***Sphingomonas pituitosa* sp. nov., an exopolysaccharide-producing bacterium that secretes an unusual type of sphingan**

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Strain EDIV^T, an exopolysaccharide-producing bacterium, was subjected to polyphasic characterization. The bacterium produced copious amounts of an extracellular polysaccharide, forming slimy, viscous, intensely yellow-pigmented colonies on Czapek–Dox (CZD) agar. The culture fluids of the liquid version of CZD medium were highly viscous after cultivation for 5 d. Cells of strain EDIV^T were Gram-negative, catalase-positive, oxidase-negative, non-spore-forming, rod-shaped and motile. Comparisons of 16S rDNA gene sequences demonstrated that EDIV^T clusters phylogenetically with the species of the genus *Sphingomonas sensu stricto*. The G+C content of the DNA (64.5 mol%), the presence of ubiquinone Q-10, the presence of 2-hydroxymyristic acid (14:0 2-OH) as the major hydroxylated fatty acid, the absence of 3-hydroxy fatty acids and the detection of *sym*-homospermidine as the major component in the polyamine pattern, together with the presence of sphingoglycolipid, supported this delineation. 16S rDNA sequence analysis indicated that strain EDIV^T is most closely related (99.4% similarity) to *Sphingomonas trueperi* LMG 2142^T. DNA–DNA hybridization showed that the level of relatedness to *S. trueperi* is only 45.5%. Further differences were apparent in the cellular fatty acid profile, the polar lipid pattern, the Fourier-transform infrared spectrum and whole-cell proteins and in a number of biochemical characteristics. On the basis of the estimated phylogenetic position derived from 16S rDNA sequence data, DNA–DNA reassociation and phenotypic differences, strain EDIV^T (= CIP 106154^T = DSM 13101^T) was recognized as a new species of *Sphingomonas*, for which the name *Sphingomonas pituitosa* sp. nov. is proposed. A component analysis of the exopolysaccharide (named PS-EDIV) suggested that it represents a novel type of sphingan composed of glucose, rhamnose and an unidentified sugar. Glucuronic acid, which is commonly found in sphingans, was absent. The mean molecular mass of PS-EDIV was approximately 3×10^6 Da.

Keywords: *Sphingomonas pituitosa* sp. nov., polyphasic characterization, exopolysaccharide, sphingan

INTRODUCTION

Interest in the genus *Sphingomonas* is becoming

Abbreviations: FT-IR, Fourier-transform infrared; PHB, poly- β -hydroxybutyrate.

The EMBL accession number for the 16S rDNA sequence of *Sphingomonas pituitosa* strain EDIV^T is AJ243751.

increasingly intense, as there have been several recent reports on the broad catabolic capabilities of strains of *Sphingomonas* species. These organisms have great potential for biotechnological applications in the degradation, bioremediation and wastewater treatment of xenobiotic pollutants (e.g. Fredrickson *et al.*, 1991, 1995; Harms *et al.*, 1995; Karlson *et al.*, 1995; Mueller *et al.*, 1990; Nohynek *et al.*, 1996a, b; Wittich

et al., 1992; Zipper *et al.*, 1996), as bacterial antagonists of phytopathogenic fungi (Berg & Balin, 1994) and for the production of industrially useful exopolysaccharides (Moorehouse, 1987; Chandrasekaran & Radha, 1995). Certain structurally related exopolysaccharides referred to as 'sphingans' (Pollock, 1993) are synthesized by several *Sphingomonas* strains, and include the following: heteropolysaccharide S-7 (Kang & McNeely, 1976), gellan (S-60), S-88, welan (S-130) (Kang & Veeder, 1982a, b, 1985; Kang *et al.*, 1982; O'Neill *et al.*, 1983), rhamsan (S-194) (Peik *et al.*, 1983), S-198 (Peik *et al.*, 1985), S-657 (Chowdhury *et al.*, 1987b), NW11 (Robison & Stipanovic, 1989), PS-P4 (Lobas *et al.*, 1994), GS-1 (Ashtaputre & Shah, 1995), I-886 (Falk *et al.*, 1996) and HWR1 (Hashimoto & Murato, 1998). Sphingans share the same carbohydrate-backbone structure ($-X\text{-glucose-glucuronic acid-glucose-X-}$, where X is either L-rhamnose or L-mannose), to which distinct side-groups are attached, influencing their rheological properties. Sphingan-producing bacteria were classified originally into diverse genera such as *Alcaligenes*, *Azotobacter*, *Pseudomonas*, *Xanthobacter* and *Xanthomonas*, although a re-examination of their phenotypic characteristics indicated that they are all closely related to each other and to *Sphingomonas* species (Pollock, 1993).

A series of polyphasic taxonomic studies has demonstrated that sphingomonads can be recognized at the genus and species level by the employment of chemotaxonomic methods (Yabuuchi *et al.*, 1990; Takeuchi *et al.*, 1993, 1995; Nohynek *et al.*, 1996a, b; Balkwill *et al.*, 1997; Kämpfer *et al.*, 1997; Denner *et al.*, 1999; Busse *et al.*, 1999; Stolz *et al.*, 2000; Yun *et al.*, 2000). The sphingomonads that have been analysed so far are characterized by the presence of ubiquinone Q-10 as the major respiratory lipoquinone and *sym*-homospermidine or spermidine as the characteristic compound in the polyamine pattern. The G+C contents of genomic DNAs range from 61.6 to 67.8 mol %. Furthermore, sphingomonads contain 2-hydroxymyristic acid (14:0 2-OH) as the major hydroxylated fatty acid, do not contain 3-hydroxy fatty acids and do not contain the lipopolysaccharide characteristic of Gram-negative bacteria. In contrast, they have sphingoglycolipids as major components in their cellular lipids and most species contain the carotenoid pigment nostoxanthin. A high degree of qualitative and quantitative variety in the polar lipid composition of sphingomonads, which is useful for their characterization and preliminary identification, was also observed. However, the presence of the unique sphingoglycolipids is a characteristic marker for the genus *Sphingomonas* and closely related genera. Combined analyses of these chemotaxonomic features appear to provide useful chemosystematic signatures for their identification at the family level and to the species level when these approaches are applied in combination (Kämpfer *et al.*, 1997; Denner *et al.*, 1999; Busse *et al.*, 1999).

In the study presented here, we report the polyphasic taxonomic characterization of a novel exopolysaccharide-producing *Sphingomonas* isolate, strain EDIV^T. The phenotypic properties, chemotaxonomic characteristics and 16S rRNA gene sequence of this organism are described and its phylogenetic position among the sphingomonads is discussed. In addition, a first component analysis of the exopolysaccharide secreted by the bacterium was also performed.

METHODS

Source of strains, cultivation and maintenance. The bacterium (designated EDIV^T) investigated in this study was isolated originally from a water sample taken from a eutrophic fountain in Vienna (Austria) in which an algal bloom was observed; isolation was by plating onto Czapek–Dox agar (CZD; Oxoid). Reference type strains *Sphingomonas echinoides* DSM 1805^T, *Sphingomonas parapaucimobilis* DSM 7463^T and *Sphingomonas paucimobilis* DSM 1098^T were obtained from the DSMZ, Braunschweig, Germany. *Sphingomonas adhaesiva* IFO 15099^T, *Sphingomonas mali* IFO 15500^T, *Sphingomonas pruni* IFO 15498^T and *Sphingomonas sanguinis* IFO 13937^T were obtained from M. Salkinoja-Salonen (Department of Applied Microbiology and Biochemistry, University of Helsinki, Helsinki, Finland). *Sphingomonas trueperi* ATCC 12417^T was obtained from G. Auling (Institute of Microbiology, University of Hannover, Hannover, Germany). All bacterial strains were stored at $-70\text{ }^{\circ}\text{C}$ in tryptone soy broth (TSB; Oxoid) containing 20% glycerol. Bacterial inocula for all experiments were taken from these glycerol stocks, streaked onto tryptone soy agar (TSA; Oxoid) and incubated aerobically at $28\text{--}30\text{ }^{\circ}\text{C}$ for 48 h; all experiments were performed twice.

Microscopy. Cell morphology and motility were examined by phase-contrast microscopy (Leitz, Diaplan) from overnight cultures grown in TSB at $28\text{--}30\text{ }^{\circ}\text{C}$ on a rotary shaker (150 r.p.m.). Cell size was estimated by DAPI (4',6-diamidino-2-phenylindole) staining according to Porter & Feig (1990), as modified by Sherr *et al.* (1992). Flagellar arrangement was examined by using the procedure of Kodaka *et al.* (1982). The Gram reaction was determined by staining and was confirmed by the KOH test as described by Smibert & Krieg (1994). For electron microscopy, cells were washed three times with a buffer (pH 7.2) containing 2 mM MgSO_4 and 75 mM sodium cacodylate and then fixed with 2% glutaraldehyde, in the same buffer, at room temperature for 1 h and washed again. The cells were prepared for transmission electron microscopy as described by Witte *et al.* (1990). Transmission electron microscopy was performed using a ZEO 912 Omega 101 transmission electron microscope operated at 80 kV in the zero-loss mode.

Cultural and biochemical characterization. Colony morphology was described from cultures grown aerobically on complex peptone-containing standard media including TSA, Luria–Bertani agar (Atlas, 1995) and nutrient agar (Merck), as well as on low-nutrient medium R2A agar (Oxoid); the individual media were incubated at $28\text{--}30\text{ }^{\circ}\text{C}$ for 24–72 h. Growth at 4, 37 and $45\text{ }^{\circ}\text{C}$ was tested for as long as 2 weeks. Susceptibility to eight antimicrobial agents of strain EDIV^T, *S. paucimobilis* DSM 1098^T and *S. trueperi* ATCC 12417^T was tested by the disc diffusion method, as described by Jorgensen *et al.* (1999), using antibiotic-impregnated discs

(Oxoid). Briefly, 100 µl of a bacterial suspension, with an optical density equivalent to that of a 0.5 McFarland standard, was plated onto Iso-Sensitest agar (Oxoid). The following antibiotics were tested (µg antibiotic per disk in parentheses): ampicillin (10), chloramphenicol (30), gentamicin (10), kanamycin (30), streptomycin (10), penicillin G (10 IU), polymyxin B (300 IU) and tetracycline (30). Any sign of growth inhibition after 48 h incubation at 28–30 °C was scored as sensitivity to that antibiotic. Resistance to an antimicrobial drug was indicated if no inhibition zone was observed.

Oxidase activity was tested for by using Bactident–Oxidase test strips (Merck) according to the manufacturer's instructions and by using the Kovac test (Smibert & Krieg, 1994). Initial identification was performed using API 20NE galleries (bioMérieux). Detailed biochemical characterization was performed by using the miniaturized assay method, as described previously (Kämpfer *et al.*, 1991; Kämpfer & Altwegg, 1992), and by conventional testing, as described by Smibert & Krieg (1994), which included the test for catalase activity, the Hugh–Leifson oxidative–fermentative test and tests for nitrate reduction, H₂S production, citrate utilization (Simmons), indole production and hydrolysis of Tween 80, starch and casein. DNase production was examined in cultures grown for 48 h on DNase test agar (Oxoid) at 28–30 °C. The accumulation of poly-β-hydroxybutyrate (PHB) was evaluated by GC from freeze-dried cell material, as described previously (Schroll *et al.*, 1996).

Chemotaxonomic investigations. Respiratory lipoquinones were extracted from 100 mg lyophilized cell material with methanol/hexane (2:1, v/v) as described by Tindall (1990). The hexane phase, containing the respiratory lipoquinones, was separated by TLC on silica-gel F₂₅₄ plates (Merck) with hexane/*tetra*-butyl methyl ether (9:1, v/v) as the solvent system and compared with commercially available ubiquinones 9 and 10 (Sigma) on the basis of *R_f* values (Collins, 1994). UV-absorbing bands corresponding to ubiquinones were removed from the plate, eluted in acetone, evaporated under a stream of nitrogen and subsequently dissolved in 100 µl hexane. A 10 µl volume of the hexane extract was analysed by HPLC (Waters Instruments), using butylchloride/methanol (1:9, v/v) as the eluant. Respiratory lipoquinones were detected by absorbance at 269 nm. Polyamines were extracted as described by Busse & Auling (1988) and were analysed according to Busse *et al.* (1997). Fatty acid methyl esters were extracted and prepared using the standard protocol of the MIDI/Hewlett Packard Microbial Identification System. Fatty acid methyl ester extracts were analysed using a Hewlett Packard (model HP6890A) GC equipped with a flame-ionization detector, an automatic sampler, an integrator and a computer, as described previously (Kämpfer & Kroppenstedt, 1996). Polar lipids were extracted from 100 mg lyophilized cell material by the modified Folch procedure devised by Bligh & Dyer (1959) and resolved in 250 µl chloroform/methanol (2:1, v/v). Extracted lipids were separated by two-dimensional TLC using 10 × 10 cm silica-gel F₂₅₄ plates (Merck). Chloroform/methanol/water (65:25:4, by vol.) was used as the first solvent system and chloroform/methanol/glacial acetic acid/water (80:12:15:4, by vol.) was used as the second solvent system. Individual lipids were detected by using the following spray reagents: ninhydrin, to detect lipids containing free amino groups (Consden & Gordon, 1948); Zinzadze reagent, to detect lipids containing phosphate esters (Dittmer & Lester, 1964); Dragendorff

reagent, to detect lipids containing quaternary nitrogen compounds (Wagner *et al.*, 1961; Beiss, 1964); and α-naphthol, to visualize carbohydrate-containing lipids (glycolipids) (Jacin & Mishkin, 1965). Molybdatophosphoric acid was used to obtain a fingerprint of the total lipid content (Gunstone & Jacobsberg, 1972). Subsequently, lipids were identified by comparing *R_f* values, using commercially prepared standards (Sigma) and lipid extracts from reference type strains.

Pigment analysis. A 100 mg quantity of cell material grown on TSA for 48 h at 28–30 °C was scraped from the agar surface and placed into a small (5 ml) Teflon-sealed glass vial. Acetone (2 ml) was added to extract acetone-soluble pigments. After centrifugation at 10000 *g* at 4 °C for 5 min, the supernatant was scanned for absorbance by using a Hitachi S-2000 spectrophotometer. Pure acetone was used as the blank and acetone extracts of the yellow pigments of *S. paucimobilis* DSM 1098^T and *S. trueperi* ATCC 12417^T grown under the same conditions were used as references.

Analysis of whole-cell protein patterns. SDS-PAGE of whole-cell proteins was performed according to Laemmli (1970). Briefly, approximately 60 mg cells (wet weight) grown on TSA for 48 h at 28–30 °C was harvested by centrifugation, resuspended in 300 µl SDS sample buffer containing 150 µl Laemmli buffer and 150 µl phosphate buffer (5 mM, pH 6.8), boiled for 10 min and centrifuged at 10000 *g* for 15 min to remove any precipitate. About 4 µl sample was loaded onto a 12% polyacrylamide gel. Proteins were visualized by staining with Coomassie blue R 250. A broad-range protein marker containing proteins from 2.3 to 212 kDa was used as a reference (New England BioLabs).

Fourier-transform infrared (FT-IR) spectroscopy. Bacterial films for FT-IR analysis were prepared from EDIV^T, as well as from reference strain *S. trueperi* ATCC 12417^T grown on TSA at 28–30 °C for 48 h, by resuspending a loopful (1 mm diameter) of cells in 80 µl deionized water and applying an aliquot of 35 µl to a preformed sample area on a zinc selenite optical plate (Bruker). Following the drying of samples under reduced pressure, spectra between wave number 4000 and 500 cm⁻¹ were recorded using an FT-IR spectrometer (type IFS 28/B; Bruker), as described by Helm *et al.* (1991a, b). Data analysis was carried out using OPUS 3.0 software (Bruker) for bacterial identification. At least six independent preparations of each strain were measured and a mean spectrum was calculated.

Determination and analysis of 16S rRNA gene sequence. Genomic DNA was prepared from individual colonies picked from agar medium, resuspended in 100 µl TE buffer and heated at 95 °C for 5 min. After cooling on ice, cell debris was pelleted in a microfuge for 30 s. Genomic DNA in the supernatant was concentrated by using Microcon-100 spin concentrators (Amicon) and was resuspended in 10 µl TE buffer. The 16S rRNA genes were targeted for amplification by PCR using a 1 µl aliquot of the DNA suspension, a forward primer hybridizing at the complement of positions 8–27 and a reverse primer hybridizing at positions 1525–1541 (*Escherichia coli* numbering) and reaction conditions described in detail previously (Karlson *et al.*, 1995). The 16S rDNA PCR products were purified by using Microcon-100 spin concentrators and were sequenced directly using a Perkin-Elmer/Applied Biosystems 373A DNA sequencer and the protocols of the manufacturer (Perkin-Elmer/Applied Biosystems) for *Taq* cycle sequencing and fluorescent dye-labelled dideoxynucleotides.

The nearly complete gene sequence of strain EDIV^T was confirmed in this study by determining the sequences of PCR products produced by two separate reactions. Additionally, all sequences have been determined by overlapping sequences in both the forward and reverse directions. Sequence data were aligned with reference rRNA (and rRNA gene) sequences (Maidak *et al.*, 1999) using evolutionarily conserved primary sequences and secondary structures as references (Gutell *et al.*, 1985). Distance and cluster analyses were carried out using programs contained in PHYLIP version 3.5c (Felsenstein, 1993).

DNA–DNA hybridization and DNA base composition. For hybridization experiments, genomic DNA of strain EDIV^T and *S. trueperi* ATCC 12417^T was isolated by hydroxyapatite chromatography by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was determined spectrophotometrically, as described by De Ley *et al.* (1970), with modifications as described by Huss *et al.* (1983) and Escara & Hutton (1980), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992). These experiments were carried out by the Identification Service of the DSMZ, Braunschweig, Germany. For determination of the DNA base composition, DNA was extracted by the method of Marmur (1961). The mean G + C content (mol%) of the genomic DNA was determined by HPLC (Tamaoka & Komagata, 1984; Kaneko *et al.*, 1986) after nuclease P₁ and alkaline phosphatase treatment.

Production, extraction, deacetylation and hydrolysis of the exopolysaccharide. For production of the exopolysaccharide, strain EDIV^T was cultivated for 5 d at 28–30 °C with rotary shaking (500 r.p.m.) in 1 litre Erlenmeyer flasks containing 300 ml of a mineral salt medium containing the following (l⁻¹ deionized water; pH 7.3 ± 0.2): 1.0 g K₂HPO₄, 0.5 g KCl, 0.01 g Fe₂(SO₄)₃, 0.5 g MgSO₄ · 7H₂O and 3.0 g NaNO₃, supplemented with 12% (w/v) sucrose. Subsequently, the extracellular material was precipitated with 3 vols 1-propanol, as described by Azeredo & Olivera (1996), after dilution (1:10) of the highly viscous broth and centrifugation at 9000 g for 30 min to remove cells. The precipitate was washed with propane three times and then lyophilized. Deacylation was performed, as described by Kang *et al.* (1982), and the deacetylated exopolysaccharide was precipitated with 2 vols 1-propanol and dried by lyophilization. For compositional analysis, the native, as well as the deacetylated, exopolysaccharides were hydrolysed with trifluoroacetic acid as described previously (Hashimoto & Murato, 1998). The remaining hydrolysed exopolysaccharide was dissolved in HPLC-grade water (Merck), after evaporation of the trifluoroacetic acid under a vacuum at room temperature, and stored at –20 °C.

Exopolysaccharide component analysis. The monosaccharides of the hydrolysed exopolysaccharide were separated by TLC on silica-gel F₂₅₄ plates (Merck). Acetone/butanol/water (40:5:5, by vol.) and butanol/acetic acid/water (4:6:1, by vol.) were used as solvent systems; 1–2 µl of the hydrolysate was applied to the TLC plate. Typical components of exopolysaccharides produced by sphingomonads (Pollock, 1993), including glucose, rhamnose, mannose and glucuronic acid, were used as references. The individual components were detected by heating the TLC plates at 110 °C for 5 min after spraying them with 10% (v/v) sulfuric acid in ethanol (Hashimoto & Murato, 1998) and α-naphthol (Jacin & Mishkin, 1965). Additionally,

hydrolysates, as well as the reference sugars (glucose, rhamnose, mannose and glucuronic acid), were separated by HPLC with an Aminex HPX87-H column, with 0.025 M H₂SO₄ as the eluant, at an operating temperature of 60 °C and a flow rate of 0.5 ml min⁻¹. A 20 µl aliquot of pre-filtered sample was injected and the components were detected by a refractive index detector (RID-10A). Monomeric sugars were identified on the basis of their retention times, using reference sugars (Sigma). The molecular mass of the exopolysaccharide was determined by gel-permeation chromatography, using a Nucleogel aqua-OH 60–8 column (Macherey-Nagel) with a pore size of 4000 Å, an exclusion size of 20 MDa and 0.02% sodium azide in water as the mobile phase. The eluent was detected by a light-scatter detector. The presence of monomeric uronic acids and sulfate esters was determined by FT-IR spectroscopy (Ishizuka *et al.*, 1973). The monomeric uronic acid content was also determined by GC-MS and the characteristic fragmentation pattern of the uronic methyl esters.

RESULTS

Phenotypic characteristics

Initially, when strain EDIV^T was isolated from CZD agar (containing sucrose as the major carbon and energy source), copious amounts of an extracellular material were produced, resulting in colonies with a slimy/viscous, intensely yellow appearance. Culture fluids of the liquid version of CZD medium were highly viscous after 5 d cultivation.

Cells of strain EDIV^T were straight rods (0.4–0.75 µm wide by 1.0–3.0 µm long) that were Gram-negative and motile by means of a single polar flagellum. After some exopolysaccharide had been produced, the cells in older cultures became slow-moving or were non-motile. Endospores were not observed under any of the growth conditions applied. Electron microscope examination of ultrathin sections demonstrated that the cell envelopes were typical of Gram-negative bacteria, there being a cytoplasmic membrane and an outer membrane (Fig. 1a); inclusion bodies containing storage material (probably polyphosphate and PHB) were also observed (Fig. 1b). GC analyses confirmed the presence of PHB in the cells. Colonies grown on complex peptone-containing standard media were as large as 1 mm in diameter, circular, entire, convex, smooth and deep-yellow as a result of a non-diffusible pigment after 48 h incubation at 28–30 °C. Good growth was also observed on the low-nutrient medium R2A (Reasoner & Geldreich, 1985). Colonies were 1.5 mm in diameter, translucent, yellow, circular, entire, low-convex and smooth on R2A agar after 2 d. No growth was observed on any media tested at 4, 37 or 45 °C.

Characterization tests, performed according to conventional methods, showed strain EDIV^T to be catalase-positive, oxidase-negative, strictly aerobic and non-fermentative. It was unable to reduce nitrate, was indole-negative and did not form H₂S. Tween 80 was hydrolysed, but starch, gelatin, casein and DNA were not. By using the API 20NE identification system,

Fig. 1. Electron micrographs of ultrathin sections of strain EDIV^T. (a) Gram-negative cell wall structure of the bacterium. Bar, 100 nm. (b) The presence of polyphosphate (black) and large PHB inclusion bodies (white) in cells grown for 5 d in a mineral salt medium containing 12% (w/v) sucrose. Bar, 500 nm.

strain EDIV^T was identified, with 99.8% (API code 0463340) probability, as *S. paucimobilis*. Strain EDIV^T formed deep-yellow colonies on agar plates. The spectral characteristics of the yellow pigment extracted in acetone (λ_{max} at 452 and 478 nm and a slight inflexion at 425 nm) corresponded to those reported for the carotenoid nostoxanthin [(2R, 3r, 2'R, 3R)- β , β -carotene-2,3,2',3'-tetrol]. Nostoxanthin was identified by Jenkins *et al.* (1979) as the yellow pigment of [*Pseudomonas*] *paucimobilis* (now *S. paucimobilis*) and was also detected in '*Pseudomonas azotocolligans*' (now *S. trueperi*) and [*Pseudomonas*] *echinoides* (now *S. echinoides*).

Antibiotic-susceptibility tests revealed that strain EDIV^T is sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin and tetracycline and resistant to penicillin G, polymyxin B and streptomycin. The type strain of *S. paucimobilis* displayed the same susceptibility pattern, except that the strain was resistant to ampicillin and tetracycline. *S. trueperi* ATCC 12417^T exhibited an antibiotic-susceptibility pattern identical to that of strain EDIV^T.

The results obtained from further biochemical testing indicated that strain EDIV^T is different from *S. paucimobilis* and is, furthermore, clearly separable from other species within the *S. paucimobilis* branch. A

detailed comparison was made between these results and all previously published biochemical data (Heumann, 1962; Yabuuchi *et al.*, 1990; Takeuchi *et al.*, 1993; Kämpfer *et al.*, 1997; Denner *et al.*, 1999; Yun *et al.*, 2000), as shown in Table 1.

Chemotaxonomic characteristics

Ubiquinone Q-10 was determined to be the major respiratory lipoquinone system in strain EDIV^T. The predominant cellular fatty acids (Table 2) were included in summed feature 7 (18:1 ω 7c, 18:1 ω 9t and/or 18:1 ω 12t). Other significant fatty acids were 14:0 2-OH, 17:1 ω 6c, 16:0, 15:0 and 15:0 2-OH. 3-Hydroxy fatty acids were not detected. The triamine *sym*-homospermidine (68.4 $\mu\text{mol g}^{-1}$, dry weight) was observed to be the major component in the polyamine pattern, with minor amounts of spermidine (3.3 $\mu\text{mol g}^{-1}$, dry weight) and spermine (1.0 $\mu\text{mol g}^{-1}$, dry weight) being detected as well. The overall polar lipid pattern was characterized by the presence of phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, sphingoglycolipid and an unidentified lipid (L1) as major compounds. Minor amounts of diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylcholine and several unknown lipids were detected as well. The total polar lipid content of

Table 1. Comparative biochemical characteristics of strain EDIV^T and type strains of species of the *S. paucimobilis* branch

Data are presented for: 1, EDIV^T; 2, *S. trueperi* ATCC 12417^T; 3, *S. asaccharolytica* IFO 15499^T; 4, *S. mali* IFO 15500^T; 5, *S. pruni* IFO 15498^T; 6, *S. paucimobilis* DSM 1098^T; 7, *S. adhaesiva* IFO 15099^T; 8, *S. parapaucimobilis* DSM 7463^T; 9, *S. sanguinis* IFO 13937^T; 10, *Sphingomonas roseiflava* IAM 14823^T; and 11, *S. echinoides* DSM 1805^T. Test results given in the table were read after 72 h incubation at 30 °C. Results are scored as: +, positive; –, negative; (+), weakly positive; ND, no data. pNA, *para*-Nitroanilide; pNP, *para*-nitrophenyl. Strain EDIV^T and all other strains gave positive results in the following tests: catalase activity; hydrolysis of bis-pNP phosphate, pNP phenyl-phosphate, 2-deoxythymidine-5'-pNP phosphate, L-alanine-pNA and L-glutamate- γ -3-carboxy-pNA; assimilation of *N*-acetylglucosamine, cellobiose, D-galactose, glucose, maltose and D-trehalose. All of the strains gave negative results for acid production from lactose, sucrose, dulcitol, adonitol, L-arabinose, raffinose and D-arabitol and for the assimilation of D-ribose, adonitol, *i*-inositol, D-mannitol, D-sorbitol, putrescine, 4-aminobutyrate, azelate, itaconate, mesaconate, β -alanine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate and phenylacetate. Data in columns 2–9 were taken from Yabuuchi *et al.* (1990), Takeuchi *et al.* (1993), Kämpfer *et al.* (1997) and this study. Data in column 10 were taken from Yun *et al.* (2000). Data in column 11 were taken from Heumann (1962), Denner *et al.* (1999) and this study.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Nitrate reduction	–	–	–	–	–	–	–	+	–	+	+
DNase production	–	–	–	–	–	+	+	+	+	+	–
Liquefaction of gelatin	–	–	–	–	–	+	–	–	+	+	–
Acid produced from:*											
Glucose	(+)	–	(+)	(+)	(+)	–	–	–	–	ND	–
D-Mannitol	–	–	+	–	–	–	–	–	–	ND	–
Salicin	–	–	(+)	–	–	+	+	–	–	ND	–
Inositol	–	–	+	–	–	–	–	–	–	ND	–
Sorbitol	–	+	–	–	–	–	–	–	–	ND	–
Rhamnose	(+)	–	(+)	–	–	–	(+)	(+)	–	ND	–
Maltose	(+)	–	–	–	–	(+)	(+)	(+)	–	ND	–
D-Xylose	(+)	–	–	–	–	+	(+)	(+)	–	ND	–
Trehalose	–	–	+	–	–	–	–	–	–	ND	–
Cellobiose	(+)	–	–	–	–	(+)	(+)	(+)	–	ND	–
Methyl α -D-glucoside	–	+	–	–	–	+	+	–	–	ND	–
Erythritol	–	–	–	–	–	–	+	–	–	ND	–
Melibiose	–	–	–	–	–	(+)	(+)	(+)	–	ND	–
D-Mannose	–	–	–	–	–	(+)	(+)	(+)	–	ND	–
Hydrolysis of:											
Aesculin	+	–	–	(+)	(+)	(+)	–	(+)	–	+	+
pNP Galactopyranoside	+	+	(+)	+	(+)	+	(+)	+	+	ND	–
pNP β -D-Glucuronide	+	+	+	+	+	–	+	+	+	ND	+
pNP Phosphorylcholine	+	+	+	+	+	–	+	+	+	ND	+
L-Glutamate- γ -3-carboxy-pNA	+	+	+	+	+	+	+	+	+	ND	(+)
L-Proline-pNA	–	–	+	+	+	–	–	+	–	ND	+
Assimilation of:											
L-Arabinose	+	+	+	+	+	+	–	+	+	+	+
<p>-Arbutin</p>	+	+	–	+	+	+	+	+	+	ND	–
D-Fructose	–	+	–	–	–	–	+	–	+	ND	–
Gluconate	–	–	–	+	–	–	–	+	–	+	–
D-Mannose	+	+	+	+	+	+	+	+	+	(+)	–
α -D-Melibiose	+	+	+	+	+	–	+	+	+	ND	–
L-Rhamnose	–	–	+	+	+	–	–	(+)	–	ND	–
Sucrose	+	+	(+)	+	+	+	+	+	+	ND	–
Salicin	+	+	(+)	+	(+)	–	+	+	+	ND	–
D-Xylose	+	+	(+)	+	+	+	–	+	+	ND	–
Maltitol	–	+	+	–	–	–	–	–	+	ND	–
Acetate	+	+	–	–	–	–	–	+	+	ND	–
Propionate	–	+	–	–	–	–	–	+	+	ND	–
<i>cis</i> -Aconitate	–	–	–	–	–	+	–	+	–	ND	–
<i>trans</i> -Aconitate	–	–	–	–	–	+	–	–	–	ND	–
Adipate	–	–	–	(+)	–	–	–	–	–	(+)	–

Table 1 (cont.)

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Citrate	—	—	—	—	—	—	—	+	—	+	—
Fumarate	+	+	(+)	+	+	+	+	—	+	ND	+
Glutarate	—	+	—	—	—	—	—	+	—	ND	—
DL-3-Hydroxybutyrate	+	+	+	+	+	—	—	+	+	ND	—
DL-Lactate	—	+	—	—	—	—	—	+	+	ND	—
L-Malate	+	+	—	—	—	+	+	+	+	+	+
Oxoglutarate	—	—	—	—	—	+	+	+	+	ND	—
Pyruvate	+	+	—	+	—	+	—	+	+	ND	—
Suberate	—	—	—	+	—	—	+	—	—	ND	—
L-Alanine	+	+	+	—	—	—	+	+	+	ND	—
L-Asparagine	—	+	+	+	+	—	+	—	+	ND	—
L-Histidine	—	—	—	+	+	—	—	—	—	ND	—
L-Leucine	—	+	—	—	—	—	+	+	+	ND	—
L-Ornithine	—	—	—	—	—	—	—	—	+	ND	—
L-Proline	+	—	+	—	—	—	+	—	+	ND	—
L-Serine	—	+	—	—	—	—	—	—	—	ND	—
4-Hydroxybenzoate	—	+	—	—	—	—	—	—	—	ND	—

* Acid production from carbohydrates by strain EDIV^T was very weak (even after prolonged incubation), as reported for other *Sphingomonas* species (Kämpfer *et al.*, 1997; Denner *et al.*, 1999); therefore these tests cannot be recommended for differentiation.

Table 2. Major fatty acid composition of strain EDIV^T and selected type strains of species of the *S. paucimobilis* branch

Data are presented for: 1, EDIV^T; 2, *S. trueperi* ATCC 12417^T; 3, *S. asaccharolytica* IFO 15499^T; 4, *S. mali* IFO 15500^T; 5, *S. pruni* IFO 15498^T; 6, *S. paucimobilis* DSM 1098^T; 7, *S. adhaesiva* IFO 15099^T; 8, *S. parapaucimobilis* DSM 7463^T; 9, *S. sanguinis* IFO 13937^T; 10, *S. echinoides* DSM 1805^T. Values are percentages of total fatty acid. Fatty acid nomenclature: for unsaturated fatty acids, the position of the double bond can be located by counting from the methyl (ω) end of the carbon chain. *cis* is indicated by the suffix *c*. Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system (Microbial ID). Summed feature 4 contained one or more of the following fatty acids: 16:1 ω 7 and 15:0 iso 2-OH. Summed feature 7 contained one or more of the following isomers: 18:1 ω 7*c*, 18:1 ω 9*t* and/or 18:1 ω 12*t* (*cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively). Data in columns 2–9 were from Kämpfer *et al.* (1997). Data in column 10 were from Denner *et al.* (1999).

Compound	1	2	3	4	5	6	7	8	9	10
Saturated fatty acids:										
14:0	—	—	—	—	—	1.4	—	1.0	1.0	0.9
15:0	2.6	—	—	—	—	—	—	—	1.1	—
16:0	11.5	9.8	13.3	11.8	16.0	8.7	6.5	9.6	13.6	10.0
17:0	—	1.6	—	—	—	—	—	—	0.5	—
18:0	—	—	—	—	—	—	—	—	0.6	—
Unsaturated fatty acids:										
16:1 ω 5 <i>c</i>	—	—	—	—	—	—	—	1.9	1.0	0.8
17:1 ω 6 <i>c</i>	13.0	13.6	—	9.3	—	3.0	21.2	3.8	2.4	2.9
17:1 ω 8 <i>c</i>	—	—	—	—	—	—	—	—	—	—
18:1 ω 5 <i>c</i>	—	4.0	—	—	—	3.2	—	3.5	3.5	2.7
Hydroxy fatty acids:										
14:0 2-OH	14.2	6.7	18.8	8.4	10.9	6.4	13.8	6.7	5.0	7.2
15:0 2-OH	2.4	—	—	—	—	—	—	—	—	—
16:0 2-OH	—	—	—	—	—	—	—	—	—	—
Summed feature 4	2.1	—	—	—	—	2.7	6.2	8.6	6.7	2.45
Summed feature 7	54.2	64.2	67.8	70.5	73.1	74.6	47.3	65.0	64.6	73.1

Fig. 2. Polar lipids of strain EDIV^T after separation by two-dimensional TLC. Major lipids: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PDE, phosphatidyl-dimethylethanolamine; PG, phosphatidylglycerol; SGL, sphingoglycolipid; L1, unidentified polar lipid. Minor lipids: PC, phosphatidylcholine; PME, phosphatidylmonomethylethanolamine; APL1, unidentified aminophospholipid; PL and PL3, unknown phospholipids; GL, unknown glycolipid. The TLC plate was sprayed with molybdatophosphoric acid.

EDIV^T after separation by two-dimensional TLC is shown in Fig. 2.

The combination of these chemotaxonomic features is consistent with the current description of the genus *Sphingomonas* and confirms the results of other studies (Takeuchi *et al.*, 1993, 1995; Kämpfer *et al.*, 1997; Denner *et al.*, 1999; Busse *et al.*, 1999).

16S rRNA gene sequence and phylogenetic inferences

PCR amplification and direct sequencing allowed the determination of 1446 nucleotide positions of the 16S rRNA gene sequence of strain EDIV^T (approx. 97% of the complete gene, as estimated by comparison with the 16S rRNA gene sequence of *E. coli*). The nearly complete 16S rRNA gene sequence determined in this study has been deposited in the EMBL database (Stoesser *et al.*, 1999). The 16S rRNA gene sequence of strain EDIV^T is seen to be clustered within those of the species of *Sphingomonas* (Fig. 3), most closely related (99.4% similarity) to *S. trueperi* LMG 2142^T (formerly '*Pseudomonas azotocolligans*' Anderson 1955) (Kämpfer *et al.*, 1997). Similarities between the 16S rRNA gene sequences of strain EDIV^T and the other validly described species of *Sphingomonas* (Table 3) ranged from 96.3% (with *Sphingomonas asaccharolytica* IFO 15099^T) to 90.8% (with *Sphingomonas terrae* IFO 15098^T). A 16S rRNA gene sequence similarity of 99.4% clearly indicates that EDIV^T and *S. trueperi* are closely related phylogenetically, but it does not allow the confident assertion that the two organisms should be considered as a single species (Fox *et al.*, 1992). The next most closely related

organisms include those of the *S. paucimobilis* lineage, giving 16S rRNA gene sequence similarities of approximately 96%, a level that clearly delineates different species. Fig. 3 is a dendrogram of estimated phylogenetic relationships inferred from comparisons and evolutionary distance calculations that were derived from 1387 nucleotide positions between positions 28 and 1465 (*E. coli* numbering) from the 16S rRNA gene sequences of strain EDIV^T and all validly published species of *Sphingomonas* and other related species of the α -4 subclass of the *Proteobacteria*. The inferred phylogenetic relationships indicated a clear delineation of the EDIV^T/*S. trueperi* branch from all other *Sphingomonas* species. A bootstrap confidence level of 100% (Felsenstein, 1985) (derived from analysis of 500 resamplings) was observed for the EDIV^T/*S. trueperi* delineation. The topography of the dendrogram was also tested by using a treeing algorithm of maximum-likelihood (fastDNAmL, version 1.0; Maidak *et al.*, 1996). This alternative analysis supported most branching points defined by the distance analysis, including the distinct delineation and branching order of the EDIV^T/*S. trueperi* lineage among the species of the *S. paucimobilis* branch (data not shown).

DNA–DNA hybridization analysis and DNA base composition

In view of the high level of 16S rRNA gene sequence similarity observed between strain EDIV^T and *S. trueperi* LMG 2142^T, a DNA–DNA hybridization analysis was performed between EDIV^T and the reference type strain, ATCC 12417^T (= LMG 2142^T). The level of DNA relatedness observed between the two strains was 45.5%, suggesting that EDIV^T and *S. trueperi* ATCC 12417^T (= LMG 2142^T) do not belong to the same species. The DNA G+C content of EDIV^T (64.5 mol%) was well within the range of values (61.6–67.8 mol%) reported for established *Sphingomonas* species.

SDS-PAGE analysis of whole-cell proteins and FT-IR spectra of whole-cell preparations

Comparison of the whole-cell protein patterns of strains EDIV^T and *S. trueperi* ATCC 12417^T following separation by SDS-PAGE, a method that is used widely in bacterial systematics as an alternative to biotyping methods for strain and species differentiation (e.g. Owen & Jackman, 1982; Jackman, 1987; Vauterin *et al.*, 1993), showed that EDIV^T differed markedly from ATCC 12417^T. In addition, FT-IR spectra of bacteria, recorded from whole cells without any preparation other than drying, provide fingerprint-like, highly reproducible patterns that distinguish strains at the subspecies level (e.g. Helm *et al.*, 1991a, b; Naumann *et al.*, 1991; Raguenes *et al.*, 1996; Huu *et al.*, 1999). The FT-IR spectra obtained from strains EDIV^T and ATCC 12417^T indicated differences in prominent, additional peaks or peak shoulders between wave numbers 1200 and 900 (data not shown);

Fig. 3. Phylogenetic dendrogram based on 16S rRNA gene sequence data, indicating the position of strain EDIV^T within the radiation of the α -4 subclass of the *Proteobacteria*, as derived by evolutionary distance calculations of pairwise comparisons of nearly complete 16S rRNA gene sequences, including the correction factor of Jukes & Cantor (1969). The dendrogram was generated by the least-squares distance algorithm of the FITCH program in PHYLIP. *Agrobacterium tumefaciens* was used as the outgroup. The bar represents estimated evolutionary distance (a mean of 1 substitution at any nucleotide position per 100 nucleotide positions). *S.*, *Sphingomonas*.

this window represents the polysaccharide region (Helm *et al.*, 1991a).

Component analysis of the exopolysaccharide

The extracellular material secreted by strain EDIV^T was found, upon hydrolysis and paper chromatography, to contain rhamnose and glucose, as well as an unidentified sugar. The mean molecular mass, as determined by gel-permeation chromatography, was approximately 3×10^6 Da. No uronic acids or sulfates could be detected using the methods applied, setting the upper limit of these glycosides to 3% of the total glycosidic content within the exopolysaccharide.

DISCUSSION

The comprehensive polyphasic taxonomic study presented here provides clear evidence for the classification of the exopolysaccharide-producing bacterial strain EDIV^T as a new species of the genus *Sphingomonas*.

The genus *Sphingomonas* (Yabuuchi *et al.*, 1990) is a heterogeneous taxon as it is currently described, and

can be divided into several phylogenetic branches within the α -4 subclass of the *Proteobacteria* together with the genera *Blastomonas*, *Rhizomonas*, *Zymomonas* and several aerobic, bacteriochlorophyll-*a*-containing genera on the basis of 16S rRNA gene sequence comparisons (Van Bruggen *et al.*, 1993; Segers *et al.*, 1994; Takeuchi *et al.*, 1994, 1995, 1999; Balkwill *et al.*, 1997; Hiraishi *et al.*, 2000). The range of 16S rRNA gene sequence dissimilarities between the species of the genus *Sphingomonas* is much too large for all described species to be included within one genus. The estimated breadth of phylogenetic relationships demonstrates clearly that a number of *Sphingomonas* species should be described as distinct, new genera and that the species of the *S. paucimobilis* branch should be considered as the genus *Sphingomonas sensu stricto*, as suggested by Takeuchi *et al.* (1995, 1999), including, at present, the species *S. paucimobilis*, *S. parapaucimobilis*, *S. sanguinis*, *S. trueperi*, *S. adhaesiva*, *S. pruni*, *S. mali*, *S. asaccharolytica* and *S. echinoides*. Interestingly, species of the *S. paucimobilis* branch share the characteristic *sym*-homospermidine as the major compound in the polyamine pattern (Busse *et al.*, 1999). In contrast, all

Table 3. Sequence similarities between the 16S rRNA genes of strain EDIV^T and members of the genus *Sphingomonas* and other species of the α -subclass of the *Proteobacteria*

Culture collections are abbreviated as: ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; HAMBI, Culture Collection of the Faculty of Agriculture and Forestry, University of Helsinki, Helsinki, Finland; IAM, Institute of Applied Microbiology, University of Tokyo, Bunkyo-ko, Tokyo, Japan; IFO, Institute for Fermentation, Yodogawa-ku, Osaka, Japan; JCM, Japan Collection of Microorganisms, Wako, Saitama, Japan; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; SMCC, Subsurface Microbial Culture Collection, Florida State University, Tallahassee, FL, USA.

Species	Strain	EMBL accession no.	Sequence similarity to strain EDIV ^T (%)
<i>S. trueperi</i>	LMG 2142 ^T	X97776	99.4
<i>S. asaccharolytica</i>	IFO 15499 ^T	Y09639	96.3
<i>S. mali</i>	IFO 15500 ^T	Y09638	96.2
<i>S. pruni</i>	IFO 15498 ^T	Y09637	96.2
<i>S. paucimobilis</i>	DSM 1089 ^T	X72722	96.0
<i>S. adhaesiva</i>	JCM 7370 ^T	X72720	95.8
<i>S. parapaucimobilis</i>	JCM 7510 ^T	X72721	95.7
<i>S. sanguinis</i>	IFO 13937 ^T	D13726	95.5
<i>S. roseiflava</i>	IAM 14823 ^T	D84520	94.9
<i>S. echinoides</i>	DSM 1805 ^T	AJ012461	94.2
<i>S. xenophaga</i>	DSM 6383 ^T	X94098	92.9
<i>Rhizomonas suberifaciens</i>	IFO 15211 ^T	D13737	92.8
<i>S. yanoikuyae</i>	JCM 7371 ^T	X72725	92.6
<i>S. aromaticivorans</i>	SMCC F199 ^T	U20756	92.5
<i>S. rosa</i>	IFO 15208 ^T	D13945	92.5
<i>S. chlorophenolica</i>	ATCC 33790 ^T	X87161	92.4
<i>S. stygia</i>	SMCC B0712 ^T	U20775	92.3
<i>S. subterranea</i>	SMCC B0478 ^T	U20773	92.3
<i>S. subarctica</i>	HAMBI 2110 ^T	X94102	91.8
<i>S. herbicidovorans</i>	DSM 11019 ^T	AB022428	91.5
<i>S. capsulata</i>	ATCC 14666 ^T	D16147	91.1
<i>Erythrobacter longus</i>	JCM 6170 ^T	D12699	91.1
<i>S. macrogoltabidus</i>	IFO 15033 ^T	D13723	91.0
<i>S. terrae</i>	IFO 15098 ^T	D13727	90.8
<i>Agrobacterium tumefaciens</i>	DSM 30150	M11223	86.1

representatives of the other branches are characterized by spermidine as the major compound. Despite this phylogenetic and chemotaxonomic heterogeneity, three species (*Blastomonas natatoria*, *Rhizomonas suberifaciens* and *Erythromonas ursincola*) have been reclassified recently in the genus *Sphingomonas* (Yabuuchi *et al.*, 1999), expanding the heterogeneity of the genus still further, because, as shown by Takeuchi *et al.* (1999), these three genera can be distinguished clearly from the four major branches of *Sphingomonas* by means of signature nucleotides in the 16S rRNA gene. As a consequence, the genus *Sphingomonas*, as it is now recognized, will ultimately be reclassified into at least three new genera, with only those species in the *S. paucimobilis* branch being maintained within the genus *Sphingomonas*. Very recently, it has been confirmed that *B. natatoria* and *Erythromonas ursincola* are distinct from the genus *Sphingomonas* (Hiraishi *et al.*, 2000). In conclusion, in view of the phylogenetic

distance from members of the *S. paucimobilis* branch, we have used the basonyms *B. natatoria* and *R. suberifaciens* in this study.

16S rRNA gene sequence analysis placed the novel exopolysaccharide-producing bacterial isolate EDIV^T within the cluster considered as the genus *Sphingomonas sensu stricto*. The G+C content of the DNA, the presence of ubiquinone Q-10, the presence of 14:0 2-OH as the major hydroxylated fatty acid, the absence of 3-hydroxylated fatty acids, the presence of sphingoglycolipid and the absorbance spectrum of the yellow pigment all supported this allocation, confirmed by the detection of *sym*-homospermidine as the major compound in the polyamine pattern.

Comparative analysis of the 16S rRNA gene sequence and estimation of the phylogenetic relationships indicated that EDIV^T is closely related (99.4% similarity) to *S. trueperi* LMG 2142^T. On the other hand, the

DNA–DNA relatedness (45.5%) between strain EDIV^T and *S. trueperi* ATCC 12417^T (= LMG 2142^T) was below the threshold value (approx. 70%) that has been suggested as delineating a bacterial species (Wayne *et al.*, 1987), indicating clearly that strain EDIV^T represents a distinct species. Comparison of the FT-IR spectra showed numerous differences in the polysaccharide regions of strains EDIV^T and ATCC 12417^T that would be consistent with differences observed in the whole-cell protein patterns of the two strains (data not shown). These results reflect differences in the genomic content of the organism, again suggesting that strain EDIV^T constitutes a separate species.

Results obtained from cellular fatty acid and polar lipid analyses were also unique and distinguished strain EDIV^T from its phylogenetic neighbour, *S. trueperi*, as well as differentiating it from other *sym*-homospermidine-containing *Sphingomonas* species. Quantitative differences in the fatty acid composition distinguished EDIV^T from *S. trueperi* as well as from other species of the *S. paucimobilis* branch (Table 2). Remarkably, strain EDIV^T possessed 2-hydroxypentadecanoic acid (15:0 2-OH), a feature unique for a member of this branch. Until now, 15:0 2-OH has been detected only in the distantly related species *S. terrae*, *Sphingomonas stygia*, *Sphingomonas subterranea* and *Sphingomonas aromaticivorans* (Kämpfer *et al.*, 1997; Stolz *et al.*, 2000), which are located on other branches of the genus *Sphingomonas* (Fig. 3), and has not been detected so far in representatives of the *S. paucimobilis* branch. The overall polar lipid pattern of EDIV^T was similar to those found in other *Sphingomonas* species (Kämpfer *et al.*, 1997; Denner *et al.*, 1999). As shown previously in our laboratory, the polar lipid composition differs qualitatively and quantitatively between *Sphingomonas* species, and identical polar lipid patterns are observed only in close relatives such as *S. asaccharolytica* and *S. mali* (Kämpfer *et al.*, 1997). Strain EDIV^T was distinguishable from its closest phylogenetic neighbour, *S. trueperi*, because of the absence of an unknown polar lipid (PL1) described for *S. trueperi* and the presence of an unknown aminolipid (APL1) not detected in *S. trueperi* (Kämpfer *et al.*, 1997). EDIV^T was also different from *S. trueperi* in a significant number of biochemical traits; for example, strain EDIV^T lacked oxidase activity, hydrolysed aesculin, assimilated L-proline and was unable to assimilate D-fructose, maltitol, propionate, glutarate, DL-lactate, L-aspartate, L-leucine, L-serine and 4-hydroxybenzoate. The absence of oxidase activity in strain EDIV^T is an almost unique biochemical trait that has been reported for only a few strains of *Sphingomonas* (Yabuuchi *et al.*, 1990). This is particularly interesting since all of the known sphingan-producing *Sphingomonas* strains are also oxidase-negative (Pollock, 1993); perhaps some of these strains are related to strain EDIV^T.

Furthermore, our first investigations of the extracellular material secreted by the bacterium revealed

that it is a heteropolysaccharide (hereafter named PS-EDIV) with a mean molecular mass of approx. 3×10^6 Da, composed of glucose and rhamnose, as well as an unidentified sugar; no uronic acids or sulfates were found in PS-EDIV. The fact that glucuronic acid (which is usually found in sphingans; Pollock, 1993) was not detected in PS-EDIV is an unusual result. To our knowledge, glucuronic acid is absent only in the sphingan PS-P4. This exopolysaccharide has been known to be produced by *S. paucimobilis* strain P4 (DSM 6418) (Lobas *et al.*, 1994), a strain that was isolated by a selective culturing method (Lobas *et al.*, 1992) from a culture of [*P.*] *paucimobilis* NCIB 11942 (Anson *et al.*, 1987). Also of particular interest is the fact that PS-EDIV does not contain L-mannose, which has been found as a constituent of sphingans S-130, S-88, S-198 and NW-11 (Jansson *et al.*, 1985, 1986; Chowdhury *et al.*, 1987a; Robison & Stipanovic, 1989). In addition, there is some similarity between exopolysaccharides produced by [*P.*] *paucimobilis* RMPD strains 17a and 17b (Hebbar *et al.*, 1992). The carbohydrate-backbone structures of these exopolysaccharides are also composed of glucose and rhamnose and also lack L-mannose and uronic acids. However, the reported chemical analyses of all of these exopolysaccharides indicated that they are quite different from PS-EDIV. There is strong evidence from our chemical analyses that the extracellular material secreted by strain EDIV^T is a novel type of exopolysaccharide within the sphingan family. Further studies on the chemical and physical properties of PS-EDIV are in progress.

In summary, this polyphasic investigation clearly demonstrates that strain EDIV^T represents a new species within the genus *Sphingomonas sensu stricto*, for which we propose the name *Sphingomonas pituitosa* sp. nov.

Description of *Sphingomonas pituitosa* sp. nov.

Sphingomonas pituitosa (pi.tu.i.to'sa. L. fem. adj. *pituitosa* slimy).

Cells are Gram-negative, rod-shaped, non-spore-forming and motile (each cell has a polar monotrichous flagellum). Cells are 0.4–0.75 µm wide and 1.0–3.0 µm long. Strictly aerobic. Yellow intracellular pigments (carotenoids) are produced; the visible absorption spectrum of the yellow pigment (acetone extract) has two peaks at λ_{max} 452 and 478 nm and a slight inflexion at 425 nm. Poly-β-hydroxybutyrate is accumulated. Colonies on complex, peptone-containing standard media are deep yellow, circular, entire, convex and smooth. Capable of growth at room temperature and at 30 °C but not at 4, 37 or 45 °C. Capable of growth on nutrient-reduced media such as Czapek–Dox and R2A. No growth on Simmons citrate agar. Positive reactions are obtained for catalase and β-galactosidase. No reactions are obtained for oxidase, arginine dihydrolase, urease, nitrate reduction, production of

indole, gelatin liquefaction or H₂S production. Tween 80 is hydrolysed but starch, casein and DNA are not. Susceptible to ampicillin, chloramphenicol, gentamicin, kanamycin and tetracycline; resistant to penicillin G, polymyxin B and streptomycin. The following compounds are assimilated: *N*-acetyl D-glucosamine, L-arabinose, *p*-arbutin, D-cellobiose, D-galactose, D-glucose, D-mannose, D-maltose, α -D-melibiose, sucrose, salicin, D-trehalose, D-xylose, acetate, fumarate, DL-3-hydroxybutyrate, L-malate, pyruvate, L-alanine and L-proline. The following compounds are not assimilated: D-fructose, gluconate, L-rhamnose, D-ribose, adonitol, *i*-inositol, maltitol, D-sorbitol, D-mannitol, putrescine, propionate, *cis*-aconitate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, glutarate, itaconate, DL-lactate, mesaconate, oxoglutarate, suberate, β -alanine, L-aspartate, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-serine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate. The following compounds are hydrolysed: aesculin, *para*-nitrophenyl (pNP) β -D-galactopyranoside, pNP β -D-glucuronide, pNP α -D-glucopyranoside, pNP β -D-glucopyranoside, pNP β -xylopyranoside, bis-pNP phosphate, pNP phenylphosphate, pNP phosphoryl-choline, 2-deoxythymidine-5'-pNP phosphate, L-alanine-*para*-nitroanilide (pNA) and L-glutamate- γ -3-carboxy-pNA. L-Proline-pNA is not hydrolysed. Weak production of acid can be observed, after prolonged incubation, from D-glucose, lactose, sucrose, L-arabinose, L-rhamnose, maltose, D-xylose and cellobiose. No acids are produced from D-mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, raffinose, trehalose, methyl α -D-glucoside, erythritol, melibiose, D-arabitol or D-mannose. Phylogenetically, *S. pituitosa* clusters with the validly published species of the genus *Sphingomonas* within the α -subclass of the *Proteobacteria* (Woese, 1987; Stackebrandt *et al.*, 1988). The G + C content of the DNA is 64.5 mol %. Ubiquinone Q-10 is the major respiratory lipoquinone. *sym*-Homospermidine is the major component in the polyamine pattern. The major polar lipids are phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol and an unknown lipid (L1). Diphosphatidylglycerol, phosphatidylmonomethanolamine and phosphatidylcholine, as well as several unknown lipids, are present in minor concentrations. Sphingoglycolipid is present. The predominant non-polar fatty acids are 18:1 and 16:0; the major 2-hydroxy fatty acid is 14:0 2-OH, but minor amounts of 15:0 2-OH are also present. Isolated from the water of a eutrophic fountain in Vienna, Austria, in which an algal bloom was observed. The type strain is EDIV^T (= CIP 106154^T = DSM 13101^T).

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