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

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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 PB172T (= ATCC BAA-413T = DSM 14914T).

**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*
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% CaCO₃ (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 0.6 × 2.1 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://ijis.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-xylene 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 3557, 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.

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Strains: 1. Paenibacillus brasilensis (PB1, PB7, PB8, PB22, PB23, PB24, PB65, PB105, PB154, PB155, PB158, PB159, PB164, PB172, 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 (Eio 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.
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<sup>T</sup> and Loutit), *P. peoriae* (NRRL BD-62, BD-54 and B-14474), *P. azotofixans* (P3L-5<sup>T</sup>) 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 the species. A protein corresponding to a molecular mass of between 90 and 10 kDa 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 rDNA 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 rDNA region was performed with primers pA and pH. Total genomic DNA from representative strains of *P. polymyxa* (LMD 24.16<sup>T</sup> and Loutit), *P. azotofixans* (P3L-5<sup>T</sup> and V22.35), *P. macerans* (LMD 24.10) and *P. peoriae* (NRRL B-14477, BD-62 and HSCC 353<sup>T</sup>) and all of the novel isolates was used. The amplicons obtained (about 1500 bp in size) were analysed by digestion with three restriction enzymes (Sau3AI, HinfI and HaeIII) 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<sup>T</sup>, 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 rDNA alleles (Vanceechoutte et al., 1992). For the determination of the phylogenetic position of the selected representative novel strain (PB172<sup>T</sup>), 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<sup>T</sup> was aligned with those of its most closely related species of the genus *Paenibacillus*.

![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 HaeIII, HinfI and Sau3AI.](image-url)
For DNA–DNA hybridization experiments, the chromosomal DNA of *P. polymyxa* 24.16T, *P. peoriae* HSCC 353T and strain PB172T 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) 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-methoxyxypir[1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.14.7]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 self-hybridization value.

**Table 2. Levels of DNA homology among the strains tested**

<table>
<thead>
<tr>
<th>Strains:</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. brasilensis</em></td>
<td>60</td>
<td>59</td>
<td>80</td>
</tr>
<tr>
<td>PB1</td>
<td>ND</td>
<td>64</td>
<td>84</td>
</tr>
<tr>
<td>PB8</td>
<td>60</td>
<td>58</td>
<td>81</td>
</tr>
<tr>
<td>PB22</td>
<td>67</td>
<td>64</td>
<td>86</td>
</tr>
<tr>
<td>PB23</td>
<td>ND</td>
<td>54</td>
<td>84</td>
</tr>
<tr>
<td>PB24</td>
<td>ND</td>
<td>50</td>
<td>78</td>
</tr>
<tr>
<td>PB154</td>
<td>56</td>
<td>54</td>
<td>ND</td>
</tr>
<tr>
<td>PB158</td>
<td>ND</td>
<td>ND</td>
<td>92</td>
</tr>
<tr>
<td>PB172T</td>
<td>66</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>PB177</td>
<td>ND</td>
<td>56</td>
<td>94</td>
</tr>
<tr>
<td><em>P. polymyxa</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMD 24.16T</td>
<td>100</td>
<td>65</td>
<td>66</td>
</tr>
<tr>
<td>PR3</td>
<td>69</td>
<td>54</td>
<td>51</td>
</tr>
<tr>
<td><em>P. azotofixans</em> P3L-5T</td>
<td>27</td>
<td>25</td>
<td>18</td>
</tr>
<tr>
<td><em>P. peoriae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSCC 353T</td>
<td>ND</td>
<td>100</td>
<td>61</td>
</tr>
<tr>
<td>NRRL B-14477</td>
<td>ND</td>
<td>97</td>
<td>61</td>
</tr>
<tr>
<td>NRRL BD-54</td>
<td>ND</td>
<td>87</td>
<td>68</td>
</tr>
<tr>
<td><em>B. subtilis</em> IS75</td>
<td>ND</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td><em>Serratia</em> sp.</td>
<td>ND</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

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 PB172T with those of some *Paenibacillus* sp. available in the databases showed that strain PB172T clustered in a monophyletic group together with *P. polymyxa*, *P. peoriae* and *P. jamiae* 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 13419T and *P. peoriae* HSCC 353T (strain PB172T/P. polymyxa = 98.8 % similarity, strain PB172T/P. peoriae = 98 % similarity). *P. jamiae* 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. polymerx* 24.16T, *P. peoriae* HSCC 353T and strain PB172T 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

**Fig. 2.** Phylogenetic tree, obtained by the neighbour-joining method, based on the alignment of the 16S rRNA gene sequence of *P. brasilensis* PB172T with the 16S rRNA 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.
type strains at levels between 50 and 67% (Table 2). The
P. azotofixans type strain showed no more than
27% hybridization to strain PB172T, 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 RB4, P.
polymyxa LMD 24.16T, P. peoriae HSCC 353T and
strain PB172T were reproducible and indicated the
likely existence of several 16S rRNA operons with
different sequences encoding these genes. Clearly,
the pattern produced by strain PB172T 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 di-
vergence 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 brasiliensis.

Description of Paenibacillus brasiliensis sp. nov.

Paenibacillus brasiliensis (bra.sil.en’sis. N.L.
adj. brasiliensis 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 & Pendino,
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 an-
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,
ad acid is produced from galactose, D-fructose, amyg-
dalin, aesculin, maltose, melibiose, sucrose and D-
raffinose, but is not produced from erythritol, L-xyllose,
adenitol, L-sorbose, L-arabinol, 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 PB172T (= ATCC BAA-413T = DSM 14914T).
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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polymyxa Loutiti), Dr L. Rabinovich (P. macerans Mac 3),
Dr L. K. Nakamura (P. peoriae NRRL B-14474, B-14477,
BD-54 and BD-62), Dr O. Shida (P. peoriae HSCC 353) and
Dr D. Dubnau (B. subtilis IS75).

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