Cellulosimicrobium terreum sp. nov., isolated from soil

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A Gram-positive, yellow-pigmented, non-motile and rod-shaped or coccoid bacterial strain, DS-61^T, was isolated from soil from Dokdo, Korea, and its taxonomic position was investigated by using a polyphasic approach. The strain grew optimally at pH 6.5–7.5 and 25 °C in the presence of 1.0% (w/v) NaCl. Strain DS-61^T had peptidoglycan of the type based on L-Lys–L-Thr–D-Asp and contained galactose as the only whole-cell sugar. MK-9(H₄) was the predominant menaquinone and anteiso-C_{15:0} and iso-C_{15:0} were the major fatty acids. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unidentified phospholipid. The DNA G + C content was 72.9 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain DS-61^T is most closely affiliated to the genus *Cellulosimicrobium*, clustering with *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei*. The levels of 16S rRNA gene sequence similarity between strain DS-61^T and the type strains of *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei* were 97.4–97.6%. DNA–DNA relatedness data and differential phenotypic properties demonstrated that strain DS-61^T is distinguishable from these two recognized *Cellulosimicrobium* species. On the basis of phenotypic, phylogenetic and genetic data, strain DS-61^T represents a novel species of the genus *Cellulosimicrobium*, for which the name *Cellulosimicrobium terreum* sp. nov. is proposed. The type strain is DS-61^T (=KCTC 19206^T=DSM 18665^T). An emended description of the genus is given.

The genus *Cellulosimicrobium* was created by the reclassification of *Cellulomonas cellulans* as *Cellulosimicrobium cellulans* (Schumann *et al.*, 2001). Subsequently, two further *Cellulosimicrobium* species, *Cellulosimicrobium variabile* (Bakalidou *et al.*, 2002) and *Cellulosimicrobium funkei* (Brown *et al.*, 2006), were described. However, *Cellulosimicrobium variabile* was reclassified within a novel genus, *Isoptericola*, as *Isoptericola variabilis* (Stackebrandt *et al.*, 2004). In this study, we report on the taxonomic characterization of a bacterial strain, DS-61^T, which is phylogenetically closely related to the genus *Cellulosimicrobium*.

Strain DS-61^T was isolated from a soil sample collected from Dokdo (37° 14' 12" N 131° 52' 07" E), Korea, by means of the standard dilution plating technique performed at 25 °C on 10 × diluted nutrient agar (Difco). *Cellulosimicrobium cellulans* DSM 43879^T and *Cellulosimicrobium funkei* DSM 16025^T, which were used as reference

strains, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The morphological, physiological and biochemical characteristics of strain DS-61^T were investigated using routine cultivation on trypticase soy agar (TSA; Difco) at 25 °C. The cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy. Flagellation was determined by using a Philips CM-20 transmission electron microscope with cells from exponentially growing cultures: for this purpose, the cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being air-dried. The Gram reaction was determined using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at various temperatures (4–40 °C) was measured on TSA. Growth in the absence of NaCl and at various NaCl concentrations (0.5 and 1.0–10.0%, w/v, using increments of 1.0%) was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was included. The pH range for growth was determined in nutrient broth (Difco) that had been

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adjusted, prior to sterilization, to various pH values (pH 4.5–10.5, using increments of 0.5 pH units) by the addition of HCl or Na₂CO₃. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on TSA and on TSA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Catalase and oxidase activities and the hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). The hydrolysis of aesculin and the reduction of nitrate were determined as described by Lanyi (1987). The utilization of substrates as sole carbon and energy sources was tested according to the method of Kämpfer *et al.* (1991). Susceptibility to antibiotics was tested on TSA plates using discs containing the following antibiotics: polymyxin B (100 U), streptomycin (50 µg), penicillin G (20 U), chloramphenicol (100 µg), ampicillin (10 µg), cephalothin (30 µg), gentamicin (30 µg), novobiocin (5 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg) and carbenicillin (100 µg). Other physiological properties and enzyme activities were tested by using the API 20E and API ZYM systems (bioMérieux).

Cell biomass for DNA extraction and for analysis of the cell-wall components, isoprenoid quinones and polar lipids was obtained from cultures grown, with shaking at 150 r.p.m., in trypticase soy broth (Difco) at 25 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by using a PCR with two universal primers, as described previously (Yoon *et al.*, 1998). The sequencing of the amplified 16S rRNA gene and the phylogenetic analysis were performed as described by Yoon *et al.* (2003). The DNA G+C content was determined according to the method of Tamaoka & Komagata (1984), with the modification that the DNA was hydrolysed and the resultant nucleotides analysed by reversed-phase HPLC. The presence or absence of diaminiopimelic acid in the peptidoglycan was determined according to the method described by Komagata & Suzuki (1987). Preparation of the cell walls and determination of the peptidoglycan structure were carried out by using the methods of Schleifer & Kandler (1972) and MacKenzie (1987) with the modification that TLC on cellulose was applied instead of paper chromatography. Whole-cell sugars were determined as described by Komagata & Suzuki (1987). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). For fatty acid methyl ester

analysis, cell mass of strain DS-61^T was harvested from TSA plates after incubation for 7 days at 25 °C, and cell mass of *Cellulosimicrobium cellulans* DSM 43879^T and *Cellulosimicrobium funkei* DSM 16025^T was harvested from TSA plates after incubation for 3 days at 28 °C. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). DNA–DNA hybridization was performed fluorometrically according to the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed using five replications for each sample: the highest and lowest values obtained in each sample were excluded and the means of the remaining three values were quoted as the DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain DS-61^T are given in the species description (see below) or are shown in Table 1. The almost-complete 16S rRNA gene sequence of strain DS-61^T determined in this study comprised 1478 nt, representing approximately 96% of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree based on the neighbour-joining algorithm, strain DS-61^T joined the cluster comprising *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei* with a bootstrap confidence value of 94.9% (Fig. 1). The relationship between strain DS-61^T and these two *Cellulosimicrobium* species was also recovered in trees based on the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain DS-61^T exhibited 16S rRNA gene sequence similarity values of 97.4 and 97.6% with respect to the type strains of *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei*, respectively.

Quantitative analysis of the peptidoglycan amino acids in the total hydrolysate, determined using gas chromatography according to MacKenzie (1987), showed that strain DS-61^T contained lysine, alanine, threonine, aspartic acid and glutamic acid in the approximate ratio 0.5:3.4:0.7:1.0:1.0. The reduced amounts of lysine and threonine are due to the occurrence of the stable peptide L-Lys–L-Thr. Under more extreme conditions (120 °C, 6 M HCl, 16 h), the peptidoglycan of strain DS-61^T contained lysine, alanine, threonine, aspartic acid and glutamic acid in the approximate ratio 0.9:2.4:1.0:1.0:1.0. From these data, it was concluded that strain DS-61^T contains peptidoglycan of the A4₂ type, based on L-Lys–L-Thr–D-Asp, as described by Schleifer & Kandler (1972). Galactose was the only whole-cell sugar detected in strain DS-61^T. The predominant isoprenoid quinone detected in strain DS-61^T was a tetrahydrogenated menaquinone with nine isoprene units [MK-9(H₄)]. The fatty acid profile of strain DS-61^T comprised large amounts of straight-chain and branched fatty acids; the major components (>10% of total fatty acids) were anteiso-C_{15:0} and iso-C_{15:0} (Table 2). The major polar lipids detected in strain DS-61^T were diphosphatidylglycerol, phosphatidylglycerol,

Table 1. Differential phenotypic characteristics of strain DS-61^T (*Cellulosimicrobium terreum* sp. nov.) and *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei*

Taxa: 1, strain DS-61^T; 2, *Cellulosimicrobium cellulans*; 3, *Cellulosimicrobium funkei*. Data are from Schumann *et al.* (2001), Brown *et al.* (2006) and this study. All are positive for the following: Gram-staining; catalase; hydrolysis of aesculin (not determined for *Cellulosimicrobium cellulans*), casein, hypoxanthine, starch (not determined for *Cellulosimicrobium funkei*) and xanthine; acid production from D-glucose and sucrose; utilization of D-glucose, D-fructose (not determined for *Cellulosimicrobium cellulans*), D-galactose (not determined for *Cellulosimicrobium cellulans*), D-cellobiose, D-mannose, trehalose (not determined for *Cellulosimicrobium cellulans*), D-xylose, L-arabinose, sucrose, maltose, acetate (not determined for *Cellulosimicrobium funkei*), pyruvate (not determined for *Cellulosimicrobium funkei*) and salicin (not determined for *Cellulosimicrobium cellulans*) (utilization data for *Cellulosimicrobium funkei* are from the type strain in this study); alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -glucosidase. All are negative for the following: hydrolysis of tyrosine; acid production from L-rhamnose, D-mannitol, inositol and D-sorbitol; utilization of citrate (not determined for *Cellulosimicrobium cellulans*) and L-malate (utilization data for *Cellulosimicrobium funkei* are from the type strain in this study); lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, α -mannosidase and α -fucosidase. Data in parentheses are for the type strain. +, Positive reaction; –, negative reaction; w, weakly positive reaction; v, variable reaction; ND, not determined.

Characteristic	1	2	3
Motility	–	–	+
Growth at 35 °C	–	+	+
Nitrate reduction	+	+	v(–)
Hydrolysis of:			
Gelatin	–	+	+
Urea	+	+	+ or w(+)
Acid production from:			
Melibiose	–	+	v(–)
L-Arabinose	+	+	v(+)
API ZYM*:			
β -Galactosidase	–	+	+
β -Glucosidase	–	–	w
N-Acetyl- β -glucosaminidase	–	+	+
Susceptibility to ampicillin	–	+	+
Major cell-wall sugar	Galactose	Rhamnose	Galactose
Peptidoglycan type	L-Lys–L-Thr–D-Asp	L-Lys–D-Ser–D-Asp	ND
DNA G + C content (mol%)	72.9	74 (<i>T_m</i>)	74.5 (<i>T_m</i>)

*Data for *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei* are from the type strains in this study.

phosphatidylinositol and an unidentified phospholipid. These fatty acid and polar lipid profiles were similar to those of *Cellulosimicrobium* species (Schumann *et al.*, 2001; Stackebrandt *et al.*, 2004; Brown *et al.*, 2006). The DNA G + C content of strain DS-61^T was 72.9 mol%.

Strain DS-61^T is most closely related phylogenetically to the genus *Cellulosimicrobium* (Fig. 1). There are no distinct phenotypic, particularly chemotaxonomic, properties that serve to differentiate strain DS-61^T from the genus *Cellulosimicrobium* (Schumann *et al.*, 2001; Brown *et al.*, 2006). Accordingly, it is appropriate to place strain DS-61^T within the genus *Cellulosimicrobium* on the basis of phylogenetic and chemotaxonomic relatedness (Schumann *et al.*, 2001; Brown *et al.*, 2006). Strain DS-61^T exhibited mean DNA–DNA relatedness values of 19 and 15 % with respect to *Cellulosimicrobium cellulans* DSM 43879^T and *Cellulosimicrobium funkei* DSM 16025^T, respectively. Strain DS-61^T differs from these two recognized *Cellulosimicrobium* species in some phenotypic

respects, as shown in Table 1. The phylogenetic and genetic distinctiveness and differential phenotypic properties of DS-61^T are sufficient to categorize it as a member of a species that is distinct from these two recognized *Cellulosimicrobium* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). On the basis of the data presented, strain DS-61^T represents a novel species of the genus *Cellulosimicrobium*, for which the name *Cellulosimicrobium terreum* sp. nov. is proposed.

Emended description of the genus *Cellulosimicrobium* Schumann *et al.* 2001

The description of the genus *Cellulosimicrobium* is as given by Schumann *et al.* (2001) and Brown *et al.* (2006), but with the following amendments. The cell-wall peptidoglycan type is A4 α , based on L-Lys–D-Ser–D-Asp or L-Lys–L-Thr–D-Asp. The DNA G + C contents are in the range 72.9–74.5 mol%.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain DS-61^T and some related taxa. Bootstrap percentages (based on 1000 replications) are shown at branch points. *Jonesia denitrificans* DSM 20603^T (GenBank accession number X83811) was used as an outgroup. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

Description of *Cellulosimicrobium terreum* sp. nov.

Cellulosimicrobium terreum (ter're.um. L. neut. adj. *terreum* of the earth).

Cells are Gram-positive, non-spore-forming rods or cocci (0.4–0.8 × 0.4–2.0 µm); in older cultures, cells are Gram-variable and most cells are cocci. Colonies on TSA are circular, convex, smooth, glistening, yellow in colour and 1.5–2.0 mm in diameter after 7 days incubation at 25 °C. Substrate hyphae are present. Optimal temperature for growth is 25 °C. Growth occurs at 4 and 34 °C, but not at 35 °C. Optimal pH for growth is 6.5–7.5; growth occurs at pH 6.0 and 9.0, but not at pH 5.5 or 9.5. Growth occurs in the presence of 0–9% (w/v) NaCl; optimal growth occurs in the presence of 1.0% (w/v) NaCl. Anaerobic growth does not occur on TSA or on TSA supplemented with nitrate. Oxidase-negative. Tweens 20, 40, 60 and 80 are

hydrolysed. H₂S and indole are not produced. L-Glutamate is utilized as a sole carbon and energy source, but succinate, benzoate and formate are not. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. Susceptible to cephalothin, chloramphenicol, neomycin, novobiocin, oleandomycin, penicillin G, streptomycin and tetracycline, but not to carbenicillin, gentamicin, kanamycin, lincomycin or polymyxin B. The cell-wall peptidoglycan type is L-Lys–L-Thr–D-Asp. The only whole-cell sugar is galactose. The predominant menaquinone is MK-9(H₄). Major fatty acids are anteiso-C_{15:0} and iso-C_{15:0}. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unidentified phospholipid. The DNA G + C content of the type strain is 72.9 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, DS-61^T (=KCTC 19206^T=DSM 18665^T), was isolated from soil from Dokdo, Korea.

Table 2. Cellular fatty acid compositions (%) for strain DS-61^T and *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei*

Strains: 1, DS-61^T; 2, *Cellulosimicrobium cellulans* DSM 43879^T; 3, *Cellulosimicrobium funkei* DSM 16025^T. Data are from this study. Fatty acids that represented <0.5% in the three strains have been omitted.

Fatty acid	1	2	3
Straight-chain fatty acids			
C _{14:0}	3.3	4.6	1.9
C _{15:0}	1.3	1.1	1.0
C _{16:0}	5.5	6.9	6.6
C _{18:0}	0.6	0.3	0.2
Branched fatty acids			
iso-C _{14:0}	4.0	2.8	0.4
iso-C _{15:0}	14.5	16.5	21.9
anteiso-C _{15:0}	52.5	35.7	44.4
iso-C _{16:0}	8.4	12.1	5.3
iso-C _{17:0}	0.8	4.5	2.9
anteiso-C _{17:0}	7.8	14.8	15.1

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