Proposal of *Novosphingobium rhizosphaerae* sp. nov., isolated from the rhizosphere

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A yellow, Gram-stain-negative, rod-shaped, non-spore-forming bacterium (strain JM-1^T) was isolated from the rhizosphere of a field-grown *Zea mays* plant in Auburn, AL, USA. 16S rRNA gene sequence analysis of strain JM-1^T showed high sequence similarity to the type strains of *Novosphingobium capsulatum* (98.9 %), *Novosphingobium aromaticivorans* (97.4 %), *Novosphingobium subterraneum* (97.3 %) and *Novosphingobium taihuense* (97.1 %); sequence similarities to all other type strains of species of the genus *Novosphingobium* were below 97.0 %. DNA–DNA hybridizations of strain JM-1^T and *N. capsulatum* DSM 30196^T, *N. aromaticivorans* SMCC F199^T and *N. subterraneum* SMCC B0478^T showed low similarity values of 33 % (reciprocal: 21 %), 14 % (reciprocal 16 %) and 36 % (reciprocal 38 %), respectively. Ubiquinone Q-10 was detected as the major respiratory quinone. The predominant fatty acid was $C_{18:1}\omega7c$ (71.0 %) and the typical 2-hydroxy fatty acid $C_{14:0}$ 2-OH (11.7 %) was detected. The polar lipid profile contained the diagnostic lipids diphosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid and phosphatidylcholine. Characterization by 16S rRNA gene sequence analysis, physiological parameters, pigment analysis, and ubiquinone, polar lipid and fatty acid composition revealed that strain JM-1^T represents a novel species of the genus *Novosphingobium*. For this species we propose the name *Novosphingobium rhizosphaerae* sp. nov. with the type strain JM-1^T (=LMG 28479^T=CCM 8547^T).

Members of the genus *Novosphingobium* are widely distributed in the environment including soil, coastal or freshwater sediments (Balkwill *et al.*, 1997; Sohn *et al.*, 2004; Liu *et al.*, 2005), surface water layers of lakes (Glaeser *et al.*, 2009; 2013a, b), activated sludge/wastewater treatment plants (Neef *et al.*, 1999; Fujii *et al.*, 2003), contaminated groundwater bioremediation reactors (Tiirola *et al.*, 2002; 2005), and associated with plants (Lin *et al.*, 2014). At the time of writing, 29 species of the genus *Novosphingobium* with validly published names had been described: *Novosphingobium acidiphilum* (Glaeser *et al.*, 2009), *N. aquaticum* (Glaeser *et al.*, 2013a), *N. aquiterrae* (Lee *et al.*, 2014b), *N. arabidopsis* (Lin *et al.*, 2014), *N. aromaticivorans* (Balkwill *et al.*, 1997), *N. barchaimii* (Niharika *et al.*, 2013), *N. capsulatum* (Yabuuchi *et al.*, 1990), *N. chloroacetimidivorans* (Chen *et al.*, 2014), *N. fuchskuhlense* (Glaeser *et al.*, 2013b), *N. hassiacum* (Kämpfer *et al.*, 2002), *N. indicum* (Yuan *et al.*, 2009), *N. kunmingense* (Xie *et al.*, 2014), *N. lentum* (Tiirola *et al.*, 2005), *N. lindaniclasticum* (Saxena *et al.*, 2013), *N. malaysiense* (Lee *et al.*, 2014a), *N. mathurense* and *N. panipatense* (Gupta *et al.*, 2009), *N. naphthalenivorans* (Suzuki & Hiraishi, 2007), *N. nitrogenifigens* (Addison *et al.*, 2007), *N. pentaromativorans* (Sohn *et al.*, 2004), *N. rosa* (Takeuchi *et al.*, 1995), *N. resinovorum* (Lim *et al.*, 2007), *N. sediminicola* (Baek *et al.*, 2011), *N. soli* (Kämpfer *et al.*, 2011), *N. stygium* (Balkwill *et al.*, 1997), *N. subarcticum* (which is a later subjective synonym of *N. resinovorum*; Lim *et al.*, 2007); *N. subterraneum* (Balkwill *et al.*, 1997), *N. taihuense* (Liu *et al.*, 2005) and *N. tardaugens* (Fujii *et al.*, 2000). A further species proposal was published recently, but the species name '*Novosphingobium ginsenosidimutans*' (Kim *et al.*, 2013) has not yet been validly published.

Strain JM-1^T was isolated from the rhizosphere of a field-grown *Zea mays* plant in Auburn, AL, USA. The strain produced single cells, which formed small yellow colonies (<0.5 mm) showing a smooth surface after 48 h at 25 °C on nutrient agar (NA). Cell morphological features were investigated by phase-contrast microscopy with cells grown on NA at 25 °C. The rod-shaped cells of strain JM-1^T were

Abbreviations: pNA *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JM-1^T is KM365125.

1.8 ± 0.4 μm long and 1.0 ± 0.2 μm wide and motile in the early growth phase, as observed by light-microscopy. Cells stained Gram-negative and were positive for cytochrome oxidase as determined by using an oxidase test (Merck). Endospores could not be detected. The strain produced catalase, after testing 24-hour-old colonies with H_2O_2.

The 16S rRNA gene of strain JM-1^T was sequenced using the universal bacterial 16S rRNA gene targeting primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′; Lane, 1991) for PCR-amplification and Sanger sequencing. The sequence was processed and manually controlled based on the electropherograms using MEGA5 (Tamura *et al.*, 2011). After unclear 5′ and 3′ ends were removed, the final sequence was 1443 nt long spanning 16S rRNA gene positions 16 to 1527 (according to Escherichia coli numbering; Brosius *et al.*, 1978). Phylogenetic analyses were performed in the software package ARB release 5.2 (Ludwig *et al.*, 2004). Therefore, the 16S rRNA gene sequence of strain JM-1^T was aligned against an expert-based reference alignment using the SILVA Incremental Aligner (SINA; v1.2.11) (Pruesse *et al.*, 2012) and incorporated into the All Species Living Tree (LTP) database. In the same manner, type strain sequences representing the genus *Novosphingobium* that were not included in the LTP database were added by using sequences published in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The alignment including all type strain sequences representing the genus *Novosphingobium* was checked manually before further analysis. Sequence similarities were calculated using the ARB neighbour-joining tool, without the application of an evolutionary model. Different treeing methods were applied for the calculation of phylogenetic trees including all type strains representing the genus *Novosphingobium*, considering 16S rRNA gene sequences between sequence positions 68 and 1450 (according to E. coli numbering; Brosius *et al.*, 1978). A maximum-likelihood tree was calculated using RAxML version 7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis, a neighbour-joining tree using ARB neighbour joining with the Jukes–Cantor correction (Jukes & Cantor, 1969), and a maximum-parsimony tree using DNAPARS v 3.6

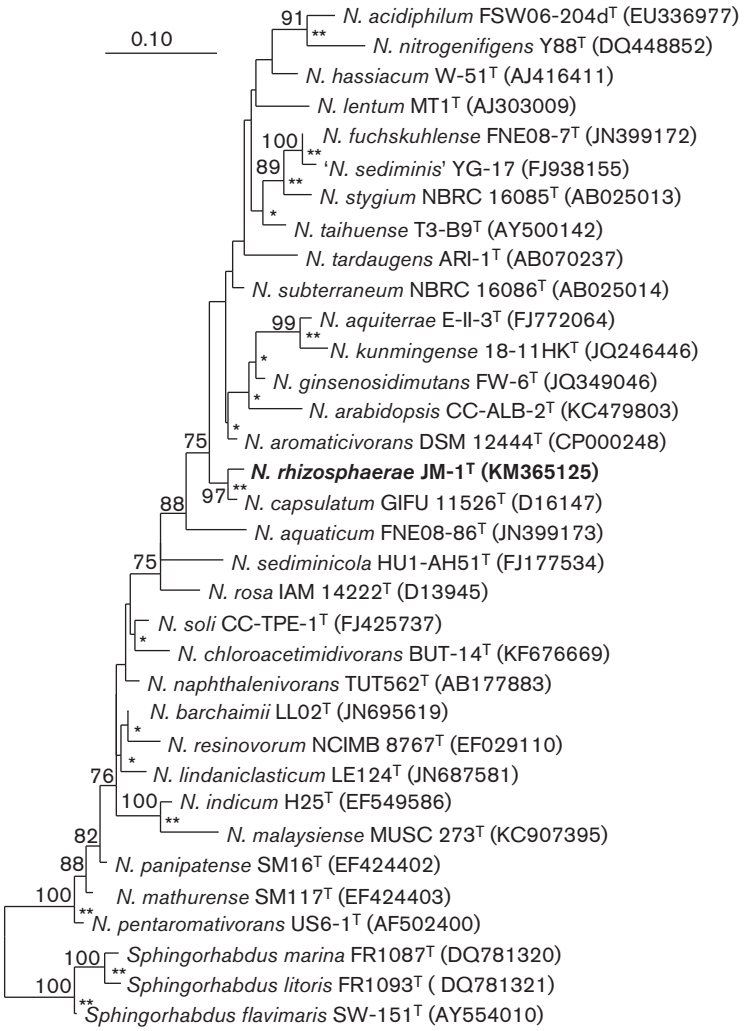

Fig. 1. Maximum-likelihood tree showing the phylogenetic position of JM-1^T among all type strains and currently proposed species of the genus *Novosphingobium*. The phylogenetic tree is based on nearly full-length 16S rRNA gene sequences and was calculated in ARB using the RAxML treeing method. Type strains of species of the genus *Sphingorhabdus* were used as an outgroup. Nodes marked with an asterisk were also conserved in the trees generated with the neighbour-joining and maximum-parsimony methods; one asterisk represents nodes that occurred with both of these alternative methods, two asterisks mark nodes that occurred in both or at least in the maximum-parsimony tree with high bootstrap values (>70 %). Numbers at nodes represent bootstrap values based on 100 resamplings; only values >70 % are depicted. Bar, 0.1 substitutions per 100 nucleotides.

(Felsenstein, 2005). All trees considered 100 resamplings (bootstap analysis; Felsenstein, 1985). Comparison of the 16S rRNA gene sequence similarities revealed highest similarity of strain JM-1^T to the type strains of *N. capsulatum* (98.9 %), *N. aromaticivorans* (97.4 %), *N. subterraneum* (97.3 %) and *N. taihuense* (97.1 %). Similarities to all other species of the genus *Novosphingobium* were below 97.0 %. The phylogenetic trees all showed a distinct clustering of strain JM-1^T with the type strain of *N. capsulatum* (Fig. 1).

DNA–DNA hybridization experiments with strain JM-1^T and the type strains of the three phylogenetically closest related species were performed as described previously (Kämpfer *et al.*, 2002) with DNA extracted using the method of Pitcher *et al.* (1989). Hybridization analysis between JM-1^T and *N. capsulatum* DSM 30196^T, *N. aromaticivorans* SMCC F199^T and *N. subterraneum* SMCC B0478^T revealed low similarity values of 33 % (reciprocal: 21 %), 14 % (reciprocal 16 %) and 36 % (reciprocal 38 %), respectively. Further characterization of strain JM-1^T was performed using a substrate assimilation panel and enzyme tests with chromogenic substrates [*p*-nitrophenyl (pNP)- and *p*-nitroanilide (pNA)-linked substrates] (Kämpfer *et al.*, 1991) and additional tests, among them the indole reaction with Ehrlich's and Kovacs' reagents, the activity of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and urease, and reduction of nitrate to nitrite (Kämpfer, 1990).

Results of the comparative characterizations are given in Table 1. The results showed that strain JM-1^T can be distinguished clearly from the most closely related species of the genus *Novosphingobium*.

Fatty acids were analysed from biomass grown on tryptone soy agar, as described by Kämpfer & Kroppenstedt (1996), and harvested in the exponential growth phase after 24 h of incubation, and the fatty acids were analysed as their methyl esters and identified using the Sherlock Microbial Identification System (Sherlock software version 2.11 and TSBA peak naming table version 4.1; MIDI).

The dominating fatty acid of JM-1^T was $C_{18:1}\omega7c$ (71 %); the characteristic 2-hydroxy fatty acid $C_{14:0}$ 2-OH (11.7 %) could be detected as well, but not $C_{15:0}$ 2-OH or $C_{16:0}$ 2-OH found in other species of the genus *Novosphingobium* (Kämpfer *et al.*, 2002; Glaeser *et al.*, 2009). The detailed fatty acid profile of strain JM-1^T is shown in Table 2.

For quinone and polar lipid analyses, cells were grown on PYE agar (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2). Biomass for analyses of the quinone system and the polar lipids was harvested after growth of strain JM-1^T in Caso-Medium for 24 h at 28 °C. The harvested biomass was lyophilized and used for the analyses.

Respiratory quinones of strain JM-1^T were extracted and separated as described by Collins *et al.* (1977) and analysed by HPLC. The HPLC system used consisted of a model PU-2080 Plus intelligent HPLC pump, a model DG 2080-54 online degasser, a model CO-2060 Plus column oven, a model AS-2057 Plus sample injector and a model UV-2070 detector (Jasco). Menaquinones were eluted from a type RP 18 column (250 mm by 4 mm i.d.) by using a solution containing acetonitrile and 2-propanol (65 : 35, v/v) at a flow rate of 1.3 ml min^{-1} and a temperature of 20 °C. The

Table 1. Physiological test results of strain JM-1^T in comparison with the most closely related species of the genus *Novosphingobium*

Strains: 1, JM-1^T; 2, *N. capsulatum* DSM 30196^T; 3, *N. aromaticivorans* SMCC F199^T; 4, *N. subterraneum* SMCC B0478^T. All data from this study. All strains were negative for acid production from dulcitol, salicin, adonitol, inositol, methyl D-glucoside and erythritol; hydrolysis of pNP-phosphorylcholine, L-glutamate-γ-3-carboxy-pNA and L-proline-pNA; assimilation of *N*-acetyl-D-galactosamine, D-ribose, salicin, adonitol, *myo*-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, acetate, propionate, *cis*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, DL-3-hydroxybutyrate, itaconate, DL-lactate, mesaconate, pyruvate, suberate, L-alanine, β-alanine, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate. All strains were positive for acid production from glucose, lactose, sucrose, raffinose and maltose; hydrolysis of aesculin, ONPG, pNP β-D-glucuronide, pNP α-D-glucopyranoside, pNP β-D-glucopyranoside, pNP β-D-xylopyranoside, bis-pNP-phosphate, pNP-phenylphosphonate, 2-deoxythymidine-5′-thymidine-pNP-phosphate and L-alanine-pNA; assimilation of *N*-acetyl-D-glucosamine, cellobiose, D-glucose, maltose and sucrose.

Characteristic	1	2	3	4
Acid production from:				
Sucrose	+	+	+	−
D-Mannitol	−	−	−	+
L-Arabinose	+	+	−	+
Rhamnose	+	−	+	+
D-Xylose	+	+	+	−
Trehalose	−	+	−	−
Cellobiose	−	+	−	−
Melibiose	+	+	+	−
D-Mannose	+	+	−	−
Assimilation of:				
L-Arabinose	+	−	−	+
p-Arbutin	+	−	+	−
D-Fructose	+	−	+	+
D-Galactose	+	+	−	−
Gluconate	+	+	−	−
D-Mannose	+	−	+	+
α-Melibiose	−	+	−	+
L-Rhamnose	+	−	+	+
Trehalose	+	−	−	−
D-Xylose	−	−	(+)	(+)
trans-Aconitate	+	−	−	−
Fumarate	+	−	−	−
Glutarate	−	−	(+)	(+)
L-Malate	+	+	+	−
Oxoglutarate	+	−	−	−

Table 2. Whole-cell fatty acid profiles of strain JM-1^T and the type strains of related species of the genus *Novosphingobium*

Strains: 1, JM-1^T; 2, *N. capsulatum* DSM 30196^T; 3, *N. aromaticivorans* SMCC F/99^T; 4, *N. subterraneum* SMCC B0478^T. Values represent the percentage of total fatty acids. Localization of double bonds by counting from the methyl (ω) end of the carbon chain. Summed features represent groups of two or three fatty acids that could not be separated by GLC in the MIDI system; summed feature 3 contains $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

Fatty acid	1	2	3	4
Saturated fatty acids				
$C_{16:0}$	13.0	9.9	7.3	7.5
Unsaturated fatty acids				
$C_{16:1}\omega5c$	–	1.3	–	–
$C_{17:1}\omega6c$	–	1.8	3.1	3.3
$C_{18:1}\omega7c$	71.0	68.8	55.2	50.5
Hydroxy fatty acids				
$C_{14:0}$ 2-OH	11.7	12.0	12.4	19.2
Summed feature 3	4.4	6.2	22.0	19.7

detection wavelength was 269 nm. Ubiquinone 10 (Q-10) was the predominant respiratory quinone. This is in agreement with data reported for the described species of the family *Sphingomonadaceae*.

Polar lipids extracted by the method of Minnikin *et al.* (1979) were identified by two-dimensional TLC as described by Collins & Jones (1980). Strain JM-1^T exhibited a complex polar lipid profile, similar to those of other species of the genus *Novosphingobium* and containing the diagnostic polar lipids phosphatidylethanolamine, diphosphatidylglycerol, sphingoglycolipid, phosphatidylcholine and traces of phosphatidylglycerol (Fig. 2). One unknown glycolipid, an unknown lipid and two unknown aminolipids were detected as well. Phosphatidyldimethylethanolamine was not detected. The hydrophobic characteristic yellow pigment observed in *N. acidiphilum*, *N. fuchskuhlense*, *N. sediminicola* and *N. stygium* was detected. This characteristic lipid profile supported the affiliation of strain JM-1^T to the genus *Novosphingobium*.

A combination of the observed phylogenetic, chemotaxonomic and physiological differences, production of the characteristic hydroxylated fatty acid and several physiological features warrant the proposal of a separate species to accommodate strain JM-1^T.

Description of *Novosphingobium rhizosphaerae* sp. nov.

Novosphingobium rhizosphaerae [rhi.zo.sphae′rae. Gr. fem. n. *rhiza* root; L. fem. n. *sphaera -ae* (from Gr. fem. n. *sphaira -as*) ball, any globe, sphere; N.L. gen. fem. n. *rhizosphaerae* of the rhizosphere].

Cells are rod-shaped, 1.8 ± 0.4 μm long and 1.0 ± 0.2 μm wide, and motile in the early exponential phase of growth.

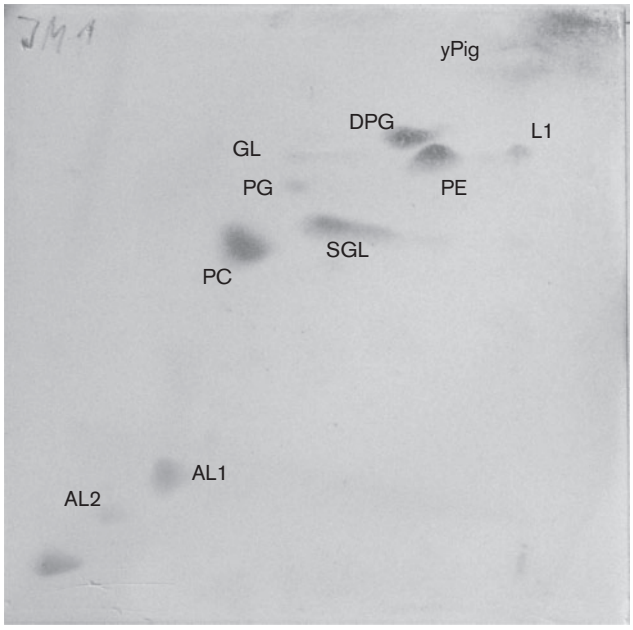

Fig. 2. Total polar lipid profile of strain JM-1^T after two-dimensional chromatography and staining with molybdatophosphoric acid. DPG, phosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SGL, sphingoglycolipid; PC, phosphatidylcholine; L1, unknown lipid; GL, glycolipid; AL1, AL2, unknown aminolipids; yPig, yellow pigment.

Chemo-organotrophic with a respiratory type of metabolism. Unable to reduce nitrate to nitrite. Catalase-positive. Good growth is observed on peptone-yeast-extract-agar (PYE), nutrient, tryptone soy and R2A agars at 25 °C. On NA, circular, yellow colonies are formed after incubation for 2–3 days at 25 °C. Cells are Gram-stain-negative and positive for cytochrome oxidase. Endospores are not observed. On NA, growth occurs between 15 and 36 °C, but not at 10 or 42 °C, and between pH 4.5 and 7.0, but not at pH 3.5 or 8.5. Negative for acid production from D-mannitol, trehalose, cellobiose, dulcitol, salicin, adonitol, inositol, methyl D-glucoside and erythritol, and positive for acid production from D-glucose, L-arabinose, L-rhamnose, raffinose, lactose, sucrose, D-xylose, maltose and melibiose. No hydrolysis of pNP-phosphorylcholine, L-glutamate-γ-3-carboxy-pNA or L-proline-pNA can by observed, but hydrolysis of aesculin, ONPG, pNP β-D-glucuronide, pNP α-D-glucopyranoside, pNP β-D-glucopyranoside, pNP β-D-xylopyranoside, bis-pNP-phosphate, pNP-phenylphosphonate, 2-deoxythymidine-5′-thymidine-pNP-phosphate and L-alanine-pNA is positive. Assimilation of *N*-acetyl-D-galactosamine, D-ribose, salicin, adonitol, *myo*-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, acetate, propionate, *cis*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, DL-3-hydroxybutyrate, itaconate, DL-lactate, mesaconate, pyruvate, suberate, L-alanine, β-alanine, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-tryptophan, 3-hydroxybenzoate,

4-hydroxybenzoate and phenylacetate is negative. *N*-Acetyl-D-glucosamine, cellobiose, D-glucose, L-arabinose, D-fructose, D-galactose, gluconate, maltose, D-mannose, trehalose, L-rhamnose, sucrose, oxoglutarate, L-malate and fumarate are utilized as sole sources of carbon. Indole formation from tryptophan, reduction of nitrate to nitrite and the activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and urease are negative. The fatty acid profile consists of major amounts of $C_{18:1}\omega7c$, and the 2-hydroxy fatty acid $C_{14:0}$ 2-OH. The polar lipid profile consists of the predominant lipids diphosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid and phosphatidylcholine. The quinone system is ubiquinone Q-10.

The type strain, JM-1^T (=LMG 28479^T=CCM 8547^T), was isolated from the rhizosphere of a *Zea mays* plant in Auburn, AL, USA.

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