Sphingomonas azotifigens sp. nov., a nitrogen-fixing bacterium isolated from the roots of *Oryza sativa*

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Three yellow-pigmented strains associated with rice plants were characterized by using a polyphasic approach. The nitrogen-fixing abilities of these strains were confirmed by acetylene reduction assay and *nifH* gene detection. The three strains were found to be very closely related, with 99.9% 16S rRNA gene sequence similarity and greater than 70% DNA–DNA hybridization values, suggesting that the three strains represent a single species. 16S rRNA gene sequence analysis indicated that the strains were closely related to *Sphingomonas trueperi*, with 99.5% similarity. The chemotaxonomic characteristics (G + C content of the DNA of 68.0 mol%, ubiquinone Q-10 system, 2-OH as the only hydroxy fatty acid and homospermidine as the sole polyamine) were similar to those of members of the genus *Sphingomonas*. Based on DNA–DNA hybridization values and physiological characteristics, the three novel strains could be differentiated from other recognized species of the genus *Sphingomonas*. The name *Sphingomonas azotifigens* sp. nov. is proposed to accommodate these bacterial strains; the type strain is Y39^T (=NBRC 15497^T = IAM 15283^T = CCTCC AB205007^T).

Oyaizu-Masuchi & Komagata (1988) reported the isolation of a number of free-living, nitrogen-fixing bacteria from the rhizosphere or roots of rice plants. These isolates were classified into ten groups on the basis of phenotypic and chemotaxonomic characteristics. Lacking phylogenetic studies, these isolates have remained unidentified. Here, we report on the classification of three strains, Y39^T (NBRC 15497^T), Y22 (NBRC 15496) and OSG47 (NBRC 15495), which belonged to group 2 in the original scheme. Takeuchi *et al.* (2001) classified these strains as representing *Sphingomonas sensu stricto*, based on 16S rRNA gene sequence analysis, and polyamines, fatty acids and some physiological traits. The strains were found to contain ubiquinone Q-10, homospermidine as the sole polyamine, a unique sphingoglycolipid and 2-hydroxy fatty acids, which are common chemotaxonomic features of the genus *Sphingomonas* (Kämpfer *et al.*, 1997; Takeuchi *et al.*, 2001). Based on the results of Oyaizu-Masuchi & Komagata (1988) and Takeuchi *et al.* (2001), together with newly obtained data in this study, we propose that the nitrogen-fixing strains Y39^T, Y22

and OSG47 represent a single novel species of the genus *Sphingomonas*.

Strains were grown at 25 °C in nitrogen-free medium (5.0 g glucose, 5.0 g lactose, 0.1 g CaCl₂·2H₂O, 0.1 g MgSO₄·7H₂O, 0.9 g K₂HPO₄, 0.1 g KH₂PO₄, 5 g CaCO₃, 10 mg FeSO₄·7H₂O, 5 mg Na₂MoO₄·2H₂O, 1 l distilled water, pH 7.3), TY medium (5.0 g tryptone, 0.75 g yeast extract, 0.454 g KH₂PO₄, 2.388 g Na₂HPO₄·2H₂O, 1.0 g CaCl₂, 1 l distilled water, pH 7.0), YMA medium (Jordan, 1984) or nutrient broth. *Sphingomonas trueperi* NBRC 100456^T and *Sphingomonas pituitosa* CIP 106154^T were grown in nutrient broth. Tolerance of salinity was determined by using YMA medium supplemented with 0–4.0% NaCl (w/v). The isolated strains and *S. trueperi* NBRC 100456^T and *S. pituitosa* CIP 106154^T were grown on TSA medium (trypticase soy agar; Becton Dickinson) for fatty acid extraction. Cellular fatty acid methyl esters were prepared, separated and identified by using the Microbial Identification system, as described by Xie & Yokota (2003). Data from acetylene reduction assays, quinone analyses and macromolecular hydrolysis were taken from Oyaizu-Masuchi & Komagata (1988). Polyamine composition was taken from Takeuchi *et al.* (2001).

DNA–DNA hybridization was performed by using the photobiotin-labelling method of Ezaki *et al.* (1989), using a multi-well plate reader (CytoFluoR; Perseptive Biosystems). The hybridization temperature was 52 °C and reciprocal

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Y39^T, Y22 and OSG47 are AB217471, AB217473 and AB217472, respectively, and that for the *nifH* gene sequence of strain Y39^T is AB217474.

A neighbour-joining tree based on *nifH* gene sequences showing the relationships between strain Y39^T and other nitrogen-fixing bacteria is available as supplementary material in IJSEM Online.

experiments were performed as follows. DNA of strain Y39^T was used as a probe for hybridization of itself, and for DNA of strains OSG47 and Y22, and *S. trueperi* NBRC 100456^T, *S. pituitosa* CIP 106154^T and a negative control (*Bacillus subtilis* IAM 12118^T). DNA of *S. trueperi* NBRC 100456^T was also used as a probe. PCR of 16S rRNA gene sequences and sequencing of the products were carried out as described previously (Xie & Yokota, 2003). A 750 bp fragment of the *nifH* gene sequence (encoding the iron protein of nitrogenase, a key enzyme in nitrogen fixation) was amplified from the extracted DNA using the forward primer IKG (5'-TACGGYAARGGBGGYATCGG-3'; Poly *et al.*, 2001) and the reverse primer R750 (5'-TCCATBGTGATGGGDCGGGATG-3'; this study) (Y = C/T; S = G/C; R = A/G; B = C/G/T). The sequences were compared with sequences obtained from GenBank and aligned using the CLUSTAL W software package (Thompson *et al.*, 1994); evolutionary distances and the K_{nuc} value (Kimura, 1980) were then calculated. Alignment gaps and ambiguous bases were excluded from the calculations. A phylogenetic tree based on comparison of 1352 bases was constructed using the neighbour-joining method (Saitou & Nei, 1987). The topology of this tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates, whereas similarity values were calculated using PAUP 4.0b1 (Swofford, 1998). Using the same methods, 415 bases of *nifH* sequences were also aligned and a phylogenetic tree was constructed.

Cells of strains Y39^T, Y22 and OSG47 are Gram-negative, straight rods and are motile by means of peritrichous flagella. Colonies are yellow–orange on agar medium and the spectral characteristics of the yellow pigment extracted in acetone had two peaks at 452 and 480 nm, corresponding to those of *S. pituitosa* DSM 13101^T (Denner *et al.*, 2001). The three isolates had only 2-hydroxy fatty acids and no 3-hydroxy fatty acids, consistent with the generic

characteristics of *Sphingomonas* (Takeuchi *et al.*, 2001). The sole cellular polyamine was homospermidine; putrescine, spermidine and agmatine were not present, which differentiates *Sphingomonas* from other genera (Takeuchi *et al.*, 2001; Kämpfer *et al.*, 1997). Similar to all species of the genus *Sphingomonas*, the three isolates contained ubiquinone Q-10. On the basis of chemotaxonomic characteristics, we consider the three strains studied to be members of the genus *Sphingomonas*.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strains OSG47, Y39^T and Y22 are affiliated with the genus *Sphingomonas* and are most closely related to *S. trueperi* NBRC 100456^T (99.5 % similarity) and *S. pituitosa* DSM 13101^T (99.0 % similarity) (Fig. 1). This sub-cluster, with a bootstrap confidence level of 100 %, is phylogenetically distant from other species of *Sphingomonas*, with not more than 96 % similarity, a level that clearly delineates different species. That the three isolates and *S. trueperi* NBRC 100456^T and *S. pituitosa* CIP 106154^T were found to have a close phylogenetic relationship does not imply that they should be considered as representing a single species (Fox *et al.*, 1992). Levels of DNA–DNA relatedness of strain Y39^T with the other isolates and *Sphingomonas* species were 78.9 % (OSG47), 80.6 % (Y22), 25.3 % (*S. trueperi* NBRC 100456^T) and 15.9 % (*S. pituitosa* CIP 106154^T). Levels of DNA–DNA relatedness of *S. trueperi* NBRC 100456^T with the three isolated strains were 29.7 % (Y39^T), 28.6 % (OSG47) and 27.7 % (Y22), and 16.5 % with *S. pituitosa* CIP 106154^T. These results suggest that strains OSG47, Y39^T and Y22 represent a single species and are distinct from *S. trueperi* NBRC 100456^T and *S. pituitosa* CIP 106154^T at the genomic level. Moreover, the three isolates could be distinguished from their close relatives by some phenotypic characteristics. When the fatty acid compositions of the strains were compared (Table 1), we found that

Fig. 1. 16S rRNA gene sequence-based phylogenetic tree generated by using the neighbour-joining method showing the relationships between the strains studied and representatives of the genus *Sphingomonas*. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 50 % are shown. Bar, 0.01 substitutions per nucleotide position.

the fatty acids of *S. trueperi* NBRC 100456^T were quite different from those of the isolated strains and *S. pituitosa* CIP 106154^T, with the former lacking 2-OH 15:0, 11-methyl 18:1 ω 7c and 19:0 cyclo ω 8c. On the other hand, the three isolates did not contain 16:1 ω 5c, in contrast to the two other strains. Moreover, the three strains could be easily distinguished from their closest phylogenetic neighbours, *S. trueperi* NBRC 100456^T and *S. pituitosa* CIP 106154^T (Table 2). *S. trueperi* NBRC 100456^T, formerly known as '*Pseudomonas azotocolligans*' Anderson 1955, has been proposed to be a diazotrophic bacterium. However, Hill & Postgate (1969) demonstrated that this organism could not fix nitrogen, and it was then reclassified as *S. trueperi* by Kämpfer *et al.* (1997).

In the present study, the *nifH* gene fragment of *S. trueperi* NBRC 100456^T was not detected by PCR (data not shown). In contrast, the three isolates showed very high acetylene-reducing activity and were found to possess the *nifH* gene, suggesting that the isolates belong to the first species of the genus *Sphingomonas* to be able to fix nitrogen. The phylogeny of the *nifH* gene has been reported to correlate well with that of the 16S rRNA gene, except for discrepancies with a few taxa (Xie & Yokota, 2004, 2005a, b; Moulin *et al.*, 2001; Rosado *et al.*, 1998; Young, 1992). Therefore, the *nifH* gene appears to fulfil the criteria for a molecule suitable for molecular phylogeny and has been successfully used as a phylogenetic marker (Ueda *et al.*, 1995). Part of the *nifH* gene of representative strain Y39^T was sequenced and a phylogenetic tree was constructed (Supplementary Fig. S1 in IJSEM Online). The highest similarities (98 %) were found with the uncultured diazotrophic bacteria (NIW samples; e.g. GenBank accession nos AF389758 and AF389759) associated with dead aboveground biomass of

Table 2. Differential characteristics of the three isolates of *S. azotifigens* sp. nov. and their closest relatives

Taxa: 1, *S. azotifigens* (results from the three isolates); 2, *S. trueperi* NBRC 100456^T (data from Denner *et al.*, 2001); 3, *S. pituitosa* CIP 106154^T (Kämpfer *et al.*, 1997); 4, *S. paucimobilis* DSM 1098^T (Kämpfer *et al.*, 1997). +, Positive; –, negative; W, weak; V, varies in different strains, but the type strain is negative; ND, not determined.

Characteristic	1	2	3	4
Nitrogen-fixing	+	–	–	–
DNase production	+	–	–	+
Liquefaction of gelatin	V	–	+	+
Oxidase	+	+	–	–
Acid produced from:				
Glucose	+	W	–	–
Sorbitol	–	–	+	W
Maltose	+	W	–	+
D-Xylose	+	W	–	+
Trehalose	+	–	–	–
Cellobiose	+	W	–	+
Melibiose	+	–	–	+
D-Mannose	+	–	–	+
Hydrolysis of:				
Aesculin	+	+	–	+
Starch	+	–	+	+
Tween 80	+	+	ND	+
Assimilation of:				
Suberate	+	–	–	–
L-Asparate	+	–	+	–
L-Alanine	–	+	+	–

Table 1. Cellular fatty acid composition (%) of *S. azotifigens* sp. nov. and its close relatives

Strains: 1, Y39^T; 2, OSG47; 3, *S. trueperi* NBRC 100456^T (data from Denner *et al.*, 2001); 4, *S. pituitosa* CIP 106154^T (Denner *et al.*, 2001).

Fatty acid	1	2	3	4
14:0	2.6	1.2	0.3	0.3
2-OH 14:0	13.3	19.3	6.6	15.3
Summed feature 3*	1.2	1.5	0.4	1.1
16:0	24.5	16.5	10.3	13.3
16:1 ω 5c	0.0	0.0	0.4	2.1
2-OH 15:0	0.9	1.1	0.0	0.1
17:1 ω 6c	1.4	1.5	1.0	1.0
18:1 ω 7c	44.7	47.1	75.0	56.4
18:1 ω 5c	1.2	1.9	4.7	1.7
18:0	3.8	2.3	1.0	0.3
11-Methyl 18:1 ω 7c	10.9	6.2	0.0	7.0
19:0 cyclo ω 8c	3.3	1.2	0.0	0.6

*Summed feature 3 contained 16:1 ω 7c and/or 15:0 iso 2-OH.

Spartina alterniflora (Lovell *et al.*, 2001), whereas there was very low similarity (less than 89 %) with other species with validly published names. The *nifH* phylogeny of the nitrogen-fixing proteobacteria consists of two distinctive monophyletic clades (*Alphaproteobacteria* and *Gamma-proteobacteria*) and one mixed group (*Alphaproteobacteria* and *Betaproteobacteria*). Strain Y39^T is closely related to the rhizobial genus *Bradyrhizobium* within the mixed group, which is distant from other common alphaproteobacteria. The diazotrophic bacteria in the mixed group are not congruent with the phylogeny of the organisms as deduced from 16S rRNA gene sequence analysis, suggesting that the nitrogen fixation of the three isolated strains occurs by lateral transfer. The possibility of lateral transfer of the *nifH* gene has been reported previously (Xiong *et al.*, 2000; Cantera *et al.*, 2004; Raymond *et al.*, 2004).

To our knowledge, the three isolates represent the first species of diazotrophic bacteria to belong to the genus *Sphingomonas*. Therefore, the name *Sphingomonas azotifigens* sp. nov. is proposed to accommodate the strains described here.

Description of *Sphingomonas azotifigens* sp. nov.

Sphingomonas azotifigens [a.zo.ti.fi'gens. French. n. *azote* (from Gr. pref. *a-* and Gr. n. *zoë*) nitrogen; N.L. n. *azotum* -i nitrogen; L. part. *figens* (from L. v. *figo*) fixing; N.L. part. adj. *azotifigens* nitrogen fixing].

The description is based on Oyaizu-Masuchi & Komagata (1988), Takeuchi *et al.* (2001) and this study. Cells are Gram-negative, aerobic, straight rods, $0.5\text{--}1.0 \times 1.0\text{--}3.0$ μm in size and motile by means of peritrichous flagella. Nitrogen-fixing. Colonies are circular, smooth, convex, opaque and yellow-orange on agar medium. The visible absorption spectrum of the acetone extract of the yellow pigment has two peaks at 452 and 480 nm. Cells contain poly- β -hydroxybutyrate granules. Optimum temperature for growth is $25\text{--}37^\circ\text{C}$; growth is inhibited at 42°C and in 2.5% NaCl. Starch, aesculin and Tween 80 are hydrolysed but not chitin. Catalase, oxidase, β -galactosidase, phosphatase and DNase are present but not indole or arginine dihydrolase. Does not produce H_2S or reduce nitrate. Acid is produced from L-arabinose, D-xylose, fructose, galactose, sucrose, maltose, lactose, trehalose, melibiose, cellobiose and D-mannose but not from D-arabinose, melezitose, adonitol, dulcitol, sorbitol, mannitol, inositol, ribose, inulin or ethanol. Assimilates acetate, L-aspartate, succinate, malate, pyruvate, arabinose, xylose, glucose, fructose, galactose, trehalose, sucrose, lactose, maltose, cellobiose, raffinose, suberate, β -hydroxybutyrate, glutamate and starch, but not citrate, malonate, *meso*-tartrate, mandelate, benzoate, *m*-hydroxybenzoate, β -aminobutyrate, proline, glycerol, gluconate, 2-ketogluconate, betaine, itaconate, adipate, mucate, L-alanine, methanol or ethanol. Major fatty acids are 18:1 ω 7 ζ , 16:0 and 11-methyl 18:1 ω 7 ζ ; there is a large amount of the hydroxy fatty acid 2-OH 14:0 and a minor amount of 2-OH 15:0, but cells do not contain 3-hydroxy fatty acid. Sphingolipids are present. Ubiquinone Q-10 is the major quinone. The G + C content of the DNA is 66.0–68.0 mol%.

The type strain is Y39^T (=NBRC 15497^T=IAM 15283^T=CCTCC AB205007^T). Strains Y39^T, OSG47 (=NBRC 15495) and Y22 (=NBRC 15496) were isolated from paddy soil and the roots of *Oryza sativa* in Japan.

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