Polyphasic assignment of an aromatic-degrading *Pseudomonas* sp., strain DJ77, in the genus *Sphingomonas* as *Sphingomonas chungbukensis* sp. nov.

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***Pseudomonas* sp. strain DJ77, which was described as an aromatic-degrading bacterium, was investigated to clarify its taxonomic position. Phylogenetic analysis based on 16S rDNA sequences indicated that the strain belonged to the genus *Sphingomonas* and formed a monophyletic clade with the type strains of *Sphingomonas chlorophenolica*, *Sphingomonas herbicidovorans* and *Sphingomonas yanoikuyae* with sequence similarity values of 98.1, 96.1 and 95.6%, respectively. Genomic relatedness based on DNA–DNA hybridization of strain DJ77 to these strains is 7–14%. Strain DJ77 contained ubiquinone 10 as the main respiratory quinone, and the G+C content of DNA was 63 mol%. The organism contained octadecenoic acid (67%) as major cellular fatty acid. Strain DJ77 can be readily differentiated from representative members of the genus *Sphingomonas* using a battery of biochemical tests. On the basis of polyphasic evidence, it is proposed that strain DJ77, previously known as *Pseudomonas* sp., be reclassified in the genus *Sphingomonas* as *Sphingomonas chungbukensis* sp. nov. The type strain is strain DJ77^T (= KCTC 2955^T = IMSNU 11152^T).**

Keywords: *Sphingomonas chungbukensis* sp. nov., polyphasic classification, 16S rDNA sequencing, DNA–DNA hybridization

INTRODUCTION

Strain DJ77^T, which degrades biphenyl and phenanthrenes, was isolated from contaminated sediment of an industrial complex near Taejeon, Republic of Korea, and subsequently identified as *Pseudomonas* sp. on the basis of the limited number of morphological and physiologic properties (Kim *et al.*, 1986). Recently, Kim (1999) discovered that the organism was capable of metabolizing a variety of monocyclic aromatic hydrocarbons such as toluene, *m*-xylene, phenol, salicylate, benzoate and *p*-cresol, in addition to polycyclic aromatic compounds. Due to its ability to degrade a broad range of aromatic hydrocarbons, additional studies of metabolic pathways, enzymology and molecular mechanisms have been carried out for

this strain (Jung *et al.*, 1996; Kim *et al.*, 1997a, b; Shin *et al.*, 1997; Hwang *et al.*, 1999; Kim *et al.*, 1999). A comparative study showed that the genes and gene orders for aromatic hydrocarbon degradation of strain DJ77^T were strikingly similar to the corresponding genes of *Sphingomonas* species, such as *Sphingomonas paucimobilis* EPA505, *Sphingomonas yanoikuyae* B1, '*Sphingomonas agrestis*' HV3 and *Sphingomonas aromaticivorans* F199 (Mueller *et al.*, 1990; Kim, 1996; Yrjälä *et al.*, 1997; Romine *et al.*, 1999). The members of the genus *Sphingomonas* have been reported to degrade various aromatic hydrocarbons (Wittich *et al.*, 1992; Ka *et al.*, 1994; Fredrickson *et al.*, 1995; Khan *et al.*, 1996). In this study, we applied a polyphasic approach to identify strain DJ77^T. On the basis of chemotaxonomic, physiological and molecular systematic evidence, we propose that *Pseudomonas* sp. strain DJ77^T be reclassified in the genus *Sphingomonas* as *Sphingomonas chungbukensis* sp. nov.

The GenBank accession number for 16S rDNA sequence of *Sphingomonas chungbukensis* DJ77^T (= KCTC 2955^T = IMSNU 11152^T) is AF159257.

Table 1. Selected physiological characteristics useful for differentiation of strain DJ77^T from the type strains of three *Sphingomonas* species

+, Positive; w, weakly positive; —, negative.

Characteristic	Strain DJ77 ^T	<i>S. chlorophenolica</i>	<i>S. herbicidovorans</i>	<i>S. yanoikuyae</i>
BIOLOG GN MicroPlate system				
Oxidation of:				
Dextrin	+	—	w	+
Glycogen	+	—	—	w
Tween 40	+	—	w	w
Tween 80	+	—	—	+
<i>N</i> -Acetyl-D-glucosamine	—	—	w	+
L-Arabinose	+	—	—	+
D-Galactose	—	—	+	+
Gentiobiose	—	—	w	+
α -D-Glucose	+	+	+	+
α -Lactose	—	—	—	+
Maltose	+	—	w	+
D-Melibiose	—	—	—	+
Methyl β -D-glucoside	—	—	—	+
D-Raffinose	—	—	—	+
L-Rhamnose	—	—	+	+
D-Sorbitol	+	—	w	—
Methylpyruvate	+	+	+	+
Acetic acid	—	—	w	+
Formic acid	+	—	—	—
D-Gluconic acid	+	—	+	+
D-Glucosaminic acid	—	—	+	—
β -Hydroxy-butyric acid	+	+	+	+
α -Keto-butyric acid	+	—	w	w
DL-Lactic acid	+	—	+	+
Quinic acid	—	—	—	+
D-Saccharic acid	—	—	—	+
Alaninamide	+	—	—	w
L-Alanyl-glycine	+	—	—	w
L-Aspartic acid	+	—	—	+
L-Glutamic acid	+	—	—	+
Glycyl-L-aspartic acid	+	—	—	+
L-Proline	+	—	+	+
API 20 NE system				
Reduction of nitrate	—	—	—	+
Acid from glucose	—	—	—	—
Arginine dihydrolase	—	—	—	+
Urease	—	—	—	+
Hydrolysis of aesculin	—	+	+	+
Hydrolysis of gelatin	—	—	—	+
pNP- β -D-galactopyranoside	—	—	—	+
Utilization of:				
Glucose	+	+	+	+
L-Arabinose	+	—	+	+
Mannose	+	—	—	+
<i>N</i> -Acetyl-glucosamine	—	—	—	+
Maltose	+	—	+	+

Table 1 (cont.)

Characteristic	Strain DJ77 ^T	<i>S. chlorophenolica</i>	<i>S. herbicidovorans</i>	<i>S. yanoikuyae</i>
Potassium gluconate	+	—	+	+
Caprate	—	—	—	+
Malate	—	w	+	+
Citrate	—	—	—	+
Tetramethyl- <i>p</i> -phenylene diamine	+	+	+	—
API 50 CHB system				
Utilization of:				
Glycerol	—	—	—	+
D-Arabinose	—	—	—	+
D-Xylose	+	—	+	+
Galactose	+	—	+	+
D-Fructose	+	—	—	+
L-Sorbose	—	—	—	+
Rhamnose	—	—	—	+
Methyl α -D-mannoside	—	—	—	+
Methyl α -D-glucoside	—	—	—	+
Amygdalin	—	+	—	+
Arbutin	—	—	—	+
Aesculin	+	+	+	+
Salicin	—	—	—	+
Cellobiose	—	+	—	+
Lactose	—	—	—	+
Melibiose	—	—	—	+
Saccharose	—	—	—	+
Trehalose	—	—	—	+
Melezitose	—	—	—	+
D-Raffinose	—	—	—	+
Amidon	+	—	—	+
D-Lyxose	+	—	+	+
D-Fucose	+	—	+	+

METHODS

Bacterial strains and cultures. Strain DJ77^T, *S. chlorophenolica* DSM 7098^T, *S. herbicidovorans* DSM 11019^T and *S. yanoikuyae* KCTC 2818^T were grown on Trypticase soy agar (TSA; BBL) at 28 °C.

Morphology. The morphological properties were examined after growth on TSA at 28 °C for 2 d by light microscopy and scanning electron microscopy (JEOL model JSM-6400).

Determination of physiological characteristics. The oxidation of 95 different carbon sources was tested using BIOLOG GN MicroPlate with an inoculum grown on TSA plates. Oxidation of individual carbon sources was detected indirectly by observing reduction of tetrazolium dye with BIOLOG computer software (GN MicroLog3) after 24 h incubation at 28 °C. Additional physiological and biochemical traits were determined with API 20NE and 50CHB strips (bioMérieux). The intensity of turbidity was measured after incubation at 28 °C for 1, 2 and 4 d.

Whole-cell fatty acids. In order to analyse fatty acid composition, all of strains were grown on TSA and incubated at 28 °C for 2 d. Cellular fatty acids of test strains were analysed as methyl esters by GLC according to the instructions of the Microbial Identification System (MIDI).

Respiratory quinones. Cells harvested after culturing for 48 h at 28 °C in LB medium were freeze-dried and respiratory quinones were isolated from 100 mg of lyophilized cells according to Collins (1985). Samples were further purified on preparative TLC (Silica Gel F₂₅₄; Merck) using hexane-diethylether (85:15, v/v) as a solvent system. The spots were scraped off and dissolved in chloroform. The samples were analysed by reverse-phase thin-layer partition chromatography using Merck HPTLC RP18F₂₅₄ as described (Collins & Jones, 1981). Ubiquinone standards were purchased from Sigma and also prepared from *S. chlorophenolica* DSM 7098^T.

Base composition of DNA. The G+C content of the DNA was determined from the mid-point value of the thermal denaturation profile using a spectrophotometer (Ultrospec 2000; Pharmacia Biotech) equipped with a programmable peltier temperature-control unit by the equation of Marmur & Doty (1962), as modified by De Ley (1970).

DNA–DNA hybridization. DIG-High Prime DNA labelling and Detection Starter Kit II (Boehringer Mannheim) was used for DNA–DNA reassociation experiments. Chromosomal DNAs were prepared following the method of Murray & Thompson (1980). Duplicate aliquots containing 200 ng of each denatured genomic DNA were transferred onto

Table 2. Whole-cell fatty acid profile of *Pseudomonas* sp. DJ77^T and reference strains

Strain	Percentage fatty acid composition (w/w, ± 2%)*							
	14:0 2-OH	Summed feature 4	16:0	17:1ω6c	Summed feature 7	16:1ω5c	18:1ω5c	16:0 2-OH
<i>Pseudomonas</i> sp. strain DJ77 ^T	9	6	12	2	67	1	3	0
<i>S. yanoikuyae</i> KCTC 2818 ^T	10	13	16	2	52	3	3	1
<i>S. chlorophenolica</i> DSM 7098 ^T	10	8	10	4	62	2	3	1
<i>S. herbicidovorans</i> DSM 11019 ^T	6	9	9	1	68	1	5	1

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

positively charged nylon membrane (Hybond-N⁺; Amersham) by using a slot blot apparatus (Bio-Rad). The membrane was air-dried and cross-linked. Chromosomal DNAs were denatured by boiling for 10 min and labelled by random priming using DIG-High Prime kit. The membrane was prehybridized in DIG Easy Hyb solution at 68 °C for 1 h. The labelled probe DNAs were then added to the hybridization solution (5 $\times$ SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% blocking reagent) and incubated at 68 °C for 16 h. The resultant membrane was washed to remove nonspecifically bound probe and signals were detected using the DIG chemiluminescent Detection Starter Kit II followed by exposure to X-ray film for periods determined by the sensitivity of the probe. All procedures were performed in accordance with the manufacturer's directions. The signal intensities were measured using a densitometer. The signal produced by self-hybridization of the probe with homologous target DNA was taken as 100% and each hybridization value was calculated as the mean of three replicates.

16S rDNA analysis. The 16S rDNA was enzymically amplified using two oligonucleotide primers, 27f (AGAGTTTGAT-CCTGGCTCAG) (Giovannoni, 1991) and 1492r (GGTT-ACCTTGTTACGACTT) (Lane, 1991). The PCR amplicons were purified by using Wizard Plus SV Minipreps DNA Purification System (Promega) and sequences were determined using Top DNA Sequencing kit and Silverstar Staining kit (BIONEER, Korea). Primers for sequencing are as follows: primer 27f, covering the sequence from positions 8–27 in the 16S rDNA nucleotide sequence of *E. coli* (Brosius *et al.*, 1978); primer Prok4 (TTACCGCGGCTGCTGGC-AC; positions 515–535); primer Prok6 (GGGCCCGCAC-AAGCGG; positions 927–942) and primer 1492r (positions 1477–1492).

The resultant sequence of strain DJ77^T was manually aligned with representatives of the genus *Sphingomonas* and related taxa using known 16S rRNA secondary structure information. Phylogenetic trees were inferred by using the neighbour-joining (Saitou & Nei, 1987), Fitch–Margoliash (Fitch & Margoliash, 1967) and maximum-parsimony (Fitch, 1972) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The trees were rooted using *Erythrobacter longus* (GenBank accession number D12699) and *Porphyrobacter neustonensis* (M96745) as outgroups. The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant tree topology

was evaluated in bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

RESULTS

Morphological properties

Strain DJ77^T was Gram-negative, non-motile curved rods, and formed yellow colonies within 2–3 d on half-strength TSA plates at 28 °C. The colonies were slimy, especially when grown at temperatures below the optimal temperature, i.e. 18 °C. The cell size is 0.5–1 $\times$ 1.5–3 μ m.

Physiological properties

Physiological characteristics of strain DJ77^T and reference strains are summarized in Table 1. Strain DJ77^T oxidized about 20 of the 95 different carbon sources tested with the BIOLOG identification system. These results were different from those obtained with *S. yanoikuyae* KCTC 2818^T, *S. chlorophenolica* DSM 7098^T and *S. herbicidovorans* DSM 11019^T, which were included for the comparative study. The reference strains showed about 31, 3 and 18 positive reactions out of the 95 carbon sources, respectively. *S. chlorophenolica* shows little response towards the majority of the substrates included in the BIOLOG and API identification kits. The substrates oxidized by strain DJ77^T in the BIOLOG system were dextrin, glycogen, Tween 40, Tween 80, L-arabinose, α -D-glucose, maltose, D-sorbitol, methylpyruvate, formic acid, D-gluconate, β -hydroxybutyric acid, α -keto-butyric acid, DL-lactic acid, alaninamide, L-alanyl-glycine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid and L-proline. Strain DJ77^T gave positive results for catalase and oxidase tests and assimilated glucose, L-arabinose, mannose, maltose, gluconate, D-xylose, galactose, D-fructose, aesculin, amidon, D-lyxose and D-fucose in the API test.

Chemotaxonomic characterization

The whole-cell fatty acid composition of strain DJ77^T and related *Sphingomonas* reference strains are given in Table 2. The dominant fatty acid in strain DJ77^T

Fig. 1. Rooted neighbour-joining tree based on nearly complete 16S rDNA sequences (1300 aligned positions) showing relationships between strain DJ77^T and members of the genus *Sphingomonas*. The numbers at the nodes indicate the levels of bootstrap support based on neighbour-joining analyses of 1000 resampled data sets. The scale bar indicates 0.01 nucleotide substitutions per nucleotide position.

was summed feature 7 (18:1 ω 7c, 18:1 ω 9t and/or 18:1 ω 12t). The other significant fatty acids were 2-hydroxymyristic acid (14:0 2-OH), hexadecanoic acid (16:0) and summed feature 4 (16:1 ω 7c and/or 15:0 iso 2-OH). Very similar profiles were detected for *S. chlorophenolica*, *S. herbicidovorans* and *S. yanoikuyae* strains. Ubiquinone 10 was the only isoprenologue found in strain DJ77^T. The ubiquinones of strain DJ77^T were identical to those of *S. chlorophenolica*

DSM 7098^T and reference ubiquinone 10 purchased from Sigma.

DNA analysis

A nearly complete 16S rRNA sequence of the strain DJ77^T (1435 bp) was determined. A preliminary comparison against the GenBank database indicated that the test strain is closely related to the members of the genus *Sphingomonas*. A rooted phylogenetic tree based on the neighbour-joining method is given in Fig. 1. Strain DJ77^T was recovered in a monophyletic clade containing the type strains of *S. chlorophenolica*, *S. herbicidovorans* and *S. yanoikuyae*. The relationship was strongly supported by 1000 bootstrap values and by the Fitch–Margoliash and maximum-parsimony treeing algorithms. *Sphingomonas suberifaciens* was recovered as a sister group to this clade in all of the treeing methods employed in this study. However, it was supported by relatively lower bootstrap value, i.e. 785. The mean 16S rRNA sequence similarity value of strain DJ77^T to sphingomonads was $92.8 \pm 1.7\%$, ranging from 91.1 (*Sphingomonas stygialis*) to 98.1% (*S. chlorophenolica*). The type strains of *S. chlorophenolica*, *S. herbicidovorans* and *S. yanoikuyae*, which were recovered in a monophyletic clade with strain DJ77^T, showed similarity values over 95% (Table 3), and were used for DNA–DNA pairing experiments. The DNA binding values of strain DJ77^T to reference sphingomonads ranged from 7 to 14% (Table 3). The G+C content of the DNA of strain DJ77^T was 63 mol%.

DISCUSSION

The genus *Sphingomonas* was created and subsequently emended by Yabuuchi *et al.* (1990, 1999) for Gram-negative, strictly aerobic, asporogenous rod- or ovoid-shaped organisms that grow as yellow to whitish brown colonies.

Phylogenetic analysis based on 16S rRNA sequences and chemotaxonomic data clearly indicated that strain DJ77^T belongs to the genus *Sphingomonas*. Among 23 valid species of the genus *Sphingomonas*, three species, namely *S. chlorophenolica*, *S. herbicidovorans* and *S.*

Table 3. Phylogenetic relationship of strain DJ77^T and related sphingomonads

	Strain DJ77 ^T	<i>S. chlorophenolica</i> DSM 7098 ^T	<i>S. herbicidovorans</i> DSM 11019 ^T	<i>S. yanoikuyae</i> KCTC 2818 ^T
16S rDNA similarity to strain DJ77 ^T (%)	100.0	98.1	96.1	95.6
Genomic homology to strain DJ77 ^T (%)*	100	13	7	14
Genomic homology to <i>S. chlorophenolica</i> DSM 7098 ^T (%)†	10	100	9	10

* Values obtained when DNA of strain DJ77^T was labelled.

† Values obtained when DNA of *S. chlorophenolica* DSM 7098^T was labelled.

yanoikuyae, were found to be closely related in terms of phylogeny. These species and strain DJ77^T formed a monophyletic clade and showed very similar cellular fatty acid profiles. However, strain DJ77^T shared very little DNA homology with three *Sphingomonas* species on the basis of DNA–DNA relatedness experiment. It is evident from the combination of 16S rDNA sequencing and DNA–DNA reassociation studies that strain DJ77^T merits a genomic species in the genus *Sphingomonas* (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987). In addition, the organism showed different physiological properties from *Sphingomonas* species (Table 1).

Old cultures of strain DJ77^T became slimy, especially when it was stored at temperatures below room temperature. This feature has been also found in *Sphingomonas subarctica* (Nohynek, 1996) and is attributed to its ability to produce extracellular polysaccharides. Strain DJ77^T produced extracellular polysaccharides that make the cells attach to one another. Because of the production of this material in excessive amounts, the pellet of strain DJ77^T frequently flows away when poured off the medium after centrifugation. This property was not shared by reference strains.

It is noteworthy that among the aromatic-degrading *Sphingomonas* strains, *S. yanoikuyae* B1 (Kim, 1996) and *Sphingomonas aromaticivorans* F199^T (Romine *et al.*, 1999) have been studied most extensively at the biochemical and molecular levels. These strains and strain DJ77^T are closely related to each other in genetic backgrounds for aromatic degradation. However, in contrast to *S. yanoikuyae* B1 where the degradative genes were located on the chromosome and *S. aromaticivorans* F199^T which originated from deep-subsurface, strain DJ77^T has degradative genes on a large plasmid (Kim, 1999) and was originally isolated from sediment.

On the basis of chemotaxonomic and physiological characteristics together with nucleotide sequence analysis of 16S rRNA and DNA–DNA homology studies, we propose that strain DJ77^T, which was previously identified as *Pseudomonas* sp., be classified in the genus *Sphingomonas* as *Sphingomonas chungbukensis* sp. nov.

Description of *Sphingomonas chungbukensis* sp. nov.

Sphingomonas chungbukensis (chung.bu.ken'sis. M.L. adj. *chungbukensis* named after Chungbuk National University).

Gram-negative, aerobic, mesophilic, asporogenous, curved rods. The cell size is 0.5–1 × 1.5–3 µm. Produces visible, circular, smooth, yellow colonies on TSA in 2–3 d. Oxidase and catalase are positive. Indole, urease, β-galactosidase and arginine dihydrolase are not produced. Aesculin and gelatin are not hydrolysed. Does not reduce nitrate. Utilizes a variety of aromatic compounds including biphenyl, naphthalene, phen-

anthrene, phenol, salicylate, *p*-cresol, *m*-xylene, toluene and benzoate. The following compounds are oxidized: dextrin, glycogen, Tween 40, Tween 80, L-arabinose, α-D-glucose, maltose, D-sorbitol, methylpyruvate, formic acid, D-gluconate, β-hydroxybutyric acid, α-keto-butyric acid, DL-lactic acid, alaninamide, L-alanyl-glycine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid and L-proline. The following compounds are assimilated: glucose, L-arabinose, mannose, maltose, gluconate, D-xylose, galactose, D-fructose, aesculin, amidon, D-lyxose and D-fucose. The major cellular fatty acids are octadecenoic, 2-hydroxymyristic, *cis*-9 hexadecenoic and hexadecanoic acids. The major respiratory quinone is ubiquinone 10. The G + C content of the DNA is 63 mol%. *Sphingomonas chungbukensis* is distinct from its phylogenetic neighbours, namely *S. chlorophenolica*, *S. herbicidovorans* and *S. yanoikuyae*, by low levels of DNA–DNA reassociation, by its ability to produce a great quantity of extracellular polysaccharide and by exhibiting different physiological characteristics. Isolated from chemically contaminated freshwater sediment in Taejeon, Republic of Korea. The type strain of the species is DJ77^T (= KCTC 2955^T = IMSNU 11152^T).

ACKNOWLEDGEMENTS

We thank Hans G. Trüper for the help with nomenclature and Sang-Jin Kim (Korea Ocean Research & Development Institute) for helpful discussion. This work was supported by grant 1998-015-D00215 of the Korea Research Foundation.

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