Sphingobacterium detergens sp. nov., a surfactant-producing bacterium isolated from soil

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A novel Gram-negative-staining strain, designated 6.2S^T, was isolated from a soil sample and identified as a biosurfactant producer. Its taxonomic position was investigated using a polyphasic approach. The cells were non-motile, non-spore-forming rods. The organism grew optimally at 30-37 °C, with 0–3 % (w/v) NaCl, and at pH 7.0. Based on 16S rRNA gene sequence analysis, strain 6.2S^T was found to be a member of the genus *Sphingobacterium* and was most closely related to four type species of the genus, showing sequence similarities of 96.8–98.9 %. Partial chaperonin 60 (*cpn60*) gene sequence analysis was useful in resolving the phylogenetic relationships between strain 6.2S^T and closely related taxa, with similarities ranging from 85.5 % (with *Sphingobacterium thalpophilum* DSM 11723^T) to 90.3 % (with *Sphingobacterium canadense* CR11^T and *Sphingobacterium multivorum* JCM 21156^T). The results of DNA–DNA hybridization experiments between the novel strain and its closest relatives gave a DNA–DNA relatedness value of less than 70 %, and consequently confirmed that this new strain did not belong to a previously described species of the genus *Sphingobacterium*. The major fatty acids were summed feature 3 (iso-$C_{15:0}$ 2 OH and/or $C_{16:1}\omega7c$); iso-$C_{15:0}$; iso-$C_{17:0}$ 3-OH and $C_{16:0}$. The G+C content of the genomic DNA was 40.0 mol%. According to its phenotypic and genotypic characteristics and the phylogenetic data, strain 6.2S^T represents a novel species of the genus *Sphingobacterium*, for which the name *Sphingobacterium detergens* sp. nov. is proposed. The type strain is 6.2S^T (=CECT 7938^T=LMG 26465^T).

During screening experiments for new biosurfactants, several soil samples from the Azores Islands were analysed. Strain 6.2S^T was selected for its ability to reduce the surface tension of the medium to 32 mN m^{-1} due to the production of a mixture of compounds with surface activity (Burgos-Díaz *et al.*, 2011). This strain was initially identified as *Sphingobacterium multivorum*, based on cell and colony morphology, as well as the results of the API 20 NE bacterial identification kit. The results of 16S rRNA gene sequence analysis showed that strain 6.2S^T belonged to the genus *Sphingobacterium*, and that its nearest relative was *Sphingobacterium siyangense*. The aim of the present study was to clarify the taxonomic position of this new strain by using a polyphasic approach, including phenotypic, chemotaxonomic, genotypic and phylogenetic analyses.

The family *Sphingobacteriaceae* belongs to the phylum *Bacteroidetes* and is composed of eight genera (*Sphingobacterium*, *Pedobacter*, *Mucilaginibacter*, *Nubsella*, *Olivibacter*, *Parapedobacter*, *Pseudosphingobacterium* and *Solitalea*) of bacteria commonly isolated from the environment (http://www.bacterio.cict.fr). The genus *Sphingobacterium* was established by Yabuuchi *et al.* (1983), based on the presence of high concentrations of sphingophospholipids in cellular lipid components. Two species of the genus *Flavobacterium* were reclassified as members of this new genus (*Sphingobacterium multivorum* and *Sphingobacterium spiritivorum*) and an additional species (*Sphingobacterium mizutae*) was recognized. Members of the genus *Sphingobacterium* are Gram-negative-staining rods, that give a positive reaction for catalase and oxidase, a negative reaction for heparinase, gelatinase and indole production and have iso-$C_{15:0}$, iso-$C_{15:0}$ 2-OH, $C_{16:1}\ \omega7c$ and $C_{17:0}$ 3-OH as the main fatty acids (Kim *et al.*, 2006; Liu *et al.*, 2008). These organisms are frequently isolated from soil, compost or activated sludge and occasionally from clinical specimens (Yabuuchi *et al.*, 1983;

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *cpn60* gene sequences of strain 6.2S^T are JN015213 and JN015214, respectively.

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

Freney *et al.*, 1987). At the time of writing, the genus *Sphingobacterium* comprises 17 recognized species: *Sphingobacterium alimentarium* (Schmidt *et al.*, 2012), *S. anhuiense* (Wei *et al.*, 2008), *S. antarcticum* (Shivaji *et al.*, 1992), *S. bambusae* (Duan *et al.*, 2009), *S. canadense* (Mehnaz *et al.*, 2007), *S. composti* (Ten *et al.*, 2006), *S. daejeonense* (Kim *et al.*, 2006), *S. faecium* (Takeuchi & Yokota, 1992), *S. kitahiroshimense* (Matsuyama *et al.*, 2008), *S. lactis* (Schmidt *et al.*, 2012), *S. mizutaii* (Yabuuchi *et al.*, 1983), *S. multivorum* (Yabuuchi *et al.*, 1983), *S. shayense* (He *et al.*, 2010), *S. siyangense* (Liu *et al.*, 2008), *S. thalpophilum* (Takeuchi & Yokota, 1992), *S. wenxiniae* (Zhang *et al.*, 2012) and the type species of the genus *S. spiritivorum* (Yabuuchi *et al.*, 1983). Eleven of these species have been described in the last seven years. Two other previously described members of the genus *Sphingobacterium* have been reclassified as members of the genus *Pedobacter* as *Pedobacter heparinus* and *Pedobacter piscium* (Steyn *et al.*, 1998).

Strain 6.2S^T, a Gram-negative-staining, aerobic, non-spore-forming bacillus, was isolated from a soil sample after cultivation in a mineral salt medium with a carbon source pool (0.5 % sodium citrate, 0.5 % C_{11-13} and 0.5 % yeast extract) for 7 days at 30 °C and 150 r.p.m. After two subcultures, a dilution (10^{-5}) was spread onto TSA. Isolated colonies were studied for surfactant production (Burgos-Díaz *et al.*, 2011) and routinely cultured on TSA plates. Stock cultures were maintained in Cryo-Beads (AES CHEMUNEX) at −80 °C.

Genomic DNA was extracted using the REALPURE genomic DNA extraction kit (Durviz). PCR amplification and sequencing of 16S rRNA and chaperonin 60 (*cpn60)* genes were performed using universal primers and previously described methods (Martínez-Murcia *et al.*, 1999; Mehnaz *et al.*, 2007). For *cpn60* gene amplification, a second pair of primers was designed using the Primer3 program (Rozen & Skaletsky, 2000) to improve PCR specificity for strain 6.2S^T. The forward and reverse primers designed were cpn60-F (5′-GCAATCGTTGC-TCCAGGTAT-3′) and cpn60-R (5′-GTTGCNANTGCC-TCACCATC-3′) to amplify a ~500 bp region of the *cpn60* gene. The nucleotide sequences obtained were compared with available gene sequences retrieved from GenBank/EMBL/DDBJ databases using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed using CLUSTAL_X software version 2.0 (Larkin *et al.*, 2007). Phylogenetic trees were constructed from a distance matrix corrected with Kimura's two-parameter model (Kimura, 1980) using the neighbour-joining method (Saitou & Nei, 1987) and MEGA4 software (Tamura *et al.*, 2007). The robustness of the tree topology was evaluated by bootstrap analysis based on 1000 replications.

An almost complete 16S rRNA gene sequence (1483 bp) of strain 6.2S^T was obtained (GenBank accession no. JN015213). Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain 6.2S^T is a member of the genus *Sphingobacterium*, which formed a robust cluster with four type strains of this genus (Fig. 1). Strain 6.2S^T was most closely related to *S. siyangense* SY1^T (98.9 % similarity), *S. canadense* CR11^T (98.8 %), *S. multivorum* JCM 21156^T (98.6 %) and *S. thalpophilum* DSM 11723^T (96.8 %). Partial chaperonin 60 (*cpn60*) gene sequence analysis was useful in resolving phylogenetic relationships between strain 6.2S^T and the most closely related taxa of the genus *Sphingobacterium* (Fig. 2). The *cpn60* sequences for isolate 6.2S^T (503 bp) and *S. siyangense* SY1^T (558 bp) were determined and have been deposited in the GenBank database under accession numbers JN015214 and JN015215, respectively. The sequence similarities of strain 6.2S^T ranged from 85.5 % with *S. thalpophilum* DSM 11723^T to 90.3 % with *S. canadense* CR11^T and *S. multivorum* JCM 21156^T. When determining the evolutionary relationships of strain 6.2S^T by sequence analysis, the interrelationships inferred from the *cpn60* gene-based phylogeny had better resolution than those based on 16S rRNA gene sequences.

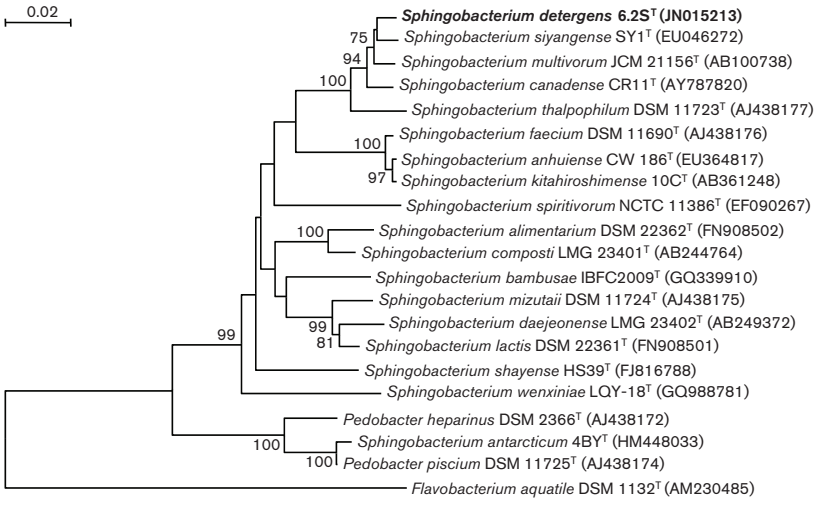

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain 6.2S^T and members of the genus *Sphingobacterium*, including two species later reclassified in the genus *Pedobacter* (*P. heparinus* and *P. piscium*). *Flavobacterium aquatile* DSM 1132^T was used as an outgroup. GenBank accession numbers are shown in parentheses. Bootstrap values (expressed as percentages of 1000 replications) >70 % are given at branch points. Bar, 0.02 substitutions per nucleotide position.

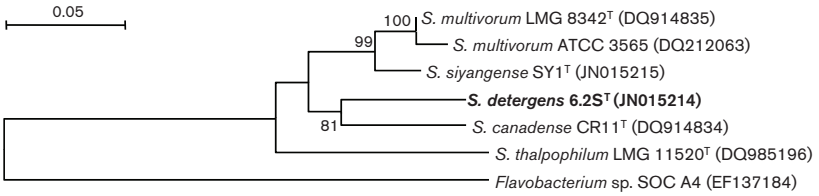

Fig. 2. Neighbour-joining phylogenetic tree based on *cpn60* gene sequences showing the position of strain 6.2S^T and its closest relatives within the genus *Sphingobacterium*. *Flavobacterium* sp. SOC A4 was used as an outgroup. GenBank accession numbers are shown in parentheses. Bootstrap values (expressed as percentages of 1000 replications) >70 % are given at branch points. Bar, 0.05 substitutions per nucleotide position.

These results also confirmed that strain 6.2S^T was a separate species of the genus *Sphingobacterium*.

For DNA–DNA hybridization experiments and determination of DNA G+C content, genomic DNA was prepared according to a modification of the procedure of Wilson (1987). DNA–DNA hybridizations were performed in the presence of 50 % formamide at 37 °C according to a modification of the method by Ezaki *et al.* (1989). The DNA base composition was determined using HPLC (Mesbah *et al.*, 1989). The G+C content of the chromosomal DNA was determined as the mean of three independent analyses. These analyses were performed by the BCCM/LMG Identification Service (Gent, Belgium). The results obtained are summarized in Table 1. The DNA–DNA hybridization values of strain 6.2S^T with the closely related type strains of the genus *Sphingobacterium* were clearly lower than 70 % (19–39 % DNA–DNA relatedness), the generally accepted limit for species delineation (Wayne *et al.*, 1987). These results confirmed that the new strain did not belong to a recognized species of the genus *Sphingobacterium*. The DNA G+C content of strain 6.2S^T was 40 mol%. This value was within the range of DNA G+C contents found previously for species of the genus *Sphingobacterium* (39–42 mol%) as reported in the second edition of *Bergey's Manual of Systematic Bacteriology* (Krieg *et al.*, 2010).

Morphological features were examined after incubation for 24 h at 30 °C on TSA by negative staining using transmission electron microscopy (J1010; JEOL). Gram staining and endospore-forming features were investigated using a light microscope (CH-2; Olympus) according to the method described by Chapin & Lauderdale (2003). Growth at different temperatures (4–42 °C) was investigated using TSA (Pronadisa) as the basal medium. Tolerance of NaCl was tested using nutrient agar (Panreac) supplemented with concentrations of NaCl of 0–6.5 % (w/v) after 5 days of incubation. Growth was assessed at pH 3–11. Growth on MacConkey agar (Oxoid) and cetrimide agar (Oxoid) was evaluated at 30 °C after 24 h incubation. Unless otherwise indicated, the phenotypic characteristics were examined using standard procedures (MacFaddin, 1980; Barrow & Feltham, 1993; Chapin & Lauderdale, 2003) and all experiments were conducted in triplicate. The following analytical procedures were performed. Gelatinase activity was studied according to the method of Pochon & Tardieux (1962). Citrate utilization was determined on Simmons' citrate agar (Oxoid). Oxidase activity was tested by determining

Table 1. DNA G+C content and DNA–DNA hybridization values between strain 6.2S^T and closely related type strains of the genus *Sphingobacterium*

Strains: 1, 6.2S^T; 2, *S. siyangense* KCTC 22131^T; 3, *S. multivorum* DSM 11691^T; 4, *S. canadense* LMG 23727^T. Results of DNA–DNA hybridization experiments are expressed as the mean of four determinations. The values given in parentheses are the differences between the reciprocal values.

Strain	DNA G+C content (mol%)	DNA–DNA relatedness (%)			
		1	2	3	4
6.2S^T	40.0	100			
S. siyangense KCTC 22131^T	40.5*	20 (3)	100		
S. multivorum DSM 11691^T	38.5*	39 (2)	20 (1)	100	
S. canadense LMG 23727^T	39.9–40.5*	19 (1)	66 (8)	21 (4)	100

*Data from Krieg *et al.* (2010).

the oxidation of (1 %) N,N,N′,N′-tetramethyl-3-*p*-phenylene-diamine solution (Merck) and catalase activity was evaluated by determining the production of oxygen bubbles in a 5 % (v/v) aqueous hydrogen peroxide solution. Single carbon-source utilization was determined as described by Kim *et al.* (2006). To this medium a trace element solution (Marqués *et al.*, 2009) and a selenite/tungstate solution (Tschech & Pfennig, 1984) were added and the pH was adjusted to 6.8. This medium was aliquoted in small tubes and filter-sterilized carbon sources were added to each tube (0.1 % w/v). Tubes were incubated at 30 °C for 7 days and growth was examined visually. Positive and negative controls were added. Other physiological characteristics were determined with an API 20 NE gallery according to the instructions of the manufacturer (bioMérieux). Antibiotic sensitivity tests were performed using the diffusion method described by Jorgensen & Turnidge (2003) with discs (bioMérieux) containing the following antibiotics (μg): ampicillin–clavulanic acid (30), ampicillin (10), aztreonam (30), amikacin (30), cephalotin (30), cefoxitin (30), ceftriaxone (30), ciprofloxacin (5), colistin (50), chloramphenicol (30), erythromycin (15), streptomycin (10), fosfomycin (50), gentamicin (10), imipenem (10), tetracycline (30), ticarcillin (75), tobramycin (10) and trimethoprim–sulfamethoxazole (1.25–23.75). After 20 h of incubation, the diameters of the inhibition zones were measured.

Strain 6.2S^T formed round, wet, beige colonies of 3–5 mm diameter when grown for 48–72 h on TSA at 30 °C. Cells of 6.2S^T were non-motile rods that were 2.5–1.8 μm long and 1.0–0.8 μm wide (see Fig. S1 in IJSEM Online). The physiological and biochemical properties that differentiate strain 6.2S^T from related species of the genus *Sphingobacterium* are shown in Table 2.

For fatty acid analysis, strain 6.2S^T was grown in TSA at 30 °C for 24 h. The cellular fatty acid profile of strain 6.2S^T was determined by using a Microbial ID system equipped with a GC (5890 Series II; Hewlett Packard) and Sherlock version 4.0 of the aerobic library (Microbial ID, 1993). The cellular fatty acids mainly comprised summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$) (43.71 %), iso-$C_{15:0}$ (20.91 %), iso-$C_{17:0}$ 3-OH (7.40 %) and $C_{16:0}$ (7.33 %). The cellular fatty acids of strain 6.2S^T are listed in Table 3 and are compared with the four type strains that formed a closely related cluster in the genus *Sphingobacterium*. The fatty acid profile of strain 6.2S^T resembled those of closely related strains of species of the genus *Sphingobacterium*. The sphingolipid assay was resolved by two-dimensional TLC as previously described (Mehnaz *et al.*, 2007). The major lipid was phosphatidylethanolamine and several unknown lipids were also detected. Sphingolipid, which is a distinct feature of the members of the genus *Sphingobacterium*, was also present (Fig. S2).

On the basis of phenotypic and chemotaxonomic properties and phylogenetic data, it is proposed that strain 6.2S^T should be classified in the genus *Sphingobacterium* as representing a novel species, for which the name *Sphingobacterium detergens* sp. nov. is proposed.

Description of *Sphingobacterium detergens* sp. nov.

Sphingobacterium detergens (de.ter′gens. L. v. *detergere* to wipe off; L. part. adj. *detergens* wiping off, modern meaning surfactant, named thus for being a good surfactant producer).

Cells are Gram-negative-staining, non-motile, non-spore-forming, strictly aerobic rods, 2.5–1.8 μm long and 1.0–0.8 μm wide. After 48 h incubation in TSA, colonies are 3–5 mm in diameter, beige, convex, circular and smooth with entire margins. Temperature range for growth is 15–37 °C (optimum, 30–37 °C); no growth occurs at 42 °C. The pH range for growth is 6.0–7.5 (optimum, pH 7) and the NaCl concentration range for growth is 0–3 % (w/v). Growth occurs on nutrient agar, TSA, cetrimide agar, MacConkey agar and Simmons' citrate agar. Gives a positive result for catalase, oxidase, lipase and *β*-galactosidase activities. Gelatin, DNA, starch, aesculin and urea are hydrolysed, but Tween 80 is not. Reduction of nitrates to nitrites gives a

Table 2. Differential characteristics between strain 6.2S^T and related members of the genus *Sphingobacterium*

Strains: 1, 6.2S^T; 2, *S. canadense* LMG 23727^T; 3, *S. siyangense* KCTC 22131^T; 4, *S. multivorum* DSM 11691^T; 5, *S. thalpophilum* LMG 11520^T. All data are from this study. All strains grow aerobically at 30 °C, give a positive result in tests for catalase, oxidase and *β*-galactosidase activities; give a positive result for the hydrolysis of DNA, starch, aesculin and urea and for the assimilation of D-glucose, L-arabinose, melibiose, D-mannose, *N*-acetylglucosamine and maltose. All strains give a positive reaction for acid production from D-glucose and sucrose. All strains give a negative result for Gram staining, sporulation, motility, indole production, Voges–Proskauer test, methyl red test, Tween 80 hydrolysis, glucose fermentation and arginine dihydrolase activity. All strains give a negative result for the assimilation of D-mannitol, L-glutamate, L-sorbitol, D-ribose, pyruvate, capric acid, adipic acid, malic acid, phenylacetic acid and for acid production from L-rhamnose, D-mannitol, sorbitol and lactose. +, Positive; −, negative. Results in parentheses indicate data from another study (Krieg *et al.*, 2010) that differed from our results.

Characteristic	1	2	3	4	5
Growth at 42 °C	−	−	−	−	+
Reduction of nitrates to nitrites	−	+	−	−	+
Hydrolysis of:					
Gelatin	+	+	−	−	+ (−)
Assimilation of:					
L-Rhamnose	+	+ (−)	+	+	− (+)
Glycerol	+	− (+)	+	+	− (+)
Acid production from:					
L-Arabinose	−	+	−	+	+
Melibiose	−	+	−	−	−

Table 3. Fatty acid composition of strain 6.2S^T and related members of the genus *Sphingobacterium*

Taxa: 1, strain 6.2S^T; 2, *S. canadense* LMG 23727^T; 3, *S. siyangense* KCTC 22131^T; 4, *S. multivorum* DSM 11691^T; 5, *S. thalpophilum* LMG 11520^T. Values are percentages of total fatty acids; components amounting to <1.0 % in all strains tested are not listed. tr, Trace (<1.0 %); –, not detected. Values in bold type are the major fatty acids.

Fatty acid	1	2	3	4	5
$C_{14:0}$	2.23	1.51	3.36	3.60	3.07
$C_{14:0}$ 2-OH	tr	tr	tr	1.32	–
$C_{16:0}$	**7.33**	**7.92**	**10.87**	**8.52**	**10.93**
$C_{16:0}$ 2-OH	tr	tr	tr	tr	2.48
$C_{16:0}$ 3-OH	5.42	4.02	6.48	7.54	3.63
iso-$C_{15:0}$	**20.91**	**20.48**	**16.67**	**17.56**	**20.62**
iso-$C_{15:0}$ 3-OH	3.40	6.35	6.81	3.44	5.58
iso-$C_{17:0}$ 3-OH	**7.40**	**8.98**	**5.37**	**5.26**	**8.67**
Summed feature 3*	**43.71**	**46.06**	**43.24**	**46.70**	**44.06**
iso-$C_{17:1}\omega 9c$	1.32	tr	tr	tr	tr

*Summed features represent groups of two fatty acids that cannot be separated by GLC. Summed feature 3 contains iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega 7c$. All data are from this study.

negative result. Acid is produced from D-glucose and sucrose but not from L-rhamnose, L-arabinose, D-mannitol, melibiose, sorbitol, inositol, amygdalin or lactose. Utilizes D-glucose, L-rhamnose, L-arabinose, melibiose, glycerol, D-mannose, *N*-acetylglucosamine and maltose, but not D-mannitol, L-glutamate, L-sorbitol, D-ribose, pyruvate, capric acid, adipic acid, malic acid, phenylacetic acid or gluconate. Resistant to ampicillin, aztreonam, amikacin, cephalotin, cefoxitin, colistin, chloramphenicol, erythromycin, streptomycin, fosfomycin, gentamicin, tetracycline and tobramycin. Intermediate levels of susceptibility with ciprofloxacin and ticarcillin; susceptible to amoxicillin–clavulanic acid, ceftriaxone, imipenem and trimethoprim–sulfamethoxazole. The predominant fatty acids are summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ $\omega 7c$) (43.71 %); iso-$C_{15:0}$ (20.91 %); iso-$C_{17:0}$ 3-OH (7.40 %) and $C_{16:0}$ (7.33 %).

The type strain 6.2S^T (=CECT 7938^T=LMG 26465^T) was isolated in 2007 from a soil sample taken near the Lagoa de Fogo, a crater lake in the centre of the island of São Miguel in the Azores archipelago, Portugal. The G+C content of the DNA of the type strain is 40.0 mol%.

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