Novosphingobium oryzae sp. nov., a potential plant-promoting endophytic bacterium isolated from rice roots

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A novel endophytic bacterium, strain ZYY112T, isolated from rice roots, was characterized by a polyphasic approach. In phylogenetic analyses based on 16S rRNA gene sequences, ZYY112T showed highest sequence similarity to Novosphingobium sediminicola HU1-AH51T (97.2 %) and less than 97 % similarity with respect to other Novosphingobium species with validly published names. The DNA G + C content of strain ZYY112T was 60.8 mol%. The level of DNA–DNA relatedness between strain ZYY112T and N. sediminicola DSM 27057T was 33.7 % (reciprocal 5.2 %), which supported the suggestion that ZYY112T represented a novel species of the genus Novosphingobium. Ubiquinone Q-10 was the unique respiratory quinone (100 %). The polar lipid profile contained diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, sphingoglycolipid, an unknown aminolipid and an unknown phospholipid. The major fatty acids of strain ZYY112T were summed feature 8 (consisting of C18 : 1ω7c and/or C18 : 1ω6c), summed feature 3 (consisting of C16 : 1ω7c and/or C16 : 1ω6c), C14 : 0 2-OH and C16 : 0. The major polyamine of ZYY112T was spermidine, which is a characteristic trait of the genus Novosphingobium. Characterization by genotypic, chemotaxonomic and phenotypic analysis indicated that strain ZYY112T represents a novel species of the genus Novosphingobium, for which the name Novosphingobium oryzae sp. nov. is proposed. The type strain is ZYY112T (=ACCC 06131T =JCM 30537T).

The genus Novosphingobium was separated from the genus Sphingomonas by Takeuchi et al. (2001) on the basis of phylogenetic and chemotaxonomic analysis. At the time of writing, the genus Novosphingobium comprised 30 species with validly published names (http://www.bacterio.net/index.html). Strains assigned to the genus Novosphingobium have been isolated from diverse habitats including freshwater, soil, sea sediments, wastewater treatment plants, bioremediation reactor of contaminated ground water and hexachlorocyclohexane-contaminated soil (Yabuuchi et al., 1990; Takeuchi et al., 1995, 2001; Balkwill et al., 1997; Köpfmer et al., 2002, 2011; Fujii et al., 2003; Sohn et al., 2004; Liu et al., 2005; Tiirloa et al., 2005; Addison et al., 2007; Lim et al., 2007; Suzuki & Hiraishi, 2007; Glaeser et al., 2009, 2013; Gupta et al., 2009; Yuan et al., 2009; Baek et al., 2011; Niharika et al., 2013) and Arabidopsis thaliana rhizosphere (Lin et al., 2014). During

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Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ZYY112T is KJ940052.

Two supplementary figures are available with the online Supplementary Material.
the study of long-term manure rotation effects on the rice endophytic microbial communities, more than 200 strains were isolated from rice roots (Zhang et al., 2013). Among those strains, ZYY112T was identified as representing a potentially novel species of the genus *Novosphingobium*. It had growth-promoting characteristics including the production of phytohormones, siderophore compounds and solubilizing phosphate (data not shown). We performed a polyphasic taxonomic study on strain ZYY112T, and here propose that it represents a novel species of the genus *Novosphingobium*.

Rice roots were taken from Qiyang County of Hunan Province, China, at tillering stage. Root tissue (5 g) was used to isolate the endophytes according to the previously described methods of Zhang et al. (2011). Routine cultivation of strain ZYY112T was performed with R2A agar (Difco) or trypticase soy agar (TSA; Difco) medium adjusted to pH 7.0 at 28 °C. For long-term storage at −80 °C, 20 % (v/v) glycerol was added. In this study, *Novosphingobium sediminicola* DSM 27057T, *Novosphingobium rosa* DSM 7285T and *Novosphingobium barchaimii* DSM 25411T were used as reference strains. Cell morphology was observed by light microscopy (CX21; Olympus) and transmission electron microscopy (H7650; Hitachi). Growth at 4, 10, 20, 25, 30, 35, 37, 40 and 45 °C was determined in trypticase soy broth (TSB; Difco) medium. Growth with 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 % (w/v) NaCl was determined in TSB medium consisting of (per litre) 17 g tryptone, 3 g soy peptone, 2.5 g glucose 2.0 % (w/v) NaCl was determined in TSB medium consisting of (per litre) 17 g tryptone, 3 g soy peptone, 2.5 g glucose and 2.5 g K2HPO4 with different concentrations of NaCl. Growth at pH 3–10 (at intervals of 1.0 pH units) was determined in TSB medium (Difco). Gram staining was performed using a Gram-stain kit (HiMedia). Catalase and oxidase activities and hydrolysis of casein, aesculin and starch were tested as described by Cowan & Steel (1965). Other tests to determine biochemical characteristics were performed by using API 20NE and API 20E strips (bioMérieux), Biolog GN MicroPlates according to the manufacturers’ instructions. Results of API tests were detected after 4 days of incubation.

Chromosomal DNA was extracted as described by Marmur (1961). The 16S rRNA gene was amplified using universal primers 27F and 1492R according to Lane (1991). A continuous stretch of 1462 bp of the 16S rRNA gene sequence of strain ZYY112T was obtained and a homology search of the 16S rRNA gene sequence was done via the EzTaxon-e server (Kim et al. 2012). Multiple alignments with sequences of related type strains were performed using CLUSTAL W (Thompson et al., 1997). Phylogenetic trees were reconstructed by the maximum-likelihood (ML), neighbour-joining (NJ) and maximum-parsimony (MP) algorithms by MEGA 6.0 (Tamura et al., 2013). For ML analysis, the best fit model for nucleotide substitution was selected from 24 models using MEGA 6.0 based on the minimum Bayesian information criterion value. The best model in this study was the Kimura two-parameter model with gamma-distributed rates plus invariant sites. The ML tree was built using the best fit model and nearest neighbour interchange for the ML heuristic method. ML, NJ and MP trees were built with partial deletion of gaps, and reliability of the phylogenetic trees was estimated using bootstrap values based on 1000 iterations (Tamura et al., 2013).

DNA–DNA hybridization assays between strain ZYY112T and *N. sediminicola* DSM 27057T were performed in triplicate on a Beckman DU 800 spectrophotometer equipped with a temperature programme controller, using the thermal denaturation and renaturation method of De Ley et al. (1970), as modified by Huss et al. (1983). The DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962) with a Beckman DU 800 spectrophotometer (Beckman Coulter); *Escherichia coli* K-12 was used as a reference strain.

For determination of fatty acid composition, cells of strain ZYY112T and the reference strains were harvested from TSA (Difco) medium at the same physiological age. Fatty acid methyl esters were separated using the method of Sasser (1990). Fatty acid analyses were carried out by the Sherlock Microbial Identification System (TSBA; library version 6.0) with the standard MIS Library Generation Software (Microbial ID) according to the manufacturer’s instructions. The quinones of the strain were analysed by HPLC (Minnikin et al. 1984). 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). Polyamines were extracted from 100 mg of lyophilized cells by boiling the cells in 0.2 M perchloric acid as described by Busse et al. (1997), and sequentially benzoylated using the methods described by Taibi et al. (2000). Detection of polyamines was performed by using a reversed-phase HPLC device equipped with a UV detector (234 nm) (Series 20 HPLC; Shimadzu) and Watcher 120 ODS-AP column (250 × 4.6 mm i.d., 5 μm particle size) (Isu Industry). Standard compounds of putrescine, spermidine and spermine were purchased from Sigma, and *Sphingosinella microcystinivorans* KCTC 12019T was used as standard for sym-homospermidine.

Cells of strain ZYY112T were rod-shaped, Gram-stain-negative, aerobic, and about 1.0 μm long and 0.7 μm wide in the mid-exponential phase (Fig. S1, available in the online Supplementary Material). Colonies were small and yellow with smooth surface after 72–96 h at 28 °C on R2A agar plates. Growth occurred at 20–40 °C (optimum: 35 °C), at pH 5.0–8.0 (optimum: 7.0) and with 0–0.8 % (w/v) NaCl (optimum: 0 %). Other physiological and biochemical characteristics are described in detail in Table 1. Most of the characteristics were in accordance with those of members of the genus *Novosphingobium*. However, other characteristics such as β-galactosidase activity, hydrolysis of starch, nitrate reduction, assimilation of D-fructose, α-D-glucose, maltose, D-mannose, L-rhamnose, D-sorbitol, sucrose, DL-lactic acid, L-proline, cellobiose and L-leucine could be used to differentiate strain ZYY112T from *N. sediminicola* DSM 27057T.
16S rRNA gene sequence similarity between strain ZYY112T and N. sediminicola HU1-AH51T was 97.2 % and lower than 97.0 % with all other Novosphingobium species. Phylogenetic analyses based on the 16S rRNA gene sequences using ML (Fig. 1), combined with the NJ and MP algorithms (data not shown), indicated that it belongs to the genus Novosphingobium in the class Gammaproteobacteria and falls within the radiation of a cluster comprising N. sediminicola HU1-AH51T. Strain ZYY112T showed 33.7 % (reciprocal 5.2 %) DNA–DNA relatedness to N. sediminicola DSM 27057T, a value significantly lower than the 70 % threshold recommended for the delineation of species (Wayne et al., 1987), indicating that ZYY112T represents a different genomic species from N. sediminicola. The DNA G+C content was 60.8 mol%, which was within the range reported for Novosphingobium species (60–65 %) (Suzuki & Hiraishi 2007). The major fatty acids (>10 %) of strain ZYY112T were summed feature 8 (consisting of C18:1ω7c and/or C18:1ω6c, 50.7 %), summed feature 3 (consisting of C16:1ω7c and/or C16:1ω6c, 14.5 %), C14:0 2-OH (13.2 %) and C16:0 (12.9 %). This profile was consistent with those of recognized Novosphingobium species (Takeuchi et al. 2001), but strain ZYY112T contained a unique fatty acid, cyclo-C17:0, which was not detected in the closely related reference strains N. sediminicola DSM 27057T, N. rosa DSM 7285T or N. barchaimii DSM 25411T (Table 2). Also, cyclo-C19:0ω8c was detected in strain ZYY112T but not in the most closely related species N. sediminicola DSM 27057T (Table 2). The quinone system consisted of ubiquinone Q-10 (100 %), which is consistent with results that Q-10 is the major quinone in members of the genus Novosphingobium (Takeuchi et al. 2001). Analysis of the polar lipids showed that strain ZYY112T contained diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and sphingoglycolipid as major components (Fig. S2), which supports the affiliation of strain ZYY112T to the genus Novosphingobium (Kämpfer et al. 2002). However, the polar lipid profile of ZYY112T was significantly different from that of N. sediminicola DSM 27057T. The major polar lipids of N. sediminicola DSM 27057T are phosphatidylethanolamine, phosphatidylglycerol and sphingoglycolipid as major components (Fig. S2), which supports the affiliation of strain ZYY112T to the genus Novosphingobium (Takeuchi et al. 2001).
In conclusion, on the basis of its morphological, physiological, biochemical, chemotaxonomic and phylogenetic characteristics, we consider that strain ZYY112T represents a novel species of the genus Novosphingobium, for which the name Novosphingobium oryzae sp. nov. is proposed.

**Description of Novosphingobium oryzae sp. nov.**

*Novosphingobium oryzae* (o.ry'zae. L. gen. n. oryzae of rice, the origin of the type strain).

Cells are rod-shaped, Gram-stain-negative, aerobic, about 1.0 μm long and 0.7 μm wide. Forms small yellow colonies
with a smooth surface after 72–96 h at 28 °C on R2A agar plates. Growth occurs at 20–40 °C, at pH 5.0–8.0 and with 0–0.8 % (w/v) NaCl. Positive for catalase, oxidase, nitrate reduction and β-galactosidase activity; negative for indole production. Aesculin is hydrolysed, whereas urea, gelatin, citrate, arginine, casein and starch are not. Positive for utilization of L-arabinose, D-arabitol, L-fucose, xylitol, trehalose, succinic acid monomethyl ester, α-ketoglutaric acid, DL-lactic acid, succinic acid and urocanic acid, weakly positive for utilization of D-galactose, α-D-glucose, D-mannose, D-sorbitol, pyruvic acid methyl ester, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketobutyric acid, succinamic acid and L-proline, but negative for L-histidine, L-leucine, L-phenylalanine, D-fructose, L-rhamnose, melibiose, malic acid, cellobiose and sucrose. The respiratory quinone is ubiquinone Q-10 (100 %). The polar lipid profile contains diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, sphingoglycolipid, an unknown aminolipid and an unknown phospholipid. The major fatty acids (> 10 %) are summed feature 8 (consisting of C18:1ω7c and/or C16:1ω6c), summed feature 3 (consisting of C16:1ω7c and/or C16:1ω6c), C14:0 2-OH and C16:0. The major polyamine is spermidine.

The type strain, ZYY112T (=ACCC 06131T=JCM 30537T), was isolated from rice roots in Hunan province, China. The DNA G+C content of the type strain is 60.8 mol%.

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