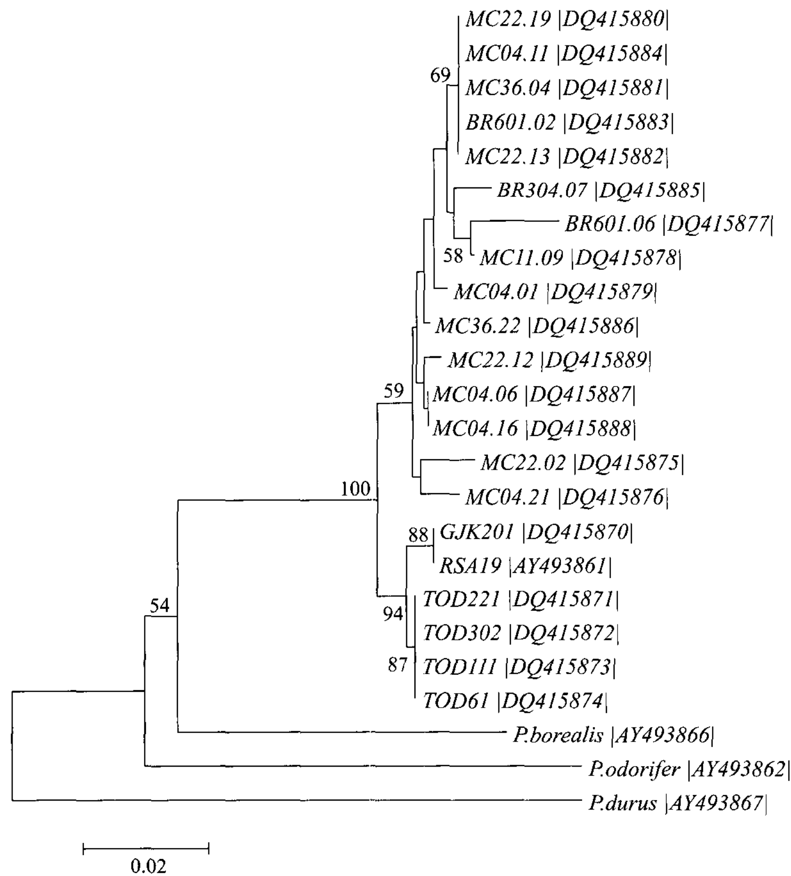

Fig. 2. Phylogenetic tree based on *rpoB* gene sequences showing the relationships of the *P. graminis* strains and related *Paenibacillus* species. This tree was constructed by neighbor-joining (Saitou and Nei, 1987) and bootstrap results greater than 50% are represented at the branch points. The GenBank accession number of each strain is enclosed in brackets.

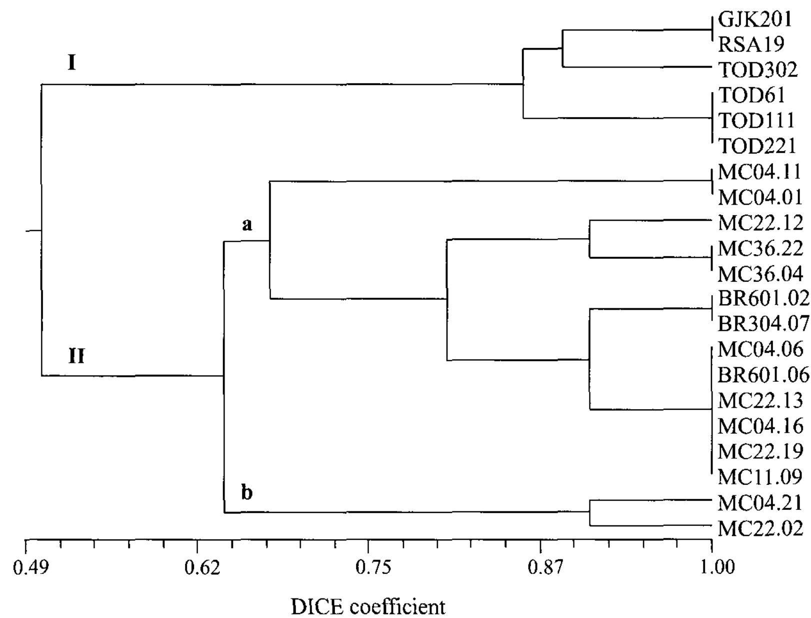

Fig. 3. Dendrogram (UPGMA) of genetic relationships among strains of *P. graminis* based on data from hybridization studies using the *gyrB* gene as a probe.

Vos, 2004; La Duc *et al.*, 2004; Mota *et al.*, 2004), avoiding the heterogeneity of the multiple copies of 16S rDNA in *Paenibacillus* genome (Nübel *et al.*, 1996; Silva *et al.*, 2003). Therefore, the probe used was obtained through the PCR amplification of the DNA of *P. graminis* RSA19^T with the primers described by Dahllöf *et al.* (2000). Three, two and three different hybridization patterns were observed within all strains tested when the endonucleases *Eco*RI, *Eco*RV, and *Hin*dIII were used, respectively (Table 2). All together, eight markers (different bands) were used to construct a dendrogram (Fig. 1). Two main groups (I and II) were formed at 49%, separating two strains of *P. graminis* described by Berge *et al.* (2002) - RSA19^T and GJK201 - from the rest of the strains. Strains isolated from wheat roots in France were grouped together with the isolates from the rhizosphere of maize and sorghum sown in Brazil. At 75% similarity, strains MC22.02, and MC04.21 were separated from the remaining strains. Both strains have been previously separated from the other strains in the hybridization experiment using the *nifKDH* as a probe (this study). Only 3 groups of strains could be observed at 100% similarity, demonstrating that, although data from homology using an *rpoB* probe is more discriminative than phenotypic data, they show a low degree of diversity among *P. graminis* strains.

rpoB sequences obtained in this study were further used to construct a phylogenetic tree (Fig. 2). All strains used (including the type-strain of *P. graminis*) constituted a monophyletic branch separated from the other *Paenibacillus* species considered close to *P. graminis* (Berge *et al.*, 2002). Considering the *P. graminis* strains studied, four clusters were observed: one group formed by the strains GJK201 and RSA19^T, a second group formed by the strains MC22.02 and MC04.21, a third group formed by the strains TOD61, TOD221, TOD302, and TOD111, and a forth group formed by all strains isolated from plants sown in Cerrado soil, Brazil (Fig. 2). The same groups have been previously observed in the hybridization experiment using the *rpoB* as probe (Fig. 1), excepting the group made up of strains isolated from wheat roots in France (TOD61, TOD111, TOD302, and TOD221).

Also as an alternative to 16S rRNA gene analysis for comparing the heterogeneity among strains of one species, a probe based on *gyrB* was used. Although this gene has already been used for taxonomic and phylogenetic studies in different bacterial species (Yamamoto and Harayama, 1995; Yamada *et al.*, 1999; Rodrigues *et al.*, 2003; Albuquerque *et al.*, 2006), it is the first time it is used within *P. graminis* strains. Four, three, and two different hybridization patterns were observed within all strains tested when the endonucleases *Eco*RI, *Eco*RV, and *Hin*dIII were used, respectively (Table 2). All together, 10 markers (different bands) were used to construct a dendrogram (Fig. 3). Two main groups (I and II) were formed at about 50%, separating all strains of *P. graminis* described by Berge *et al.* (2002), including strains RSA19^T and GJK201, from the rest of the strains. Both strains showed to be genetically closely related in all approaches studied here and, although they have been isolated from plants cultivated in different coun-

tries (Table 1), they may have originated from a common ancestor. Strains isolated from both sorghum and maize sown in the same soil (Cerrado) in Brazil were grouped together (group II), therefore we can speculate that the diversity of these strains is being more affected by the soil type than the plant from where they have been isolated. The same has been observed among strains of *P. durus* isolated from the rhizosphere of maize planted in two different soils (Seldin *et al.*, 1998). At 64% similarity, strains MC22.02 and MC04.21 were separated from the remaining strains (group b). Interestingly, both strains were separated again from the other Brazilian strains. The remaining isolates from the rhizosphere of maize and sorghum sown in Brazil were clustered in group a. At 100% similarity, 10 groups of strains could be observed. Some of these groups consisted of a single isolate, whereas others encompassed two or more strains (Fig. 3). Therefore, data from *gyrB* revealed a higher degree of diversity among *P. graminis* strains than *rpoB* homology results. Yamada *et al.* (1999) have already demonstrated the good discriminative power of this gene when they were able to separate *Bacillus cereus* from *B. thuringiensis* strains.

The methods used here were sensitive indicators of diversity among the strains tested and have potential to help in understanding the structure of *P. graminis* community and possible interactions in the ecosystem. Furthermore, exploring the diversity of strains belonging to this new cyclodextrin-producing species may also represent a potential source for the discovery of novel bioactive compounds with different physicochemical properties and new applications. Further physicochemical and molecular studies of the CGTase produced by *P. graminis* strains are necessary and are being developed in our laboratory.

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