**Bacillus oryzisoli** sp. nov., isolated from rice rhizosphere

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The taxonomy of strain 1DS3-10ᵀ, a Gram-staining-positive, endospore-forming bacterium isolated from rice rhizosphere, was investigated using a polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequences demonstrated that the novel strain was grouped with established members of the genus *Bacillus* and appeared to be closely related to the type strains *Bacillus benzoevorans* DSM 5391ᵀ (97.9 %), *Bacillus circulans* DSM 11ᵀ (97.7 %), *Bacillus novalis* JCM 21709ᵀ (97.3 %), *Bacillus soli* JCM 21710ᵀ (97.3 %), *Bacillus oceanisediminis* CGMCC 1.10115ᵀ (97.3 %) and *Bacillus nealsonii* FO-92ᵀ (97.1 %). The fatty acid profile of strain 1DS3-10ᵀ, which showed a predominance of iso-C₁₅:₀ and anteiso-C₁₅:₀, supported the allocation of the strain to the genus *Bacillus*. The predominant menaquinone was MK-7 (100 %). The major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylethanolamine and unknown aminolipids. Cell-wall peptidoglycan contained meso-diaminopimelic acid. DNA–DNA hybridization values between strain 1DS3-10ᵀ and the type strains of closely related species were 25–33 %, which supported that 1DS3-10ᵀ represented a novel species in the genus *Bacillus*. The results of some physiological and biochemical tests also allowed the phenotypic differentiation of strain 1DS3-10ᵀ from the most closely related recognized species. On the basis of the phylogenetic and phenotypic evidence, strain 1DS3-10ᵀ represents a novel species of the genus *Bacillus*, for which the name *Bacillus oryzisoli* sp. nov. is proposed. The type strain of the novel species is 1DS3-10ᵀ (=ACCC 19781ᵀ = DSM 29761ᵀ).

In 1872, Cohn proposed the genus *Bacillus*, with *Bacillus subtilis* as the type species. Members of the genus *Bacillus* form endospores, the most common Gram reaction is positive, they generally have meso-diaminopimelic acid in the peptidoglycan, the major menaquinone present is MK-7, the major fatty acids present are iso-C₁₅:₀ and anteiso-C₁₅:₀, and the DNA G+C content varies from 32 to 66 mol % (Logan & De Vos, 2009). At the time of writing, nearly 300 species with validly published names had been described (http://www.bacterio.net/). Members of the genus *Bacillus* have been isolated from a wide variety of environments, including soils, water, food and plants. Some species of the genus *Bacillus* isolated from rhizosphere, phylloplanes and endophytic sites may help to promote plant growth (Madhaiyan et al., 2010; Bibi et al., 2011; Lin et al., 2015; Liu et al., 2015). In this study, we report on the taxonomy of a novel strain, 1DS3-10ᵀ, which was isolated from rice rhizosphere during studies of the effects of long-term winter growth of green manure plants on microbial diversity.

Rice roots were taken from the Key Field Monitoring Experimental Station for Red Soil Eco-environment of Ministry of Agriculture, located in Qiyang County of Hunan Province of China. Roots were completely recovered from the paddy field and shaken vigorously to remove loosely adhered soil. Soil tightly adhered to the roots was considered as rhizosphere soil. Sterile water was used to wash the roots and the suspension was centrifuged at a high speed...
(10 000 g, 10 mins); the sediment containing the rhizosphere soil was collected. Five grams of sediment was diluted with sterile water using the standard dilution plating technique and then incubated at 28°C for 4 days. More than 100 strains were isolated from plates of tryptase soy agar (Difco) based on colony characteristics, and identified by sequencing of the 16S rRNA gene. All strains were stored at −80°C as glycerol suspensions (20%, v/v) and at −4°C in freeze-drying ampoules. Among those strains, strain 1DS3-10T was identified as a representative of a potentially novel species of the genus *Bacillus*.

The 16S rRNA gene was amplified using universal primers 27F and 1492R according to the method of Lane et al. (1991). Amplified products were purified and cloned into vector PMD-18 (Takara). Cloned 16S rRNA genes were sequenced by Invitrogen (Shanghai, China). The 16S rRNA gene sequence of strain 1DS3-10T was for determining the similarities to its phylogenetic neighbours using the EzTaxon-e database (Kim et al., 2012). Sequences were aligned and analysed using MEGA 6.0 software (Tamura et al., 2013). For maximum-likelihood (ML) (Felsenstein, 1981) analysis, the best-fit model for nucleotide substitution was selected using MEGA 6.0. The best model in this study was the general time reversible model with gamma-distributed rates plus invariant sites. For neighbour-joining (NJ) (Saitou & Nei, 1987) analysis, the average pairwise Jukes–Cantor distance was chosen to determine whether the sequence data were suitable for estimating NJ trees. The average distance of 16S rRNA gene sequences in this study was 0.04, a value suitable for making NJ trees (Nei & Kumar, 2000). The NJ and minimum-evolution (ME) trees were built using the Jukes–Cantor model method, and the rate variation among sites was modelled with a gamma distribution (gamma parameter=5). ML, NJ and ME trees were built with partial deletion of gaps (95%), and reliability of the phylogenetic trees was estimated using bootstrap values based on 1000 iterations (Felsenstein, 1985). The length of the 1DS3-10T 16S rRNA gene sequence was 1511 bp. It shared 97.9% sequence similarity with *Bacillus benzoaevarans* DSM 5391T, and 97.7%, 97.3%, 97.3%, 97.3% and 97.1% similarity with type strains *Bacillus circulans* DSM 11T, *Bacillus novalis* JCM 21709T, *Bacillus soli* JCM 21710T, *Bacillus oceannisediminis* CGMCC 1.10115T and *Bacillus nealsonii* FO-92T, respectively. Phylogenetic analyses based on the separate 16S rRNA gene sequences using neighbour-joining, minimum-evolution and maximum-likelihood trees showed that all the algorithms produced a highly similar phylogenetic topology (Figs 1, S1 and S2, available in the online Supplementary Material). The phylogenetic trees indicated that strain 1DS3-10T belongs to the genus *Bacillus* in the class *Bacilli* and falls within the radiation of a cluster comprised of type strains *B. benzoaevarans* DSM 5391T, *B. circulans* DSM 11T and *B. nealsonii* FO-92T.

Genomic DNA was prepared according to the method of Marmur (1961), and the purity was checked spectrophotometrically. The DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962) with a Beckman DU 800 spectrophotometer (Beckman Coulter), using *Escherichia coli* K-12 as a reference strain. The DNA G+C content of strain 1DS3-10T was 33.5 mol%, which falls within the range of DNA G+C contents observed for other members of the genus *Bacillus*, namely 32–66 mol% (Logan & De Vos, 2009). DNA–DNA hybridization assays between strain 1DS3-10T and *B. benzoaevarans* DSM 5391T, *B. circulans* DSM 11T, *B. novalis* JCM 21709T, *B. soli* JCM 21710T, *B. nealsonii* FO-92T and *B. oceannisediminis* CGMCC 1.10115T were performed on a Beckman DU 800 spectrophotometer equipped with a controlled temperature programme, using the thermal denaturation and renaturation method of De Ley et al. (1970) as modified by Huss et al. (1983). Means of the three most confident readings were taken for calculation purposes. Strain 1DS3-10T exhibited 33.4% DNA–DNA relatedness to *B. benzoaevarans* DSM 5391T, 25.8% to *B. circulans* DSM 11T, 25.0% to *B. novalis* JCM 21709T, 21.9% to *B. soli* JCM 21710T, 27.8% to *B. nealsonii* FO-92T and 17.9% to *B. oceannisediminis* CGMCC 1.10115T. These values were significantly lower than 70%, the threshold value recommended for the delineation of species (Wayne et al., 1987), which supported the conclusion from the 16S rRNA gene sequence data that 1DS3-10T represents a novel species in the genus *Bacillus*.

Cells of strain 1DS3-10T and the reference strains were grown on TSA (Difco) medium at 28°C for 36–48 h and harvested at a similar physiological age for chemical analysis. Fatty acid methyl esters were separated using a previously described method (Sasser, 1990). The fatty acid analyses were carried out by using the Sherlock Microbial Identification System (TSBA; version, 6.0 library) with the standard MIS Library Generation Software (Microbial ID) according to the manufacturer’s instructions. The major cellular fatty acids of strain 1DS3-10T were iso-C15:0 and anteiso-C15:0, which are the predominant cellular fatty acids found in all recognized members of the genus *Bacillus* (Logan & De Vos, 2009). The fatty acid profiles of strain 1DS3-10T and the reference strains are shown in Table 1. Strain 1DS3-10T produced major amounts of anteiso-C15:0 and iso-C15:0, which was consistent with its close relatives, but could be differentiated by the production of major amounts of iso-C14:0 and C16:1ω7c.

The quinones of the novel strain were analysed by HPLC (Minnikin et al., 1984); the respiratory quinone of 1DS3-10T was MK-7 (100%), which is one of the typical characteristics of the genus *Bacillus*. Polar lipid profiles have proved to be one of the most useful tools for the classification of members of the family *Bacillaceae* in recent years (Zhou et al., 2009). In this study, polar lipids were analysed following the polar lipid extraction procedure and tested by two-dimensional TLC according to the methods of Minnikin et al. (1984). Cells of 1DS3-10T were grown on TSA (Difco) medium at 28°C for 48 h and harvested for polar lipid analysis. Polar lipids of 1DS3-10T consisted of...
diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and unknown aminolipid (Fig. S3). The polar lipid profile of \textit{B. subtilis} has been shown to be characteristic of the genus \textit{Bacillus} and should form part of the genus description (Kämpfer et al., 2006). The polar lipid profile of \textit{B. subtilis} comprises DPG, PG, PE, unknown aminolipid

\begin{table}[h]
\centering
\caption{Cellular fatty acid profiles of strain 1DS3-10$^\text{T}$ and type strains of closely related species of the genus \textit{Bacillus}}
\begin{tabular}{|l|c|c|c|c|c|c|c|}
\hline
Fatty acids & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\
\hline
C14:0 & 2.5 & 1.5 & 0.3 & 0.5 & 0.7 & 5.8 & 2.0 \\
C16:0 & 6.0 & 9.5 & 1.0 & 0.8 & 1.3 & 11.4 & 3.9 \\
iso-C13:0 & 3.6 & 0.3 & 0.1 & 0.2 & 0.3 & 1.3 & 0.2 \\
iso-C14:0 & 12.4 & 3.4 & 2.3 & 1.7 & 3.6 & 6.7 & 5.5 \\
iso-C15:0 & 23.2 & 35.9 & 41.5 & 37.2 & 45.4 & 22.9 & 27.9 \\
iso-C16:0 & 2.4 & 8.3 & 13.4 & 1.1 & 3.6 & 6.0 & 4.8 \\
iso-C17:0 & 0.5 & 10.9 & 9.4 & 2.1 & 1.2 & 2.8 & 1.4 \\
antieiso-C15:0 & 30.9 & 23.0 & 3.2 & 36.4 & 34.0 & 33.1 & 12.3 \\
antieiso-C16:0 & 1.1 & 5.6 & 1.4 & 1.7 & 2.7 & 7.0 & 2.4 \\
C16:1ω7c alcohol & 1.3 & – & 15.2 & 2.2 & 2.0 & 0.1 & 10.7 \\
C16:1ω11c & 10.1 & 0.1 & 2.5 & 2.9 & 1.7 & 0.2 & 5.9 \\
C18:1ω9c & 0.6 & 0.2 & 0.2 & 0.3 & 0.2 & 0.4 & 1.6 \\
iso-C17:1 & 0.7 & – & 5.1 & 7.9 & 1.0 & – & 2.7 \\
\hline
\end{tabular}
\end{table}
an unknown aminophospholipid and β-gentiobiosyldiacetyl-
glycerol (Kämpfer et al., 2006). The polar lipid profile of
strain 1DS3-10$^T$ was similar to that for members of the
genus Bacillus. The cell-wall peptidoglycan was isolated after
disruption of the cells by shaking with glass beads and
subsequent total hydrolysis (4 M HCl, 100 °C, 16h). The
whole-cell sugar composition and diagnostic isomers of dia-
minopimelic acid were analysed by TLC as described by
Lechevalier & Lechevalier (1980). Strain 1DS3-10$^T$
contained the cell-wall peptidoglycan meso-diaminopimelic
acid, which is the most common murein cross-linkage
type in genus Bacillus (Fig. S4).

Cell morphology was observed by light microscopy (CX21;
Olympus). Growth at 4, 10, 20, 30, 37, 40, 45 °C and 50 °C
was determined in TSB (Difco) medium. Growth at 0, 1.0
2.0, 3.0, 4.0 and 5.0 % (w/v) NaCl was determined in TSB
medium consisting of 17 g tryptone l$^{-1}$, 3 g soy peptone
1$^{-1}$, 2.5 g glucose 1$^{-1}$ and 2.5 g K$_2$HPO$_4$ 1$^{-1}$ with different
concentrations of NaCl. Growth at pH 5.0–11.0 (at intervals
of 0.5 pH units) was determined in TSB medium (Difco)
adjusted by adding 1 M HCl or NaOH. Gram staining was
determined by adding 1 M HCl or NaOH. Gram staining was
subsequently tested as described by Dong & Cai (2001). Other tests to
determine biochemical characteristics were performed by
using API ZYM, API 20E and API 50CH strips (Bio-
Mérieux). Results of API tests were detected after 4 days of
incubation at 28 °C. Comparison of the characteristics of the
novel strain 1DS3-10$^T$ with those of closely related spe-
cies of the genus Bacillus is shown in Table 2. Detailed
results of physiological and biochemical analyses are given
in the species description. Several phenotypic characteristics
differentiated strain 1DS3-10$^T$ from the phylogenetically
related species. For example, cells of 1DS3-10$^T$ were rod
shaped (Fig. S5), but cells of the most closely related species,
B. benzoeverans, were filamentous. Growth occurred in the
presence of 4 % but not 5 % NaCl. Starch was not
hydrolysed. Acids were produced from D-glucose, D-fructose,
inositol, D-mannitol, methyl α-D-glucoside, N-
acetylglicosamine, maltose, sucrose and D-tagatose, but not
cellobiose or arbutin.

**Table 2.** Differential characteristics of strain 1DS3-10$^T$ and closely related species of genus Bacillus

<table>
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<tr>
<th>Characteristic</th>
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<tr>
<td>Cell shape</td>
<td>Rods</td>
<td>Filamentous</td>
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<td>Growth with NaCl (5 %)</td>
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<td>Growth at pH 11</td>
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<td>Growth at 50 °C</td>
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<td>Hydrolysis of:</td>
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<td>Casein</td>
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<td>Starch</td>
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<td>+</td>
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<td>Use of citrate</td>
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<td>Urease</td>
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<td>Arginine dihydrodase</td>
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<td>Acid production from:</td>
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<td>L-Arabinose</td>
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<td>D-Glucose</td>
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<td>D-Fructose</td>
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<td>Inositol</td>
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<td>D-Mannitol</td>
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<td>Methyl α-D-glucoside</td>
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<td>N-Acetylglucosamine</td>
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<td>Maltose</td>
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<td>Cellobiose</td>
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<td>Arbutin</td>
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<td>DNA G+C content (mol%)</td>
<td>33.5</td>
<td>41.3</td>
<td>35.7</td>
<td>40.5</td>
<td>40.1</td>
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<td>44.8</td>
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Gram-stain-positive, aerobic and forms endospores. Cells are rod-shaped. Forms circular colonies, creamy in colour, on TSA at 30 °C. Catalase- and oxidase-negative. Growth occurs at pH 6.0–11.0 and is optimal at pH 7–8. Temperature range for growth is 10–45 °C, optimum growth temperature is 30 °C. Grows in the presence of 4 % but not 5 % NaCl. Nitrate is reduced. Casein is hydrolysed, while starch and gelatin are not hydrolysed. Negative for H₂S and indole production. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, chymotrypsin, acid phosphatase, α-glucosidase, β-galactosidase and urease. Acid is produced from l-arabinose, D-glucose, D-fructose, inositol, D-mannitol, methyl α-D-glucoside, N-acetyl-glucosamine, aesculin, maltose, sucrose and D-tagatose. The major cellular fatty acids are iso-C₁₅:0 and anteiso-C₁₅:0; iso-C₁₄:0; C₁₆:1ω11c and C₁₆:0 (>5 % of total cellular fatty acids). The major menaquinone is MK-7 (100%). Polar lipids consist of diphasphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and an unknown aminolipid. Cell-wall peptidoglycan contains meso-diaminopimelic acid.

The type strain, 1DS3-10T (=ACCC 19781T =DSM 29761T), was isolated from rice rhizosphere in Qiyang, Hunan Province, China. The DNA G+C content of the type strain is 33.5 mol%.

Acknowledgements

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References


