Tenacibaculum litoreum sp. nov., isolated from tidal flat sediment

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A rod-shaped bacterium, designated CL-TF13^T, was isolated from a tidal flat in Ganghwa, Korea. Analysis of the 16S rRNA gene sequence revealed an affiliation with the genus *Tenacibaculum*. The sequence similarities between CL-TF13^T and type strains of members of the genus *Tenacibaculum* were from 94.2 to 97.4 %. Cells were motile by means of gliding. Strain CL-TF13^T grew on solid medium as pale-yellow colonies with an irregular spreading edge. The strain was able to grow in NaCl at a range of 3–5 %. They grew within a temperature range of 5–40 °C and at pH range of 6–10. The major fatty acids were summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 19.6 %), iso-C_{15:0} (18.8 %) and iso-C_{17:0} 3-OH (13.6 %). Fatty acids such as C_{18:3}ω6c (6,9,12) (1.5 %) and summed feature 4 (iso I- and/or anteiso B-C_{17:1}, 1.3 %) were uniquely found in minor quantities in CL-TF13^T among *Tenacibaculum* species. The DNA G+C content was 30 mol%. According to physiological data, fatty-acid composition and 16S rRNA gene sequence, CL-TF13^T could be assigned to the genus *Tenacibaculum* but distinguished from the recognized species of the genus. Therefore, strain CL-TF13^T (=KCCM 42115^T=JCM 13039^T) represents a novel species, for which the name *Tenacibaculum litoreum* sp. nov. is proposed.

Since the genus *Tenacibaculum* was proposed by Suzuki *et al.* (2001), six species have been isolated from various marine sources. Two species, *Tenacibaculum maritimum* and *Tenacibaculum ovolyticum*, were reclassified from the former [*Flexibacter*] *maritimus* isolated from diseased red sea bream fingerling (Wakabayashi *et al.*, 1986) and [*Flexibacter*] *ovolyticus* isolated from halibut egg (Hansen *et al.*, 1992). Two other species, *Tenacibaculum mesophilum* and *Tenacibaculum amyolyticum*, were isolated from homogenates of sponge and surfaces of marine macroalgae, respectively (Suzuki *et al.*, 2001). *Tenacibaculum skagerrakense* and *Tenacibaculum lutimaris* were recently isolated from sea water (Frette *et al.*, 2004) and tidal flat sediment (Yoon *et al.*, 2005), respectively.

In this study, strain CL-TF13^T, related to the genus *Tenacibaculum*, was isolated from tidal flat sediment in Ganghwa, Korea. The sediment slurry was spread onto a plate containing marine agar 2216 (MA; Difco) and the plate was incubated at 30 °C for 1 week. Strain CL-TF13^T was isolated on the plate and subsequently purified four times on MA at

30 °C. The strain was maintained both on MA at 4 °C and in marine broth 2216 (MB; Difco) supplemented with 30 % (v/v) glycerol at –80 °C.

The 16S rRNA gene was amplified from a single colony by PCR with *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). The PCR product was purified using the AccuPrep PCR Purification kit (Bioneer) and cloned using the pCR2.1 TOPO TA Cloning kit (Invitrogen). Sequencing of the 16S rRNA gene was performed with an Applied Biosystems automatic sequencer (ABI 3730XL) at Macrogen (Seoul, Korea). The almost-complete 16S rRNA gene sequence of strain CL-TF13^T (1446 bp) was obtained and compared with 16S rRNA gene sequences available in GenBank using BLASTN (Altschul *et al.*, 1990) searches. The sequence of strain CL-TF13^T was manually aligned with those of the type strains of six *Tenacibaculum* species and with the type species of other genera in the family *Flavobacteriaceae* obtained from GenBank and Ribosomal Database Project (Cole *et al.*, 2003) databases using known 16S rRNA secondary structure information. Phylogenetic trees were obtained by neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of tree topologies was assessed by bootstrap analyses based on 1000

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Abbreviations: ASW, artificial sea water; ECL, equivalent chain-length.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CL-TF13^T is AY962294.

replications for neighbour-joining and maximum-parsimony methods and 100 replications for the maximum-likelihood method. Alignment analysis was carried out using the jPHYDIT program (version 1.0; <http://chunlab.snu.ac.kr/jphydit/>) and phylogenetic analysis was carried out using MEGA3 (Kumar *et al.*, 2004) and PAUP* 4.0 (Swofford, 1998). Likelihood parameters were estimated by the hierarchical ratio tests in MODELTEST, version 3.04 (Posada & Crandall, 1998). Sequence similarity indicated that the closest relatives of strain CL-TF13^T were *T. lutimaris* (97.4%), *T. mesophilum* (97.1%), *T. skagerrakense* (96.5%), *T. amylolyticum* (96.0%), *T. ovolyticum* (95.4%) and *T. maritimum* (94.2%). Phylogenetic analyses based on the 16S rRNA gene sequence showed that strain CL-TF13^T formed a robust cluster with species of the genus *Tenacibaculum* (Fig. 1). Thus, it is clear that our isolate belongs to the genus *Tenacibaculum*. The DNA G + C content was determined using the thermal denaturation method (Mandel & Marmur, 1968) and was found to be 30 mol%, at the lower limit of the range reported in other *Tenacibaculum* species (Table 1). As the similarities between strain CL-TF13^T and two species (*T. lutimaris* and *T. mesophilum*) were close to the theoretical threshold (97%) for the delineation of bacterial species based on 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994), the relatedness of genomic DNA was determined by dot-blot hybridization. Probe DNA labelling was performed using a nick translation kit (Roche) and hybridization and detection were done using the DIG labelling and detection kit (Roche) according to the manufacturer's instruction. The DNA–DNA relatedness between CL-TF13^T and *T. lutimaris* was 11%, while that for *T. mesophilum* was 46%. These values are below the currently accepted limit of DNA relatedness (70%) for the phylogenetic definition of a species (Stackebrandt & Goebel, 1994) and therefore provide evidence that the isolate represents a novel species in the genus *Tenacibaculum*.

Morphological and physiological analyses were performed. Gram staining was performed as described by Smibert & Krieg (1994). Cell morphology was examined by phase-contrast microscopy and transmission electron microscopy (JEOL EX2) with cells grown at 30 °C in MB. Gliding motility was observed by the hanging-drop method (Suzuki *et al.*, 2001). Anaerobic growth was checked on MA using the GasPak anaerobic system (BBL). Catalase and oxidase activities were determined according to the protocols described by Smibert & Krieg (1994), and gelatinase, amylase, DNase and nitrate reductase activities and degradation of Tween 80 were examined as described by Hansen & Sørheim (1991). Cells are rod-shaped (Fig. 2) and motile by means of gliding. Spherical cells were rarely observed in ageing cultures of CL-TF13^T (Fig. 2b). Colony size, shape and colour were determined on MA after 5 days incubation at 30 °C. Colonies were pale yellow in colour, greenish glistening and irregular with spreading edges on MA. Colonies were 5–10 mm in diameter and not adherent to the agar. Other morphological characteristics are shown in Table 1.

The temperature range for growth was determined on the basis of colony formation on MA plates that were incubated at 5–45 °C. The pH range (5–11) for growth was determined by changes in OD₆₀₀ with time in MB. The final pH was adjusted using NaOH and HCl solutions. Tolerance of CL-TF13^T to NaCl and sea salts (Sigma) was determined using the synthetic ZoBell broth (5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate, 1 l distilled water) with various concentrations [0, 1, 3, 5, 7, 10, 15, 20, 25% (w/v)] of NaCl or sea salts. Nitrate reduction, production of indole, arginine dihydrolase, urease, gelatinase and β -galactosidase, acid production from glucose and hydrolysis of aesculin were tested using API 20NE kit (bioMérieux) according to the

Fig. 1. Neighbour-joining tree based on rRNA gene sequences showing relationships between strain CL-TF13^T, members of the genus *Tenacibaculum* and other related genera belonging to the family *Flavobacteriaceae*. Only bootstrap values above 70% are shown (1000 resamplings) at the branching points. Solid circles indicate that the corresponding nodes are also recovered in maximum-likelihood and maximum-parsimony trees. *Flexibacter flexilis* ATCC 23079^T (M62794) was used as an outgroup (not shown). Bar, 0.02 nt substitution per site.

Table 1. Differential phenotypic characteristics of strain CL-TF13^T and other *Tenacibaculum* species

Strains: 1, *T. litoreum* sp. nov. CL-TF13^T; 2, *T. lutimaris* TF-26^T; 3, *T. skagerrakense* D30^T; 4, *T. amylolyticum* MBIC 4355^T; 5, *T. mesophilum* MBIC 1140^T; 6, *T. ovolyticum* IAM 14318^T; 7, *T. maritimum* NCIMB 2154^T. Data from Yoon *et al.* (2005), Frette *et al.* (2004), Suzuki *et al.* (2001) or this study. +, Positive; –, negative; W, weakly positive; opt., optimal; NA, not available; NG, no growth with NaCl only. All species are Gram-negative, rod-shaped and positive for catalase and oxidase. Data for API ZYM were obtained in this study.

Characteristic	1	2	3	4	5	6	7
Origin	Tidal flat	Tidal flat	Pelagic	Macroalgae	Sponge, macroalgae	Halibut egg	Diseased red sea bream fingerling
Colony morphology:*							
Shape	Irregular, spreading edge	Irregular, spreading edge	Circular, convex, spreading edge	Circular, spreading edge	Irregular, spreading edge	Regular edge	Uneven edge
Diameter at 5 days (mm)	5–10	10–20	5–20	23–27	30–60	NA	<5
Colour	Pale yellow	Pale yellow	Bright yellow	Yellow	Yellow	Pale yellow	Pale yellow
Cell size (µm)	0.3–0.5 × 2–35	0.5 × 2–10	0.5 × 2–15	0.4 × 2–5	0.5 × 1.5–10	0.5 × 2–20	0.5 × 2–30
Spherical cells	Very rare	Very rare	Frequent	Very rare	Very rare	Not observed†	Frequent†
Gliding motility	+	+	–	+	+	+	+
Tolerance to salinity (%):							
NaCl	3–5	<8 (opt. 2–3)	NG	3 (W)	1–7	NG	NG
Sea water	25–250‡	25–175‡§	25–150‡	50–100	10–100	70–100	30–100
Temperature range (°C)	5–40	10–39	10–40	20–35	15–40	4–25	15–34
Optimal temperature (°C)	35–40	30–37	25–37	27–30	28–35	ND	30
pH range	6–10	7–8 (opt.)	6–9	5.3–8.3	5.3–9	5.9–8.6	5.9–8.6
Growth at pH 5	–	+	–	–	–	–	–
DNA G+C content (mol%)	30	32.3–32.8	35.2	30.9	31.6–32.0	30.3–32.0	31.3–32.5
Nitrate reduction	+	–	+	W	–	+	+
Degradation of:							
Starch	+	–	+	+	–	–	–
Gelatin	+	+	NA	+	+	+	+
Tween 80	+	–	–	+	+	+	+
DNA	+	NA	NA	+	+	+	+
API ZYM:							
Esterase lipase (C8)	+	W	NA	NA	NA	NA	+
Lipase (C14)	–	–	NA	NA	NA	NA	W
Trypsin	+	+	NA	NA	NA	NA	W
Naphthol-AS-BI-phosphohydrolase	+	+	NA	NA	NA	NA	W
Carbon utilization:							
Citrate	–	NA	+	–	–	–	–
L-Leucine	–	–	W	–	–	–	–
Sucrose	–	–	+	–	–	–	–
L-Proline	+	–	+	+	+	–	–
L-Glutamate	–	–	+	+	+	–	W
DL-Aspartate	–	–	+	–	+	–	–
D-Glucose	–	–	+	NA	NA	NA	NA
D-Mannose	–	NA	+	NA	NA	NA	NA

*For analyses of colony morphology, CL-TF13^T and *T. lutimaris* were grown on MA at 30 °C, *T. skagerrakense* was grown on ZoBell plate at 20 °C. The other strains were grown on 1/5 LBM agar (Suzuki *et al.*, 2001) at 20 °C.

†J.-F. Bernardet, personal communication.

‡Percentage of sea-water strength was calculated using a relation of 100 % sea water = 40 g sea salts l^{–1} (Frette *et al.*, 2004).

§Data from this study.

Fig. 2. Transmission electron micrograph of negatively stained cells of strain CL-TF13^T. Bars, 2 μm (a) and 1 μm (b).

manufacturer's instruction, except that the cell suspension was prepared using artificial sea water (ASW; 24 g NaCl, 5.1 g MgCl₂, 4 g Na₂SO₄, 1.1 g CaCl₂, 0.7 g KCl, 0.2 g NaHCO₃, 0.1 g KBr, 0.027 g H₃BO₃, 0.024 g SrCl₂, 0.003 g NaF, 1 l distilled water; Lyman & Fleming, 1940) as a suspension medium. Other enzyme activities were also assayed by using the API ZYM kit (bioMérieux) and ASW as a suspension medium. Carbon utilization was tested on basal medium agar (0.2 g NH₄Cl, 0.2 g NaNO₃, 0.05 g yeast extract, 15 g Bacto agar, 1 l ASW) containing 0.4 % of the carbon source. Incubation was prolonged for 1 month and growth was scored as negative when growth was equal to or less than that in the negative control, without any carbon source. For the analyses of API 20NE, API ZYM and carbon utilization tests, *T. lutimaris* TF-26^T and *T. maritimum* NCIMB 2514^T were employed as reference strains. In contrast to the other *Tenacibaculum* species, CL-TF13^T grew optimally at 35–40 °C and was able to grow at pH 10, with 3–5 % (w/v) NaCl and with 1–10 % (w/v) sea salts (Table 1). The strain showed positive responses in tests for nitrate reductase, gelatinase, DNase, amylase, catalase, cytochrome oxidase and degradation of Tween 80 (Table 1). The other results of biochemical and physiological tests are given in Table 1 and the species description.

Isoprenoid quinone was isolated according to Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). The major isoprenoid quinone in CL-TF13^T was menaquinone-6 (MK-6). Pigments were extracted from cells cultured in the dark for 1 day by using methanol and analysed by HPLC system. Flexirubin pigments were detected by a colour-shift test using 20 % (w/v) KOH solution (Reichenbach, 1992). CL-TF13^T contained zeaxanthin as the major carotenoid pigment but did not contain flexirubin pigments. Thus, the characteristics of isoprenoid quinone and pigments were the same as other *Tenacibaculum* species. The fatty acid methyl esters in whole cells, which were grown on MA at 30 °C for 1 day, were analysed by gas chromatography according to the instructions of the

Microbial Identification system (MIDI) at the Korean Culture Center of Microorganisms (Seoul, Korea). Similar to other members of the genus, the fatty-acid profile of CL-TF13^T was dominated by summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 19.6 %), iso-C_{15:0} (18.8 %), iso-C_{17:0} 3-OH (13.6 %), iso-C_{15:1} (8.2 %), iso-C_{16:0} 3-OH (6.8 %) and iso-C_{15:0} 3-OH (6.6 %) (Table 2). However, the relative proportions of

Table 2. Cellular fatty-acid content of strain CL-TF13^T and other *Tenacibaculum* species

Values are percentages of total fatty acids. Strains: 1, *T. litoreum* sp. nov. CL-TF13^T; 2, *T. lutimaris* TF-26^T; 3, *T. skagerrakense* D30^T; 4, *T. mesophilum* MBIC 1140^T; 5, *T. maritimum* NCIMB 2154^T. No fatty acid data are available for *T. ovolyticum* or *T. amylolyticum*. Data from Yoon *et al.* (2005) and this study. Fatty acids that represented <0.5 % in all strains were omitted. ND, Not detected; –, ND or <0.5%. ECL, Equivalent chain-length. Fatty-acid analyses were performed using cells cultivated on the same medium and at the same temperature, but incubation time was different between CL-TF13^T (1 day) and the other strains (2 days).

Fatty acid	1	2	3	4	5
Straight-chain					
C _{15:0}	2.7	8.9	4.9	3.6	2.9
C _{16:0}	0.9	0.6	0.6	0.7	0.3
C _{18:0}	0.3	ND	ND	ND	1.4
Branched					
iso-C _{13:0}	1.4	0.7	0.2	0.8	1.8
iso-C _{14:0}	0.7	1.7	0.9	0.8	0.8
iso-C _{15:0}	18.8	17.2	9.5	13.2	16.8
iso-C _{15:1}	8.2	5.3	8.2	7.1	7.6
anteiso-C _{15:0}	1.8	0.7	ND	1.1	0.8
iso-C _{16:0}	2.3	3.8	1.3	1.7	0.3
iso-C _{16:1}	1.3	1.7	1.7	0.8	ND
iso-C _{17:1} ω9c	1.6	0.4	ND	0.6	ND
Unsaturated					
C _{15:1} ω6c	1.7	4.2	ND	1.6	2.2
C _{17:1} ω6c	0.9	1.5	1.2	0.9	0.3
C _{18:3} ω6c (6,9,12)	1.5	–	–	–	–
Hydroxylated					
C _{15:0} 2-OH	0.7	1.2	2.5	1.1	1.1
C _{15:0} 3-OH	ND	3.4	8.6	2.9	3.8
iso-C _{15:0} 3-OH	6.6	4.6	7.8	8.0	19.8
C _{16:0} 3-OH	1.6	1.3	2.1	3.2	1.5
iso-C _{16:0} 3-OH	6.8	12.8	12.2	9.0	5.0
C _{17:0} 2-OH	0.9	0.2	ND	0.8	ND
C _{17:0} 3-OH	0.3	0.9	2.5	0.7	0.6
iso-C _{17:0} 3-OH	13.6	8.4	11.7	14.9	13.7
ECL 13.565	1.3	–	–	–	–
ECL 16.582	1.3	0.7	0.6	1.0	1.0
Summed feature 3*	19.6	18.1	22.5	24.4	17.9
Summed feature 4†	1.3	–	–	–	–

*C_{16:1}ω7c/iso-C_{15:0} 2-OH.

†Iso I-/anteiso B-C_{17:1}.

the dominant fatty acids in CL-TF13^T were different from other *Tenacibaculum* species and three fatty acids, C_{18:3}ω6c (6,9,12) (1.5%), summed feature 4 (iso I- and/or anteiso B-C_{17:1}, 1.3%) and an unknown fatty acid (ECL 13.565) (1.3%), were uniquely found in minor quantities in CL-TF13^T among *Tenacibaculum* species. Therefore, the fatty-acid pattern of strain CL-TF13^T differed significantly from those of previously described *Tenacibaculum* species.

In conclusion, physiological features, fatty-acid profiles, phylogenetic analyses based on 16S rRNA gene sequences and DNA–DNA relatedness suggest that strain CL-TF13^T represents a novel species of the genus *Tenacibaculum*, for which the name *Tenacibaculum litoreum* sp. nov. is proposed.

Description of *Tenacibaculum litoreum* sp. nov.

Tenacibaculum litoreum (li.to.re'um. L. neut. adj. *litoreum* of the shore).

Gram-negative, strictly aerobic and straight rods, approximately 0.3–0.5 × 2–35 µm in size. Cells are motile by means of gliding. On MA solid medium colonies are pale yellow, irregular with spreading edges and greenish glistening. After 5 days on MA at 30 °C colonies are approximately 5–10 mm in diameter. Growth occurs within the temperature range of 5–40 °C (optimum 35–40 °C) and at pH values of 6–10. Growth occurs in NaCl concentrations of 3–5% (w/v) and in sea salt concentrations of 1–10% (w/v). Positive for catalase, cytochrome oxidase, amylase, gelatinase, DNase and nitrate reductase and degradation of Tween 80. Major fatty acids are summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 19.6%), iso-C_{15:0} (18.8%) and iso-C_{17:0} 3-OH (13.6%). Also contains minor amounts of C_{18:3}ω6c (6,9,12) (1.5%) and summed feature 4 (iso I- and/or anteiso B-C_{17:1}, 1.3%). According to API 20NE tests, activities for nitrate reductase and gelatinase are present, whereas activities for indole production, acid production from glucose, arginine dihydrolase and urease, hydrolysis of aesculin and β-galactosidase are absent. According to API ZYM tests, activities for alkaline phosphatase, esterase (C4 and C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, whereas activities for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Growth occurs on Casamino acids, tryptone, yeast extract, peptone, L-arginine, L-ornithine, L-proline and L-lysine. No growth occurs on acetate, benzoate, citrate, maleic acid, ethanol, glycerol, L-leucine, tartrate, pyruvic acid, succinate, sucrose, L-glutamate, D-ribose, DL-aspartate, N-acetylglucosamine, L-arabinose, D-xylose, D-fructose, D-glucose, D-mannose, D-trehalose, inulin, D-mannitol, D-sorbitol, D-salicin, D-raffinose, D-galactose, urea or lactose. The DNA G + C content is 30 mol%.

The type strain, CL-TF13^T (= KCCM 42115^T = JCM 13039^T), was isolated from a tidal flat sediment in Ganghwa, Korea.

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