**Paenibacillus riograndensis** sp. nov., a nitrogen-fixing species isolated from the rhizosphere of *Triticum aestivum*

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A bacterial strain designated SBR5\(^T\) was isolated from the rhizosphere of *Triticum aestivum*. A phylogenetic analysis based on the 16S rRNA gene sequence placed the isolate within the genus *Paenibacillus*, being most closely related to *Paenibacillus graminis* RSA19\(^T\) (98.1 % similarity). The isolate was a Gram-reaction-variable, motile, facultatively anaerobic bacterium, with spores in a terminal position in cells. Starch was utilized and dihydroxyacetone and catalase were produced. Strain SBR5\(^T\) displayed plant-growth-promoting rhizobacteria characteristics: the ability to fix nitrogen and to produce siderophores and indole-3-acetic acid. The DNA G+C content was 55.1 mol%. Chemotaxonomic analysis of the isolated strain revealed that MK-7 was the predominant menaquinone, while the major fatty acid was anteiso-C\(_{15}\) :0. DNA–DNA hybridization values between strain SBR5\(^T\) and *P. graminis* RSA19\(^T\), *Paenibacillus odorifer* TOD45\(^T\) and *Paenibacillus borealis* KK19\(^T\) were 43, 35 and 28 %, respectively. These DNA relatedness data and the results of phylogenetic and phenotypic analyses showed that strain SBR5\(^T\) should be considered as the nitrogen-fixing type strain of a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus riograndensis* sp. nov. is proposed. The type strain is SBR5\(^T\) (=CCGB 1313\(^T\) = CECT 7330\(^T\)).

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The bacilli are a wide group of micro-organisms that are characterized by endospore formation. Currently, this group includes several families and genera, many of which formerly belonged to the genus *Bacillus*, which has been separated into several novel genera that belong to several families. This is the case for the genus *Paenibacillus*, which was proposed by Ash et al. (1994) and belongs to the family 'Paenibacillaceae'. Bacteria belonging to the genus *Paenibacillus* are among the most widely distributed micro-organisms and play significant roles in microbial communities (Reva et al., 1995). They can be found associated with plants or freely in soils and have potential applications in different fields of agricultural biotechnology as inoculants for crop production.

Nitrogen fixation has been described in several species of the genus *Paenibacillus*, such as *P. polymyxa* (Grau & Wilson, 1962), *P. macerans*, *P. durus* (*P. azotofixans*), *P. peoriae* (Montefusco et al., 1993), *P. borealis* (Elo et al., 2001), *P. graminis*, *P. odorifer* (Berge et al., 2002), *P. brasiliensis* (von der Weid et al., 2002), *P. massiliensis* (Roux & Raoult, 2004), *P. wynii* (Rodríguez-Díaz et al., 2005), *P. sabinae* (Ma et al., 2007a), *P. zanthoxyli* (Ma et al., 2007b), ‘*P. donghaensis*’ (Choi et al., 2008) and *P. forsythiae* (Ma & Chen, 2008). Some of these bacteria are promising candidates for crop inoculation, not only for their nitrogen-fixing ability, but also for their capacity to promote plant growth through the production of phytohormones (auxins and cytokinins) and antimicrobial substances (Rosado et al., 1996). Although several species of plant-growth-promoting rhizobacteria (PGPR) have already been described (Lebuhn et al., 1997; Timmusk & Wagner, 1999; Timmusk et al., 1999; Helbig, 2001; von der Weid et al., 2003), the vast majority of rhizospheric bacterial species present in many soils remain unknown, and their identification could be useful in the formulation of new inoculants to improve crop production.

In the present report, we describe the morphological, phylogenetic and physiological characteristics of a novel PGPR, strain SBR5\(^T\), isolated from the rhizosphere of *Triticum aestivum* cultivated in Rio Grande do Sul State, Brazil.
Aliquots of serially diluted pasteurized (10 min, 80 °C) rhizosphere suspensions of wheat (Triticum aestivum) were inoculated onto thiamine-biotin agar (TB, nitrogen-free medium; Seldin et al., 1983) and incubated in anaerobic jars (Permut) for 7 days at 28 °C. Anaerobic bacilli colonies were transferred to fresh TB agar plates for another period of anaerobic incubation. Single colonies were then transferred to aerobic GB broth (Seldin et al., 1983). A bacterial strain, designated SBR5T, was isolated and a pure culture was maintained in a glycerol suspension (20 %) at −20 °C.

The morphology of cells was examined by phase-contrast microscopy. Flagellum and spore types were examined with a scanning electron microscope (XL-30, Philips) using cells cultured for 48 h in GB broth. Cells were fixed according to Borges et al. (2004). Gram behaviour was ascertained by staining (Doetsch, 1981). Motility was verified by the SIM (hydrogen-sulfide, indole, motility; MacFaddin, 2000) test.

The SBR5T isolate was also evaluated for the presence of desirable PGPR characteristics. To assess indole-3-acetic acid, indolepyruvic acid, and indoleaceticamide production (collectively referred to as indolic compounds), the method described by Glickmann & Dessaux (1995) was used. To determine the nitrogen-fixing capability of this strain, an assay based on sulfur digestion and distillation with 10 mol NaOH l−1, as described by Keeney & Bremner (1966), was performed together with PCR amplification of a 360 bp fragment of the nifH gene using the universal degenerate primers PoIF and PoIR, described by Poly et al. (2001). Strain SBR5T was also analysed for its siderophore production capacity in Petri dishes containing King B medium (Glickmann & Dessaux, 1995) supplemented with a chrome azurol S complex [CAS/iron(III)/hexadeciltrimethyl ammonium bromide], as described by Schwyn & Neilands (1987).

Chromosomal DNA was extracted and purified according to standard methods (Sambrook & Russell, 2001). The 16S rRNA gene sequence of strain SBR5T was amplified using primers 5′-AGAGTTTGATCCTGCTCAG-3′ and 5′-AGAAAAAGGTGATCCAGCC-3′ under conditions described by Rivas et al. (2003). The corresponding positions in the Escherichia coli small-subunit rRNA gene sequence for these primers are 8–27 and 1498–1522, respectively. The 16S rRNA gene sequence was determined as described by De Ley et al. (1970).

DNA–DNA hybridization and DNA G+C content were determined according to the standard methods described by Claus & Berkeley (1986).

Cells of strain SBR5T were Gram-reaction-variable, rod-shaped, sporulating and motile. The isolate produced ellipsoidal spores with a regular stripe pattern (Fig. 2). Regarding PGPR abilities, the SBR5T isolate produced 213.7 and 269.4 µg indolic compounds ml−1 after 72 and 144 h of incubation, respectively, and was able to fix 8 µg N ml−1. Strain SBR5T produced a yellow halo in the blue-green media, which indicated its ability to produce siderophores.

Strain SBR5T is phylogenetically related to members of the genus Paenibacillus. As shown in Fig. 1, the most closely related recognized type strains were P. graminis RSA19T (98.1 % similarity), P. odorifer TOD45T (95.8 %) and P. borealis KK19T (96.3 %). Phylogenetic analysis based on nifH sequences revealed that the SBR5T strain also clustered together with species of the genus Paenibacillus (Fig. 3). The novel strain showed high levels of the nifH gene sequence similarity with P. graminis (78 %), P. wynnii (79 %), P. odorifer (77 %) and P. borealis (74 %).

The values for DNA–DNA hybridization between strain SBR5T and P. graminis RSA19T, P. odorifer TOD45T and P. borealis KK19T were 43, 35 and 28 %, respectively. In terms of DNA–DNA hybridization, the threshold value for the
definition of a species is considered to be 70 % (Wayne et al., 1987); consequently, our results indicate that the strain isolated in this study does not belong to any of the known species of the genus Paenibacillus. The DNA G + C content of strain SBR5T was 55.1 mol%. Although this is higher than those described for the majority of species of the genus Paenibacillus (Shida et al., 1997), it is similar to that obtained for Paenibacillus stellifer (55.6 mol%, Suominen et al., 2003), another nitrogen-fixing member of the genus.

Unsaturated menaquinone with seven isoprene units (MK-7) was the predominant isoprenoid quinone found in strain SBR5T. The major polar lipids present were diphasatidylglycerol, phosphatidylglycerol and one unknown phospholipid that could not be identified. The total hydrolysate (4 M HCl, 16 h, 100 °C) of the peptidoglycan contained the amino acids Lys, Glu and Ala in a molar ratio of approximately 1.0 : 1.0 : 3.3. The partial hydrolysate (4 M HCl, 0.75 h, 100 °C) of the peptidoglycan contained (in addition to the amino acids Lys, Glu and Ala) the peptides L-Ala-Lys, L-Ala-L-Ala-L-Lys, L-Ala-L-ALA, L-ALA-L-Lys →α-D-Glu, L-ALA-L-ALA-L-Lys, L-lys →α-D-Glu, L-ALA-L-Lys →α-D-Glu, L-ALA-L-ALA-L-Lys →α-D-Glu and L-ALA-L-ALA-L-ALA. From these data it was concluded that strain SBR5T shows the peptidoglycan type A3α (http://www.dsmz.de). The predominant fatty acids in strain SBR5T were anteiso-C15:0 and C16:0 comprising 45.7 and
17.6% of the total, respectively. According to these results, the fatty acid composition of strain SBR5$^T$ is similar to those reported for species of the genus *Paenibacillus* (Shida et al., 1997).

Details of phenotypic characteristics that differentiate strain SBR5$^T$ and phylogenetically related species are given in Table 2. Other characteristics determined are given in the species description. Strain SBR5$^T$ differed from *P. graminis* with respect to growth at 40°C, gas production from D-glucose and nitrate reduction, from *P. odorifer* with respect to nitrate reduction and acid production from D-mannitol, from *P. wynnii* with respect to spore position and nitrate reduction, and from *P. borealis* with respect to casein hydrolysis and growth at pH 10. Strain SBR5$^T$ differed from all of these species with respect to aesculin hydrolysis.

On the basis of the phylogenetic and phenotypic data, we propose that isolate SBR5$^T$ (CCGB 1313$^T$ = CECT 7330$^T$) represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus riograndensis* sp. nov. is proposed.

**Description of *Paenibacillus riograndensis* sp. nov.**

*Paenibacillus riograndensis* (ri.o.gran.den’sis. N. L. masc. adj. riograndensis referring to Rio Grande do Sul, the state located in Southern Brazil, where the strain was isolated). Cells are rod-shaped, 0.65–0.8 μm by 3.8–4.5 μm, Gram-stain-variable, motile and facultatively anaerobic. Spores are in a terminal position in cells. Colonies on GB medium are circular, convex, white and translucent.
Table 2. Phenotypic characteristics that differentiate Paenibacillus riograndensis SBR5T from its closest relatives in the genus Paenibacillus

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Typically 1–2 mm in diameter within 24 h at 28°C. Optimal growth at 28°C and pH 7. Cannot grow in the presence of 5% NaCl. Catalase-positive and oxidase-negative. DNA G+C content of the type strain is 55.1 mol%. The main fatty acid is anteiso-C_{15:0} and the predominant menaquinone is MK-7. Gas is not produced from D-glucose. Acid is produced from D-glucose, sucrose, D-mannose, lactose, raffinose, maltose, D-xylose, mannitol, L-arabinose, galactose, glycerol, D-fructose and trehalose, but not from dulcitol or myo-inositol. Does not utilize citrate as a carbon source for growth. Starch is hydrolysed. Casein and aesculin are not hydrolysed and acetoin is not produced. Gelatinase, urease, phenylalanine deaminase, indole, hydrogen sulhide and acetoin (in Voges-Proskauer medium) are not produced. Nitrate is not reduced to nitrite. Displays PGPR characteristics: able to fix nitrogen, produces siderophores and indole-3-acetic acid.

The type strain, SBR5T (=CCGB 1313T =CECT 7330T), was isolated from the rhizosphere of wheat (Triticum aestivum) in Rio Grande do Sul State, Southern Brazil.

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References


