Sphingomonas phyllosphaerae sp. nov., from the phyllosphere of *Acacia caven* in Argentina

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Two bacterial strains (FA1 and FA2^T) were isolated from the phyllosphere of a leguminous tree, *Acacia caven*, in central Argentina. The strains were Gram-negative, strictly aerobic, rod-shaped, motile and formed yellow-pigmented colonies on nutrient agar. The two-primer RAPD patterns of the two strains were identical, suggesting that they belong to the same species. The complete 16S rRNA gene sequences of the two strains were obtained and comparisons demonstrated that they cluster phylogenetically with the species of the genus *Sphingomonas sensu stricto*. Strain FA2^T was most closely related (97.6%) to *Sphingomonas adhaesiva*. 16S rRNA gene sequence similarities to all other established *Sphingomonas* species ranged from 94.4% (to *Sphingomonas echinoides*) to 97.6% (to *S. adhaesiva*). Strains FA1 and FA2^T were catalase-positive and oxidase-negative. Aesculin was hydrolysed, gelatin and urea were not. β -Galactosidase was produced. From 51 compounds tested 21 were used as single sources of carbon. The major respiratory lipoquinone was ubiquinone-10. The predominant cellular fatty acids were 16:0, 18:1 ω 7c and 16:1 ω 7c (from summed feature 3). Hydroxy fatty acids 14:0 2-OH and 15:0 iso 2-OH were present as well (from summed feature 4). The polar lipids detected in strain FA2^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, sphingoglycolipid and two unidentified phospholipids. The DNA G + C content of strain FA2^T was 61 mol%. DNA–DNA hybridization experiments showed 27.6% relatedness between strain FA2^T and *S. adhaesiva* DSM 7418^T. Based upon phenotypic and molecular evidence, a novel species of the genus *Sphingomonas* is proposed, *Sphingomonas phyllosphaerae* sp. nov., with strain FA2^T (=LMG 21958^T=CECT 5832^T) as the type strain.

The genus *Sphingomonas sensu stricto* encompasses species phylogenetically related to *Sphingomonas paucimobilis* according to the nomenclature proposed by Takeuchi *et al.* (2001). Many *Sphingomonas* species are associated with plants and produce yellow or orange pigments (Busse *et al.*, 2003). Recently, several novel species of this genus have been described: *Sphingomonas melonis* causes brown spots on yellow melon fruits (Buonaurio *et al.*, 2002) and *Sphingomonas aurantiaca*, *Sphingomonas areolata* and *Sphingomonas faenia* were isolated from dust and air (Busse *et al.*, 2003). During a study of phyllospheric bacteria of leguminous trees in central Argentina, we isolated two strains (FA1 and FA2^T) from *Acacia caven* that produce a yellow pigment.

Strain isolation from the phyllosphere was performed according to the methods of Behrendt *et al.* (2003). The strains were grown on nutrient agar (Difco) for 48 h at 22 °C, developing opaque, slimy and yellow colonies. Motility was checked by phase-contrast microscopy using a Nikon HFX microscope. For electron microscopy the cells were grown in nutrient broth (Difco) for 2 days at 180 r.p.m. and at 28 °C. The samples were stained with uranyl acetate and examined at 80 kV with a Zeiss EM 209 transmission electron microscope. The strains were Gram-negative, rod-shaped and motile (1.5–1.9 $\times$ 0.6–0.8 μ m) by means of a polar flagellum (data not shown).

Both strains were subjected to two-primer RAPD fingerprinting according to the methods of Rivas *et al.* (2001); they had the same pattern (available as Supplementary Fig. A in IJSEM Online), indicating that they belong to the same species.

The nearly complete 16S rRNA gene sequences (1481 nucleotides) for strains FA1 and FA2^T were obtained according to the methods of Rivas *et al.* (2003), showing 100% similarity,

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains FA2^T and FA1 are AY453855 and AY563441, respectively.

RAPD and polar lipid data and a full phylogenetic tree are available as supplementary material in IJSEM Online.

and compared with those from GenBank using BLAST (Altschul *et al.*, 1990). This indicated that the novel strains were phylogenetically related to *Sphingomonas* species. 16S rRNA gene sequence similarities to all established *Sphingomonas* species ranged from 94.3% (to *Sphingomonas echinoides*) to 97.6% (to *Sphingomonas adhaesiva*). The phylogenetic tree obtained with Kimura's two-parameter correction and the neighbour-joining method (Fig. 1; a fuller phylogenetic tree is available as Supplementary Fig. B in IJSEM Online) placed strain FA2^T in a separate group together with *S. adhaesiva* GIFU 2395^T (DSM 7418^T). The 16S rRNA gene sequence of strain FA2^T contained the nucleotide signatures that define the genus *Sphingomonas* according to Takeuchi *et al.* (2001).

Phenotypic analyses were performed according to the methods of Yabuuchi *et al.* (1990). Supplementary API 20NE and API 50CH galleries (bioMérieux) were used for characterization. The novel strains had the same phenotypic characteristics. Differential phenotypic characteristics between these strains and the phylogenetically closest species (*Sphingomonas sensu stricto*) are shown in Table 1. The strains isolated from rhizosphere of *Acacia caven* differ from *S. adhaesiva*, the most closely related species, in gelatinase production, acid production from glycerol, salicin and galactose and assimilation of mannose, maltose and citrate.

Non-polar and hydroxy fatty acid analyses were performed from a culture of strain FA2^T grown for 24 h in tryptic soy agar (TSA; Merck) at 28 °C. The main non-polar fatty acids detected were 16:0, 18:1 ω 7c and 16:1 ω 7c (from summed feature 3). The hydroxy fatty acids detected were 14:0 2-OH and 15:0 iso 2-OH (from summed feature 4). The fatty acid

Fig. 1. Dendrogram based on 1440 nt 16S rRNA gene sequences showing the phylogenetic position of *S. phyllosphaerae* sp. nov. FA2^T within the genus *Sphingomonas*. Nucleotide substitution rates (Kimura, 1980) were determined using CLUSTAL X (Thompson *et al.*, 1997) and the neighbour-joining method (Saitou & Nei, 1987) was used to construct the phylogenetic tree. Alignment gaps and unidentified base positions were excluded from the analysis. Bootstrap values (1000 replicates) are shown as percentages at each node for values $\geq 55\%$. Bar, 1 nt substitution per 100 nt. *Erythrobacter longus* was used as the outgroup.

profile of strain FA2^T is characteristic of strains from *Sphingomonas sensu stricto* (Kämpfer *et al.*, 1997; Takeuchi *et al.*, 2001). Main differences between strain FA2^T and *S. adhaesiva* are the presence of 14:0, 15:0 and 11-methyl 18:1 ω 7c, which were not detected in *S. adhaesiva* (Kämpfer *et al.*, 1997; Takeuchi *et al.*, 2001). Quantitative differences in the non-polar saturated fatty acid 16:0 and in the unsaturated acid 17:1 ω 6c were observed.

Quinone and lipid compositions were determined by HPLC (Tindall, 1990). Similarly to other *Sphingomonas* species (Busse *et al.*, 1999; Takeuchi *et al.*, 2001; Yabuuchi *et al.*, 2002), the major respiratory lipoquinone in strain FA2^T was ubiquinone Q-10. The polar lipid pattern of strain FA2^T after separation by two-dimensional TLC is available as Supplementary Fig. C. The polar lipids detected were di-phosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and sphingoglycolipid and two unidentified phospholipids, PL1 and PL2. Phosphatidylmonomethylethanolamine and phosphatidyl dimethylethanolamine were detected in small amounts. This polar lipid pattern is in good agreement with those found in other *Sphingomonas* species (Busse *et al.*, 1999), especially with that of *S. adhaesiva* according to the data of Kämpfer *et al.* (1997). The two unidentified phospholipids in FA2^T have only been detected infrequently in a few *Sphingomonas* species (Busse *et al.*, 1999).

DNA G+C content was determined according to the methods of Chun & Goodfellow (1995); the G+C content of strain FA2^T was 61 mol%, which is similar to other *Sphingomonas* species (Yabuuchi *et al.*, 2002). DNA–DNA hybridization analyses were done as described by Huß *et al.* (1983) and Escara & Hutton (1980). Renaturation rates were computed with the TRANSFER.BAS software by Jahnke (1992). DNA–DNA relatedness was tested in 2 $\times$ SSC plus 10% (v/v) DMSO at 68 °C. The results of DNA–DNA hybridization showed 100% relatedness between strains FA1 and FA2^T and 27.6% between strain FA2^T and *S. adhaesiva* DSM 7418^T, indicating that the novel strains do not belong to *S. adhaesiva* when a threshold value of 70% DNA–DNA relatedness is adopted for defining species (Wayne *et al.*, 1987). On the basis of phylogenetic, chemotaxonomic and phenotypic data, we propose that strain FA2^T classified as the type strain of *Sphingomonas phyllosphaerae* sp. nov.

Description of *Sphingomonas phyllosphaerae* sp. nov.

Sphingomonas phyllosphaerae (phyl.lo.sphaer'ae. N.L. gen. fem. n. *phyllosphaerae* of the phyllosphere).

Gram-negative, strictly aerobic, non-spore-forming, rod-shaped cells, 1.5–1.9 μ m long and 0.6–0.8 μ m in diameter. Motile by polar flagellation. Colonies on nutrient agar are circular convex, slimy, yellow, opaque and usually 1–2 mm in diameter within 2 days of growth at 25 °C. Major respiratory lipoquinone is ubiquinone Q-10. Major non-polar fatty acids detected in the type strain are 16:0, 18:1 ω 7c and

Table 1. Differential characteristics among *S. phyllosphaerae* sp. nov. and closely related species

+, Positive; –, negative; W, weak; ND, no data available. Data are from this study (*S. phyllosphaerae*) and from Yabuuchi *et al.* (1990), Kämpfer *et al.* (1997), Takeuchi *et al.* (2001) and Buonauro *et al.* (2002).

Characteristic	<i>S. phyllosphaerae</i> *	<i>S. adhaesiva</i>	<i>S. parapaucimobilis</i>	<i>S. roseiflava</i>	<i>S. sanguinis</i>	<i>S. paucimobilis</i>
Phenotypic						
Nitrite from nitrate	–	–	+	+	–	–
Gelatinase	–	+	–	–	+	+
Acid from:						
Glycerol	–	+	–	–	+	–
Maltose	+	+	–	+	+	+
Rhamnose	+	+	+	–	+	–
Salicin	–	+	–	–	–	+
Galactose	+	–	+	+	+	+
Melezitose	–	–	+	–	+	+
Assimilation of:						
Mannose	–	+	+	–	+	+
Maltose	–	+	+	+	+	+
<i>N</i> -Acetylglucosamine	+	+	+	–	+	+
Gluconate	–	–	+	+	+	+
Adipate	+	–	–	–	–	+
Citrate	–	–	+	–	–	+
Chemotaxonomic						
Saturated fatty acids:						
14:0	2.1	–	1.0	ND	1	1.4
15:0	2.9	–	–	ND	1.1	–
16:0	15.6	6.5	9.6	ND	13.6	8.7
17:0	–	–	–	3.0	0.5	–
18:0	–	–	–	ND	0.6	–
Unsaturated fatty acids:						
16:1 ω 5 <i>c</i>	–	–	1.9	ND	1.1	–
17:1 ω 6 <i>c</i>	1.5	21.2	3.8	ND	2.4	2.0
18:1 ω 5 <i>c</i>	–	–	3.5	ND	3.5	3.2
18:1 ω 7 <i>c</i>	14.2	–	–	ND	–	–
Hydroxy fatty acids:						
14:0 2-OH	14.2	13.8	6.7	16.0	5.0	6.4
Summed feature 4†	18.3	6.2	8.6	ND	6.7	2.7
Summed feature 7†	–	47.3	65.0	ND	64.6	74.6
Polar lipids‡	PE, PG, DPG, PC, SGL, (PME), (PDE), PL1, PL2	(PME), PE, PG, DPG, (PDE), PC, SGL, (APL1), (PL1), PL2, (PL3), (GL2)	(PME), PE, PG, DPG, (PDE), PC, (SGL), (PL2), (PL3), GL1, (GL2)	ND	PE, PG, DPG, (PDE), PC, (SGL); (PL3), (GL1), (GL2)	PE, PG, (DPG), PC, SGL, (PL3), (GL2)

*Fatty acid analyses were performed for strain FA2^T.

†Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 4 contained 16:1 ω 7*c* and/or 15:0 iso 2-OH. Summed feature 7 contained 18:1 ω 7*c*, 18:1 ω 9*t* and/or 18:1 ω 12*t*.

‡APL_n, Unidentified aminophospholipid; DPG, diphosphatidylglycerol; GL_n, unidentified glycolipid; PC, phosphatidylcholine; PDE, phosphatidyltrimethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL_n, unidentified phospholipid; PME, phosphatidylmonomethylethanolamine; SGL, sphingoglycolipid. Components in parentheses were detected in small amounts.

16:1 ω 7*c* (from summed feature 4). Hydroxy fatty acids detected are 14:0 2-OH and 15:0 iso 2-OH (from summed feature 4). Polar lipids detected in the type strain are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and sphingoglycolipid

and two unidentified phospholipids (PL1 and PL2). Oxidizes glucose in media containing ammonium nitrate as nitrogen source, but is unable to ferment glucose in the same media. Produces catalase and β -galactosidase, and oxidase weakly, but not gelatinase, urease, arginine

dehydrogenase, tryptophan deaminase, indol or H₂S. Aesculin is hydrolysed. Utilizes L-arabinose, D-xylose, galactose, D-fructose, D-fucose, melibiose, methyl α -D-glucoside, L-rhamnose, amygdalin, arbutin, cellobiose, lactose, sucrose, trehalose, D-raffinose, N-acetylglucosamine, turanose, adipate and malate as sole carbon sources. Does not grow in D-arabinose, L-xylose, ribose, L-sorbose, L-lyxose, L-fucose, inositol, mannitol, adonitol, glycerol, D-arabitol, L-arabitol, xylitol, caprate, gluconate, 2-ketogluconate, citrate, methyl β -xyloside, methyl α -D-mannoside, salicin, inulin, melezitose, starch, glycogen, erythritol, sorbitol, dulcitol, maltose, β -gentiobiose, D-tagatose, 5-ketogluconate or phenylacetate.

Strains FA1 and FA2^T were isolated from *Acacia caven* phyllosphere. The type strain FA2^T (=LMG 21958^T=CECT 5832^T) has a DNA G+C content of 61 mol%.

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