Sphingobacterium kitahiroshimense sp. nov., isolated from soil

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A novel exopolysaccharide-degrading bacterium, designated strain 10C^T, was isolated from soil from Kitahiroshima city, Hokkaido, Japan. The novel isolate was Gram-negative, strictly aerobic and chemoheterotrophic. The DNA G+C content was 36.9 mol%. Major fatty acids were $C_{16:1}\omega7c$, iso-$C_{15:0}$ 2-OH, iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. 16S rRNA gene sequence analysis and chemotaxonomic and morphological data indicated that the novel strain clearly belonged to the genus *Sphingobacterium*. Based on phenotypic properties and DNA–DNA hybridization data, the new isolate was assigned to the genus *Sphingobacterium* as *Sphingobacterium kitahiroshimense* sp. nov. The type strain is 10C^T (=JCM 14970^T=NCIMB 14398^T).

There have been many reports of micro-organisms that produce exopolysaccharides (EPS). In this study, we attempted to isolate novel polysaccharide-degrading bacteria and to obtain the oligosaccharides in order to elucidate the structure of the EPS. Isolate 10C^T had the ability to degrade polysaccharides and was found to be a novel *Sphingobacterium*-like strain. The genus *Sphingobacterium* can be distinguished from the genus *Flavobacterium* by the presence of high concentrations of sphingophospholipids (Yabuuchi *et al.*, 1983). In this study, we examined the physiological, biochemical and chemotaxonomic characteristics and phylogeny of strain 10C^T. DNA–DNA relatedness data showed that the strain should be classified as a novel species of the genus *Sphingobacterium*.

In order to isolate polysaccharide-degrading bacteria, we analysed a soil sample that was collected from Kitahiroshima, Hokkaido, Japan. The sample was suspended in 10 ml medium containing (l^{-1}): 0.1 g KNO_3, 1 g yeast extract, 0.1 g EPS produced by *Halomonas* sp. strain su3k3 and 10 ml trace element solution (Patel, 1984), at pH 7.8. Strain su3k3 was isolated from a sediment sample taken from the Pacific Ocean as a polysaccharide producer (unpublished data). This medium was incubated at 25 °C with shaking at 150 r.p.m. After incubation for several days, a portion of the suspension was transferred into 10 ml fresh medium and reincubated. After three successive transfers, the suspension was plated onto solid medium to isolate pure cultures. The resulting isolates were checked to determine whether they degraded EPS by gel chromatography (HW-40S; Toyopearl) of both the cultural and enzyme reaction broth. Of the strains isolated, strain 10C^T, which showed good EPS degradation, was selected for further study. To investigate the morphological and physiological characteristics of the novel isolate, strain 10C^T was cultivated aerobically at 25 °C on nutrient agar (Difco). Cell morphology was observed using a scanning electron microscope.

Strain 10C^T was tested for a range of phenotypic properties using standard procedures (Komagata, 1985). Growth at different temperatures (4–40 °C) was tested using nutrient broth (Difco). Additional biochemical tests were performed with the API 20NE test kit (bioMérieux) and the Biolog GN MicroPlate method as described by the manufacturers. The analysis of quinones was carried out by the identification services of TechnoSuruga Laboratory Co., Ltd, Shizuoka, Japan.

Sphingolipid analysis was performed according to Kawahara *et al.* (2000). Cellular lipids were extracted by chloroform/methanol (2 : 1, v/v) and chloroform/methanol (1 : 3, v/v) and both fractions were combined. An aliquot of this lipid fraction was hydrolysed with 0.1 M NaOH (100 °C, 30 min). The alkaline-stable lipids were extracted

Abbreviations: EI, electron ionization; EPS, exopolysaccharides.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Sphingobacterium kitahiroshimense* sp. nov. 10C^T is AB361248.

with acetone and the acetone-insoluble fraction was prepared. The non-treated lipid fraction and acetone-insoluble fraction were methanolysed with 1 M HCl/methanol (100 °C, 5 h) and the sphingosine molecules liberated were peracetylated by pyridine/acetic anhydride (1 : 1, v/v). The peracetylated derivatives were analysed by a GLC equipped with a CBP1 capillary column with a temperature programme of 200 °C for 5 min and heating (at 5 °C min^{-1}) to 300 °C.

Fatty acid analysis was carried out by TechnoSuruga Laboratory Co., Ltd, Shizuoka, Japan. For the fatty acid analysis, strain 10C^T was grown in nutrient broth at 25 °C for 24 h. The cellular fatty acid profile of isolate 10C^T was determined by using a Microbial ID system equipped with a GC and version 5.0 of the aerobic library (Microbial ID, 1993) according to a standard protocol (Paisley, 1996).

DNA was prepared from bacterial cells according to the method of Marmur (1961). The G+C content of the DNA was determined using the method of Tamaoka & Komagata (1984). Levels of DNA–DNA relatedness were determined fluorometrically according to the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microplates.

The 16S rRNA gene was amplified by using the PCR method with primers 9F and 1510R. The PCR product, approximately 1.5 kb in size, was sequenced by the dideoxynucleotide chain-termination method, using a BigDye Terminator v.3.0 cycle sequencing ready kit (Applied Biosystems) and a DNA sequencer (ABI Prism 3100). Primers 9F, 339F, 785F, 1099F, 536R, 802R and 1242R were used in the gene sequencing reaction (Nakagawa & Kawasaki, 2001). Multiple alignments of the sequences were performed and the nucleotide substitution rate (K_{nuc} value) was calculated. A phylogenetic tree was constructed by the neighbour-joining method (Kimura, 1980; Saitou & Nei, 1987) using the CLUSTAL W program (Thompson *et al.*, 1994). Sequence similarity was calculated by using the GENETYX program (Software Development).

Isolate 10C^T formed round, wet, off-white colonies which turned yellow after a few days. Cells of the bacterium were short rods, 0.5–0.6 μm wide and 0.6–0.8 μm long and were non-motile. The predominant menaquinone detected was MK-7. The results of some physiological tests for strain 10C^T are given in the species description. Phenotypic properties of strain 10C^T and reference strains of the genus *Sphingobacterium* are compared in Table 1.

For sphingolipid analysis, peracetylated derivatives from the methanolysate were analysed by GLC-MS. A peak with a retention time two minutes earlier than the standard peracetylated 18 : 0 dihydrosphingosine gave the electron ionization (EI)-mass spectrum shown in Fig. 1. The fragment ion at *m/z* 144 was assigned to the C-1/C-2

Table 1. Differential phenotypic characteristics of strain 10C^T and the type strains of other species of the genus *Sphingobacterium*

Strains: 1, *Sphingobacterium kitahiroshimense* sp. nov. 10C^T; 2, *S. faecium* JCM 21820^T; 3, *S. canadense* CR11^T; 4, *S. daejeonense* TR6-04^T; 5, *S. spiritivorum* NBRC 14948^T; 6, *S. multivorum* NBRC 14947^T; 7, *S. mizutaii* NBRC 14946^T; 8, *S. thalpophilum* NBRC 14963^T; 9, *S. antarcticum* MTCC 675^T; 10, *S. heparinum* NBRC 12017^T; 11, *S. piscium* NBRC 14985^T. +, Positive; −, negative; V, variable; ND, no data available. Data for taxa 2–11 are from the descriptions given by Mehnaz *et al.* (2007).

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Growth at:											
5 °C	+	+	−	−	−	−	−	−	+	+	+
41 °C	−	−	−	+	−	−	−	+	−	−	−
Hydrolysis of:											
Aesculin	+	+	+	−	+	+	+	+	+	+	+
Gelatin	−	−	+	−	−	−	−	−	+	−	−
Urease	+	+	+	−	+	+	+	+	+	−	−
Assimilation of:											
L-Rhamnose	−	+	−	−	+	+	−	+	+	ND	ND
L-Arabinose	−	+	+	−	−	+	V	+	+	+	−
D-Mannitol	−	−	−	−	+	−	−	−	+	ND	ND
Melibiose	+	+	+	+	+	+	+	+	−	ND	ND
Glycerol	−	+	+	−	V	−	−	+	+	ND	ND
L-Glutamate	+	+	−	−	−	+	+	−	+	ND	ND
Acid production from:											
L-Rhamnose	−	−	−	−	−	V	−	+	−	ND	ND
L-Arabinose	+	+	+	−	−	+	+	+	−	+	V
Sucrose	+	+	+	−	+	+	+	+	−	+	+
DNA G+C content (mol%)	36.9	37.3	40.5	38.7	39	39.9–40.5	39.3–40.0	44.0–44.2	39.3	42.3	41.1–42.1

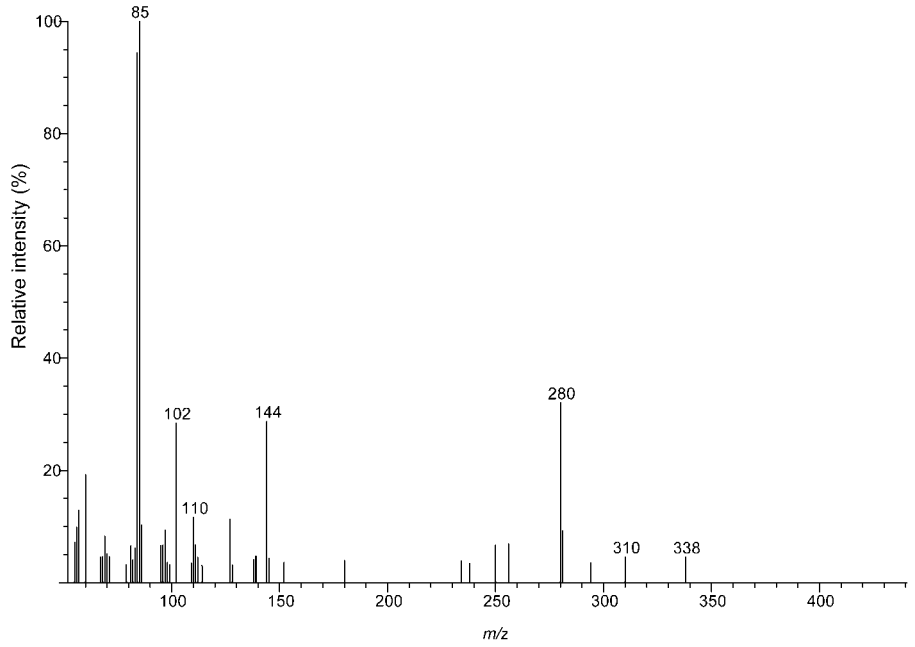

Fig. 1. EI-MS spectrum of the peracetylated derivative of branched-17 : 0 dihydrosphingosine of *Sphingobacterium kitahiroshimense* sp. nov. 10C^T. This was analysed by a GLC equipped with a capillary column CBP1 with a temperature program of 200 °C for 5 min followed by heating (at 5 °C min^{-1}) to 300 °C.

moiety of the peracetylated derivative. The fragment ion at *m/z* 280 was deduced to be derived from the cleavage of C-1 and C-2 and the subtraction of acetic acid (60 Da). These results indicated that the derivative detected by GLC-MS was peracetylated branched-17 : 0 dihydrosphingosine, which is the main sphingosine usually found in species of the genus *Sphingobacterium*.

The fatty acid content of strain 10C^T was as follows: iso-$C_{15:0}$ 2-OH and $C_{16:1}\omega7c$ (summed feature 3), 40.28 %; iso-$C_{15:0}$, 28.89 % and iso-$C_{17:0}$ 3-OH, 12.83 %. The fatty acid profile of strain 10C^T clearly resembled those previously determined for other strains of the genus *Sphingobacterium* (Mehnaz *et al.*, 2007).

The almost-complete 16S rRNA gene sequence of strain 10C^T, which consisted of 1490 bp, was compared with all other known 16S rRNA gene sequences and a phylogenetic tree was constructed using related taxa. The phylogenetic tree indicated that strain 10C^T belonged to the genus *Sphingobacterium* (Fig. 2). The 16S rRNA gene sequence similarity of strain 10C^T to *Sphingobacterium faecium* JCM 21820^T was 98.6 %. According to 16S rRNA gene sequence analysis, strain 10C^T was a member of the genus *Sphingobacterium*.

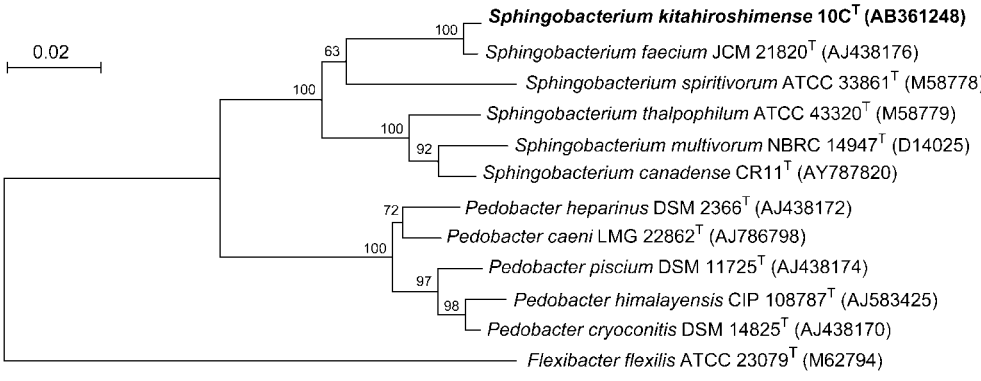

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequence data for *Sphingobacterium kitahiroshimense* sp. nov. 10C^T, other species of the genus *Sphingobacterium* and other related organisms. Bar, 0.02 K_{nuc}.

The genomic relatedness between strain 10C^T and the most closely related strain, *S. faecium* JCM 21820^T (Takeuchi & Yokota, 1992), was determined by DNA–DNA hybridization analysis. The DNA–DNA relatedness of strain 10C^T with *S. faecium* JCM 21820^T was 43.6 % and the reciprocal value was 31.3 %. On the basis of the above results, strain 10C^T was considered to represent a novel species.

Strain 10C^T differed from *S. faecium*, in that the novel strain gave a negative result for the following phenotypic characteristics: utilization of L-arabinose, L-rhamnose and glycerol and detection of esterase (C4), cystine arylamidase, chymotrypsin and β-galactosidase.

The DNA G+C content of strain 10C^T, determined using a HPLC method, was 36.9 mol%. This value was almost consistent with the G+C content of members of the genus *Sphingobacterium*, which ranges between 37.3 and 44.2 mol% (Mehnaz *et al.*, 2007).

On the basis of this polyphasic taxonomic analysis, the new isolate is deemed to represent a novel species for which the name *Sphingobacterium kitahiroshimense* sp. nov. is proposed.

Description of *Sphingobacterium kitahiroshimense* sp. nov.

Sphingobacterium kitahiroshimense (kita.hi.ro.shim.en′se. N.L. neut. adj. *kitahiroshimense* pertaining to Kitahiroshima city, where the type strain was isolated).

Aerobic, Gram-negative, oxidase- and catalase-positive, non-spore-forming, rod-shaped cells. Cells are 0.6–0.8 μm long, 0.5–0.6 μm in diameter and are non-motile. Circular, entire, low convex, smooth colonies develop on nutrient agar after 2 days. A yellow or creamy white, non-fluorescent pigment is produced on nutrient agar. The temperature range for growth is 4–37 °C, no growth occurs at temperatures of 42 °C or above. Gives a positive result in tests for the hydrolysis of aesculin. Urease, alkaline phosphatase, acid phosphatase, α-glucosidase, β-glucosidase, α-galactosidase and α-mannnosidase activities are detected. Positive result in the GN2 Biolog test for the following substrates: dextrin, Tween 40, Tween 80, cellobiose, *N*-acetylglucosamine, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, maltose, D-mannose, melibiose, raffinose, L-rhamnose, sucrose, trehalose, turanose, α-ketovaleric acid, L-alanine, L-glutamic acid, L-threonine, L-proline and L-serine. Acid is produced from trehalose, raffinose, L-arabinose and L-xylose. Gives a negative result in tests for indole production, hydrolysis of gelatin and nitrate reduction. Arginine dihydrolase activity is not detected. The activities of DNase, esterase (C4), lipase (C4), cystine arylamidase, trypsin, chymotrypsin, β-galactosidase and fucosidase are not detected. Cannot utilize citrate, L-arabinose, L-histidine, adonitol, erythritol, L-fucose, D-sorbitol, xylitol, formic acid, D-gluconic acid, DL-lactic acid, malonic acid, inosine or glycerol. Acid is not produced from rhamnose. Contains branched-17 : 0 dihydrosphingosine as the main sphingosine. Predominant fatty acids are iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$ (summed feature 3), 40.28 %; iso-$C_{15:0}$, 28.89 % and iso-$C_{17:0}$ 3-OH, 12.83 %. The DNA G+C content of the type strain is 36.9 mol% (HPLC method).

The type strain, 10C^T (=JCM 14970^T=NCIMB 14398^T), was isolated from soil from Kitahiroshima city, Japan.

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