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

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A bacterial strain designated SBR5⁵ 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 RSA19⁵ (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 SBR5⁵ 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-C₁₅ : ₀. DNA–DNA hybridization values between strain SBR5⁵ and P. graminis RSA19⁵, Paenibacillus odorifer TOD45⁵ and Paenibacillus borealis KK1⁵ were 43, 35 and 28 %, respectively. These DNA relatedness data and the results of phylogenetic and phenotypic analyses showed that strain SBR5⁵ 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 SBR5⁵ (=CCGB 1313⁵ =CECT 7330⁵).

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. wynnii (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 SBR5⁵, 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 (Permution) 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 SBR5\(^T\), 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 staining (Doetsch, 1981). Motility was verified by the SIM (hydrogen-sulfide, indole, motility; MacFaddin, 2000) test.

DNA–DNA hybridization and DNA G+C content were determined as described by De Ley et al. (1970).

The predominant fatty acids were analysed by GLC as described in the MIS operating manual (MIDI, 2001). The results shown in Table 1 were determined under the same conditions for all strains used for comparison.

Phenotypic characterization was performed according to standard methods described by Claus & Berkeley (1986).

Cells of strain SBR5\(^T\) 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 SBR5\(^T\) 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 SBR5\(^T\) produced a yellow halo in the blue-green media, which indicated its ability to produce siderophores.

Strain SBR5\(^T\) is phylogenetically related to members of the genus Paenibacillus. As shown in Fig. 1, the most closely related recognized type strains were P. graminis RSA19\(^T\) (98.1 % similarity), P. odorifer TOD45\(^T\) (95.8 %) and P. borealis KK19\(^T\) (96.3 %). Phylogenetic analysis based on nifH sequences revealed that the SBR5\(^T\) strain also clustered together with species of the genus Paenibacillus (Fig. 3). The novel strain showed high levels of nifH gene sequence similarity with P. graminis (78 %), P. wynii (79 %), P. odorifer (77 %) and P. borealis (74 %).

The values for DNA–DNA hybridization between strain SBR5\(^T\) and P. graminis RSA19\(^T\), P. odorifer TOD45\(^T\) and P. borealis KK19\(^T\) were 43, 35 and 28 %, respectively. In terms of DNA–DNA hybridization, the threshold value for the
A 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-D-Glu, L-Ala-L-Lys, L-Ala-L-Ala-L-Lys, L-Lys-D-Ala, L-Ala-L-Lys-D-Ala, L-Ala-L-Ala-L-Lys-D-Ala and L-Ala-D-Ala. From these data it was concluded that strain SBR5T shows the peptidoglycan type A3a L-Lys r L-Ala r L-Ala (type A11.5 according to http://www.dsmz.de). The predominant fatty acids in strain SBR5T were anteiso-C15:0 and C16:0, comprising 45.7 and

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**Fig. 1.** Rooted phylogenetic tree based on 16S rRNA sequence comparisons of strain SBR5T and selected members of the genus *Paenibacillus*. GenBank accession numbers are given in parentheses. Sequence of *Lactobacillus delbrueckii* subsp. *lactis* was used to root the dendrogram. Bootstrap analyses were based on 1000 replications.
Table 1. Cellular fatty acid composition of strain SBR5T and phylogenetically related species of the genus Paenibacillus

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straight-chain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>8.0</td>
<td>10.3</td>
<td>4.7</td>
<td>6.0</td>
<td>18.8</td>
</tr>
<tr>
<td>C16:0</td>
<td>17.6</td>
<td>28.5</td>
<td>14.7</td>
<td>32.2</td>
<td>12.5</td>
</tr>
<tr>
<td>Iso-branched</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>3.9</td>
<td>ND</td>
<td>4.3</td>
<td>5.3</td>
<td>4.6</td>
</tr>
<tr>
<td>C15:0</td>
<td>10.3</td>
<td>6.4</td>
<td>14.4</td>
<td>5.7</td>
<td>13.2</td>
</tr>
<tr>
<td>C16:0</td>
<td>9.0</td>
<td>11.5</td>
<td>5.5</td>
<td>4.9</td>
<td>9.0</td>
</tr>
<tr>
<td>C17:0</td>
<td>2.4</td>
<td>ND</td>
<td>2.9</td>
<td>1.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Anteiso-branched</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C15:0</td>
<td>45.7</td>
<td>43.3</td>
<td>49.3</td>
<td>33.1</td>
<td>37.0</td>
</tr>
<tr>
<td>C17:0</td>
<td>3.16</td>
<td>ND</td>
<td>2.3</td>
<td>&gt;1</td>
<td>2.3</td>
</tr>
</tbody>
</table>

17.6% of the total, respectively. According to these results, the fatty acid composition of strain SBR5T is similar to those reported for species of the genus Paenibacillus (Shida et al., 1997).

Details of phenotypic characteristics that differentiate strain SBR5T and phylogenetically related species are given in Table 2. Other characteristics determined are given in the species description. Strain SBR5T 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. wynii with respect to spore position and nitrate reduction, and from P. borealis with respect to casein hydrolysis and growth at pH 10. Strain SBR5T differed from all of these species with respect to aesculin hydrolysis.

On the basis of the phylogenetic and phenotypic data, we propose that isolate SBR5T (CCGB 1313T = CECT 7330T) 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-reaction-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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore position</td>
<td>T</td>
<td>T</td>
<td>C or S</td>
<td>S or T</td>
<td></td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Aesculin hydrolysis</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Arabinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gas production from glucose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth in the presence of 5 %</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth at 40 °C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth at pH 10</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Urease activity</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
</tr>
</tbody>
</table>

All strains are rod-shaped with ellipsoidal spores, motile, grow anaerobically and are positive for starch hydrolysis, catalase and acid production from D-glucose, fructose, galactose, lactose, maltose, sucrose, D-xylose and trehalose. All strains are negative for growth at 132 °C, acid production from dulcitol, use of citrate as a carbon source, oxidase activity, gelatin hydrolysis, production of hydrogen sulphide and Voges–Proskauer and indole tests. C, Central or paracentral; S, subterminal; T, terminal.

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-C15:0 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, mannohexose, 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 ascinulin are not hydrolysed and acetoin is not produced. Gelatinase, urease, phenylalanine deaminase, indole, hydrogen sulfide 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.

Acknowledgements

This work was supported by a grant and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

References


