Tenacibaculum soleae sp. nov., isolated from diseased sole (*Solea senegalensis* Kaup)

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A novel Gram-negative, rod-shaped, gliding bacterial strain designated LL04 12.1.7^T was isolated from diseased sole (*Solea senegalensis* Kaup) in Galicia, Spain. Colonies were yellow-pigmented with uneven edges and did not adhere to the agar. The DNA G + C content of the strain was 29.8 mol%. 16S rRNA gene sequence similarity analysis indicated that strain LL04 12.1.7^T is a member of the genus *Tenacibaculum* in the family *Flavobacteriaceae*. Sequence similarities between the isolate and the type strains of other members of the genus were 96.7–94.8 %. The major fatty acids (>10 % of total fatty acids) were iso-C_{15:0} (23.1 %), iso-C_{15:0} 3-OH (10.6 %), C_{15:1}ω6c (12.2 %) and summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 11.0 %). Genotypic and phenotypic data distinguished strain LL04 12.1.7^T from the 11 recognized *Tenacibaculum* species, indicating that it represents a novel species, for which the name *Tenacibaculum soleae* sp. nov. is proposed. The type strain is strain LL04 12.1.7^T (=CECT 7292^T =NCIMB 14368^T).

The genus *Tenacibaculum* (Suzuki *et al.*, 2001) in the family *Flavobacteriaceae* (Reichenbach, 1992a, b; Bernardet *et al.*, 1996, 2002) currently comprises 11 species derived from different marine ecosystems and intensive aquaculture systems. *Tenacibaculum maritimum* and *Tenacibaculum discolor* were isolated from diseased fish (Wakabayashi *et al.*, 1986; Piñeiro-Vidal *et al.*, 2008); *Tenacibaculum ovolyticum* was isolated from fish eggs (Hansen *et al.*, 1992); *Tenacibaculum litopenaei* (Sheu *et al.*, 2007) and *Tenacibaculum gallaicum* (Piñeiro-Vidal *et al.*, 2008) were from seawater of shrimp- and turbot-holding tanks, respectively; *Tenacibaculum amyolyticum* and *Tenacibaculum mesophilum* were from marine macroalgae and sponge, respectively (Suzuki *et al.*, 2001); *Tenacibaculum skagerrakense* was from seawater (Frette *et al.*, 2004); and *Tenacibaculum lutimaris* (Yoon *et al.*, 2005), *Tenacibaculum litoreum* (Choi *et al.*, 2006) and *Tenacibaculum aestuarii* (Jung *et al.*, 2006) were from tidal flat sediment.

During the characterization of bacteria isolated from a diseased cultured sole (*Solea senegalensis* Kaup), strain LL04 12.1.7^T was recovered on plates of *Flexibacter maritimus* medium (FMM) (Pazos *et al.*, 1996). Subcultivation was done on FMM or marine agar 2216 (MA; Difco) at 25 °C for 48 h. Strains were preserved at

–80 °C in both marine broth 2216 (MB; Difco) supplemented with 15 % (v/v) glycerol and Microbank tubes (Prolab Diagnostics). Experimental infection assays have demonstrated that strain LL04 12.1.7^T is virulent for fingerlings of sole and turbot, but not for mice (data not shown).

Morphological, physiological and biochemical tests were performed as described by Bernardet *et al.* (2002). The Gram reaction was tested by using the bioMérieux Gram stain kit according to the manufacturer's instructions and the non-staining KOH method (Buck, 1982). Gliding motility was determined by phase-contrast microscopic examination of a fresh MB culture by the hanging drop technique as recommended by Bernardet *et al.* (2002). The presence of flexirubin-type pigments was determined by using the KOH test as described by Reichenbach (1989). Catalase and oxidase activities were determined as described by Cowan & Steel (1965). The capacity of the strain to grow under anaerobic conditions was tested on MA using the GasPak anaerobic system (BBL). The optimal pH and the pH range for growth were determined in FMM broth adjusted to pH 4–10 according to Suzuki *et al.* (2001). Growth at various temperatures (8, 15, 18, 22, 25, 30, 37 and 44 °C) was determined on FMM agar plates. Tolerance to salinity was tested in FMM broth containing 10, 20, 30, 50, 70 or 100 % seawater or 0.8, 1, 3, 5, 7 or 10 % (w/v) NaCl. Indole and H₂S production were tested on FMM broth supplemented with 1 % (w/v) tryptone or 5 %

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LL04 12.1.7^T is AM746476.

(w/v) peptone, respectively. The Voges–Proskauer reaction was evaluated in seawater with 0.7% (w/v) peptone and 0.5% (w/v) (+)-D-glucose. The capacity of the strain to degrade casein (1%), gelatin (1%), starch (1%) and Tween 80 (1%) was evaluated in FMM medium (Suzuki *et al.*, 2001). Utilization of carbon sources was tested on basal agar medium (0.2 g NaNO₃, 0.2 g NH₄Cl, 0.05 g yeast extract and 15 g agar in 1 l artificial seawater) containing 0.4% carbon source [sucrose, (–)-D-ribose, (+)-D-galactose, (+)-D-glucose, L-proline, L-glutamate or L-tyrosine] as described by Suzuki *et al.* (2001). The absence of growth after 1 month of incubation was scored as a negative result. Other enzyme activities were evaluated in the API ZYM system (bioMérieux) following the manufacturer's instructions, except that sterile seawater was used as the suspension medium.

For analysis of fatty acid methyl esters, strain LL04 12.1.7^T was grown on MA plates for 48 h at 25 °C. Cell harvesting, saponification of lipids, methylation of fatty acids, extraction of fatty acid methyl esters, washing of extracts and GC analysis were performed according the standardized procedures of the Microbial Identification system (MIDI; Microbial ID) (Sasser, 1990).

Determination of the DNA G + C content and sequencing of the 16S rRNA gene of the isolate were carried out by the identification service of the DSMZ, Braunschweig, Germany. The 1506 bp sequence of strain LL04 12.1.7^T was automatically aligned using CLUSTAL W (Thompson *et al.*, 1994) with those of the type strains of the 11 *Tenacibaculum* species and of other representative members of the family *Flavobacteriaceae* obtained from GenBank/EMBL. Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods (Fig. 1). The evolutionary distance matrix for the neighbour-joining method was generated according to Kimura's two-parameter model (Kimura, 1980). To evaluate phylogenetic trees, a bootstrap analysis with 1000 sample replications was performed with the SEQBOOT and CONSENSE programs

in the PHYLIP 3.67 package (Felsenstein, 2007). The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Results of morphological, physiological and biochemical tests are given in Table 1 and in the species description.

Fatty acid composition data for strain LL04 12.1.7^T and type strains of other *Tenacibaculum* species are detailed in Table 2. The main difference between the novel isolate and the other *Tenacibaculum* type strains was the high content of unsaturated fatty acids (13.9%) in strain LL04 12.1.7^T. The cellular fatty acid profile of strain LL04 12.1.7^T was dominated by iso-C_{15:0} (23.1%), iso-C_{15:0} 3-OH (10.6%), iso-C_{16:0} 3-OH (8.4%), C_{15:1}ω6c (12.2%) and summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 11.0%) (Table 2).

The DNA G + C content of strain LL04 12.1.7^T was 29.8 mol%, the lowest reported value within the genus *Tenacibaculum*. Comparison of the 16S rRNA gene sequence of strain LL04 12.1.7^T with those of the type strains of the 11 *Tenacibaculum* species and other members of the family *Flavobacteriaceae* demonstrated that strain LL04 12.1.7^T formed a robust cluster with the *Tenacibaculum* species. The phylogenetic tree based on 16S rRNA gene sequences is shown in Fig. 1. The closest relatives of strain LL04 12.1.7^T were the type strains of *T. ovolyticum* and *T. aestuarii* (96.7% sequence similarity), *T. lutimaris* and *T. mesophilum* (96.4%), *T. gallaicum* (96.2%), *T. amylolyticum* (96.0%), *T. litoreum* (95.9%), *T. discolor* (95.8%), *T. skagerrakense* (95.7%), *T. litopenaei* (95.0%) and *T. maritimum* (94.8%). All these values are lower than the theoretical threshold (97%) for the delineation of bacterial species based on 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006). According to the phenotypic and genetic data obtained in this study, it is concluded that strain LL04 12.1.7^T represents a novel species within the genus *Tenacibaculum*, for which the name *Tenacibaculum soleae* sp. nov. is proposed.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain LL04 12.1.7^T, other members of the genus *Tenacibaculum* and related genera of the family *Flavobacteriaceae*. Bootstrap values >50% (based on 1000 replications) are shown at branching points. Solid circles indicate that the corresponding nodes are also recovered in the maximum-parsimony tree. Bar, 0.01 substitutions per site.

Table 1. Differential phenotypic characteristics of strain LL04 12.1.7^T and the type strains of other *Tenacibaculum* species

Strains: 1, *T. soleae* sp. nov. LL04 12.1.7^T; 2, *T. gallaicum* DSM 18841^T; 3, *T. discolor* DSM 18842^T; 4, *T. mesophilum* MBIC1140^T; 5, *T. lutimaris* KCTC 12302^T; 6, *T. skagerrakense* ATCC BAA-458^T; 7, *T. amyolyticum* MBIC4355^T; 8, *T. ovolyticum* IAM 14318^T; 9, *T. maritimum* NCIMB 2154^T; 10, *T. litoreum* JCM 13039^T; 11, *T. aestuarii* KCTC 12569^T; 12, *T. litopenaei* BCRC 17590^T. Data from Wakabayashi *et al.* (1986), Hansen *et al.* (1992), Suzuki *et al.* (2001), Bernardet *et al.* (2002), Frette *et al.* (2004), Yoon *et al.* (2005), Choi *et al.* (2006), Jung *et al.* (2006), Sheu *et al.* (2007), Piñeiro-Vidal *et al.* (2008) and this study. +, Positive; −, negative; w, weakly positive; ND, no data available; NG, no growth.

Character	1	2	3	4	5	6	7	8	9	10	11	12
Colour	Yellow	Bright yellow	Bright yellow	Yellow	Pale yellow	Bright yellow	Yellow	Pale yellow	Pale yellow	Pale yellow	Pale yellow	Yellow
Cell size (μm)	2–25 × 0.5	2–30 × 0.5	2–30 × 0.5	1.5–10 × 0.5	2–10 × 0.5	2–15 × 0.5	2–5 × 0.4	1.5–10 × 0.5	2–30 × 0.5	2–35 × 0.3–0.5	2–3.5 × 0.3	2–10 × 0.3–0.5
Spherical cells in stationary phase	Frequent	Rare	Rare	Very rare	Very rare	Frequent	Very rare	ND	Frequent	Very rare	Very rare	Very rare
Gliding motility	+	+	+	+	+	−	+	+	+	+	+	+
Growth with:												
NaCl (%)	NG	NG	NG	1–7	<8 (2–3)	NG	3	NG	NG	3–5	2–3	2–10
Seawater (%)	55–100	30–100	30–100	10–100	25–175	25–150	50–100	70–100	30–100	25–250	ND	ND
Temperature range (°C)	14–30	14–38	14–38	15–40	10–39	10–40	20–35	4–25	15–34	5–40	9–41	ND
Optimal growth temperature (°C)	22–25	25–30	25–30	28–35	30–37	25–37	27–30	ND	25–30	35–40	30–37	28–37
pH range	6.0–8.0	6.0–8.0	6.0–8.0	5.3–9	7–8	6–9	5.3–8.3	5.9–8.6	5.9–8.6	6.0–10.0	5.5–8.5	7.0–8.0
Degradation of:												
Gelatin	+	+	+	−	+	+	−	−	+	−	+	+
Starch	−	−	−	+	−	+	+	+	+	+	−	+
Tween 80	−	−	−	−	−	−	+	+	+	+	+	−
Carbon source utilization												
(+)-D-Galactose	−	−	−	ND	ND	−	ND	ND	−	−	ND	ND
(+)-D-Glucose	−	−	−	ND	−	+	ND	ND	−	−	−	+
L-Proline	−	+	+	+	−	+	+	−	−	+	−	+
L-Glutamate	−	+	+	+	−	+	+	−	w	−	−	+
L-Tyrosine	−	−	−	ND	ND	ND	ND	ND	−	ND	ND	ND
API ZYM results												
Esterase lipase (C8)	+	+	+	ND	ND	ND	ND	ND	+	−	+	+
Trypsin	−	+	+	ND	ND	ND	ND	ND	+	+	−	−
α-Chymotrypsin	−	+	+	ND	ND	ND	ND	ND	+	+	+	−

Table 2. Cellular fatty acid compositions (%) of strain LL04 12.1.7^T and the type strains of other *Tenacibaculum* species

Strains: 1, *T. soleae* sp. nov. LL04 12.1.7^T; 2, *T. mesophilum* MBIC1140^T; 3, *T. lutimaris* KCTC 12302^T; 4, *T. skagerrakense* ATCC BAA-458^T; 5, *T. maritimum* NCIMB 2154^T; 6, *T. litoreum* JCM 13039^T; 7, *T. aestuarii* KCTC 12569^T; 8, *T. litopenaei* BCRC 17590^T. Data from Yoon *et al.* (2005), Jung *et al.* (2006), Choi *et al.* (2006), Sheu *et al.* (2007) and this study. —, Not detected; tr, trace (<1%); ECL, equivalent chain-length. No data were available for *T. ovolyticum*, *T. gallaicum* or *T. discolor*. Fatty acids amounting to less than 1% of the total fatty acids in all strains tested are not listed.

Fatty acid	1	2	3	4	5	6	7	8
Straight chain								
C _{15:0}	4.8	3.6	8.9	4.9	2.9	2.7	6.1	—
C _{16:0}	tr	tr	tr	tr	tr	tr	tr	1.8
Branched chain								
iso-C _{13:0}	tr	tr	tr	tr	1.8	1.4	1.3	tr
iso-C _{14:0}	2.4	tr	1.7	tr	tr	2.2	2.2	tr
iso-C _{15:0}	23.1	13.2	17.2	9.5	16.8	18.8	18.9	22.0
anteiso-C _{15:0}	tr	1.1	tr	—	tr	1.8	2.0	tr
iso-C _{15:1}	5.7	7.1	5.3	8.2	7.6	8.2	8.7	8.7
iso-C _{16:0}	1.7	1.7	3.8	1.3	tr	2.3	2.3	1.8
iso-C _{16:1}	2.4	tr	1.7	1.7	—	1.3	tr	1.6
iso-C _{17:1ω9c}	—	tr	tr	tr	—	1.6	1.3	1.6
Unsaturated								
C _{15:1ω6c}	12.2	1.6	4.2	—	2.2	1.7	3.0	1.6
C _{17:1ω6c}	1.7	tr	1.5	1.2	tr	tr	1.6	1.9
C _{18:3ω6c}	—	—	—	—	—	1.5	—	—
Hydroxylated								
iso-C _{15:0} 3-OH	10.6	8.0	4.6	7.8	19.8	6.6	6.1	4.6
C _{15:0} 2-OH	tr	1.1	1.2	2.5	1.1	tr	tr	tr
C _{15:0} 3-OH	3.2	2.9	3.4	8.6	3.8	—	4.2	2.7
iso-C _{16:0} 3-OH	8.4	9.0	12.8	12.2	5.0	6.8	12.3	3.4
C _{16:0} 3-OH	2.2	3.2	1.3	2.1	1.5	1.6	1.0	5.4
iso-C _{17:0} 3-OH	2.9	14.9	8.4	11.7	13.7	13.6	9.6	12.7
C _{17:0} 3-OH	—	tr	tr	2.5	—	tr	tr	1.0
Unknown fatty acids								
ECL 13.565	tr	tr	—	—	—	—	—	1.9
ECL 16.582	tr	1.0	tr	tr	1.0	1.0	1.0	tr
Summed features*								
Summed feature 3	11.0	24.4	18.1	22.5	17.9	19.6	11.9	21.3
Summed feature 4	—	—	—	—	—	1.3	—	—

*Summed feature are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprises C_{16:1ω7c} and/or iso-C_{15:0} 2-OH. Summed feature 4 comprises iso-C_{17:1} I and/or anteiso-C_{17:1} H.

Description of *Tenacibaculum soleae* sp. nov.

Tenacibaculum soleae [so.le'ae. L. gen. n. *soleae* of a sole, in reference to the source of the isolate, a cultured sole (*Solea senegalensis* Kaup)].

Cells are Gram-negative rods, 0.5 µm in diameter and 2–25 µm in length, motile by gliding. Spherical cells are

observed in ageing cultures. Colonies on FMM agar and MA 2216 (Difco) are flat and yellow with uneven edges and do not adhere to the agar. The yellow pigment does not belong to the flexirubin type. Strictly aerobic. Growth occurs in media containing 50–100% seawater, but not in media supplemented with NaCl only. Growth occurs at 14–30 °C (optimum 22–25 °C) and pH 6.0–8.0. Catalase and cytochrome oxidase activities are present. Gelatin and casein are hydrolysed, but Tween 80 and starch are not. The Voges–Proskauer test is negative. No acid is produced from carbohydrates. H₂S and indole are not produced. L-Proline, L-glutamate, sucrose, (–)-D-ribose, (+)-D-galactose, (+)-D-glucose and L-tyrosine are not utilized. In the API ZYM system, alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase and cystine arylamidase activities are present, but trypsin, α-chymotrypsin and all enzymes related to the metabolism of carbohydrates are absent.

The type strain is LL04 12.1.7^T (=CECT 7292^T =NCIMB 14368^T), isolated from a diseased sole (*Solea senegalensis*) reared in Galicia (north-western Spain).

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