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NOTE

Paenibacillus brasilensis sp. nov., a novel nitrogen-fixing species isolated from the maize rhizosphere in Brazil

Irene von der Weid,[1] Gabriela Frois Duarte,[1] Jan Dirk van Elsas[2] and Lucy Seldin[1]

[1] Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

[2] Plant Research International–PRII, Wageningen, The Netherlands

Author for correspondence: Lucy Seldin. Tel: +55 21 2562 6741. Fax: +55 21 2540 8492. e-mail: lseldin@micro.ufrj.br or seldin@marlin.com.br

Sixteen nitrogen-fixing strains isolated from the rhizosphere of maize planted in Cerrado soil, Brazil, which showed morphological and biochemical characteristics similar to the gas-forming *Paenibacillus* spp., were phenotypically and genetically characterized. Their identification as members of the genus *Paenibacillus* was confirmed by using specific primers based on the 16S rRNA gene. SDS-PAGE of whole-cell proteins, API 50CH, morphological and biochemical tests, amplified rDNA-restriction analysis (ARDRA), DNA-relatedness analyses, denaturing-gradient gel electrophoresis (DGGE) and 16S rRNA gene sequence determinations were performed to characterize the novel isolates and to compare them to strains of other nitrogen-fixing *Paenibacillus* spp. Phenotypic analyses showed that the 16 strains were very homogeneous and shared a high level of relatedness with *Paenibacillus polymyxa* and *Paenibacillus peoriae*. However, none of the novel isolates was able to ferment glycerol (positive test for *P. polymyxa*), L-arabinose or D-xylose (positive tests for *P. polymyxa* and *P. peoriae*) or utilize succinate (positive test for *P. peoriae*). Genetic approaches also indicated a high level of similarity among the novel isolates and *P. polymyxa* and *P. peoriae*, but the novel strains clearly could not be assigned to either of these two recognized species. On the basis of the features presented in this study, the 16 novel isolates were considered to represent members of a novel species within the genus *Paenibacillus*, for which the name *Paenibacillus brasilensis* is proposed. The type strain is PB172^T (= ATCC BAA-413^T = DSM 14914^T).

Keywords: *Paenibacillus brasilensis*, taxonomy, nitrogen-fixing *Paenibacillus*

In a comparative analysis of the 16S rRNA gene sequences of different species of the genus *Bacillus*, Ash *et al.* (1991, 1993) defined a genus named *Paenibacillus*. At that time, the genus *Paenibacillus* encompassed 11 species, and *Paenibacillus polymyxa* was considered the type species of the genus. The gas-forming species *Bacillus azotofixans* and *Bacillus macerans* were also incorporated into the genus *Paenibacillus* (Ash *et al.*, 1993), and Heyndrickx *et al.* (1996) later transferred *Bacillus peoriae* to it. At the time of writing, the genus *Paenibacillus* comprises 33 species and nitrogen fixation has already been described in *Paenibacillus azotofixans* (Seldin *et al.*, 1984), *P. polymyxa* (Grau & Wilson, 1962) and *Paenibacillus macerans* (Witz *et al.*, 1967), while acetylene reduction (an indication of nitrogen-fixing capacity) has also been detected in *Paenibacillus peoriae* (strain NRRL BD-62, this study). Recently, three novel gas-forming and nitrogen-fixing species of *Paenibacillus*, *Paenibacillus borealis* (Elo *et al.*, 2001), *Paenibacillus*

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Abbreviations: ARDRA, amplified rDNA-restriction analysis; DIG, digoxigenin-11-dUTP.

The GenBank accession number for the 16S rRNA gene sequence of *Paenibacillus brasilensis* PB172^T is AF273740.

A phase-contrast micrograph showing the position of the spores of *P. brasilensis* PB172^T within its cells can be found as supplementary data in IJSEM Online (http://ijs.sgmjournals.org).

graminis and *Paenibacillus odorifer* (Berge *et al*., 2002), have been described.

Identification of strains belonging to the nitrogen-fixing group within the genus *Paenibacillus* used to be virtually restricted to phenotypic characterization. *P. polymyxa* was traditionally considered to be phenotypically very homogeneous and readily recognizable, as strains that make up this species tend to give the same reaction to each test applied (Gordon *et al*., 1973). However, many authors have described the isolation of variants of *P. polymyxa* or other known *Paenibacillus* spp. (Budi *et al*., 1999; Rennie *et al*., 1982; Rhodes-Roberts, 1981; Shishido *et al*., 1995; Wullstein *et al*., 1979). In the same manner, we have isolated, from the rhizosphere of maize, a range of strains that showed morphological and biochemical characteristics similar to the gas-forming *Paenibacillus* spp. which were tentatively assigned to *P. polymyxa* (von der Weid *et al*., 2000).

The current study was undertaken in an attempt to elucidate the taxonomic position of the aforementioned strains. We used extensive phenotypic characterization studies (including SDS-PAGE of whole-cell proteins), DNA-relatedness analyses and 16S rRNA gene sequence determinations and amplified rDNA-restriction analysis (ARDRA), as well as other genetic approaches, to characterize the novel strains. Representative strains of *P. azotofixans*, *P. macerans*, *P. polymyxa* and *P. peoriae* were included in most of the analyses. Our isolates were also compared phenotypically to *P. borealis*, *P. graminis* and *P. odorifer*.

The 16 strains studied here (Table 1) were isolated from the rhizosphere of maize planted in Cerrado soil, Brazil, and have been tentatively identified as *P. polymyxa* (von der Weid *et al*., 2000). These novel isolates and all of the *Paenibacillus* strains used in this study were kept on slants containing glucose broth (GB) agar supplemented with 1% $CaCO_3$ (Seldin *et al*., 1983). GB (*P. polymyxa*, *P. macerans* and *P. peoriae*) or thiamine/biotin/nitrogen (TBN) (*P. azotofixans*; Seldin *et al*., 1984) liquid media were used to propagate cultures (16–24 h at 32 °C, without shaking). The different media were supplemented with 1·2% agar to obtain solid media. Most biochemical tests were performed by using the methods and media described by Gordon *et al*. (1973). For all tests that required complex media (temperature range of growth, growth inhibition by NaCl and by pH 5·7, hydrolysis of starch, resistance to lysozyme and liquefaction of gelatin), appropriately adjusted GB solid or liquid media were employed. The basal medium of Gordon *et al*. (1973) was used to test for acid and gas production from carbohydrates. Tests for utilization of organic acids used acetate, citrate and succinate. The results obtained are shown in Table 1 and in the species description. Cellular morphology, form and position of spores, and swelling of the sporangia were observed by using a Zeiss phase-contrast microscope. Cellular motility was observed for the novel isolates in fresh wet-mounts of young (24 h-old) bacterial cultures in GB broth. All of the novel strains were Gram-positive or Gram-variable, and cells were rod-shaped (type strain measuring $0{\cdot}6 \times 2{\cdot}1$ μm) and motile. Spores of these cells were ellipsoidal, distending the sporangia and located in the central to subterminal position in the cell [phase-contrast micrograph available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org)]. Colonies of the novel isolates were translucent to white, convex and mucoid when grown on GB agar and bright yellow when grown on TBN agar. On the basis of the cultural and biochemical tests recommended by Gordon *et al*. (1973), the novel strains were considered to be similar to *P. polymyxa*. However, none of these strains was able to produce acid from glycerol, D-xylose and L-arabinose, which are diagnostic characteristics for *P. polymyxa*.

Strains were also characterized by using API tests (API 50CH, composed of 49 different carbohydrates; bioMérieux) as described by Seldin & Penido (1986). Data from API tests were recorded as described previously (Rosado *et al*., 1998). Phenotypic characteristics that differentiate the novel isolates from the other seven known gas-forming and nitrogen-fixing *Paenibacillus* spp. are presented in Table 1. All isolated strains produced acid from ribose, galactose, glucose, fructose, mannose, mannitol, methyl α-D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, raffinose, starch and glycogen. None of the 16 novel strains were able to utilize 27 of the other carbohydrates tested. This included glycerol, which had initially differentiated these strains from *P. polymyxa*. Fermentation of the remaining three carbohydrates (trehalose, β-gentiobiose and D-turanose) varied among strains; these compounds were utilized by 81, 75 and 81% of the novel strains, respectively. When the fermentation patterns of the novel isolates were compared with those of other closely related *Paenibacillus* spp., it became clear that these isolates could not be considered to represent typical members of any one of these well-established species (Table 1).

Nitrogenase activity was determined using the acetylene-reduction assay by measuring the ethylene production of cultures in 18 ml vials, as described previously (Seldin *et al*., 1983). The novel isolates, together with the *P. peoriae* strains NRRL B-14474, B-14477, BD-54, BD-62 and HSCC 353^T, were also tested for their capacity to fix nitrogen by assaying their nitrogenase activity. All of the novel strains effectively reduced acetylene, and the values obtained were equivalent to those obtained for some *P. azotofixans* strains (approx. 100–200 nmol ethylene $ml^{-1}\,h^{-1}$; data not shown). However, the *P. peoriae* strains were generally negative in this test and only *P. peoriae* strain NRRL BD-62 showed a positive result.

Total-protein profiles obtained by using PAGE have been used for the characterization and differentiation

Table 1. Phenotypic characteristics that differentiate *Paenibacillus brasilensis* from other nitrogen-fixing *Paenibacillus* spp.

Strains: 1, *Paenibacillus brasilensis* (PB1, PB7, PB8, PB22, PB23, PB24, PB65, PB105, PB154, PB155, PB158, PB159, PB164, PB172^T, PB174 and PB177); 2, *P. polymyxa* (Gordon *et al*., 1973; Seldin & Penido, 1986; this study); 3, *P. peoriae* (Montefusco *et al*., 1993; Heyndrickx *et al*., 1996; this study); 4, *P. macerans* (Gordon *et al*., 1973; Seldin & Penido, 1986; this study); 5, *P. azotofixans* (Seldin *et al*., 1984; Seldin & Penido, 1986); 6, *P. borealis* (Elo *et al*., 2001); 7, *P. graminis* (Berge *et al*., 2002); 8, *P. odorifer* (Berge *et al*., 2002). ND, Not described in the papers cited here. −, 0–15 % of all strains tested produced positive results; +, 86–100 % of all strains tested produced positive results; v, 16–85 % of all strains tested produced positive results.

Characteristic	1	2	3	4	5	6	7	8
Growth at 45 °C	−	−	−	+	−	−	−	−
Growth in 0·001 % lysozyme	+	v	+	−	−	−	ND	ND
Voges–Proskauer (VP) reaction	+	+	+	−	+	−	ND	ND
Reduction of nitrate to nitrite	+	+	+	+	−	−	+	+
Liquefaction of gelatin	v	+	+	−	−	−	−	ND
Production of dihydroxyacetone	−	+	−	−	−	−	ND	ND
Production of crystalline dextrins	−	−	−	+	−	ND	ND	ND
Hydrolysis of starch	+	+	+	+	v	−	+	+
Nitrogen fixation	+	v	v	v	+	+	+	+
Decomposition of casein	+	+	+	−	−	+	−	ND
Utilization of citrate	+	−	+	−	−	−	−	ND
Utilization of succinate	−	−	+	−	−	ND	−	ND
Acid from:								
Glycerol	−	+	v	+	−	+	+	v
D-Arabinose	−	−	−	v	−	−	−	−
L-Arabinose	−	+	+	+	−	+	+	+
Ribose	+	+	+	+	−	−	−	+
D-Xylose	−	+	+	+	−	+	+	+
Methyl β-xyloside	−	+	+	v	−	+	+	+
D-Mannose	+	+	+	+	+	+	+	v
Rhamnose	−	v	v	+	−	−	−	−
Dulcitol	−	−	−	−	v	−	−	−
Inositol	−	−	−	v	−	−	−	−
Mannitol	+	+	+	+	+	+	+	−
Sorbitol	−	−	−	v	−	v	−	−
Methyl α-D-mannoside	−	−	+	v	−	−	−	−
Methyl α-D-glucoside	+	+	−	v	+	v	+	+
N-Acetylglucosamine	−	−	v	v	−	+	+	+
Arbutin	+	+	+	+	v	+	+	+
Salicin	+	+	+	+	v	+	+	+
Cellobiose	+	+	+	+	v	+	+	+
Lactose	+	+	+	+	−	+	+	+
Trehalose	v	+	−	+	+	+	+	+
Inulin	−	v	−	+	+	+	v	+
Melezitose	−	v	−	+	+	+	+	−
Starch	+	+	+	+	v	+	+	+
Glycogen	+	+	+	+	v	+	+	+
Xylitol	−	−	−	−	−	v	−	−
β-Gentiobiose	v	v	+	+	+	+	+	+
D-Turanose	v	+	v	+	+	+	+	+
D-Lyxose	−	−	−	−	−	v	−	−
D-Tagatose	−	−	−	v	v	v	−	−
D-Fucose	−	−	−	−	−	−	v	−
L-Fucose	−	−	−	v	−	−	−	v
D-Arabitol	−	−	−	v	−	+	−	−
Gluconate	−	v	v	v	−	−	v	−

of several microbial species, including *Paenibacillus* spp. (Heyndrickx *et al*., 1996). To perform SDS-PAGE analyses on whole-cell proteins extracted from all of the novel strains and from representative strains of *P. polymyxa* (LMD 24.16^T and Loutit), *P. peoriae* (NRRL BD-62, BD-54 and B-14474), *P. azotofixans* (P3L-5^T) and *P. macerans* (Mac 3), cells were harvested after 18 h growth in GB broth at 32 °C. Proteins were extracted and analysed by SDS-PAGE (12% polyacrylamide) using the method described by Laemmli (1970). Protein concentration was determined using the Lowry method. Electrophoresis was carried out at 100 V for 2 h and polypeptides were stained using Coomassie brilliant blue R-250. Broad-range SDS-PAGE standards (Gibco-BRL) were used in all electrophoresis experiments. The whole-cell-protein profiles of *P. polymyxa*, *P. peoriae* and *P. azotofixans* were very similar, with bands of between 90 and 10 kDa seen. Minor variations were observed within the profiles of each group of strains belonging to the same species. A protein corresponding to a molecular mass of about 90 kDa was absent in the strain of *P. azotofixans* tested. The profile observed for *P. macerans* was quite different from that of the other strains tested, exhibiting specific proteins and confirming the distinctness of this species.

Genetic approaches were combined with the phenotypic studies to assist in the determination of the taxonomic position of the 16 novel isolates. Total DNA was extracted from all of the strains of *Paenibacillus* studied here using the method of Marmur (1961) modified as described previously (Seldin *et al*., 1998). DNA concentrations were determined by using a Gene Quant apparatus (Pharmacia) and diphenylamine as described by Johnson (1981). Strains had their identification as members of the genus *Paenibacillus* confirmed by PCR amplification of a 16S rRNA gene fragment with the specific forward primer PAEN515F and the universal reverse primer 1377R proposed by Shida *et al*. (1997). They all produced the expected fragment of 860 bp, characteristic for members of the genus *Paenibacillus* (data not shown).

ARDRA has been used extensively to discriminate between bacterial species (Berge *et al*., 2002; Heyndrickx *et al*., 1996). The procedure described by Massol-Deya *et al*. (1995) was employed here for PCR amplification of the 16S rRNA gene fragments using a pair of universal primers (pA and pH). Total genomic DNA from representative strains of *P. polymyxa* (LMD 24.16^T and Loutit), *P. azotofixans* (P3L-5^T and V22.35), *P. macerans* (LMD 24.10) and *P. peoriae* (NRRL B-14477, BD-62 and HSCC 353^T) and all of the novel isolates was used. The amplicons obtained (about 1500 bp in size) were analysed by digestion with three restriction enzymes (*Sau*3AI, *Hin*fI and *Hae*III) as described by Massol-Deya *et al*. (1995). A similarity matrix was then produced by comparisons between pairs of strains using the simple-matching coefficient. For these analyses, the NTSYS software package (version 2.02J; Exeter Software, Setauket, NY, USA) was used. A dendrogram was constructed for the ARDRA profiles by using the unweighted pair group method with arithmetic means (UPGMA). The ARDRA patterns of the 16 novel strains were internally consistent and exhibited high levels of similarity, but also differences, with those of *P. polymyxa* and *P. peoriae*. Such levels of similarity could not be observed when the ARDRA patterns of the novel strains were compared with those of *P. azotofixans* and *P. macerans* (data not shown). Two isolates, PB159 and PB172^T, were selected for further analysis and a dendrogram was constructed on the basis of the data from their ARDRA patterns. The dendrogram showed that the two novel isolates were linked at a level of similarity of 97%, and were separated from *P. polymyxa* and *P. peoriae* at 65% similarity. *P. azotofixans* and *P. macerans* were separated from all of the other strains tested (including the novel ones) at 51% similarity (Fig. 1). The small differences observed in the ARDRA patterns of strains of the same group or species (Fig. 1) were probably caused by additional restriction sites present in the 16S rRNA alleles (Vaneechoutte *et al*., 1992).

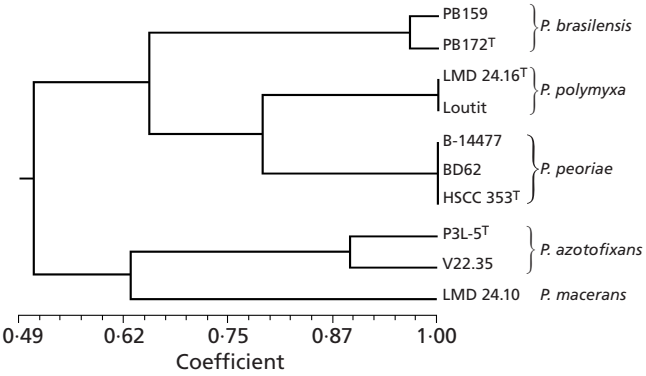

Fig. 1. Dendrogram (UPGMA) based on data from the ARDRA profiles of the different gas-forming *Paenibacillus* spp., generated by digestion of the 16S rDNA with *Hae*III, *Hin*fI and *Sau*3AI.

For the determination of the phylogenetic position of the selected representative novel strain (PB172^T), amplification of the 16S rDNA region was performed using primers pA and pH (Massol-Deya *et al*., 1995). The amplification product was purified by using the GeneClean II kit (Bio 101) and cloned into vector pCRII, in accordance with the manufacturer's instructions (Invitrogen). Plasmid DNA was purified by using Wizard resin spin columns (Promega) and was used as a template in sequencing reactions with a thermo sequenase fluorescently labelled primer cycle sequencing kit, 7-deaza-dGTP and an automatic sequence analyser (model ALF DNA sequencer; Amersham-Pharmacia Biotech). The 16S rDNA sequence of strain PB172^T was aligned with those of its most closely related species of the genus *Paenibacillus*

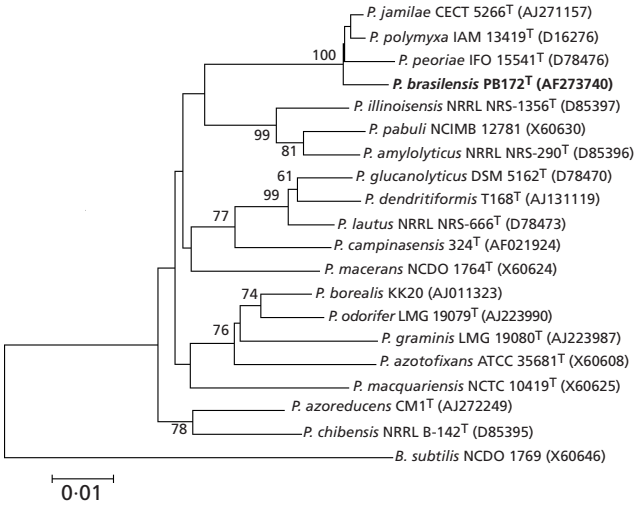

Fig. 2. Phylogenetic tree, obtained by the neighbour-joining method, based on the alignment of the 16S rRNA gene sequence of *P. brasilensis* PB172^T with the 16S rRNA gene sequences of 18 recognized species of the genus *Paenibacillus*. *B. subtilis* was used as an outgroup. Only bootstrap values occurring in > 50 % of the 2000 trees generated are shown.

by using the CLUSTAL W program (Thompson *et al.*, 1994). Phylogenetic distances were calculated according to the neighbour-joining method (Saitou & Nei, 1987) with the one-parameter model of Jukes & Cantor (1969). To obtain a robust neighbour-joining tree, 2000 bootstrap replications were performed as described by Kumar *et al.* (1993). The MEGA program (Kumar *et al.*, 1993) was used to carry out neighbour-joining analysis for investigating the tree topology. Alignment gaps and unidentified base positions were not taken into account for the calculations. The comparison of the 16S rDNA sequence of strain PB172^T with those of some *Paenibacillus* spp. available in the databases showed that strain PB172^T clustered in a monophyletic group together with *P. polymyxa*, *P. peoriae* and *P. jamilae* in 100 % of the trees obtained after bootstrap analysis (Fig. 2). The phylogenetic similarity indicated by the 16S rDNA data was in agreement with the levels of rDNA sequence similarity obtained with the novel strain and *P. polymyxa* IAM 13419^T and *P. peoriae* HSCC 353^T (strain PB172^T/*P. polymyxa* = 98·8 % similarity, strain PB172^T/*P. peoriae* = 98 % similarity). *P. jamilae* was recently described as an exopolysaccharide-producing bacterium that is able to grow in olive-mill wastewater and has characteristics not shared with our strains (Aguilera *et al.*, 2001).

For DNA–DNA hybridization experiments, the chromosomal DNA of *P. polymyxa* 24.16^T, *P. peoriae* HSCC 353^T and strain PB172^T was used to construct probes; these probes were digoxigenin-11-dUTP (DIG) labelled by using the protocol provided by Boehringer Mannheim Biochemicals (BMB). The DNA from the strains used in the hybridization studies

Table 2. Levels of DNA homology among the strains tested

Strains: 1, *P. polymyxa* LMD 24.16^T; 2, *P. peoriae* HSCC 353^T; 3, *P. brasilensis* PB172^T. ND, Not determined. Results were obtained with material from at least two experiments.

Source of unlabelled DNA	Homology with DIG-labelled DNA (%) from		
	1	2	3
P. brasilensis			
PB1	60	59	80
PB8	ND	64	84
PB22	60	58	81
PB23	67	64	86
PB24	ND	54	84
PB154	ND	50	78
PB158	56	54	ND
PB164	ND	ND	92
PB172^T	66	63	100
PB177	ND	56	94
P. polymyxa			
LMD 24.16^T	100	65	66
PR3	69	54	51
P. azotofixans P3L-5^T	27	25	18
P. peoriae			
HSCC 353^T	ND	100	61
NRRL B-14477	ND	97	61
NRRL BD-54	ND	87	68
B. subtilis IS75	ND	14	10
Serratia sp.	ND	9	5

(Table 2) was loaded onto positively charged nylon membranes (BMB) as described by Seldin & Dubnau (1985). Pre-hybridization and hybridization conditions using the DIG-labelled probes were those described by the BMB manual for the DIG Nucleic Acid detection kit. After hybridization, the blots were subjected to stringent washing steps, after which the chemiluminescence detection kit (BMB) based on disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD) was used. The levels of homology were determined by scanning the X-ray membranes, and slot intensities were determined by using the 1D Image Analysis Software, version 3.0 (Kodak Digital Science). The self-hybridization values were considered to represent 100 % of the maximal achievable signal and the values obtained with the other strains were compared to this standard. When the DNA of strain PB172^T was used as a probe, all of the novel strains studied (10 of the 16 isolates) displayed 78–100 % DNA relatedness to the probe. However, the level of homology with this probe varied from 51 to 66 % among *P. polymyxa* strains and from 61 to 68 % among *P. peoriae* strains (Table 2). The novel isolates showed homologies to the probes representing the *P. polymyxa* and *P. peoriae*

type strains at levels between 50 and 67% (Table 2). The *P. azotofixans* type strain showed no more than 27% hybridization to strain PB172^T, *P. polymyxa* and *P. peoriae*. DNA from *Bacillus subtilis* IS75 and *Serratia* sp. was used as a negative control; the DNA of both of these strains showed less than 14% homology to the other strains tested.

Denaturing-gradient gel electrophoresis (DGGE) analysis of the 16S rDNA samples obtained after amplification by PCR using the eubacterial primers 968F and 1401R (Heuer & Smalla, 1997; Heuer *et al.*, 1997) was performed using the Universal Mutation Detection System (DCode; Bio-Rad). Polyacrylamide (6%) gels with gradients of between 45 and 65% denaturants (urea and formamide) were prepared in accordance with the method of Muyzer *et al.* (1993). The running time and voltage were 16 h and 100 V, respectively. After electrophoresis, the gels were stained for 30–60 min with SYBR green I nucleic acid gel stain (Molecular Probes). The banding patterns obtained after DGGE analysis of the PCR-amplified fragments of 16S rDNA from *P. azotofixans* RBN4, *P. polymyxa* LMD 24.16^T, *P. peoriae* HSCC 353^T and strain PB172^T were reproducible and indicated the likely existence of several 16S rRNA operons with different sequences encoding these genes. Clearly, the pattern produced by strain PB172^T was different from that produced by strains of the other three species (data not shown). The presence of multiple rRNA operons in most bacterial species and sequence divergence in these genes within one organism have already been widely demonstrated (Mylvaganam & Dennis, 1992; Nübel *et al.*, 1996).

Combining all of the data presented here, it is clear that the 16 novel isolates form a very homogeneous group which is different from all other related species within the genus *Paenibacillus*. Therefore, we propose that they represent a novel species of the genus *Paenibacillus*, *Paenibacillus brasilensis*.

Description of *Paenibacillus brasilensis* sp. nov.

Paenibacillus brasilensis (bra.sil.en′sis. N.L. adj. *brasilensis* referring to Brazil, the country where the strains were isolated).

Cells are straight, motile rods. Spores are oval to ellipsoidal and predominantly central to subterminal and distend the sporangium. Young GB (Seldin *et al.*, 1983) broth cultures are Gram-positive or Gram-variable. On GB agar, colonies are 15–30 mm in diameter, whitish, circular to slightly irregular, convex and mucoid. On TBN (Seldin *et al.*, 1984) agar, colonies are about 10 mm in diameter, bright yellow, circular, convex, with entire margins and they adhere to the agar. TY broth and agar (van Elsas & Penido, 1981) permit poor growth. In GB or TBN broth, strains grow abundantly, forming a mucous pellet at the bottom of the tube. The maximum temperature for growth is 42 °C; the optimum is 30–32 °C. Grows at pH 5·7, in the presence of 2% NaCl, but not 5% NaCl, and in the presence of lysozyme. Facultatively anaerobic. Catalase-positive. Voges–Proskauer-positive. Dihydroxyacetone is not produced from glycerol. No crystalline dextrins are formed in rolled-oat medium. Acid and gas are produced from glucose. Other characteristics of the species can be found in Table 1. In addition to the carbohydrates shown in Table 1, acid is produced from galactose, D-fructose, amygdalin, aesculin, maltose, melibiose, sucrose and D-raffinose, but is not produced from erythritol, L-xylose, adonitol, L-sorbose, L-arabitol, 2-ketogluconate or 5-ketogluconate. Nitrogen fixation (acetylene reduction) detected. Isolated from the rhizosphere of maize sown in Cerrado soil, Minas Gerais, Brazil. The type strain is PB172^T (= ATCC BAA-413^T = DSM 14914^T). The description of the type strain is the same as that given for the species, except for the following results for variable characteristics: positive for liquefaction of gelatin; positive for fermentation of trehalose and D-turanose; negative for fermentation of β-gentiobiose.

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References

Aguilera, M., Monteoliva-Sanchez, M., Suarez, A., Guerra, V., Lizama, C., Bennasar, A. & Ramos-Cormenzana, A. (2001). *Paenibacillus jamilae* sp. nov., an exopolysaccharide-producing bacterium able to grow in olive-mill wastewater. *Int J Syst Evol Microbiol* **51**, 1687–1692.

Ash, C., Farrow, J. A. E., Wallbanks, S. & Collins, M. D. (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small subunit-ribosomal RNA sequences. *Lett Appl Microbiol* **13**, 202–206.

Ash, C., Priest, F. G. & Collins, M. D. (1993). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie van Leeuwenhoek* **64**, 253–260.

Berge, O., Guinebretière, M.-H., Achouak, W., Normand, P. & Heulin, T. (2002). *Paenibacillus graminis* sp. nov. and *Paenibacillus odorifer* sp. nov., isolated from plant roots, soil and food. *Int J Syst Evol Microbiol* **52**, 607–616.

Budi, S. W., van Tuinen, D., Martinotti, G. & Gianinazzi, S. (1999). Isolation from *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorhiza development and antagonistic towards soilborne fungal pathogens. *Appl Environ Microbiol* **65**, 5148–5150.

Elo, S., Suominen, I., Kämpfer, P., Juhanoja, J., Salkinoja-Salonen, M. & Haahtela, K. (2001). *Paenibacillus borealis* sp. nov., a nitrogen-fixing species isolated from spruce forest humus in Finland. *Int J Syst Evol Microbiol* **51**, 535–545.

Gordon, R. E., Haynes, W. C. and Pang, H.-N. (1973). The genus *Bacillus*. In *Agriculture Handbook* no. 427. Washington, DC: Agricultural Research Service, US Department of Agriculture.

Grau, F. H. & Wilson, P. W. (1962). Physiology of nitrogen-fixation by *Bacillus polymyxa*. *J Bacteriol* **83**, 490–496.

Heuer, H. & Smalla, K. (1997). Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In *Modern Soil Microbiology*, pp. 353–373. Edited by J. D. van Elsas, E. M. H. Wellington & J. T. Trevors. New York: Marcel Dekker.

Heuer, H., Krsek, M., Baker, P., Smalla, K. & Wellington, E. M. H. (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl Environ Microbiol* **63**, 3233–3241.

Heyndrickx, M., Vandemeulebroecke, K., Scheldeman, P., Kersters, K., de Vos, P., Logan, N. A., Aziz, A. M., Ali, N. & Berkeley, R. C. W. (1996). A polyphasic reassessment of the genus *Paenibacillus*, reclassification of *Bacillus lautus* (Nakamura 1984) as *Paenibacillus lautus* comb. nov. and *Bacillus peoriae* (Montefusco *et al*., 1993) as *Paenibacillus peoriae* comb. nov., and emended descriptions of *P. lautus* and of *P. peoriae*. *Int J Syst Bacteriol* **46**, 988–1003.

Johnson, J. L. (1981). Genetic characterization. In *Manual of Methods for General Microbiology*, pp. 450–472. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kumar, S., Tamura, K. & Nei, M. (1993). MEGA: Molecular evolutionary genetics analysis, version 1.0. Pennsylvania State University, PA, USA.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* **227**, 680–685.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 205–218.

Massol-Deya, A. A., Odelson, D. A., Hichey, R. P. & Tiedje, J. M. (1995). Bacterial community fingerprinting of amplified 16S and 16–23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In *Molecular Microbial Ecology Manual*, pp. 3.3.2, 1–8. Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. de Bruijn. Dordrecht: Kluwer Academic.

Montefusco, A., Nakamura, L. K. & Labeda, D. P. (1993). *Bacillus peoriae* sp. nov. *Int J Syst Bacteriol* **43**, 388–390.

Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695–700.

Mylvaganam, S. & Dennis, P. P. (1992). Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaebacterium *Haloarcula marismortui*. *Genetics* **130**, 399–410.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W. & Backhaus, H. (1996). Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J Bacteriol* **178**, 5636–5643.

Rennie, R. J., Freitas, J. R. & Ruschel, A. P. (1982). Isolation and identification of N_2-fixing bacteria associated with sugar cane (*Saccharum* sp.). *Can J Microbiol* **28**, 462–467.

Rhodes-Roberts, M. E. (1981). The taxonomy of some nitrogen-fixing *Bacillus* species with special reference to nitrogen fixation. In *The Aerobic Endospore-forming Bacteria: Classification and Identification*, pp. 315–335. Edited by R. C. W. Berkeley & M. Goodfellow. London: Academic Press.

Rosado, A. S., de Azevedo, F. S., da Cruz, D. W., van Elsas, J. D. & Seldin, L. (1998). Phenotypic and genetic diversity of *Paenibacillus azotofixans* strains isolated from the rhizoplane or rhizosphere soil of different grasses. *J Appl Microbiol* **84**, 216–226.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Seldin, L. & Dubnau, D. (1985). Deoxyribonucleic acid homology among *Bacillus polymyxa*, *Bacillus macerans*, *Bacillus azotofixans*, and other nitrogen-fixing *Bacillus* strains. *Int Syst Bacteriol* **35**, 151–154.

Seldin, L. & Penido, E. G. C. (1986). Identification of *Bacillus azotofixans* using API tests. *Antonie van Leeuwenhoek* **52**, 403–409.

Seldin, L., van Elsas, J. D. & Penido, E. G. C. (1983). *Bacillus* nitrogen fixers from Brazilian soils. *Plant Soil* **70**, 243–255.

Seldin, L., van Elsas, J. D. & Penido, E. G. C. (1984). *Bacillus azotofixans* sp. nov., a nitrogen-fixing species from brazilian soils and grass roots. *Int J Syst Bacteriol* **34**, 451–456.

Seldin, L., Rosado, A. S., da Cruz, D. W., Nobrega, A., van Elsas, J. D. & Paiva, E. (1998). Comparison of *Paenibacillus azotofixans* strains isolated from rhizoplane, rhizosphere and non-root-associated soil from maize planted in two different Brazilian soils. *Appl Environ Microbiol* **64**, 3860–3868.

Shida, O., Takagi, H., Kadowaki, K., Nakamura, L. K. & Komagata, K. (1997). Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus*. *Int J Syst Bacteriol* **47**, 289–298.

Shishido, M., Loeb, B. M. & Chanway, C. P. (1995). External and internal root colonization of lodgepole pine seedlings by two growth-promoting *Bacillus* strains originated from different root microsites. *Can J Microbiol* **41**, 703–713.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (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.

van Elsas, J. D. & Penido, E. G. C. (1981). Isolation and characterization of *Bacillus pumilus* phages from Brazilian soil. *Zentbl Bakteriol Parasitenkd Infektkrankh Hyg Abt I Orig* **2 136**, 581–589.

Vaneechoutte, M., Rossau, R., de Vos, P. & 7 other authors (1992). Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiol Lett* **93**, 227–234.

von der Weid, I., Paiva, E., Nobrega, A., van Elsas, J. D. & Seldin, L. (2000). Diversity of *Paenibacillus polymyxa* strains isolated from the rhizosphere of maize planted in Cerrado soil. *Res Microbiol* **151**, 369–381.

Witz, D. F., Detroy, R. W. & Wilson, P. W. (1967). Nitrogen fixation by growing cells and cell-free extracts of the *Bacillaceae*. *Arch Mikrobiol* **55**, 369–381.

Wullstein, L. H., Bruening, M. L. & Bollen, W. B. (1979). Nitrogen fixation associated with sand grain root sheaths (rhizosheaths) of certain xeric grasses. *Physiol Plant* **46**, 1–4.