Sphingomonas formosensis sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from agricultural soil

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In the present study, a yellow-pigmented, Gram-negative, short rod-shaped novel bacterium that was capable of degrading a wide range of polycyclic aromatic hydrocarbons (naphthalene, phenanthrene and pyrene) was isolated from agricultural soil located in Yunlin County, Taiwan. Comparative 16S rRNA gene sequence analysis positioned the novel strain in the genus *Sphingomonas* as an independent lineage adjacent to a subclade containing *Sphingomonas fennica* K101^T, *Sphingomonas histidinilytica* UM2^T, *Sphingomonas wittichii* RW1^T and *Sphingomonas haloaromaticamans* A175^T. 16S rRNA gene sequence analysis of strain CC-Nfb-2^T showed highest sequence similarity to *S. fennica* K101^T (96.2 %), *S. histidinilytica* UM2^T (96.1 %), *S. wittichii* RW1^T (95.9 %), *S. haloaromaticamans* A175^T (95.7 %), and *Sphingobium ummariense* RL-3^T (94.7 %); lower sequence similarities were observed with strains of all other *Sphingomonas* species. The strain contained phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingoglycolipid and diphosphatidylglycerol. The predominant fatty acids were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) $C_{16:0}$ and 11-methyl $C_{18:1}\omega7c$; $C_{14:0}$ 2-OH was the major 2-hydroxy fatty acid. Previously, these lipids have been found to be characteristic of members of the genus *Sphingomonas*. The serine palmitoyl transferase gene (*spt*) was also detected and sphingolipid synthesis was confirmed. The predominant isoprenoid quinone system was ubiquinone (Q-10) and the isolate contained *sym*-homospermidine as the major polyamine. The DNA G+C content of the isolate was 62.8 ± 0.8 mol%. On the basis of chemotaxonomic, phenotypic and phylogenetic data, strain CC-Nfb-2^T represents a novel species within the genus *Sphingomonas*, for which the name *Sphingomonas formosensis* sp. nov. is proposed; the type strain is CC-Nfb-2^T (=BCRC 80272^T=DSM 24164^T).

The genus *Sphingomonas*, which belongs phylogenetically to the *Alphaproteobacteria* (Anzai *et al.*, 2000; Lee *et al.*, 2005), was first described by Yabuuchi *et al.* (1990). Based on refined phylogenetic, chemotaxonomic and physiological analyses, the former sphingomonads have now been divided into five genera: *Novosphingobium*, *Sphingobium*, *Sphingomonas*, *Sphingopyxis* and *Sphingosinicella* (Takeuchi *et al.*, 2001; Maruyama *et al.*, 2006). Sphingomonads are Gram-negative, rod-shaped, strictly aerobic, orange-, yellow- or whitish-brown-pigmented bacteria, and most of them contain sphingoglycolipid in their outer membrane and lack lipopolysaccharides (White *et al.*, 1996; Yim *et al.*, 2010). Members of the genus *Sphingomonas* can be characterized chemotaxonomically, with fatty acid profiles that contain $C_{18:1}\omega7c$ as the major fatty acid and $C_{14:0}$ 2-OH as main hydroxylated fatty acid. They contain Q-10 as the main respiratory quinone and *sym*-homospermidine as the major polyamine. The polar lipid pattern consists of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), sphingoglycolipid (SGL), phosphatidylethanolamine (PE), phosphatidyldimethylethanolamine and phosphatidylcholine (PC) (Busse *et al.*, 1999; Wittich *et al.*, 2007).

The genus *Sphingomonas* contains environmental isolates that play important roles in the bioremediation and biodegradation

Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; SGL, sphingoglycolipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CC-Nfb-2^T is HM193517.

Five supplementary figures are available with the online version of this paper.

of organic pollutants. Because of their metabolic diversity, pollutant-degrading *Sphingomonas* strains can serve as potential microbial agents in the remediation of contaminated soils (White *et al.*, 1996). At the time of writing, the genus comprised 45 recognized species (Zhang *et al.*, 2011). In the present study, the taxonomic characterization of a yellow-pigmented isolate, CC-Nfb-2^T, was carried out using a polyphasic approach and a novel species belonging to the genus *Sphingomonas* was proposed.

In the present study, strain CC-Nfb-2^T was isolated from agricultural soil sampled from Yunlin County, Taiwan. The organism was able to grow on nutrient agar (Hi-Media), tryptone soy agar (Difco), PYE agar (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2) and R2A agar (Oxoid). This isolate was subsequently cultivated on PYE agar at 30 °C for 48 h to determine its morphological characteristics (Young *et al.*, 2007). On the same medium, strain CC-Nfb-2^T was tested for growth at 20–50 °C. Salt tolerance was determined by cultivating the organism in PYE medium supplemented with NaCl at final concentrations of 0–5.0 % (w/v). Gram-staining was performed as described by Murray *et al.* (1994). Catalase activity was determined by assessing bubble production by cells in 3 % (v/v) H_2O_2 and oxidase activity was determined by using 1 % (w/v) *N*,*N*,*N*′,*N*′,-tetramethyl-1,4-phenylenediamine reagent (bioMérieux). Carbon source utilization patterns were determined by using Biolog GN2 (bioMérieux) and acid production from 49 carbohydrates was determined by using API 50 CH strips (bioMérieux). Nitrate reduction, indole production, β-galactosidase and urease activities, hydrolysis of aesculin and gelatin, and assimilation of 12 substrates were tested with API 20 NE strips. The activities of various enzymes were detected by using the API ZYM system (bioMérieux). Physiological tests were performed according to Lin *et al.* (2009). The physiological characteristics that can be used to differentiate strain CC-Nfb-2^T from other *Sphingomonas* species are given in Table 1.

The morphology of negatively stained cells was determined by transmission electron microscopy. For thin sections, the cells were prefixed with 2 % (v/v) glutaraldehyde and 3 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at room temperature, and washed three times in the same buffer. Thin sections were prepared and examined. For scanning electron microscopy, 50–200 µl culture was placed onto a Millipore filter (0.45 µm) and fixed with 2.5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. Then the samples were rinsed with buffer, dehydrated in a graded series of ethanol and critical-point-dried. The filters were coated with gold/palladium and observed by field emission scanning electron microscopy (JEOL JSM-7401F). Morphology of strain CC-Nfb-2^T, revealing the presence of a polar flagellum, was determined by electron microscopy (see Figs S1 and S2).

Bacterial genomic DNA was isolated by using UltraClean Microbial Genomic DNA Isolation kits (MO BIO) according to the manufacturer's instructions. DNA extracted by the above methods was used as a template to amplify the 16S rRNA gene (Hung *et al.*, 2005). PCR was performed with bacterial universal primers 1F (5′-GAGTTTGATCATGGCTCAGA-3′) and 9R (5′-AAGGAGGTGATCCAACCGCA-3′). 16S rRNA gene cycle sequencing was performed by using the Bigdye terminator kit (Heiner *et al.*, 1998) and determination of the nucleotide sequence of PCR product was performed by an automatic DNA sequencer (ABI PRISM 310; Applied Biosystems) (Watts & MacBeath, 2001). The 16S rRNA gene sequences of type strains of species of the genus *Sphingomonas* were retrieved from NCBI/GenBank and sequence alignments were carried out by using the program CLUSTAL_X (1.83) (Thompson *et al.*, 1997). Phylogenetic analysis was performed with the software MEGA4 (Tamura *et al.*, 2007) and an evolutionary tree was inferred using neighbour-joining (Saitou & Nei, 1987) methods. Topology of the resultant tree was evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings. Comparative 16S rRNA gene sequence analysis positioned the novel strain in the genus *Sphingomonas* as an independent lineage adjacent to a subclade containing *Sphingomonas fennica* K101^T, *Sphingomonas histidinilytica* UM2^T, *Sphingomonas wittichii* RW1^T, and *Sphingomonas haloaromaticamans* A175^T. Strain CC-Nfb-2^T revealed highest similarity to K101^T (96.2 %) (Wittich *et al.*, 2007), *S. histidinilytica* UM2^T (96.1 %) (Nigam *et al.*, 2010), *S. wittichii* RW1^T (95.9 %) (Yabuuchi *et al.*, 2001), *S. haloaromaticamans* A175^T (95.7 %) (Wittich *et al.*, 2007), and *Sphingobium ummariense* RL-3^T (94.7 %) (Singh & Lal, 2009); other species showed lower levels of similarity. Sequence divergence values of 3 % or greater are considered to be strong evidence that organisms are not related at the species level (Stackebrandt & Goebel, 1994). The phylogenetic tree reconstructed by neighbour-joining method is shown in Fig. 1.

Two genus-specific PCR primer pairs targeting a *Sphingomonas*-specific sphingolipid synthesis gene were designed by aligning the serine palmitoyl transferase gene (*spt*) sequences of several *Sphingomonas* strains and other sphingolipid-containing alphaproteobacteria (Yim *et al.*, 2010). The first set of the *Sphingomonas spt*-specific primers was Sph-*spt* 295f (forward, 5′-CGATCCCTTCGCGATCGTG-3′) and Sph-*spt* 713r (reverse, 5′-TGGCGGAAGCGGACGATC-3′), and the second specific primer set contained Sph-*spt* 694f (forward, 5′-GAGATCGTCCGCTTCCGC-3′) and Sph-*spt* 983r (reverse, 5′-CCGACCGATTTGGAGAAG-3′). The first and the second Sph-*spt* primer sets targeted the *Sphingomonas spt* gene producing DNA fragments of 408 and 289 bp, respectively. In this study, both primer pairs amplified the expected fragment with the template DNA isolated from strain CC-Nfb-2^T. The sequences of both fragments match the *Sphingomonas chungbukensis spt* gene with 84 % and 87 % similarity; this demonstrated that strain CC-Nfb-2^T also possesses sphingolipid synthesis capability which is unique to the genus *Sphingomonas*.

Table 1. Differential characteristics of *Sphingomonas formosensis* and related strains

Strains: 1, *Sphingomonas formosensis* CC-Nfb-2^T; 2, *S. fennica* K101^T; 3, *S. haloaromaticamans* A175^T; 4, *S. histidinilytica* UM2^T; 5, *S. wittichii* RW1^T. Data for columns 1–3 are from this study; data for columns 4–5 are from Nigam *et al.* (2010). NA, Data not available.

Characteristic	1	2	3	4	5
Cell size (μm)	0.4 × 1.4	0.5–0.9 × 0.9–1.5*	0.4–0.6 × 0.8–1.5*	1.4 × 0.4	0.4–0.6 × 0.9–1.5
Colour†	LY	Y	Y	W	LY
Nitrate reduction	−	−	−	+	−
Oxidase	+	+	+	−	+
DNase	−	−	−	NA	−
Growth temperature (°C)	25–37	20–30*	30–37*	4–40	30
pH range	5–9	5–8*	5–8*	4–10	NA
NaCl (%, w/v)	0–1	<1	0–2	0–4	NA
API ZYM system:					
Trypsin	+	−	+	NA	NA
α-Chymotrypsin	+	−	+	NA	NA
β-Glucuronidase	+	−	−	NA	NA
β-Glucosidase	+	−	+	NA	NA
Activity of:					
Urease	−	+	+	−	NA
β-Glucosidase	+	−	+	NA	NA
β-Galactosidase	+	−	+	NA	NA
Gelatin hydrolysis	+	−	−	−	+
Assimilation of:					
D-Glucose	+	−	+	−	+
L-Arabinose	+	−	+	+	−
N-Acetylglucosamine	+	−	+	NA	−
Maltose	+	−	+	NA	−
Biolog GN2:					
Melibiose	−	−	+	+	+
D-Galactose	+	−	+	−	+
Trehalose	+	−	+	−	+
Sucrose	+	−	+	−	+
L-Arabinose	+	−	−	+	−
D-Galactose	+	−	+	−	+
DNA G+C content (mol%)	62.8 ± 0.8	63.6*	66.1*	66.9	67.0

*Data from Wittich *et al.* (2007).
†LY, Light yellow; Y, yellow; W, white.

For analysis of DNA G+C content, a DNA sample was prepared and degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated via HPLC. The analysis showed DNA G+C content of strain CC-Nfb-2^T was 62.8 ± 0.8 mol%. Fatty acid methyl esters were prepared, separated and identified according to the standard protocol (Paisley, 1996) of the Microbial Identification System (MIDI) (Sasser, 1990) by GC (Agilent 7890A) fitted with a flame-ionization detector. The culture was grown on PYE agar for 72 h at 30 °C. Grown culture was scraped from the plate and subjected to saponification, methylation and extraction (Miller, 1982). Identification and comparison were made by using the Aerobe (RTSBA6) database of the MIDI System (Sherlock version 6.0). The major fatty acids in strain CC-Nfb-2^T were $C_{16:0}$ (14.9 %), $C_{14:0}$ 2-OH (8.4 %), 11-methyl $C_{18:1}\omega7c$ (9.5 %), and $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$ (56.9 %), which are generally present in sphingomonads (Wittich *et al.*, 2007). However, strain CC-Nfb-2^T showed minor qualitative and quantitative differences in fatty acid methyl ester profiles when compared with *S. fennica* DSM 13665^T and *S. haloaromaticamans* DSM 13477^T. The details of fatty acid profiles of strain CC-Nfb-2^T and other closely related species are given in Table 2.

Polar lipids were extracted and analysed by two-dimensional TLC, and isoprenoid quinones were purified according to Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). Polyamines were extracted as described by Scherer & Kneifel (1983) and analysed by one-dimensional TLC and HPLC. Bacterial cells were hydrolysed in 0.2 M perchloric acid ($HClO_4$) at 100 °C (30 min) with shaking once after 15 min. After centrifugation, 200 μl supernatant was incubated with 300 μl Na_2CO_3 solution (100 mg ml^{-1}

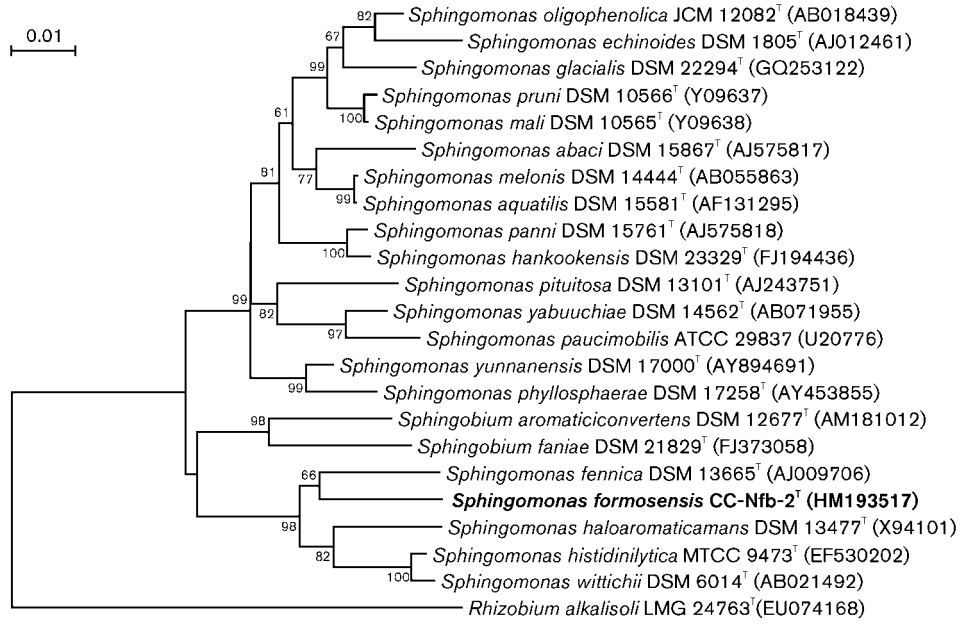

Fig. 1. Phylogenetic analysis of *Sphingomonas formosensis* CC-Nfb-2^T and strains of closely related species was performed by using the neighbour-joining method based on 16S rRNA gene sequences. Bootstrap percentages based on 1000 replications are given at branch points. Bar, 0.01 substitutions per nucleotide position.

Table 2. Fatty acid composition (%) of *Sphingomonas formosensis* and related strains

Strains: 1, *Sphingomonas formosensis* CC-Nfb-2^T; 2, *S. fennica* K101^T; 3, *S. haloaromaticamans* A175^T; 4, *S. histidinilytica* UM2^T; 5, *S. wittichii* RW1^T. Data from columns 1–3 are from this study; data for columns 4–5 are from Nigam *et al.* (2010). –, Not detected; tr, trace (less than 1 %).

Fatty acid	1	2	3	4	5
Saturated					
$C_{14:0}$	tr	1.2	tr	1.4	2.5
$C_{16:0}$	14.9	11.4	13.3	12.1	12.1
$C_{18:0}$	1.4	tr	tr	tr	1.5
Unsaturated					
$C_{16:1}\omega5c$	3.5	tr	1.2	4.4	4.5
$C_{17:1}\omega6c$	tr	2.6	3.3	9.1	1.0
$C_{18:1}\omega5c$	1.5	tr	tr	2.1	1.5
$C_{18:1}\omega7c$ 11-methyl	9.5	2.1	1.4	4.4	3.9
$C_{19:0}$ cyclo $\omega8c$	–	2.1	4.1	15.5	11.3
Hydroxy					
$C_{14:0}$ 2-OH	8.4	10.1	9.9	7.6	10.3
iso-$C_{15:0}$ 3-OH	–	tr	1.8	–	–
iso-$C_{17:0}$ 3-OH	–	3.5	–	–	–
Summed features*					
3	tr	10.2	10.1	8.1	5.1
8	56.9	47.7	53.8	32.9	41.6

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 consists of $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 8 consists of $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

in water) and 800 µl dansyl chloride solution (7.5 mg ml^{-1} in dry acetone) in 0.5 dram vials with Teflon-lined caps (20 min at 60 °C). One hundred microlitres of a proline solution (50 mg ml^{-1} in water) was added to bind excess dansyl chloride (10 min at 60 °C). After cooling in a refrigerator (to 5 °C), the mixture was shaken with 100 µl toluene. Ten microlitres of the extracted sample was applied on TLC plate (Silica gel 60 F_{254}, 20 × 20 cm; Merck 5554) and the running solvent used was ethyl acetate/cyclohexane (2 : 3, v/v). The dansyl derivatives were separated by using a Hitachi L-2130 chromatograph equipped with a Hitachi AS-4000 injector, Hitachi L-2485 fluorescence detector (excitation at 360 nm and emission at 520 nm) and a reverse-phase C18 column (Phenomenex Synergi 4µ Fusion-RP80 250 × 4.60 mm). A linear gradient (40–80 %) of acetonitrile/water at 40 °C with the flow rate of 1 ml min^{-1} was used. The major polar lipids of strain CC-Nfb-2^T were PC, PE, PG, SGL and PG. Furthermore, moderate amounts of five unidentified phospholipids (PL1–5) and unknown aminolipids (AL1–2) were also found (Fig. S3). The polar lipid profile of strain CC-Nfb-2^T was similar to those of *S. fennica*, *S. haloaromaticamans*, *S. histidinilytica* and *S. wittichii*, which comprised PC, PE, PG and SGL in these closest phylogenetic neighbours. The predominant quinone system was ubiquinone Q-10. The polyamine pattern showed a predominance of *sym*-homospermidine and trace amounts of spermidine in the cell walls. This important characteristic feature demonstrated that strain CC-Nfb-2^T belongs to the genus *Sphingomonas* (Figs S4 and S5).

In phenotypic studies, the following carbon sources were utilized by strain CC-Nfb-2^T in the Biolog GN2 system: Tween 80, D-fructose, D-galactose, gentiobiose, α-D-glucose, sucrose, trehalose, pyruvic acid methyl ester, D-glucuronic acid, β-hydroxybutyric acid, propionic acid, glucuronamide, L-alanylglycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-ornithine, L-phenylalanine, L-proline and urocanic acid. Strain CC-Nfb-2^T, *S. fennica* K101^T and *S. haloaromaticamans* A175^T utilized α-D-glucose as a carbon source, but acetic acid and citric acid were not used. In the API ZYM system, the activities of alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase and β-glucosidase were present, but esterase (C4), esterase lipase (C8), lipase (C14), α-galactosidase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were absent. Trypsin, α-chymotrypsin and β-glucosidase were present in strain CC-Nfb-2^T and *S. haloaromaticamans* A175^T, but absent in *S. fennica* K101^T. In the API 20NE system, β-glucosidase, gelatinase and β-galactosidase were present, but D-glucose, L-arabinose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, malic acid, trisodium citrate, D-mannose, capric acid, adipic acid and phenylacetic acid were not assimilated. Compared to other most closely related species, D-glucose, L-arabinose, *N*-acetylglucosamine, maltose, potassium gluconate, malic acid, and trisodium citrate were assimilated by strain CC-Nfb-2^T and *S. haloaromaticamans* A175^T, but not by *S. fennica* K101^T. On the basis of morphological, chemotaxonomic and physiological data, strain CC-Nfb-2^T represents a novel species in the genus *Sphingomonas*.

Description of *Sphingomonas formosensis* sp. nov.

Sphingomonas formosensis [for.mo.sen′sis. N.L. fem. adj. *formosensis* of or pertaining to Formosa (Taiwan), the beautiful island].

Cells are Gram-negative, short rod-shaped, 1.4 μm in length and 0.4 μm in diameter. Colonies are smooth, shiny, convex and light-yellow-coloured after 2 days incubation. The growth temperature ranges from 25 to 37 °C (optimum is 30 °C), grows at pH 5.0–9.0 (optimum pH is 7.0) and tolerates less than 3 % (w/v) NaCl concentration. Oxidase and catalase are positive. Able to utilize D-galactose, α-D-glucose, sucrose and trehalose as sole carbon sources, but not α-lactose, maltose, D-mannose or glycerol. Nitrate and nitrite are not reduced. Positive reactions for alkaline phosphatase, phosphatase and β-glucosidase, but negative for DNase test. The fatty acid profile consists mainly of straight-chain saturated and unsaturated fatty acids, with $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$ (summed feature 8) as the major fatty acid. Minor fatty acids are $C_{16:0}$, $C_{18:0}$, $C_{16:1}\omega5c$, $C_{18:1}\omega5c$ and 11-methyl $C_{18:1}\omega7c$. Major hydroxy fatty acids are $C_{14:0}$ 2-OH. The main polar lipids are PC, PE, PG, SGL and DPG; the minor constituents are unknown aminolipids and unidentified phospholipids. The predominant quinone system is ubiquinone Q-10 and *sym*-homospermidine is the major polyamine.

The type strain is CC-Nfb-2^T (=BCRC 80272^T=DSM 24164^T), isolated from agricultural soil. The DNA G+C content of this type strain is 62.8±0.8 mol%.

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