Tenacibaculum discolor sp. nov. and *Tenacibaculum gallaicum* sp. nov., isolated from sole (*Solea senegalensis*) and turbot (*Psetta maxima*) culture systems

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Two Gram-negative, rod-shaped, gliding bacterial strains, designated strain LL04 11.1.1^T and strain A37.1^T, were isolated from a diseased sole (*Solea senegalensis*) and from seawater from a holding tank for turbot (*Psetta maxima*), respectively. The strains grew on solid media as bright yellow colonies with uneven edges; the colonies did not adhere to the agar. The bacteria were able to grow at temperatures in the range 14 to 38 °C and from pH 6.0 to pH 8.0. The DNA G+C contents of strains LL04 11.1.1^T and A37.1^T were 32.1 and 32.7 mol%, respectively. Analysis of the 16S rRNA gene sequences indicated that strains LL04 11.1.1^T and A37.1^T were members of the genus *Tenacibaculum* in the family *Flavobacteriaceae*. The sequence similarities of the two isolates with respect to the type strains of recognized members of the genus ranged from 94.2 to 99.4 %. DNA–DNA hybridization studies revealed that the strains studied represent two distinct novel species of the genus *Tenacibaculum*, for which the names *Tenacibaculum discolor* sp. nov. [type strain LL04 11.1.1^T (=NCIMB 14278^T=DSM 18842^T)] and *Tenacibaculum gallaicum* sp. nov. [type strain A37.1^T (=NCIMB 14147^T=DSM 18841^T)] are proposed.

The genus *Tenacibaculum*, proposed by Suzuki *et al.* (2001), belongs to the family *Flavobacteriaceae* (Reichenbach, 1992a, b; Bernardet *et al.*, 1996, 2002) and currently comprises nine species: *Tenacibaculum maritimum* from diseased fish (Wakabayashi *et al.*, 1986), *T. ovolyticum* from fish eggs (Hansen *et al.*, 1992), *T. amyolyticum* from the surfaces of marine macroalgae and *T. mesophilum* from sponges (Suzuki *et al.*, 2001), *T. skagerrakense* isolated from seawater (Frette *et al.*, 2004), *T. lutimaris*, *T. litoreum*, *T. aestuarii* from tidal flat sediment (Yoon *et al.*, 2005; Choi *et al.*, 2006; Jung *et al.*, 2006) and *T. litopenaei* isolated from a shrimp mariculture pond (Sheu *et al.*, 2007).

During the characterization of bacteria isolated from the kidney of a diseased sole (*Solea senegalensis*) and from seawater from holding tanks for turbot (*Psetta maxima*), strains LL04 11.1.1^T and A37.1^T were recovered on plates of *Flexibacter maritimus* medium (FMM; Pazos *et al.*, 1996) at 25 °C (*T. maritimum* was previously misclassified within the genus *Flexibacter*). The diseased fish showed the typical

signs observed in fish affected by marine flexibacteriosis caused by *T. maritimum* (i.e. eroded mouth, rotten fins, shallow skin lesions and paleness of internal organs). Pathogenicity assays demonstrated that the isolated bacteria were virulent for turbot and sole (Piñeiro-Vidal *et al.*, 2007). The isolated bacteria were subcultured on FMM agar at 25 °C for 48 h and maintained at –80 °C both in FMM broth supplemented with 15 % (v/v) glycerol and in Microbank tubes (Prolab Diagnostics).

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 FMM broth culture and 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 ability of the strains to grow under anaerobic conditions was tested in FMM agar by using the GasPak anaerobic system (BBL). To assess their growth at different pH values,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LL04 11.1.1^T and A37.1^T are AM411030 and AM746477, respectively.

the strains were cultured in FMM broth adjusted to pH values ranging from 4 to 10. The temperature range for growth was determined on FMM agar plates incubated at 8, 15, 18, 22, 25, 30, 37 and 44 °C. Tolerance of salinity was tested in FMM broth containing seawater strengths of 10, 20, 30, 50, 70 and 100 % or at NaCl concentrations of 0.8, 1, 3, 5, 7 and 10 % (w/v). Indole production was tested in FMM broth supplemented with 1 % (w/v) tryptone, H₂S production was determined in FMM broth supplemented with 5 % (w/v) peptone and the Voges–Proskauer reaction was determined in seawater supplemented with 0.7 % (w/v) peptone and 0.5 % (w/v) D-glucose. The ability of the novel strains to degrade casein (1%), gelatin (1%), starch (0.4%) and Tween 80 (1%) and to produce nitrate reductase were evaluated in FMM medium supplemented with substrate concentrations as reported previously (Suzuki *et al.*, 2001). The utilization of carbon sources was tested on basal agar medium [containing, 1⁻¹ artificial seawater (Sigma), 0.2 g NaNO₃, 0.2 g NH₄Cl, 0.05 g yeast extract, 15 g agar] supplemented with 0.4 % carbon source [(+)-D-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 incubation in the media for 1 month was scored as a negative result. Other enzymic activities were evaluated using the API ZYM system (bioMérieux) according to the manufacturer's instructions, except that sterile seawater was used as the suspension medium.

The results of the morphological, physiological and biochemical tests are given in the species descriptions and in Table 1.

Sequencing of the 16S rRNA gene of the isolates was carried out by the Identification Service of the Colección Española de Cultivos Tipo (Universidad de Valencia, Valencia, Spain). The resulting sequences of strains LL04 11.1.1^T (1512 bp) and A37.1^T (1518 bp) were aligned automatically (using CLUSTAL W; Thompson *et al.*, 1994) with those of the type strains of the nine species of the genus *Tenacibaculum* and other representative members of the family *Flavobacteriaceae* obtained from GenBank. The 16S rRNA gene sequence similarities were determined using MEGA, version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were constructed by using 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 the Kimura two-parameter model (Kimura, 1980). The robustness of the phylogenetic trees was determined by means of bootstrap analyses based on 1000 replications. In both trees, strains LL04 11.1.1^T and A37.1^T formed a robust cluster with the species of the genus *Tenacibaculum*. Data from the sequence similarity analysis indicated that the closest relatives of strains LL04 11.1.1^T and A37.1^T were *T. litoreum* (99.4 and 98.4 %, respectively), *T. mesophilum* (97.4 and 96.7 %, respectively), *T. aestuarii* (97.3 % for both isolates), *T. lutimaris* (97.3 and 96.8 %, respectively), *T. skagerrakense* (96.4 and 95.9 %, respectively), and *T.*

amylolyticum and (96.0 and 96.3 %, respectively). The 16S rRNA gene sequence similarity between strains LL04 11.1.1^T and A37.1^T was 98.4 %.

The DNA G + C content was determined using the method described by Cashion *et al.* (1977). As the 16S rRNA gene sequence similarities between strains LL04 11.1.1^T and A37.1^T and between them and the type strain of *T. litoreum* were close to 99 %, DNA–DNA hybridization experiments were performed using the method described by De Ley *et al.* (1970), as modified by Huß *et al.* (1983). The experiment was performed at 45 °C in 2 × SSC (1 × SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate at pH 7.0) using a Cary 100 Bio UV/visible spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian). The DNA G + C content determinations and the DNA–DNA hybridization experiments were performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The DNA G + C contents of strains LL04 11.1.1^T and A37.1^T were 32.1 and 32.7 mol%, respectively (Table 1). The DNA–DNA relatedness between strains LL04 11.1.1^T and A37.1^T was 42.8 %, and the strains shared 39.6 and 40.2 % relatedness, respectively, with their closest relative, *T. litoreum*. Therefore, we conclude that strains LL04 11.1.1^T and A37.1^T represent two novel bacterial species within the genus *Tenacibaculum*, for which we propose the names *Tenacibaculum discolor* sp. nov. and *Tenacibaculum gallaicum* sp. nov., respectively.

Description of *Tenacibaculum discolor* sp. nov.

Tenacibaculum discolor (dis.co'lor. L. neut. adj. *discolor* of different colours, referring to the colours of the colonies).

Cells are Gram-negative rods that are 0.5 µm in diameter, 2–30 µm in length and motile by gliding. Spherical cells are rarely observed in ageing cultures. Colonies on FMM agar medium are flat, bright yellow, have uneven edges and do not adhere to the agar. On marine agar 2216 (Difco), colonies also appear bright yellow from the front, but appear bright green when viewed at an angle of 30–45 °C. The yellow pigment is not of the flexirubin type. Strictly aerobic. Growth occurs in media containing seawater at strengths in the range 30 to 100 % but not in media supplemented with NaCl alone. Growth occurs at 14–38 °C (optimum, 25–30 °C) and at pH 6.0–8.0. Catalase and cytochrome oxidase activities are present. Nitrate is reduced. Gelatin and casein are hydrolysed, but Tween 80 and starch are not. The Voges–Proskauer test gives a negative result. No acid is produced from carbohydrates. H₂S and indole are not produced. L-Proline and L-glutamate are utilized, but (+)-D-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, cystine arylamidase, trypsin, α-chymotrypsin,

Table 1. Differential phenotypic characteristics of strains LL04 11.1.1^T and A37.1^T and the type strains of species of the genus *Tenacibaculum*

Taxa: 1, strain LL04 11.1.1^T; 2, strain A37.1^T; 3, *T. mesophilum* MBIC1140^T; 4, *T. lutimaris* KCTC 12302^T; 5, *T. skagerrakense* ATCC BAA-458^T; 6, *T. amylolyticum* MBIC4355^T; 7, *T. ovolyticum* IAM 14318^T; 8, *T. maritimum* NCIMB 2154^T; 9, *T. litoreum* JCM 13039^T; 10, *T. aestuarii* KCTC 12569^T; 11, *T. litopenaei* BCRC 17590^T. Data are 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) and this study. +, Positive; –, negative; w, weakly positive; NT, not tested; NG, no growth.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Origin	Diseased sole	Seawater from holding tanks for turbot	Sponges, macroalgae	Tidal flat sediment	Seawater	Macroalgae	Halibut eggs	Diseased marine fish	Tidal flat sediment	Tidal flat sediment	Shrimp mariculture pond
Cell size (μm)	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	2–20 × 0.5	2–30 × 0.5	2–35 × 0.3–0.5	2.0–3.5 × 0.3	2–10 × 0.3–0.5
Gliding motility	+	+	+	+	–	+	+	+	+	+	+
NaCl concentration (%) for growth	NG	NG	1–7	<8	NG	3	NG	NG	3–5	<7	2–10
Seawater strength (%) for growth	30–100	30–100	10–100	25–175	25–150	50–100	70–100	30–100	25–250	NT	NT
Temperature range (°C)	14–38	14–38	15–40	10–39	10–40	20–35	4–25	15–34	5–40	9–41	10–39
Optimal temperature (°C)	25–30	25–30	28–35	30–37	25–37	27–30	NT	25–30	35–40	30–37	28–37
pH range	6.0–8.0	6.0–8.0	5.3–9	7.0–8.0	6.0–9.0	5.3–8.3	5.9–8.6	5.9–8.6	6.0–10.0	5.5–8.5	5–10
Nitrate reduction	+	+	–	–	+	w	+	+	+	–	–
Degradation of:											
Starch	–	–	–	–	+	+	–	–	+	–	+
Gelatin	+	+	+	+	NT	+	+	+	+	+	+
Tween 80	–	–	+	–	–	+	+	+	+	+	–
Carbon utilization:											
(+)–D–Sucrose	–	–	–	–	+	–	–	–	–	–	–
(–)–D–Ribose	–	–	–	NT	NT	–	–	–	–	–	NT
(+)–D–Galactose	–	–	NT	NT	NT	NT	NT	–	–	–	–
(+)–D–Glucose	–	–	NT	–	+	NT	NT	–	–	–	+
L–Proline	+	+	+	–	+	+	–	–	+	–	+
L–Glutamate	+	+	+	–	+	+	–	w	–	–	+
L–Tyrosine	–	–	NT	NT	NT	NT	NT	–	NT	NT	NT
DNA G + C content (mol%)	32.1	32.7	31.6–32.0	32.3–32.8	35.2	31.6–32.0	30.3–32.0	31.3–32.5	30	33.6	35.2

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strains LL04 11.1.1^T and A37.1^T and members of the genus *Tenacibaculum* and other related genera belonging to the family *Flavobacteriaceae*. Bootstrap percentages (based on 1000 replications) are shown at the branching points. Filled circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. Bar, 0.01 nucleotide substitutions per site.

acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present, but activities for all of the enzymes relating to the metabolism of carbohydrates are absent.

The type strain, LL04 11.1.1^T (=NCIMB 14278^T=DSM 18842^T), was isolated from the kidney of a diseased sole (*Solea senegalensis*) in Galicia in north-west Spain.

Description of *Tenacibaculum gallaicum* sp. nov.

Tenacibaculum gallaicum (gal.lai'cum. L. neut. adj. *gallai-cum* of Galicia, a north-western province of Spain, referring to the place of isolation).

Cells are Gram-negative rods that are 0.5 µm in diameter, 2–30 µm in length and motile by gliding. Spherical cells are rarely observed in ageing cultures. Colonies on FMM agar and marine agar 2216 media are flat, bright yellow, have uneven edges and do not adhere to the agar. Strictly aerobic. Growth occurs in media containing seawater strengths of 30–100 %, but not in media supplemented with NaCl alone. Growth occurs at 14–38 °C (optimum, 25–30 °C) and at pH 6.0–8.0. Catalase and cytochrome oxidase activities are present. Nitrate is reduced. Gelatin and casein are hydrolysed, but Tween 80 and starch are not hydrolysed. The Voges–Proskauer test gives a negative result. No acid is produced from carbohydrates. H₂S and indole are not produced. L-Proline and L-glutamate are utilized but (+)-D-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, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but all of the enzymes relating to the metabolism of carbohydrates are absent.

The type strain, A37.1^T (=NCIMB 14147^T=DSM 18841^T), was isolated from seawater from a holding tank for turbot (*Psetta maxima*), in Galicia in north-west Spain.

Acknowledgements

This investigation was supported by grant PGIDIT 04 RMA 003E from the Xunta de Galicia, Spain. The authors acknowledge Stolt Sea Farm for collaboration during this study, Dr J. P. Euzéby (Laboratoire de Bactériologie, École Nationale Vétérinaire, Toulouse, France) for his help with species names, and the referees for constructive suggestions.

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