Sphingomonas hankookensis sp. nov., isolated from wastewater

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A Gram-negative, non-motile, rod-shaped bacterial strain, ODN7^T, was isolated from a wastewater treatment plant in Korea, and its taxonomic position was investigated by use of a polyphasic taxonomic approach. Strain ODN7^T grew optimally at around pH 7.5 and 30 °C and in the presence of 0–0.5 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain ODN7^T fell within the cluster comprising species of the genus *Sphingomonas*, clustering with *Sphingomonas panni* C52^T, with which it shared highest 16S rRNA gene sequence similarity (98.9 %). The chemotaxonomic properties of strain ODN7^T were consistent with those of the genus *Sphingomonas*. The predominant ubiquinone was Q-10, and the major fatty acids were C_{18:1}ω7c, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, and C_{16:0}. Major polar lipids were sphingoglycolipid, phosphatidylcholine, phosphatidylglycerol and phosphatidylethanolamine. The DNA G+C content of strain ODN7^T was 67.2 mol%. Strain ODN7^T exhibited levels of DNA–DNA relatedness of 15–32 % to the type strains of phylogenetically related *Sphingomonas* species and could be differentiated from these species based on differences in phenotypic characteristics. On the basis of the data presented, strain ODN7^T is considered to represent a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas hankookensis* sp. nov. is proposed. The type strain is ODN7^T (=KCTC 22579^T=CCUG 57509^T).

The genus *Sphingomonas*, first described by Yabuuchi *et al.* (1990), belongs phylogenetically to the *Alphaproteobacteria* (Anzai *et al.*, 2000; Lee *et al.*, 2005). At the time of writing, the genus comprises at least 39 recognized species, including the recently described species *Sphingomonas insulae* (Yoon *et al.*, 2008), *Sphingomonas sanxanigenens* (Huang *et al.*, 2009) and *Sphingomonas japonica* (Romanenko *et al.*, 2009). In the present study, we report the taxonomic characterization of a *Sphingomonas*-like bacterial strain, ODN7^T, which was isolated from a wastewater treatment plant in Korea.

Strain ODN7^T was isolated by means of the standard dilution plating technique on 10 × diluted nutrient agar (Difco) at 30 °C. The type strains of five recognized *Sphingomonas* species were used as reference strains for DNA–DNA hybridization and phenotypic characterizations: *Sphingomonas panni* KACC 12169^T and *Sphingomonas desiccabilis* KACC 13216^T were obtained from the Korean Agricultural Culture Collection, Suwon, Korea; *Sphingomonas mucosissima* DSM 17494^T was

obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany; *Sphingomonas molluscorum* JCM 14122^T was obtained from the Japan Collection of Microorganisms (JCM), Saitama, Japan, and *Sphingomonas dokdonensis* DS-4^T was obtained from the study of Yoon *et al.* (2006). The morphological, physiological and biochemical characteristics of strain ODN7^T were investigated by using routine cultivation on trypticase soy agar (TSA; Difco) at 30 °C. Cell morphology was examined by light microscopy (E600; Nikon) by using cells from exponentially growing cultures. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at various temperatures (4, 15, 20, 25, 28, 30, 35, 37, 40 and 45 °C) was measured on TSA. The pH range for growth was determined in nutrient broth (Difco) adjusted, prior to sterilization, to various pH values (4.5–10.5 at intervals of 0.5 pH units) by the addition of HCl or Na₂CO₃. Growth at various NaCl concentrations (0–5.0 %, w/v, at increments of 1.0 %) was investigated by using trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded from the medium formula. Growth under

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ODN7^T is FJ194436.

anaerobic conditions was determined after incubation in an anaerobic chamber on TSA and on TSA supplemented with potassium nitrate (0.1 %, w/v), both of which had been prepared anaerobically under a nitrogen atmosphere. Catalase and oxidase activities and hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and reduction of nitrate were studied as described by Lányi (1987). Susceptibility to antibiotics was tested on TSA plates by using antibiotic discs containing the following

compounds: polymyxin B (100 U), streptomycin (50 µg), penicillin G (20 U), chloramphenicol (100 µg), ampicillin (10 µg), cephalothin (30 µg), gentamicin (30 µg), novobiocin (5 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg) and carbenicillin (100 µg). Utilization of various substrates, enzyme activities and other physiological and biochemical properties were tested by using the API 20E, API 20NE and API 50CH systems (bioMérieux). Cells were suspended in AUX medium according to the manufacturer's instructions to inoculate the API 50CH system.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain ODN7^T among other related taxa. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-likelihood algorithm. *Rhodospirillum rubrum* ATCC 11170^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Cell biomass for DNA extraction and for analysis of isoprenoid quinones and polar lipids was obtained from cultivation in trypticase soy broth (Difco) at 30 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by PCR by using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). The isomer type of the diamino acid in the cell-wall peptidoglycan was analysed by TLC according to Komagata & Suzuki (1987). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and were analysed by using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. For cellular fatty acid analysis, cell mass of strain ODN7^T and the five reference strains was harvested from TSA plates after incubation for 3 days at 30 °C. Fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The presence of phosphatidylcholine was determined by spraying with Dragendorff's reagent (Sigma). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984) with a modifica-

tion that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. DNA-DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) by using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the mean of the remaining three values was quoted as the level of DNA-DNA relatedness.

The almost-complete 16S rRNA gene sequence of strain ODN7^T determined in this study comprised 1442 nt (approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence). Comparative 16S rRNA gene sequence analysis showed that strain ODN7^T was phylogenetically most closely related to the genus *Sphingomonas*. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain ODN7^T fell within the cluster comprising *Sphingomonas* species, joining *S. panni* C52^T at a bootstrap resampling value of 99.9 % (Fig. 1). The relationship between strain ODN7^T and *S. panni* C52^T was also maintained in the trees constructed via the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain ODN7^T exhibited 98.9 % 16S rRNA gene sequence similarity to *S. panni* C52^T and 93.8–98.0 % similarity to the type strains of other recognized species of the genus *Sphingomonas*.

Strain ODN7^T contained ubiquinone-10 as the predominant isoprenoid quinone. The cellular fatty acid profile of strain ODN7^T is given in Table 1, together with those of the type strains of the five *Sphingomonas* species also analysed

Table 1. Cellular fatty acid profiles of strain ODN7^T and the type strains of phylogenetically related *Sphingomonas* species

Strains: 1, ODN7^T; 2, *S. panni* KACC 12169^T; 3, *S. desiccabilis* KACC 13216^T; 4, *S. molluscorum* JCM 14122^T; 5, *S. mucosissima* DSM 17494^T; 6, *S. dokdonensis* DS-4^T. All data are from the present study. Values are percentages of the total fatty acids; components that represented <0.5 % in all strains were omitted. —, Not detected.

Fatty acid	1	2	3	4	5	6
Straight-chain						
C _{14:0}	1.1	1.2	0.4	1.2	1.5	0.58
C _{15:0}	0.5	—	0.4	0.9	0.8	0.26
C _{16:0}	11.5	11.7	10.3	10.9	15.4	13.7
Unsaturated						
C _{16:1} ω5c	5.4	4.4	2.7	2.5	2.5	2.6
C _{16:1} ω11c	—	—	—	—	1.2	—
C _{17:1} ω6c	3.7	0.37	4.3	4.7	3.3	1.6
C _{17:1} ω8c	0.5	—	—	0.6	0.3	—
C _{18:1} ω5c	1.2	1.5	2.5	1.8	1.6	1.0
C _{18:1} ω7c	53.9	54.5	58.9	53.6	43.7	47.2
Hydroxy						
C _{14:0} 2-OH	5.6	6.6	7.9	7.9	9.9	11.9
C _{15:0} 2-OH	0.2	—	0.4	0.7	0.4	—
11-Methyl C _{18:1} ω7c	—	—	3.9	4.9	5.7	5.0
Summed feature 3*	16.2	19.2	7.2	9.3	12.3	15.8

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 comprised C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

in this study. Strain ODN7^T had large amounts of unsaturated, straight-chain and hydroxy fatty acids; the major components (>10 % of the total) were C_{18:1}ω7c, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, and C_{16:0}. This fatty acid profile was similar to those of the five *Sphingomonas* species studied, although 11-methyl C_{18:1}ω7c was not

detected in strain ODN7^T or in *S. panni* KACC 12169^T (Table 1). Major polar lipids found in strain ODN7^T were sphingoglycolipid, phosphatidylcholine, phosphatidylglycerol and phosphatidylethanolamine; small amounts of diphosphatidylglycerol, an unidentified phospholipid and an unidentified lipid were also detected. This polar lipid

Table 2. Differential phenotypic characteristics between strain ODN7^T and phylogenetically related *Sphingomonas* species

Taxa: 1, strain ODN7^T; 2, *S. panni*; 3, *S. desiccabilis*; 4, *S. molluscorum*; 5, *S. mucosissima*; 6, *S. dokdonensis*. Data for reference species are from Busse *et al.* (2005), Reddy & Garcia-Pichel (2007), Romanenko *et al.* (2007), Reddy & Garcia-Pichel (2007) and Yoon *et al.* (2006) unless indicated otherwise. +, Positive reaction; –, negative reaction; w, weakly positive reaction. All are Gram-negative rods. All are positive for catalase, hydrolysis of aesculin and Tween 80 (data from the present study) and for the utilization of glucose, aesculin, cellobiose and trehalose. All are negative for utilization of erythritol, D-arabinose, ribose, L-xylose, adonitol, methyl β-D-xyloside, sorbose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, inulin, glycogen, xylitol, D-lyxose, D-tagatose, D-arabitol, L-arabitol, 2-ketogluconate, caprate and phenylacetate.

Characteristic	1	2	3	4	5	6
Colony colour*	Y	Y	Y	Y	LO	Y
Motility	–	–	–	–	–	+
Oxidase	+	–	+	+	+	+
β-Galactosidase	–	+	+	+	–	–
Nitrate reduction	–	–†	+	–	–	–
Growth at 3 % (w/v) NaCl	–	–†	+	+	–	+
Hydrolysis of:†						
Casein	–	–	–	–	–	+
Gelatin	–	–	–	–	–	+
Starch	–	+	+	+	+	+
Utilization of:†						
N-Acetylglucosamine	+	–	+	+	–	–
Adipate	+	–	–	–	+	+
Amygdalin	+	–	+	+	–	–
L-Arabinose	+	w	+	+	–	–
Arbutin	+	–	+	+	–	–
Citrate	+	–	–	–	–	–
Fructose	+	+	+	+	–	+
D-Fucose	+	–	–	–	–	–
L-Fucose	+	–	–	–	–	–
Galactose	w	+	+	+	–	–
Gentiobiose	+	+	+	+	–	–
Gluconate	+	–	+	+	+	–
Glycerol	–	–	–	–	+	+
5-Ketogluconate	+	–	–	–	–	–
Lactose	+	+	+	+	–	–
Malate	+	–	–	–	–	+
Maltose	+	+	+	+	–	–
Mannose	+	+	+	+	–	+
Melezitose	+	–	+	+	–	–
Melibiose	+	–	+	+	–	–
Methyl α-D-glucoside	+	–	+	+	–	–
Raffinose	+	+	+	+	–	–
Rhamnose	+	w	+	+	–	–
Salicin	+	–	+	+	–	–
Starch	+	+	–	–	–	–
Sucrose	+	+	+	+	–	–
Turanose	+	–	+	+	–	–
D-Xylose	+	w	+	+	–	–

*Data from the present study; all were grown at 30 °C on TSA; Y, yellow; LO, light orange.

†Data from the present study.

profile was similar to that of *S. panni* (Busse *et al.*, 2005), the closest phylogenetic neighbour to strain ODN7^T. The DNA G+C content of strain ODN7^T was 67.2 mol%. The results obtained from chemotaxonomic analyses, showing the characteristic properties shared by species of the genus *Sphingomonas*, were in agreement with the results of phylogenetic analysis, i.e. that strain ODN7^T may be a member of the genus *Sphingomonas*.

Strain ODN7^T exhibited levels of DNA–DNA relatedness of 15–32% to the type strains of the five phylogenetically related *Sphingomonas* species studied herein, namely *S. panni* (32%), *S. desiccabilis* (23%), *S. molluscorum* (28%), *S. mucosissima* (25%) and *S. dokdonensis* (15%). Strain ODN7^T was distinguishable from *S. panni* and other phylogenetically related *Sphingomonas* species based on differences in several phenotypic characteristics (Table 2). The phylogenetic and genetic distinctiveness and differential phenotypic properties of strain ODN7^T are sufficient to categorize it as a member of a species that is distinct from recognized *Sphingomonas* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, strain ODN7^T is considered to represent a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas hankookensis* sp. nov. is proposed.

Description of *Sphingomonas hankookensis* sp. nov.

Sphingomonas hankookensis (han.kook.en'sis. N.L. fem. adj. *hankookensis* of Hankook, the Korean name for South Korea from where the type strain was isolated).

Cells are Gram-negative, non-motile rods (0.3–0.6 × 0.9–2.8 µm). Colonies on TSA are circular, convex, smooth, yellow and 0.8–1.0 mm in diameter after incubation for 3 days at 30 °C. Growth occurs at 4 and 37 °C, but not at 40 °C. Optimal pH for growth is around 7.0. Growth occurs at pH 5.0 and 10.0, but not at pH 4.5 or 10.5. Growth occurs in the presence of 0–1% (w/v) NaCl (optimum 0–0.5%, w/v). Negative for arginine dihydrolyase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase. H₂S and indole are not produced. Tweens 20, 40 and 60 are hydrolysed, but xanthine, hypoxanthine and tyrosine are not. Susceptible to chloramphenicol, gentamicin, neomycin, novobiocin, oleandomycin and tetracycline and weakly susceptible to kanamycin and polymyxin B. Resistant to ampicillin, carbenicillin, cephalothin, lincomycin, penicillin G and streptomycin. The predominant ubiquinone is Q-10. The major fatty acids (>10% of the total) are C_{18:1}ω7c, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, and C_{16:0}. Other phenotypic characteristics are given in Table 2.

The type strain, ODN7^T (=KCTC 22579^T=CCUG 57509^T), was isolated from a wastewater treatment plant in Taejeon, Korea. The DNA G+C content of the type strain is 67.2 mol% (determined by HPLC).

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