Proposal of *Sphingomonas wittichii* sp. nov. for strain RW1^T, known as a dibenzo-*p*-dioxin metabolizer

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A polyphasic taxonomic study was performed on *Sphingomonas* sp. strain RW1^T. The organism was isolated from water of the River Elbe and has been known as a potent metabolizer of dibenzo-*p*-dioxin and its relatives. TLC of a mild alkaline hydrolysate of extractable cellular lipids of strain RW1^T and type strains of 21 *Sphingomonas* species gave a spot of sphingoglycolipid (SGL)-1 (glucuronosyl ceramide), which is characteristic of sphingomonads. In addition, strain RW1^T and type strains of three *Sphingomonas* species (*Sphingomonas yanoikuyae*, *Sphingomonas terrae* and *Sphingomonas macrogoltabidus*) showed a second spot of SGL (SGL-1') identified as galacturonosyl ceramide. The presence of SGL-1 in cellular lipids suggested that strain RW1^T is a member of the genus *Sphingomonas*. DNA–DNA reassociation rates between strain RW1^T and each type strain of 14 *Sphingomonas* species including *Sphingomonas paucimobilis*, type species for the genus, revealed that strain RW1^T is independent from these species. Results of phylogenetic analysis of 16S rDNA sequences of strain RW1^T and type strains of 21 named *Sphingomonas* species verified that strain RW1^T belongs to the genus *Sphingomonas*. Strain RW1^T could be differentiated from named species of the genus by phenotypic characteristics and has been assigned to a new species, *Sphingomonas wittichii* sp. nov. The type strain is DSM 6014^T (= JCM 10273^T = EY 4224^T). DNA G+C content is 67 mol%.

Keywords: dibenzo-*p*-dioxin metabolizer, *Sphingomonas* sp. strain RW1^T, *Sphingomonas wittichii* sp. nov.

INTRODUCTION

From enrichment cultures inoculated with a water sample of the River Elbe, Wittich *et al.* (1992) isolated a strain that was able to grow with either dibenzo-*p*-dioxin (DD) or dibenzofuran (DF) as a sole source of carbon and energy; the strain was named *Sphingomonas* sp. strain RW1^T. In addition to mineralization of DD and DF, strain RW1^T co-metabolized mono- and dichloro-derivatives of these compounds (Megharaj *et al.*, 1997; Wilkes *et al.*, 1996). The

enzymology of dioxygenation by strain RW1^T and its genetic study have been reported previously (Armengaud *et al.*, 1998; Bünz *et al.*, 1993; Bünz & Cook, 1993).

DD, DF and other related compounds are now known to be metabolized by certain species or strains of aerobic and anaerobic bacteria and fungi. Biotechnological methods can be used to exploit the degrading potential of such micro-organisms, which may be useful for the bioremediation of contaminated soil and water.

For the biodegradation of dioxin and related compounds, bacteriologists, mycologists, biochemists and geneticists are doing extensive work. Taxonomists

Abbreviations: DD, dibenzo-*p*-dioxin; DF, dibenzofuran; SGL, sphingoglycolipid.

have to support this research by polyphasic taxonomic techniques to establish nomenclatural standing of the organisms concerned.

The presence of the second spot of sphingoglycolipid (SGL), SGL-1', on TLC of a mild alkaline hydrolysate of cellular extractable lipids is a characteristic finding of *S. yanoikuyae* strains. This alkali-stable lipid was identified as galacturonosyl ceramide (Naka *et al.*, 2000), which is also found in type strains of *Sphingomonas wittichii* sp. nov., *Sphingomonas terrae* and *Sphingomonas macrogoltabidus*.

METHODS

Bacterial strains. Since TLC of cellular lipids of strain RW1^T (= DSM 6014^T = EY 4224^T), type strains of *S. terrae* EY 4207^T and *S. macrogoltabidus* EY 4304^T revealed a spot of SGL-1' (galacturonosyl ceramide) similar to that of *Sphingomonas yanoikuyae* EY 4208^T, these four strains were used, together with *S. paucimobilis* EY 2395^T, for comparative study of phenotypic characteristics. Strains were grown on either Bacto-heart infusion agar or half-concentration nutrient broth supplemented with 1.5% agar; incubations were at 30 °C, unless otherwise stated.

For DNA–DNA hybridization, in addition to the above described five strains, the type strains of ten other *Sphingomonas* species (*Sphingomonas sanguinis*, *Sphingomonas parapaucimobilis*, *Sphingomonas adhaesiva*, *Sphingomonas capsulata*, *Sphingomonas trueperi*, *Sphingomonas chlorophenolica*, *Sphingomonas natatoria*, *Sphingomonas rosa*, *Sphingomonas echinoides* and *Sphingomonas mali*), and *Escherichia coli* SMUM 344^T as a control strain, were used (Table 1).

Morphological, biochemical and physiological characterization. For strain RW1^T, motility was tested by microscopy of a wet mount preparation of young broth culture. Motile organisms were taken from diffuse growth on a 0.3% agar plate, inoculated with heart infusion broth; flagellation was confirmed by photomicroscopy of a flagella-stained preparation by Leifson's method. Transmission electron microscopy of cells negatively stained with 1% potassium phosphotungstate was performed under a JEOL JEM-1200 electron microscope.

Tests for characterization listed in Table 2 were performed by previously described methods (Yabuuchi *et al.*, 1990). Utilization of citrate and malonate were determined by growth and alkali production on Simmons' citrate agar and in malonate broth (Eiken Chemicals), respectively. Oxidative acid production was tested by using Bacto-OF basal medium supplemented with a total of 26 carbohydrates and sugar alcohols (1%) and ethanol (3%). Results were read for up to 4 weeks. Biotype 100 and Biotype medium 1 or 2 (bioMérieux) were used to establish nutritional profiles of test organisms following the manufacturer's instructions.

Drug susceptibilities. Susceptibilities of the organisms were tested with 36 kinds of drug-containing Senci-discs and ready-to-use Mueller–Hinton medium II (Becton–Dickinson) as described previously (Yabuuchi *et al.*, 1999).

Cellular lipids and fatty acids analysis. Cells grown on agar media were harvested, the cellular lipids were extracted twice, washed and hydrolysed with 0.5 M NaOH as described previously (Yabuuchi *et al.*, 1990, 1999). The total extractable lipids and their alkaline hydrolysates were analysed by TLC with an acidic solvent system composed of chloroform/methanol/acetic acid/water (100:20:12:5, by vol.). Purified SGL-1 of *S. paucimobilis*

Table 1 Histories and corresponding numbers of the type strains of 15 *Sphingomonas* species and *Escherichia coli*

For comparative characterization, species nos 1–5 were used.

No.	Species	Corresponding no.*							Received from:*	Received as:
		ATCC	DSM	GIFU	IFO	JCM	NCTC	SMUM		
1	<i>Sphingomonas</i> sp. RW1 ^T		6014			10273		2128	DSM	<i>Sphingomonas</i> sp. RW1 ^T
2	<i>S. yanoikuyae</i>	51230	7462	9882	15102	7371		2121	Bruun	<i>Sphingobacterium</i> sp. AB 1105
3	<i>S. terrae</i>	51381			15098	10195		2120	JCM	<i>Sphingomonas terrae</i>
4	<i>S. macrogoltabidus</i>	51380			15033	10192		3667	JCM	<i>Sphingomonas macrogoltabidus</i>
5	<i>S. paucimobilis</i> †	29837	1098	2395	13935	7516	11030	2114	NCTC	<i>Pseudomonas paucimobilis</i>
6	<i>S. sanguinis</i>	51382		2397	13937	7514	11032	2115	GIFU	<i>Sphingomonas</i> genospecies 1
7	<i>S. parapaucimobilis</i>	51231		11387	15100	7510		2122	GIFU	<i>Sphingomonas parapaucimobilis</i>
8	<i>S. adhaesiva</i>	51229		11458	15099	7370		2123	GIFU	<i>Sphingomonas adhaesiva</i>
9	<i>S. capsulata</i>	14666	30196	11526	12533	7508		2124	GIFU	<i>Sphingomonas capsulata</i>
10	<i>S. trueperi</i>	12417				10278		2125	ATCC	<i>Sphingomonas trueperi</i>
11	<i>S. chlorophenolica</i>	33790				10275		2126	ATCC	<i>Sphingomonas chlorophenolica</i>
12	<i>S. natatoria</i>	35951	3183			10396		2127	ATCC	<i>Blastomonas natatoria</i>
13	<i>S. rosa</i>	51837			15208	10277		2131	JCM	<i>Sphingomonas rosa</i>
14	<i>S. echinoides</i>	14820	50409		15742	10637		3668	JCM	<i>Sphingomonas echinoides</i>
15	<i>S. mali</i>	51840			15500	10193		3669	IFO	<i>Sphingomonas mali</i>
	<i>Escherichia coli</i>	11775	30083	12542		1649	9001	344	GIFU	<i>Escherichia coli</i>

* ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; GIFU, Department of Microbiology, Gifu University School of Medicine, Gifu, Japan; IFO, Institute of Fermentation Osaka, Osaka, Japan; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Saitama, Japan; NCTC, National Collection of Type Cultures, London, UK; SMUM, St Marianna University School of Medicine, Kanagawa, Japan; Bruun, E. Bruun, Copenhagen, Denmark; EY, Eiko Yabuuchi, Aichi Medical University, Aichi, Japan.

† Nomenclatural type species for the genus *Sphingomonas*.

Table 2 Comparative characterization of *Sphingomonas* sp. RW1^T and four other *Sphingomonas* species

All five strains were Gram-negative, rod-shaped and positive for motility, growth at 30 °C, oxidase, catalase and hydrolysis of aesculin. All five strains were negative for growth on MacConkey agar, fermentation of glucose, nitrate to nitrite, acylamidase, phenylalanine deaminase, lysine, ornithine decarboxylase and arginine dihydrolase in decarboxylase base Møller, urease in Christensen's medium. ND, Not done.

Characteristic	<i>Sphingomonas</i> sp. strain RW1 ^T (= EY 4224 ^T)	<i>S. yanoikuyae</i> EY 4208 ^T	<i>S. terrae</i> EY 4207 ^T	<i>S. macrogoltabidus</i> EY 4304 ^T	<i>S. paucimobilis</i> EY 2395 ^T *
Flagellation	Single polar/subpolar	Single polar	ND	ND	Single polar
Deep yellow pigmentation	—	—	—	—	+
Utilization of:					
Citrate (Simmons)	—	+	+	+	+
Malonate broth	+	—	+	—	—
Hydrolysis of:					
Gelatin	+	—	+	—	+
Starch	—	—	—	—	+
Tween 80	—	—	—	+	+
Deoxyribonuclease	—	+	+	+	+
G + C (mol %)	67†	61·7	63·0‡	63·9	63·7

* Type species for the genus.

† Wittich *et al.* (1992).

‡ Takeuchi *et al.* (1993).

Fig. 1. Electron micrograph of a negatively stained cell of *Sphingomonas* sp. strain RW1^T with a single polar flagellum. Cells were stained with 1% potassium phosphotungstate and observed under a JEOL JEM-1200 transmission electron microscope. Bar, 0·5 µm

EY 2395^T was used as a standard. To detect spots and their colour reaction, 50% sulfuric acid solution, Dittmer's reagent, ninhydrin solution and Anthrone reagent were used for all lipids, phospholipids, free amino bases and glycolipids, respectively. Cellular fatty acid analysis was performed by a method described previously (Yabuuchi *et al.*, 1990, 1999).

Preparation of DNA. Bacterial cells growing in nutrient broth (Difco) in shaking culture for 24 h at 30 °C were collected and resuspended in 50 ml S-TE buffer [10 mM Tris/HCl buffer, 1 mM EDTA (disodium), 0·15 M NaCl, pH 8·0]

containing lysozyme (0·1 mg ml⁻¹). The suspension, supplemented with 3% SDS, was incubated at 60 °C for 10 min. An equal volume of phenol mixture [phenol/chloroform/3-methyl-1-butanol (25:24:1, by vol.)] was added to the suspension, which was then mixed vigorously and centrifuged at 10000 *g* for 10 min. The centrifuged supernatant fluid was transferred to a new tube and 2 vol. ethanol was added. The precipitated DNA dissolved in TE buffer (10 mM Tris/HCl buffer, 1 mM EDTA, pH 8·0) was incubated with RNase A (10 µg ml⁻¹) for 30 min at 35 °C and further incubated with proteinase K (10 µg ml⁻¹) and 0·3% SDS for 60 min at 50 °C. Following phenol mixture treatment and ethanol precipitation, the purified DNA was dissolved in TE buffer and used as a reference for DNA–DNA hybridization.

Preparation of DNA plate and labelled DNA. Immobilization of the reference DNA to a microplate was performed by a previously described protocol (Ezaki *et al.*, 1989). The heat-denatured reference DNA (10 µg ml⁻¹) was dissolved in PBS (0·145 M NaCl, 0·15 M sodium phosphate) containing 0·1 M MgCl₂. A portion (100 µl) of the solution was distributed into wells of the microplate (Cliniplate EB; Labsystems). The plates were kept for 16 h at 30 °C. After discarding the solution, the DNA plate was kept in a dry condition. The DNA was labelled using a commercial kit with a photo-activatable form of biotin (PHOTOPROBE Long Arm Biotin, SP-1020; Vector Laboratories).

Hybridization. Blocking solution [2 × SSC (0·3 M NaCl and 0·03 M sodium citrate) containing 2% blocking reagent (Boehringer Mannheim)] was distributed to wells of the DNA plate and kept for 30 min at room temperature. After discarding the blocking solution, 100 µl hybridization solution (2 × SSC, 50% formamide, 1% blocking reagent; pH 7·2–7·6) containing labelled single-stranded DNA was distributed to wells of the DNA plate. After 16 h hybridization at 50 °C, the wells were washed three times

Fig. 2. Flagellar morphology of *Sphingomonas* sp. strain RW1^T stained by Leifson's method. Three images (a, b and c) were taken from the same smear. (a) A single polar flagellum of normal type wave; (b) a single subpolar flagellum of normal type wave; (c) a single polar flagellum of a single long curvature.

with 1 × SSC solution. Alkaline phosphatase–streptavidin (Vector Laboratories) was diluted 1000-fold with PBS containing blocking agent (Block Ace Powder; Dainippon

Seiyaku). The diluted mixture was distributed to the wells (100 µl in each). After 30 min incubation at 35 °C, the wells were washed three times with PBS and filled with 100 µl substrate solution (BluePho Microwell Phosphatase Substrate system; Kirkegaard & Perry Laboratories). The plate was incubated at room temperature and the OD₆₃₀ of each well was measured. The relative relatedness among the reference DNAs was calculated using the OD₆₃₀ values and the protocol of colorimetric microplate hybridization (Kusunoki *et al.*, 1991).

16S rDNA gene sequences and phylogenetic analysis.

Deposited data on 16S rDNA sequences obtained from the DNA Database of Japan (DDBJ, Institute of Genetics, Mishima, Shizuoka, Japan) were used for the phylogenetic analysis. In addition to the five strains listed in Table 1, data of type strains of 17 *Sphingomonas* species and two subspecies of *Zymomonas mobilis* were used. *Brevundimonas diminuta* DSM 1635^T was used as an outgroup organism. Sources of sequence data of a total of 25 strains are shown in parentheses following each type strain number in the phylogenetic tree (Fig. 4).

For the phylogenetic analysis, alignment gaps and unidentified base positions were not taken into consideration for the calculation. Each 1210 base sequence of all 25 strains (Fig. 4) was used for the calculation. Nucleotide substitution rates (K_{nuc}) (Kimura, 1980) were determined by the CLUSTAL

Table 3 Oxidative acid production from 26 carbohydrates, sugar alcohols and ethanol by *Sphingomonas* sp. strain RW1^T and type strains of five other *Sphingomonas* spp.

The first six rows of the table show tests useful for differentiation of strain RW1^T. +, Positive reaction within 3 d; +*, 4 d or more required for positive reaction; –, negative reaction during 4 week incubation; NG, no growth.

Substrate	Strain RW1 ^T (= EY 4224 ^T)	<i>S. yanoikuyae</i> EY 4208 ^T	<i>S. mali</i> EY 4340 ^T	<i>S. terrae</i> EY 4207 ^T	<i>S. macrogoltabidis</i> EY 4304 ^T	<i>S. paucimobilis</i> EY 2395 ^{T†}
Inulin	+*	–	–	–	+	–
Sorbitol	+	–	–	–	–	–
Mannitol, D-ribose	+	–	+	+	–	–
Fructose, galactose	+	+	–	–	–	+
Dulcitol	–	–	–	+	–	+*
Xylose	–	+	–	–	–	+
Glycerol	+	+	–	–	–	+*
Maltose	+	+	+	+	–	+*
Glucose	+	+	–	+*	–	+*
Salicin	+	+	–	+	–	+*
Rhamnose	+	+	+	+	–	–
D-Ribose	+	–	+*	+	–	–
Cellobiose	+*	+	+*	+	+	+*
Sucrose	+*	+	+*	+*	+	+*
Mannose, melezitose	+*	+	+*	+	–	+
Trehalose	+*	+	+	+	+	+*
D-Arabinose	–	+	+	–	–	+*
L-Arabinose	–	+	+*	–	+	+*
Melibiose	–	+	+	+*	+	–
Ethanol	–	+	–	+*	NG	+
Raffinose	–	+	–	+*	–	+*
Inositol	–	–	–	+*	–	–
Adonitol	–	–	–	–	–	–
Lactose	–	+	–	–	–	+*

† Type species of the genus.

w program (Thompson *et al.*, 1994). The neighbour-joining method (Saitou & Nei, 1987) was used to reconstruct a phylogenetic tree from the distance matrices by NJPLOT written by Manolo Gouy (Laboratoire de Biometrie, University of Lyon, Villeurbanne, France). To evaluate the topology of the phylogenetic tree, a bootstrap analysis was performed. Bootstrap values, out of a total of 1000 replications, are indicated at the branching points.

RESULTS

Phenotypic characteristics

Colonies of strain RW1^T (= EY 4224^T) were 1 mm in diameter, smooth and glistening with entire margins and faintly yellow-pigmented after 3 d incubation at 30 °C. Compared with *S. yanoikuyae* EY 4208^T, *S. macroglotabidus* EY 4304^T and *S. paucimobilis* EY

2395^T, growth of strain RW1^T and *S. terrae* EY 4207^T was slower; 3 d were required for pin-point colony production. Cells of strain RW1^T were Gram-negative straight rods with rounded ends. Soma size was 0.4–0.6 × 0.9–1.5 µm. Cells were actively motile with a single polar or subpolar flagellum (Figs 1 and 2).

A comparison of phenotypic characteristics of type strains of five *Sphingomonas* species is given in Table 2. Strain RW1^T produced acid oxidatively from 18 of 26 carbohydrates, sugar alcohols and ethanol in OF basal medium (Difco). Positive reactions were observed for 12 carbohydrates, including glucose and inulin, within 3 d of inoculation. Acid was slowly produced from the remaining eight carbohydrates. Oxidative acid production in OF medium is summarized in Table 3.

Results of carbon assimilation using Biotype 100 (99

Table 4 Assimilation test (Biotype 100) results for *Sphingomonas* sp. strain RW1^T and the type strains of four *Sphingomonas* species

The names of the four *Sphingomonas* species used are given in Table 2. All five strains were positive for: aesculin, DL-β-hydroxybutyrate, L-glutamate, D(+)trehalose, maltotriose and maltose. All five strains were negative for: D(+)arabitol, L(–)arabitol, xylitol, dulcitol, D-sorbitol, adonitol, malonate, D-lyxose, *i*-erythritol, hydroxyquinoline-β-glucuronate, L(+)tartrate, tryptamine, itaconate, L(+)sorbitol, α-ketoglutarate, *meso*-tartrate, D(+)malate, D-galacturonate, 2-keto-D-gluconate, 5-ketogluconate, L-tryptophan, *m*-hydroxybenzoate, benzoate, 3-phenylpropionate, *m*-coumarate, trigonellin, putrescine, histamine, caprylate, L-histidine, DL-α-amino-n-valerate, ethanolamine and α-ketogluconate.

Substrate	Result of strain RW1 ^T (= EY 4224 ^T)	EY no. of type strain giving the result:	
		+	–
D-Tagatose, <i>myo</i> -inositol, tricarballoylate, phenylacetate, DL-glycerate, D-glucosamine	+	4208, 4207, 4304, 2395	
α-D(+)Glucose, β-D(+)fructose, D(+)xylose, α-D(+)melibiose, D(+)raffinose, D(+)galactose, α-lactose, D(+)cellobiose, β-gentiobiose, DL-lactate, L-aspartate, L(+)arabinose, sucrose, D(+)turanose, <i>N</i> -acetyl-D-glucosamine, maltitol, 1- <i>o</i> -methyl-β-glucopyranoside	+	4208, 4304, 2395	4207
L-Proline	+	4208, 4207, 4304	2395
L-Serine, L-alanine	+	4207, 4304, 2395	4208
L(–)Malate, succinate, fumarate	+	4208, 4207, 2395	4304
L-Tyrosine	+	4304, 2395	4208, 4207
1- <i>o</i> -Methyl-α-D-glucopyranoside	+	4208, 2395	4207, 4304
D(+)Mannose, (–)quinine	–		
Glycerol, D-gluconate	+	4208, 4304	4207, 2395
Citrate, D-glucuronate, gentisate	+	4208	4207, 4304, 2395
D-Alanine	+	4207	4208, 4304, 2395
D-Mannitol, DL-α-amino-n-butyrate	+	4304	4208, 4207, 4304
<i>cis</i> -Aconitate, <i>trans</i> -aconitate	+	2395	4208, 4207, 4304
Propionate	–	4208, 4207, 4304	2395
Paratinose, protocatechuate,	–	4208, 4304, 2395	4207
L(–)Fucose, D(+)melezitose, <i>p</i> -hydroxybenzoate	–	4208, 2395	4207, 4304
D-Gluconate, glycerol	–	4208, 4304	4207, 2395
α-L-Rhamnose, D-saccharate, caprate, mucate	–	4208	4207, 4304, 2395
D(–)Tartrate	–	4207	4208, 4304, 2395
D-Ribose	–	4304	4208, 4207, 2395
Betaine, glutarate	–	2395	4208, 4207, 4304

Table 5 Susceptibilities of *Sphingomonas* sp. strain RW1^T and four other *Sphingomonas* species against 36 antibacterial agents by the agar disk diffusion method

S, Susceptible; R, resistant; IM, intermediate.

Drug	Strain RW1 ^T	<i>S. yanoikuyae</i> EY 4208 ^T	<i>S. terrae</i> EY 4207 ^T	<i>S. macrogoltabidus</i> EY 4304 ^T	<i>S. paucimobilis</i> EY 2395 ^T	No. resistant strains
Imipenem, tetracycline, doxycycline, minocycline	S	S	S	S	S	0
Amikacin, gentamicin, dibekacin, panipenem	S	S	S	S	S	0
Amoxicillin/clavulanic acid	S	S	S	IM	S	0
Cefaclor, ceftazidime, cefotaxim, cefoperazon	S	S	IM	R	S	1
Penicillin, ampicillin, amoxicillin, meropenem	S	S	S	R	S	1
Sparfloxacin, levofloxacin, ciprofloxacin, ofloxacin	R	S	S	S	S	1
Tosufloxacin, norfloxacin, erythromycin	R	IM	IM	S	S	1
Polymyxin B	IM	S	R	S	IM	1
Clarithromycin, sulfamethoxazole/trimethoprim	R	S	R	S	S	2
Roxithromycin	R	R	R	IM	S	3
Flomoxef, carmonum	S	R	R	R	S	3
Piperacillin, cefazolin	S	R	S	R	R	3
Cefmetazole	S	R	R	R	R	4
Aztreonam	R	R	R	R	IM	4
Moxalactam, trimethoprim	R	R	R	R	R	5
Total number of drugs						
Resistant	13	9	10	16	5	
Intermediate	1	3	7	2	2	
Susceptible	22	24	19	18	29	
Susceptible + Intermediate	23	27	26	20	31

Fig. 3. TLC of cellular lipids of strain RW1^T (= EY 4224^T), *S. yanoikuyae* EY 4208^T, *S. terrae* EY 4207^T, *S. macrogoltabidus* EY 4304^T, *S. paucimobilis* EY 2395^T and purified SGL-1 of EY 2395^T as standard (from left to right). Solvent system: chloroform/methanol/acetic acid/water (100:20:12:5, by vol.). For each strain, lane 1 indicates a crude lipid and lane 2 indicates an alkali-stable lipid.

Table 6 Cellular fatty acid composition of *Sphingomonas* sp. strain RW1^T and type strains of four *Sphingomonas* species

—, Not detected; TR, trace (less than 1 %).

Fatty acid	Strain RW1 ^T (= EY 4224 ^T)	<i>S. yanoikuyae</i> EY 4208 ^T	<i>S. terrae</i> EY 4207 ^T	<i>S. macrogoltabidus</i> EY 4304 ^T	<i>S. paucimobilis</i> EY 2395 ^{T*}
14:0	TR	TR	—	TR	1
15:0	—	—	2	TR	—
<i>cis</i> 9-16:1	6	14	4	29	2
16:1†	2	2	TR	3	—
16:0	12	9	2	10	17
17:1	—	—	8	—	—
17:0Δ	TR	TR	45	TR	—
17:0	—	TR	4	—	—
<i>cis</i> 11- and <i>trans</i> 9-18:1	54	52	13	43	60
18:1‡	TR	2	TR	TR	—
18:0	—	TR	TR	TR	1
19:1	—	—	TR	—	—
19:0Δ	3	—	1	—	TR
2-OH 14:0	10	14	2	7	18
2-OH 15:0	—	—	13	2	—
2-OH 16:1	—	TR	—	—	—
2-OH 16:0	—	2	TR	TR	—
Others	11	3	2	3	TR

* Type species of the genus.

† Except *cis*9-16:1.

‡ Except *cis*11- and *trans*9-18:1.

Table 7 DNA–DNA reassociation rates (%) among the type strains of 15 *Sphingomonas* species and *Escherichia coli* SMUM 344

SMUM, Department of Microbiology, St Marianna University School of Medicine, Kanagawa, Japan; EY, Eiko Yabuuchi, Department of Microbiology, Aichi Medical University, Aichi, Japan.

EY no.	Species	DNA from:														
		4224 ^T	4208	4207	4304	2395	2397	4213	4215	4216	4218	4219	4220	4227	4340	4341
4224 ^T	<i>Sphingomonas</i> sp. strain RW1 ^T	100	3	2	3	1	5	3	3	3	16	3	0	10	5	5
4208	<i>S. yanoikuyae</i>	0	100	0	2	2	5	3	18	8	3	16	0	11	3	5
4207	<i>S. terrae</i>	0	1	100	2	27	1	1	0	1	0	1	0	1	1	0
4304	<i>S. macrogoltabidus</i>	0	2	22	100	1	1	1	0	2	0	3	3	3	1	1
2395	<i>S. paucimobilis</i> *	0	4	2	1	100	33	24	1	10	9	4	0	6	4	6
2397	<i>S. sanguinis</i>	0	7	4	0	29	100	35	1	3	4	5	3	7	4	5
4213	<i>S. parapaucimobilis</i>	1	7	1	0	36	42	100	0	2	5	5	0	7	4	6
4215	<i>S. adhaesiva</i>	6	12	0	0	11	13	7	100	6	5	14	0	6	8	5
4216	<i>S. capsulata</i>	0	6	6	0	1	2	2	0	100	0	0	0	4	1	1
4218	<i>S. trueperi</i>	1	4	6	0	5	5	2	0	3	100	6	0	2	2	2
4219	<i>S. chlorophenolica</i>	0	8	5	0	1	1	1	0	3	0	100	0	6	2	1
4220	<i>S. natatoria</i>	0	3	4	0	1	2	2	0	32	0	4	100	6	1	1
4227	<i>S. rosa</i>	1	3	0	2	1	6	2	0	6	0	3	0	100	1	2
4340	<i>S. echinoides</i>	0	3	8	2	4	7	2	0	3	3	8	8	3	100	4
4341	<i>S. mali</i>	5	3	7	1	14	9	1	0	1	8	2	0	4	2	100
4342	<i>Escherichia coli</i> SMUM 344	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Type species of the genus.

Fig. 4. Phylogenetic position of *Sphingomonas* sp. strain RW1^T among *Sphingomonas* and *Zymomonas* species obtained by the neighbour-joining analysis of 16S rDNA. Figures in parentheses indicate the accession numbers of 16S rDNA sequence data. Bar, 10 nucleotide substitutions per 1000 nucleotides of 16S rDNA sequence. Bootstrap values from 1000 analyses are shown at the branch points.

substrates and negative control) are shown in Table 4. Whereas *S. paucimobilis* EY 2395^T and *S. yanoikuyae* EY 4208^T assimilated 44 and 47 substrates, respec-

tively, strain RW1^T assimilated 50. *S. terrae* EY 4207^T and *S. macrogoltabidus* EY 4304^T assimilated 16 and 38 substrates, respectively.

Strain RW1^T was susceptible to penicillins, cepheems, macrolides and aminoglycosides, whereas it was characteristically resistant to all seven new quinolone chemotherapeutics tested (Table 5).

Cellular lipids and fatty acids analysis

Major and ubiquitous cellular lipids of all 15 strains tested were phosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin (data not shown). As shown in Fig. 3, which was developed by an acidic solvent system, spots of SGL-1' were seen in the alkali-stable lipids of RW1^T, *S. yanoikuyae* EY 4208^T, *S. terrae* EY 4207^T and *S. macrogoltabidus* EY 4304^T.

The major cellular fatty acid was 18:1 in four strains, with the exception of *S. terrae* EY 4207^T in which 17:0 cyclopropanoic acid was the major fatty acid. The major 2-hydroxy acid in *S. terrae* EY 4207^T was 2-OH 15:0, whereas 2-OH 14:0 was the major acid in the four other strains. None of the five strains contained 3-hydroxy fatty acids (Table 6).

DNA–DNA reassociation rates and phylogenetic analysis of 16S rDNA sequences

As shown Table 7, DNA–DNA reassociation rates between strain RW1^T and each type strain of the 14 *Sphingomonas* species, including three species (*S. yanoikuyae*, *S. terrae* and *S. macrogoltabidus*) which contain SGL-1' in their cellular lipids, ranged from 0 to 10%. Reassociation rates among the 14 species ranged from 0 to 42% and also reconfirmed them as independent species.

The phylogenetic tree (Fig. 4) illustrates that strain RW1^T falls within the genus *Sphingomonas* and shows clear distinction from the 14 named species of this genus. The type strains of the four SGL-1'-containing species did not form a cluster.

DISCUSSION

Since the transfer of *Pseudomonas paucimobilis* (Holmes *et al.*, 1977) to a new genus, *Sphingomonas*, biodegradation or utilization of DD, DF and other various related compounds by strains of *Sphingomonas* species have been reported. Examples of such compounds are diphenyl ether (Schmidt *et al.*, 1992), 4,4'- and 2,4-dihalodiphenyl ethers (Schmidt *et al.*, 1993), DD (Wittich *et al.*, 1992), chlorinated DF (Wilkes *et al.*, 1996; Strubel *et al.*, 1989), chloro- and bromo-biphenyls (Davison *et al.*, 1996), polycyclic aromatic hydrocarbons (Khan *et al.*, 1996; Dagher *et al.*, 1997), pentachlorophenol (Nohynek *et al.*, 1995; Karlson *et al.*, 1996; McCarthy *et al.*, 1996), herbicides (Nickel *et al.*, 1997), polyethylene glycol (Takeuchi *et al.*, 1993; Zipper *et al.*, 1996) and γ -hexachlorocyclohexane (Thomas *et al.*, 1996).

Enzymes (Armengaud & Timmis, 1997; Bünz *et al.*, 1993; Bünz & Cook, 1993; Happe *et al.*, 1993; McCarthy *et al.*, 1996), plasmids (Yrjala *et al.*, 1997, 1998; Romine *et al.*, 1999) and genetics (Armengaud

et al., 1998; Masai *et al.*, 1999; Miyauchi *et al.*, 1998; Peng *et al.*, 1998) of such degradations have also been reported.

Most of the organisms studied in the above-described literature are named as *Sphingomonas* sp., without establishing their nomenclatural standing as a species. *Sphingomonas* sp. strain RW1^T has been known as the most potent organism to degrade and even utilize DD and DF as sole sources of carbon and energy (Wittich *et al.*, 1992). DNA–DNA reassociation rates between the type strains of each of 14 *Sphingomonas* species studied revealed that strain RW1^T is independent from these species. The presence of SGL-1 (Fig. 3) and 2-OH myristic acid, and the absence of 3-OH fatty acids (Table 6) in cellular lipids suggests that RW1^T is a member of the genus *Sphingomonas*; this is confirmed by phylogenetic analysis of the 16S rDNA sequence (Fig. 4). Among the 22 *Sphingomonas* species, only *S. terrae*, *S. macrogoltabidus*, *S. yanoikuyae* and strain RW1^T contained SGL-1' (galacturonosyl ceramide) in their cellular lipids (Fig. 3). These four organisms did not form a cluster in 16S rDNA phylogenetic analysis (Fig. 4) and could be phenotypically differentiated from each other (Table 8). Although it has been previously reported (Naka *et al.*, 2000) that *S. mali* EY 4206 possesses SGL-1' as its cellular lipid component, the strain was identified as *S. macrogoltabidus* by DNA–DNA hybridization. In addition, cellular lipids of *S. mali* EY 4341^T, obtained from IFO as IFO 15500^T, did not contain SGL-1'. These findings indicate that *S. mali* EY 4206 used in the previous study (Naka *et al.*, 2000) was, in fact, a strain of *S. macrogoltabidus* and that *S. mali* does not have SGL-1'.

On the standpoint of toxicity and environmental pollution, DD is one of the most important toxic substances for all life on Earth. Strain RW1^T is thus far a single strain of a new species, for which the name *Sphingomonas wittichii* sp. nov. is herein proposed. It is expected that by establishing the nomenclatural standing of this organism, research work on dioxin-degrading bacteria will be accelerated.

Description of *Sphingomonas wittichii* sp. nov.

Sphingomonas wittichii (wi.tti.chi.i. L. gen. n. *wittichii* of Wittich, referring to Rolf-Michael Wittich, the German bacteriologist who first isolated this potent metabolizer of dibenzo-*p*-dioxin from the water of the River Elbe and described the metabolism of dibenzo-*p*-dioxin by this organism).

Colonies are pin-point after 2 d incubation and, after a further 2–3 d are 1 mm in diameter. They are smooth, shiny, convex, semi-translucent and initially greyish-white, becoming faintly yellow after 3 d incubation at 30 °C. Gram-negative rods with rounded ends. Soma size is 0.4–0.6 × 0.9–1.5 µm. Actively motile with single polar or subpolar flagellum. Flagellar wave morphology is regular (Fig. 2a, b) or with only one long curve (Fig. 2c). Able to grow at 30 °C. Oxidase- and catalase-positive. Metabolism is respiratory, never

Table 8 Phenotypic characteristics for differentiating *Sphingomonas* sp. strain RW1^T from the type strains of four other *Sphingomonas* species

+, Positive reaction; —, negative reaction; R, resistant; S, susceptible.

Characteristic	Strain RW1 ^T (= EY 4224 ^T)	<i>S. yanoikuyae</i> EY 4208 ^T	<i>S. terrae</i> EY 4207 ^T	<i>S. macrogoltabidus</i> EY 4304 ^T	<i>S. paucimobilis</i> EY 2395 ^{T*}
Galacturonosyl ceramide	+	+	+	+	—
Deep yellow pigmentation	—	—	—	—	—
Utilization of citrate (Simmons)	—	+	+	—	—
Hydrolysis of gelatin	+	—	+	—	—
DNase	—	+	+	—	+
Acid from:					
Inulin	+	—	—	+	—
Sorbitol, mannitol	+	—	+	—	—
Assimilation of:					
Malonate	+	—	—	—	—
Caprate	—	+	—	—	—
DL- α -Amino-n-butyrate	—	—	—	+	—
D(—) Tartrate	—	—	+	—	—
Susceptibility to:					
Penicillin	S	S	S	R	S
Piperacillin	S	R	S	R	R
Cefmetazole	S	R	R	R	R
Clarisharomycin	R	S	R	S	S
Ofloxacin, sparflaxacin	R	S	S	S	S

* Type species of the genus.

fermentative or photosynthetic. Hydrolyses aesculin, liquefies gelatin and alkalinizes malonate broth, but does not alkalinize Simmons' citrate medium and does not hydrolyse starch or Tween 80. Failed to grow on MacConkey agar medium. The following tests are negative: deoxyribonuclease, nitrate reduction to nitrite, acylamidase, phenylalanine deaminase, lysine and ornithine decarboxylase and arginine dihydrolase. In OF medium, oxidative acidity observed from D-arabinose, D-ribose, fructose, galactose, glucose, maltose, glycerol, sorbitol, mannitol, rhamnose, salicin and inulin within 3 d. Among the 99 substrates of Biotype 100 (bioMérieux), only two carbohydrates, α -L(—)fucose and D-glucuronate are assimilated; the amino acids phenylalanine, L-glutamate, L-proline, D-alanine, L-alanine, L-aspartate and L-tyrosine and the organic acids gentisate, DL-lactate, succinate, fumarate, DL- β -hydroxybutyrate and malonate are assimilated. Resistant to macrolide and new quinolone antibiotics. Major fatty acid of cellular lipid is 18:1; the only hydroxy acid is 2-OH 14:0. Alkali-stable cellular lipids contain both SGL-1 and SGL-1'. DNA G + C content is 67 mol % (Wittich *et al.*, 1992). Type strain is DSM 6014^T (= JCM 10273^T = EY 4224^T).

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