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Tenacibaculum lutimaris sp. nov., isolated from a tidal flat in the Yellow Sea, Korea

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Four Gram-negative, rod-shaped bacterial strains, TF-26^{T}, TF-28, TF-42 and TF-53, were isolated from a tidal flat in the Yellow Sea, Korea, and their taxonomic positions were determined by a polyphasic characterization. The strains grew optimally in the presence of 2–3 % (w/v) NaCl and at 30–37 °C. The predominant menaquinone detected in the four strains was MK-6. These strains contained large amounts of fatty acids $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH, iso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH, $C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The DNA G+C contents of the four strains were 32·3–32·8 mol%. Strains TF-26^{T}, TF-28, TF-42 and TF-53 showed 16S rRNA gene sequence similarity levels of 99·8–100 % and DNA–DNA relatedness levels of 82–87 %. The four strains exhibited 16S rRNA gene sequence similarity levels of 95·0–98·0 % to the type strains of the five current *Tenacibaculum* species, and DNA–DNA relatedness levels between the four strains and two phylogenetic relatives, *Tenacibaculum mesophilum* DSM 13764^{T} and *Tenacibaculum skagerrakense* DSM 14836^{T}, were less than 21 %. On the basis of phenotypic, phylogenetic and genetic data, strains TF-26^{T}, TF-28, TF-42 and TF-53 were classified in the genus *Tenacibaculum* as members of a novel species, for which the name *Tenacibaculum lutimaris* sp. nov. (type strain, TF-26^{T}=KCTC 12302^{T}=DSM 16505^{T}) is proposed.

The genus *Tenacibaculum* was proposed by reclassification of two species that had been assigned to the genus *Flexibacter*, *Flexibacter maritimus* (Wakabayashi *et al.*, 1986) and *Flexibacter ovolyticus* (Hansen *et al.*, 1992), as *Tenacibaculum maritimum* and *Tenacibaculum ovolyticum*, and with two novel species, *Tenacibaculum mesophilum* and *Tenacibaculum amylolyticum* (Suzuki *et al.*, 2001). One further *Tenacibaculum* species, *Tenacibaculum skagerrakense*, has been described recently (Frette *et al.*, 2004). The genus *Tenacibaculum* is characterized chemotaxonomically by having MK-6 as the predominant menaquinone and by DNA G+C contents of 30·3–35·2 mol% (Suzuki *et al.*, 2001; Frette *et al.*, 2004). Phylogenetic analyses based on 16S rRNA gene sequences showed that the genus is phylogenetically related to the *Cytophaga–Flavobacterium–Bacteroidetes* group (Suzuki *et al.*, 2001; Frette *et al.*, 2004). In this study, we describe four *Tenacibaculum*-like strains, TF-26^{T}, TF-28, TF-42 and TF-53, which were isolated from a tidal flat in the Yellow Sea in Korea. The aim of the present work was to determine the taxonomic positions of the four strains by the detailed taxonomic characterization that combined phenotypic, chemotaxonomic, phylogenetic and genetic analyses.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TF-26^{T}, TF-28, TF-42 and TF-53 are AY661691, AY661692, AY661693 and AY661694, respectively.

Bacterial strains were isolated from tidal sediments collected from Daepo Beach in the Yellow Sea, Korea. Strains TF-26^{T}, TF-28, TF-42 and TF-53 were isolated from different specimens by the standard dilution plating technique at 30 °C on marine agar 2216 (MA; Difco). To investigate their morphological and physiological characteristics, strains TF-26^{T}, TF-28, TF-42 and TF-53 were routinely cultivated at 30 °C on MA. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy (TEM; Philips model CM-20). Presence of flagella was examined by TEM using cells from exponentially growing cultures. Gram reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Gliding motility was determined as described by Bowman (2000). Growth at various temperatures (4–45 °C) was measured on MA. Growth at various NaCl concentrations was investigated in marine broth 2216 (MB; Difco) or in trypticase soy broth (Difco) lacking NaCl and in trypticase soy broth. The pH range for growth was determined in MB (Difco) that was adjusted to various pH values (pH 4·5–9·5 at intervals of 0·5 pH units). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with MA that had been prepared anaerobically using nitrogen. Catalase and oxidase activities and hydrolysis of casein, starch and

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Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and nitrate reduction were studied as described previously (Lanyi, 1987) with the modification that artificial sea water was used for preparation of media. The artificial sea water contained (per litre distilled water) 23·6 g NaCl, 0·64 g KCl, 4·53 g $MgCl_2.6H_2O$, 5·94 g $MgSO_4.7H_2O$ and 1·3 g $CaCl_2.2H_2O$ (Bruns *et al.*, 2001). H_2S production was tested as described previously (Bruns *et al.*, 2001). Presence of flexirubin pigment was investigated as described by Reichenbach (1992). Congo red adsorption was determined as described by Bernardet *et al.* (2002). Acid production from carbohydrates was determined as described by Leifson (1963). Growth on several substrates was tested in a basal medium containing 0·2 g $NaNO_3$, 0·2 g NH_4Cl and 0·05 g yeast extract in 1000 ml artificial sea water (Bruns *et al.*, 2001) as described by Suzuki *et al.* (2001).

Cell biomass for isoprenoid quinone analysis and for DNA extraction was obtained from cultivation for 1–2 days in MB at 30 °C. Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. Chromosomal DNA isolation and purification were performed according to the method described by Yoon *et al.* (1996), with the exception that ribonuclease T1 was used together with ribonuclease A to minimize the contamination of RNA. For fatty acid methyl ester (FAME) analysis, cell mass of the four strains was harvested from agar plates after incubation for 2 days on MA at 30 °C. The FAMEs were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with a modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

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). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded, and the remaining three values were used to calculate similarity values. The DNA relatedness values quoted are the means of the three values.

Strains TF-26^T, TF-28, TF-42 and TF-53 grew optimally at 30–37 °C and pH 7·0–8·0 and in the presence of 2–3 % (w/v) NaCl. The four strains were similar in most phenotypic characteristics. Differential characteristics of the four strains were as follows: strains TF-26^T and TF-53 grew at pH 5·0, but strains TF-28 and TF-42 did not; strains TF-26^T, TF-28 and TF-42 grew weakly at 40 °C, but strain TF-53 did not; strains TF-26^T, TF-28 and TF-42 did not grow in the presence of greater than 8 % (w/v) NaCl, but strain TF-53 did not grow in the presence of greater than 7 % (w/v) NaCl; strains TF-28 and TF-42 grew under anaerobic conditions on MA supplemented with nitrate, but strains TF-26^T and TF-53 did not; strains TF-28 and TF-42 reduced nitrate to nitrogen, but strains TF-26^T and TF-53 did not. Other phenotypic characteristics are shown in Table 1 or given in the species description (see below).

Strains TF-26^T, TF-28, TF-42 and TF-53 contained menaquinone-6 (MK-6) as the predominant isoprenoid quinone at peak area ratio of approximately 93–97 %. The four strains had similar fatty acid profiles that contained large amounts of straight-chain, branched, unsaturated and hydroxy fatty acids; the major components were $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, iso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH, $C_{15:0}$ and iso-$C_{17:0}$ 3-OH (Table 2). These fatty acid profiles were similar to those of three *Tenacibaculum* species analysed in this study (Table 2). There were some differences in the compositions of fatty acids between the four strains and three *Tenacibaculum* species, particularly between the four strains and *T. maritimum* JCM 8137^T (Table 2). The DNA G + C contents of strains TF-26^T, TF-28, TF-42 and TF-53 were 32·6, 32·8, 32·6 and 32·3 mol%, respectively.

The 16S rRNA gene sequences of strains TF-26^T, TF-28, TF-42 and TF-53 determined in this study comprised 1473 nucleotides, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. The 16S rRNA gene sequences of strains TF-26^T, TF-28 and TF-42 were identical. Strain TF-53 showed three nucleotide differences to the other three strains. The phylogenetic trees based on 16S rRNA gene sequences showed that strains TF-26^T, TF-28, TF-42 and TF-53 fall within the radiation of the cluster comprising *Tenacibaculum* species (Fig. 1). Strains TF-26^T, TF-28, TF-42 and TF-53 exhibited 16S rRNA gene sequence similarity levels of 97·2–98·0 % to the type strains of *T. mesophilum* and *T. skagerrakense* and of 95·0–97·0 % to the type strains of the other *Tenacibaculum* species. The four strains exhibited 16S rRNA gene sequence similarity levels of 92·4–93·1 % to *Polaribacter* species and of less than 89·2 % to other species included in the phylogenetic analysis. DNA–DNA hybridization was performed to determine the genomic relatedness among strains TF-26^T, TF-28, TF-42 and TF-53 and between the four novel strains and the type strains of *T. mesophilum* and *T. skagerrakense* that showed 16S rRNA gene sequence similarity levels of greater than 97 % to the four strains. Strains TF-26^T, TF-28, TF-42 and TF-53 exhibited mean levels of DNA–DNA relatedness of 82–87 %, when their DNAs were used individually as labelled DNA probes for cross-hybridization. These values indicate that the four strains are members of the same genomic species. DNA–DNA relatedness levels between the four strains and *T. mesophilum* DSM 13764^T and *T. skagerrakense* DSM 14836^T were in the range of 9–21 %.

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Table 1. Differential phenotypic characteristics of *Tenacibaculum* species

Species: 1, *T. lutimaris* sp. nov.; 2, *T. skagerrakense*; 3, *T. amylolyticum*; 4, *T. mesophilum*; 5, *T. ovolyticum*; 6, *T. maritimum*. Data are from Wakabayashi *et al.* (1986), Hansen *et al.* (1992), Suzuki *et al.* (2001), Frette *et al.* (2004) and this study. +, Positive; −, negative; W, weakly positive; V, variable reaction; ND, not determined; NG, no growth in the presence of NaCl only; *n*, number of strains. Data in parentheses are for the type strain. All species are Gram-negative and rod-shaped. All species are positive for catalase, oxidase and degradation of casein.

Characteristic	1 (*n*=4)	2 (*n*=2)	3 (*n*=1)	4 (*n*=4)	5 (*n*=3)	6 (*n*=2)
Origin	Tidal flat, Korea	Pelagic, Denmark	Macroalgae, Japan	Sponge and macroalgae, Japan	Halibut egg, Norway	Diseased Red Sea bream fingerling, Japan
Cell size (μm)	0·5 × 2–10	0·5 × 2–15	0·4 × 2–4	0·5 × 1·5–10	0·5 × 2–20	0·5 × 2–30
Colony morphology						
Shape	Irregular, spreading edge	Circular, spreading edge	Circular, spreading edge	Irregular, spreading edge	Regular edge	Uneven edge
Diameter at 5 days (mm)	10–20	5–20	23–27	30–60	ND	<5
Colour	Pale yellow	Bright yellow	Yellow	Yellow	Pale yellow	Pale yellow
Spherical cells	Very rare	Frequent	Very rare	Very rare	ND	+*
Temperature range (°C)	10–39	10–40	20–35	15–40	4–25	15–34
Optimal temperature (°C)	30–37	25–37	27–30	28–35	ND	30
Growth on:						
Casamino acids	+	ND	+	+	V	+
Sucrose	−	+	−	−	−	−
D-Ribose	−	ND	−	−	−	−
DL-Aspartate	−	+	−	+	−	−
L-Proline	−	+	+	+	−	−
L-Glutamate	−	+	+	+	−	W
L-Leucine	−	W	−	−	−	−
Degradation of:						
Starch	−	+	+	−	−	−
Gelatin	+	ND	+	+	+	+†
Tween 80	−	−	+	+	+	+
Nitrate reduction	V(−)	+	W	−	+	+
DNA G+C content (mol%)	32·3–32·8	35·2	30·9	31·6–32·0	30·3–32·0	31·3–32·5

*Spherical cells may appear in old cultures (Wakabayashi *et al.*, 1986; Bernardet *et al.*, 2002).
†Data from Wakabayashi *et al.* (1986); different result was obtained by Suzuki *et al.* (2001).

In the phylogenetic trees, inferred from comparison of 16S rRNA gene sequences, the four strains were phylogenetically affiliated to the genus *Tenacibaculum* (Fig. 1). The predominant isoprenoid quinone type was in agreement with the result of phylogenetic classification based on 16S rRNA gene sequences. The menaquinone (MK-6) detected in the four strains was the same as that for the genus *Tenacibaculum* (Suzuki *et al.*, 2001). The fatty acid profiles of the four strains were similar to those of the type strains of the three *Tenacibaculum* species that were analysed in this study (Table 2). These fatty acid profiles were similar to those of *Polaribacter* species, some *Cellulophaga* species and *Flavobacterium aquatile* (Gosink *et al.*, 1998; Bernardet *et al.*, 1996) but were distinguished from those of some other phylogenetically related taxa, although the observation may be caused by different experimental conditions. Hydroxy fatty acids were major components in the four strains and *Tenacibaculum* species, while they were not detected or were minor components in the genera *Psychroserpens* and *Gelidibacter* (Bowman *et al.*, 1997). The fatty acid iso-$C_{13:0}$, which was a minor component in the four strains and *Tenacibaculum* species, was one of the major components in the genus *Coenonia* (Vandamme *et al.*, 1999). The four strains were similar phylogenetically and genetically as well as phenotypically. Strains TF-26^T, TF-28, TF-42 and TF-53 were distinguished from the five *Tenacibaculum* species by differences in some phenotypic properties as shown in Table 1. The genetic distinctiveness, together with 16S rRNA gene sequence similarity data, were enough to categorize the four strains as a species that is distinct from the five current *Tenacibaculum* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, strains TF-26^T, TF-28, TF-42 and TF-53 should be classified in the genus *Tenacibaculum* as members of a novel species, for which the name *Tenacibaculum lutimaris* sp. nov. is proposed.

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Table 2. Cellular fatty acid compositions (%) of strains TF-26T, TF-28, TF-42 and TF-53 and the type strains of three *Tenacibaculum* species

Strains: 1, strain TF-26T; 2, strain TF-28; 3, strain TF-42; 4, strain TF-53; 5, *T. skagerrakense* DSM 14836T; 6, *T. mesophilum* DSM 13764T; 7, *T. maritimum* JCM 8137T. Fatty acids that represented less than 0·5 % in all strains were omitted.

Fatty acid	1	2	3	4	5	6	7
Straight-chain fatty acids							
$C_{15:0}$	8·9	10·0	8·3	8·5	4·9	3·6	2·9
$C_{16:0}$	0·6	0·4	0·6	0·6	0·6	0·7	0·3
$C_{18:0}$	–	–	–	–	–	–	1·4
Branched fatty acids							
iso-$C_{13:0}$	0·7	0·5	0·6	0·5	0·2	0·8	1·8
iso-$C_{14:0}$	1·7	1·1	1·5	1·2	0·9	0·8	0·8
iso-$C_{15:0}$	17·2	17·1	17·8	14·9	9·5	13·2	16·8
iso-$C_{15:1}$	5·3	5·2	4·4	5·1	8·2	7·1	7·6
anteiso-$C_{15:0}$	0·7	0·9	1·1	0·8	–	1·1	0·8
iso-$C_{16:0}$	3·8	2·6	3·0	3·3	1·3	1·7	0·3
iso-$C_{16:1}$	1·7	1·7	1·7	1·5	1·7	0·8	–
iso-$C_{17:1}\omega 9c$	0·4	0·5	0·4	0·4	–	0·6	–
Unsaturated fatty acids							
$C_{15:1}\omega 6c$	4·2	5·3	4·4	4·3	–	1·6	2·2
$C_{17:1}\omega 6c$	1·5	2·1	1·6	1·8	1·2	0·9	0·3
Hydroxy fatty acids							
$C_{15:0}$ 2-OH	1·2	1·2	1·4	1·5	2·5	1·1	1·1
$C_{15:0}$ 3-OH	3·4	4·1	3·9	3·8	8·6	2·9	3·8
iso-$C_{15:0}$ 3-OH	4·6	5·2	5·7	4·5	7·8	8·0	19·8
$C_{16:0}$ 3-OH	1·3	1·3	1·7	1·8	2·1	3·2	1·5
iso-$C_{16:0}$ 3-OH	12·8	11·3	13·0	13·0	12·2	9·0	5·0
$C_{17:0}$ 2-OH	0·2	0·3	0·3	0·4	–	0·8	–
$C_{17:0}$ 3-OH	0·9	1·2	1·1	1·2	2·5	0·7	0·6
iso-$C_{17:0}$ 3-OH	8·4	8·8	8·6	9·0	11·7	14·9	13·7
Unknown fatty acid (ECL* 16·582)	0·7	0·6	0·4	0·7	0·6	1·0	1·0
Summed feature 3†	18·1	18·1	17·4	19·7	22·5	24·4	17·9

*ECL, Equivalent chain-length.

†Summed feature represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH.

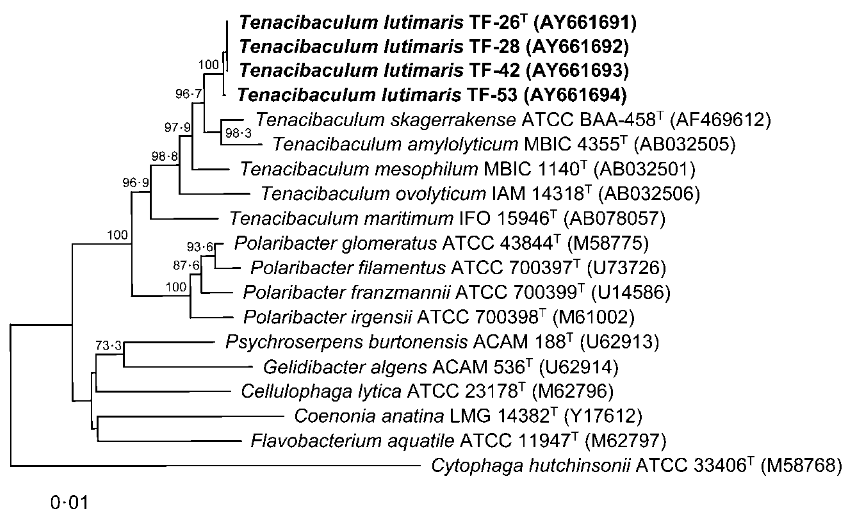

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strains TF-26T, TF-28, TF-42 and TF-53 and some other related taxa. The numbers on the branches indicate the bootstrap value of 1000 resamplings (greater than 50 %). Bar, 0·01 substitution per nucleotide position.

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Description of *Tenacibaculum lutimaris* sp. nov.

Tenacibaculum lutimaris (lu.ti.ma′ris. L. n. *lutum* mud; L. gen. n. *maris* of the sea, marine; N.L. gen. n. *lutimaris* of a marine mud).

Cells are Gram-negative and non-flagellated. Motile by means of gliding. Colonies are irregular, smooth, glistening and pale yellow in colour on MA at 30 °C. Adherence of colonies to MA is observed. Growth occurs at 10 and 39 °C with an optimum temperature of 30–37 °C; growth does not occur at 4 °C or above 41 °C. Optimal pH for growth is between 7·0 and 8·0; no growth is observed at pH 4·5. Optimal growth occurs in the presence of 2–3 % (w/v) NaCl; growth does not occur in the absence of NaCl. Flexirubin-type pigments are absent. Tyrosine is hydrolysed. Aesculin, hypoxanthine, Tweens 20, 40 and 60, xanthine and urea are not hydrolysed. H_2S is not produced. Growth under anaerobic conditions does not occur on MA. Growth under anaerobic conditions on MA supplemented with nitrate is variable (negative for type strain). Growth occurs on peptone and tryptone as the sole carbon and nitrogen sources, but does not occur on D-glucose. No acid is produced from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, melibiose, D-melezitose, D-raffinose, L-rhamnose, D-ribose, sucrose, D-trehalose, D-xylose, adonitol, D-sorbitol, *myo*-inositol or D-mannitol. Predominant menaquinone is MK-6. Major fatty acids are $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, iso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH, $C_{15:0}$ and iso-$C_{17:0}$ 3-OH. DNA G+C content is 32·3–32·8 mol%. Other phenotypic properties are given in Table 1.

The type strain, TF-26^T (=KCTC 12302^T=DSM 16505^T), was isolated from a tidal flat on Daepo Beach in the Yellow Sea, Korea. Reference strains are TF-28, TF-42 and TF-53.

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