Tenacibaculum geojense sp. nov., isolated from seawater

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A Gram-negative, non-flagellated, non-spore-forming bacterium, designated YCS-6^T, that was motile by gliding, was isolated from seawater on the southern coast of Korea. Strain YCS-6^T grew optimally at 30 °C and with 2 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YCS-6^T fell within the genus *Tenacibaculum* and was most closely associated with *Tenacibaculum litopenaei* B-1^T, with which the isolate exhibited 95.9 % 16S rRNA gene sequence similarity. Sequence similarity between strain YCS-6^T and other members of the genus *Tenacibaculum* was 93.8–95.7 %. Strain YCS-6^T contained menaquinone-6 (MK-6) as the predominant respiratory quinone and iso-C_{15:0}, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1} ω7c), iso-C_{15:0} 3-OH and iso-C_{15:1} G as the major fatty acids. The DNA G + C content was 32.7 mol%. Differential phenotypic properties and phylogenetic distinctiveness distinguished strain YCS-6^T from all other members of the genus *Tenacibaculum*. On the basis of our phenotypic, chemotaxonomic and phylogenetic data, strain YCS-6^T is considered to represent a novel species of the genus *Tenacibaculum*, for which the name *Tenacibaculum geojense* sp. nov. is proposed. The type strain is YCS-6^T (=KCTC 23423^T =CCUG 60527^T).

The genus *Tenacibaculum*, a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes* (Bernardet & Nakagawa, 2006), was established by Suzuki *et al.* (2001) with the reclassification of two bacterial species previously allocated to the genus *Flexibacter* and the description of two novel species. At the time of writing, the genus *Tenacibaculum* comprises 15 species with validly published names (Euzéby, 1997). All members of the genus have been isolated from marine environments and marine organisms (Suzuki *et al.*, 2001; Frette *et al.*, 2004; Yoon *et al.*, 2005; Sheu *et al.*, 2007; Heindl *et al.*, 2008; Wang *et al.*, 2008; Lee *et al.*, 2009). In this study, we describe a bacterial strain, designated YCS-6^T, which was isolated from seawater on the southern coast of Korea and found to be phylogenetically affiliated with the genus *Tenacibaculum* on the basis of comparative 16S rRNA gene sequence analysis.

Strain YCS-6^T was isolated by the dilution-plating technique using marine agar 2216 (MA; Difco) at 25 °C. *Tenacibaculum litopenaei* LMG 23706^T was used as a reference strain for phenotypic characterization and fatty acid analysis. The morphological, physiological and biochemical characteristics of strain YCS-6^T were investigated after routine

cultivation on MA at 30 °C. Cell morphology was examined by light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips). Flagellation was assessed by transmission electron microscopy using cells from an exponentially growing culture on MA. For this purpose, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. Gliding motility was investigated as described by Bowman (2000). The Gram reaction was determined using the bioMérieux Gram-stain kit, according to the manufacturer's instructions. Growth with 0, 0.5, 1.0, 2.0 and 3.0 % (w/v) NaCl was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was replaced with 0.45 % (w/v) MgCl₂ · 6H₂O or 0.06 % (w/v) KCl. Growth with 2.0–10.0 % (w/v) NaCl (in increments of 1.0 %) was measured in marine broth 2216 (MB; Difco). Growth at 10–45 °C (in increments of 5 °C), 28 and 37 °C was measured on MA. Growth at pH 4.5–9.5 (in increments of 0.5 pH units) was measured in MB using sodium acetate/acetic acid and Na₂CO₃ to adjust the pH. The pH was verified after autoclaving. Growth under anaerobic conditions was determined by incubation in a Forma anaerobic chamber on MA and on MA supplemented with potassium nitrate (0.1 %, w/v), both of which had been prepared anaerobically

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain YCS-6^T is HQ401023.

under a nitrogen atmosphere. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, starch, hypoxanthine, tyrosine and xanthine was tested on MA, using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin, Tweens 20, 40, 60 and 80 and urea and reduction of nitrate were investigated as described by Lányi (1987) with the modification that media were prepared with artificial seawater [containing (per l distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂ · 6H₂O, 5.94 g MgSO₄ · 7H₂O and 1.3 g CaCl₂ · 2H₂O; Bruns *et al.*, 2001]. H₂S production was tested as described by Bruns *et al.* (2001). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). Acid production from carbohydrates was determined as described by Leifson (1963). Susceptibility to antibiotics was tested on MA using antibiotic discs (Advantec) containing the following (µg per disc unless otherwise stated): polymyxin B (100 U), streptomycin (50), penicillin G (20 U), chloramphenicol (100), ampicillin (10), cephalothin (30), gentamicin (30), novobiocin (5), tetracycline (30), kanamycin (30), lincomycin (15), oleandomycin (15), neomycin (30) and carbenicillin (100). Other physiological and biochemical tests were performed with the API 20 E and API ZYM systems (bioMérieux) at 30 °C for 2 days and 8 h, respectively. The morphological, cultural, physiological and biochemical characteristics of strain YCS-6^T are given in the species description and Table 1. Strain YCS-6^T was distinguishable from its closest phylogenetic neighbour, *T. litopenaei* LMG 23706^T, by differences in several phenotypic characteristics, most of which were determined under the same conditions and methods (Table 1).

Cell biomass of strain YCS-6^T for DNA extraction and isoprenoid quinone analysis was obtained from cultures grown for 2 days in MB at 30 °C. Chromosomal DNA was isolated and purified according to the method described previously (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 PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). The almost-complete 16S rRNA gene sequence of strain YCS-6^T determined in this study comprised 1442 nt (approx. 96 % of the *Escherichia coli* 16S rRNA sequence). In the neighbour-joining phylogenetic tree, strain YCS-6^T fell within the genus *Tenacibaculum* and was most closely associated with *T. litopenaei* B-I^T (97.6 % bootstrap support), and this relationship was also recovered in trees reconstructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain YCS-6^T exhibited 95.9 % 16S rRNA gene sequence similarity to *T. litopenaei* B-I^T, 93.8–95.7 % to other members of the genus *Tenacibaculum* and <93.0 % to the other taxa used in the phylogenetic analysis.

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)

Table 1. Differential phenotypic characteristics of strain YCS-6^T and its closest phylogenetic neighbour in the genus *Tenacibaculum*

Data were taken from this study unless otherwise indicated. Both strains are Gram-negative rods and positive for gliding motility*, hydrolysis of gelatin* and starch*, activities of alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase and susceptibility to chloramphenicol, lincomycin, novobiocin, oleandomycin and tetracycline. Both strains are negative for flagellation*, production of flexirubin-type pigments*, nitrate reduction*, production of H₂S* and indole*, hydrolysis of urea*, acid production from L-arabinose, cellobiose, D-fructose, myo-inositol, lactose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol, trehalose and D-xylose, activities of lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α- and β-galactosidases, β-glucuronidase, α- and β-glucosidases, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase and susceptibility to ampicillin, gentamicin, kanamycin, neomycin, penicillin G, polymyxin B and streptomycin. +, Positive; w, weakly positive; –, negative.

Characteristic	YCS-6 ^T	<i>T. litopenaei</i> LMG 23706 ^T
Catalase	+	–*
Oxidase	–	+
Hydrolysis of:		
Aesculin	+	–*
Tween 80	+	–*
Acid production from:		
D-Galactose	–	+
D-Glucose	w	–
Maltose	w	+
Sucrose	+	–
Enzyme activity		
Esterase (C4)	+	–
Esterase lipase (C8)	+	–
Susceptibility to:		
Carbenicillin	+	–
Cephalothin	+	–
DNA G + C content (mol%)	32.7	35.2*

*Data for *T. litopenaei* B-I^T taken from Sheu *et al.* (2007).

column. The predominant isoprenoid quinone detected in strain YCS-6^T was menaquinone-6 (MK-6), which is in line with all members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006). For fatty acid methyl ester analysis, cell mass of strain YCS-6^T and *T. litopenaei* LMG 23706^T was harvested from MA after incubation for 2 days at 30 °C. Fatty acids were saponified, methylated and extracted using the standard protocol of the Sherlock Microbial Identification System, version 4.0 (MIDI). The fatty acid methyl esters were analysed by GC (model 6890; Hewlett Packard) and identified using the TSBA40 database of the Microbial Identification System (Sasser, 1990). The cellular fatty acid compositions of strain YCS-6^T and *T. litopenaei* LMG 23706^T are shown in Table 2. The major

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain YCS-6^T, members of the genus *Tenacibaculum* and members of some other related genera. Bootstrap values (>70%) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Cellulophaga lytica* ATCC 23178^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 2. Cellular fatty acid compositions of strain YCS-6^T and its closest phylogenetic neighbour in the genus *Tenacibaculum*

Data were obtained in this study. Values are percentages of total fatty acids; fatty acids that represented <0.5% in both strains are not shown. tr, Trace (<0.5%); –, not detected; ECL, equivalent chain-length.

Fatty acid	YCS-6 ^T	<i>T. litopenaei</i> LMG 23706 ^T
Straight-chain		
C _{15:0}	2.3	1.8
C _{16:0}	tr	1.0
Unsaturated		
C _{15:1} ω6c	2.5	0.8
C _{17:1} ω6c	0.6	1.0
Branched		
iso-C _{13:0}	1.5	tr
iso-C _{14:0}	1.5	tr
iso-C _{15:1} G*	10.2	3.0
iso-C _{15:0}	23.5	13.1
anteiso-C _{15:0}	0.6	tr
iso-C _{16:0}	1.2	1.1
iso-C _{16:1} H*	1.1	–
iso-C _{17:1} ω9c	0.9	0.9
Hydroxy		
C _{15:0} 2-OH	tr	1.1
C _{15:0} 3-OH	2.5	3.6
iso-C _{15:0} 3-OH	10.5	7.2
C _{16:0} 3-OH	1.0	7.4
iso-C _{16:0} 3-OH	8.4	5.6
C _{17:0} 2-OH	tr	0.8
C _{17:0} 3-OH	tr	1.3
iso-C _{17:0} 3-OH	9.9	19.0
Summed features†		
2	–	1.1
3	16.4	25.8
Unknown fatty acids		
ECL 13.565	0.7	0.8
ECL 16.582	0.6	0.8

*The position of the double bond indicated by the capital letter is unknown.

†Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 2 consisted of C_{14:0} 3-OH and/or iso-C_{16:1} I. Summed feature 3 consisted of iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

fatty acids (>10% of the total) in strain YCS-6^T were iso-C_{15:0}, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), iso-C_{15:0} 3-OH and iso-C_{15:1} G. The fatty acid composition of strain YCS-6^T was similar to that of *T. litopenaei* LMG 23706^T, but the two strains could be differentiated by differences in the proportions of some fatty acids. The fatty acid composition of strain YCS-6^T was also similar to those of other members of the genus *Tenacibaculum* (Lee *et al.*, 2009). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. The DNA G+C content of strain YCS-6^T was 32.7 mol%, which is in the range reported for members of the genus *Tenacibaculum* (29.8–35.2 mol%; Lee *et al.*, 2009). The chemotaxonomic data supported the phylogenetic inference that strain YCS-6^T belonged to the genus *Tenacibaculum* (Suzuki *et al.*, 2001; Frette *et al.*, 2004; Yoon *et al.*, 2005; Choi *et al.*, 2006; Jung *et al.*, 2006; Sheu *et al.*, 2007; Heindl *et al.*, 2008; Piñeiro-Vidal *et al.*, 2008; Wang *et al.*, 2008; Lee *et al.*, 2009).

The phylogenetic distinctiveness of strain YCS-6^T, together with the differential phenotypic properties, is sufficient to show that this strain is separate from all other members of the genus *Tenacibaculum* (Stackebrandt & Goebel, 1994). Therefore, strain YCS-6^T is considered to represent a novel species, for which the name *Tenacibaculum geojense* sp. nov. is proposed.

Description of *Tenacibaculum geojense* sp. nov.

Tenacibaculum geojense (ge.o.jen'se. N.L. neut. adj. *geojense* of Geoje, Korea, from where the type strain was isolated).

Cells are Gram-negative, non-spore-forming, non-flagellated rods (0.2–0.4 µm wide and 1.0–9.0 µm long; a few cells longer than 10 µm are also observed). Motile by gliding. Degenerative spherical cells are observed in ageing cultures. Flexirubin-type pigments are not produced. Colonies on MA are circular to irregular, flat, smooth, glistening, yellow, non-adherent and 2.0–5.0 mm in diameter after incubation for 2 days at 30 °C. Grows at 15–40 °C (optimum 30 °C), but not at 10 or 45 °C, at pH 5.5 (optimum pH 7.0–8.0), but not at pH 5.0, and with 0–7.0% (w/v) NaCl (optimum 2% NaCl). Mg²⁺ ions are required for growth. Growth does not occur under anaerobic conditions on MA or on MA supplemented with nitrate. Catalase-positive and oxidase-negative. H₂S is not produced. Nitrate is not reduced. Casein, tyrosine and Tweens 20, 40 and 60 are hydrolysed, but hypoxanthine and xanthine are not. The predominant menaquinone is MK-6. The major fatty acids (>10% of total) are iso-C_{15:0}, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), iso-C_{15:0} 3-OH and iso-C_{15:1} G. Other phenotypic properties are shown in Table 1. The DNA G+C content of the type strain is 32.7 mol% (HPLC).

The type strain, YCS-6^T (=KCTC 23423^T =CCUG 60527^T), was isolated from seawater collected from the South Sea near the city of Geoje, Korea.

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