Lutibacter litoralis gen. nov., sp. nov., a marine bacterium of the family *Flavobacteriaceae* isolated from tidal flat sediment

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A rod-shaped marine bacterium, designated strain CL-TF09^T, isolated from a tidal flat in Ganghwa, Korea, was characterized based on its physiological and biochemical features, fatty acid profile and phylogenetic position. 16S rRNA gene sequence analysis revealed a clear affiliation with the family *Flavobacteriaceae*. Strain CL-TF09^T showed the closest phylogenetic relationship with the genera *Tenacibaculum* and *Polaribacter*; sequence similarities between CL-TF09^T and the type strains of *Tenacibaculum* and *Polaribacter* species ranged from 90.7 to 91.8%. Cells of strain CL-TF09^T were non-motile and grew on solid media as yellow colonies. The strain grew in the presence of 1–5% sea salts, within a temperature range of 5–30 °C and at pH 7–8. The strain had iso-C_{15:0} 3-OH (17.4%), iso-C_{15:0} (16.7%), anteiso-C_{15:0} (15.1%) and iso-C_{16:0} 3-OH (13.4%) as predominant fatty acids. The DNA G + C content was 33.9 mol%. Based on the physiological, fatty acid composition and phylogenetic data presented, strain CL-TF09^T is considered to represent a novel genus and species of the family *Flavobacteriaceae*, for which the name *Lutibacter litoralis* gen. nov., sp. nov. is proposed. The type strain is CL-TF09^T (=KCCM 42118^T = JCM 13034^T).

The family *Flavobacteriaceae* is one of the major branches of the phenotypically diverse phylum *Bacteroidetes* (Garrity & Holt, 2001). Since the proposal of the family *Flavobacteriaceae* by Jooste (1985) and subsequent validation and emended description (Reichenbach, 1992; Bernardet *et al.*, 1996, 2002), more than 40 genera have been assigned to the family *Flavobacteriaceae*, about half of which have been described since 2004. The family *Flavobacteriaceae* includes species from various habitats, including freshwater, brackish and marine waters, soil and epibenthic fauna, and members of the family are known to be proficient in degrading various biopolymers such as cellulose, chitin and pectin (Kirchman, 2002). Recently, phylogenetic analyses have revealed that many marine species in the family clustered together into a well-defined ‘marine clade’ (Bowman & Nichols, 2005). These marine flavobacteria are known to be important members of the bacterial community in many aquatic environments and have a specialist role in using high-molecular-mass dissolved organic matter (Kirchman, 2002).

In this study, a bacterium, designated strain CL-TF09^T, affiliated with the ‘marine clade’ of the family *Flavobacteriaceae*, was isolated from tidal flat sediment in Ganghwa, Korea. Sediment slurry was spread on a marine agar 2216 (MA; Difco) plate, which was then incubated at 30 °C for 1 week. Strain CL-TF09^T was isolated and subsequently purified on MA at 30 °C four times. 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 (ABI3730XL) at Macrogen, Seoul, Korea. The almost complete 16S rRNA gene sequence of strain CL-TF09^T (1444 bp) was obtained. The sequence was compared with those available in the GenBank using BLAST-N searches (Altschul *et al.*, 1990). The 16S rRNA gene sequence of strain CL-TF09^T was manually aligned with those of the type strains of species belonging to genera phylogenetically related to CL-TF09^T and of the type species of other genera in the family *Flavobacteriaceae* obtained from GenBank and the Ribosomal Database Project (Cole *et al.*, 2003) databases using known 16S rRNA gene secondary structure information. Phylogenetic

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CL-TF09^T is AY962293.

The complete fatty acid profile of strain CL-TF09^T is given in Supplementary Table S1 available in IJSEM Online.

trees were obtained by use of the 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 replications for the 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, available at <http://chunlab.snu.ac.kr/jphydit/>), and phylogenetic analyses were carried out using MEGA 3 (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-TF09^T belonged to the genera *Tenacibaculum* (90.6–91.8%) and *Polaribacter* (91.0–91.5%). Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain CL-TF09^T formed a very robust clade with *Tenacibaculum* and *Polaribacter* species, but could not be linked to any of the known genera in the family *Flavobacteriaceae* (Fig. 1). Thus, strain CL-TF09^T was recognized as representing a new genus. The DNA G + C content was determined by HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989) after DNA purification following the method of Marmur (1961). The DNA G + C content was 33.9 mol%.

Morphological and physiological analyses were also 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, nitrate reductase activities and degradation of Tween 80 were examined as described by Hansen & Sørheim (1991). Cells of strain CL-TF09^T were rods 0.3–0.8 µm in width and 1.0–5.7 µm in length when in the exponential growth phase, but irregular rods or spherical cells were observed in older cultures (Fig. 2). Cells were non-motile (Table 1). Colonies on MA were circular, smooth, entire, convex, shining and yellow. After 1 week incubation, colonies were about 2 mm in diameter.

The temperature range for growth was determined on the basis of colony formation on MA plates 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-TF09^T to sea salts was determined using synthetic ZoBell broth (5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate, per litre distilled water) with various concentrations (0, 1, 3, 5, 7, 10, 15, 20, 25 %, w/v) of sea salts (Sigma). Growth in a medium with NaCl as the sole salt was tested in synthetic ZoBell agar medium supplemented with 3 % NaCl. Nitrate reduction, production of indole, arginine dihydrolase, urease, gelatinase and β-galactosidase, acid production from glucose, and hydrolysis of aesculin were tested using the API 20NE kit (bioMérieux) according to the

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain CL-TF09^T and other related species belonging to the 'marine clade' of the family *Flavobacteriaceae*. Only bootstrap values above 60% 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* (M62794) was used as an outgroup (not shown). Bar, 0.02 nucleotide substitutions per site.

Fig. 2. Transmission electron micrographs of negatively stained cells of strain CL-TF09^T. Cells were grown at 30 °C in marine broth for 1 day (a) and on marine agar for 4 days (b).

manufacturer's instructions, except that 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, per litre distilled water; Lyman & Fleming, 1940] as a suspension medium. Carbon utilization was tested on basal agar medium supplemented with yeast extract (23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O, 1.3 g CaCl₂·2H₂O, 0.2 g NaNO₃, 0.2 g NH₄Cl, 15 g Bacto agar, 0.05 g yeast extract, per litre distilled water; Bruns *et al.*, 2001) containing 0.2 % of the carbon source. Incubation was prolonged for 1 month and growth was scored as positive when visible colonies were observed. Growth of CL-TF09^T was observed at temperatures of 5–30 °C, with optimum growth at 25–30 °C. Growth occurred from pH 7 to 8. The strain could grow at sea salt concentrations from 1 to 5 % but could not grow on agar plates with 3 % NaCl as the sole salt. Strain CL-TF09^T could degrade starch, gelatin and aesculin (Table 1). However, it was not able to reduce

nitrate to nitrite. CL-TF09^T was negative for oxidase and weakly positive for β-galactosidase.

Isoprenoid quinones were isolated according to the method of Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). The major isoprenoid quinone in CL-TF09^T was menaquinone-6 (MK-6). Pigments were extracted using 90 % acetone from cells cultured in the dark, and then examined by spectroscopy. Flexirubin-type pigments were detected by using a colour shift test using a 20 % (w/v) KOH solution (Reichenbach, 1992). The spectrum of CL-TF09^T showed two peaks at 450 and 475 nm that are typical for carotenoids. The strain did not contain flexirubin-type pigments. Fatty acid methyl esters in whole cells were analysed by GC according to the instructions of the Microbial Identification System (MIDI) at the Korean Culture Center of Microorganisms in Korea. The fatty acid profile for strain CL-TF09^T was dominated by iso-C_{15:0} 3-OH (17.4 %), iso-C_{15:0} (16.7 %), anteiso-C_{15:0} (15.1 %) and iso-C_{16:0} 3-OH (13.4 %) (Table 1; the complete fatty acid profile of CL-TF09^T is given in Supplementary Table S1 available in IJSEM Online).

In terms of its phenotypic features, strain CL-TF09^T could be differentiated from members of the closely related genera *Tenacibaculum* and *Polaribacter* based on colony morphology, pH and temperature range for growth, and biochemical characteristics (Table 1). In particular, hydrolysis of aesculin, a negative response for oxidase, no growth at pH 6, and utilization of pyruvic acid and succinate distinguish strain CL-TF09^T from members of the genus *Tenacibaculum*; and growth at 25 °C and utilization of citrate, L-leucine, tartrate, pyruvic acid and succinate distinguish it from members of the genus *Polaribacter* (Table 1). In addition, the fatty acid profile of strain CL-TF09^T clearly distinguishes it from members of the genera *Tenacibaculum* and *Polaribacter* (Table 1). In this regard, the major distinctive difference is a large fraction of anteiso-C_{15:0} in strain CL-TF09^T. In addition, they were clearly differentiated by the proportions of several fatty acids, including iso-C_{16:0} 3-OH, iso-C_{15:1}-G and summed feature 3 (C_{16:1}ω7c/iso-C_{15:0} 2-OH).

In conclusion, phylogenetic analyses based on 16S rRNA gene sequences, fatty acid profiles and phenotypic features suggest that strain CL-TF09^T should be classified as the type strain of a novel genus and species, for which the name *Lutibacter litoralis* gen. nov., sp. nov. is proposed.

Description of *Lutibacter* gen. nov.

Lutibacter (Lu.ti.bac'ter. L. n. *lutum* mud; N.L. masc. n. *bacter* rod; N.L. masc. n. *Lutibacter* rod from mud).

Cells are Gram-negative and rod-shaped. Growth is heterotrophic and aerobic. Catalase-positive and oxidase-negative. The predominant menaquinone is MK-6. Dominant fatty acids are iso-C_{15:0} 3-OH, iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{16:0} 3-OH. Cells contain carotenoids but no flexirubin-type pigments. The genus is a member of the

Table 1. Selected differential characteristics of strain CL-TF09^T and other phylogenetically related genera

Taxa: 1, strain CL-TF09^T; 2, *Tenacibaculum*; 3, *Polaribacter*. Data from Gosink *et al.* (1998), Suzuki *et al.* (2001), Frette *et al.* (2004), Yoon *et al.* (2005), Choi *et al.* (2006) and this study. +, Positive; –, negative; V, variable between species; NA, not available.

	1	2	3
Habitat	Tidal flat	Sea water, tidal flat, saprophytic or parasitic	Sea water or saprophytic
Pigment production	+	V*	+
Gliding motility	–	V*	–
Colony:			
Colour	Yellow	Yellow, pale yellow	Orange, salmon pink
Shape	Entire	Uneven edge	Entire
Sea salt requirement	+	V	V
Growth at:			
pH 6	–	+	NA
25 °C	+	+	–
37 °C	–	V	–
Hydrolysis of:			
Starch	+	V	+
Gelatin	+	+†	V
Aesculin	+	–	V
Nitrate reductase activity	–	V	V‡
Oxidase activity	–	+	V
Major fatty acids (%):			
iso-C _{15:1} -G	4	ND	6–12
anteiso-C _{15:0}	15	1–2	4–6
iso-C _{16:0} 3-OH	13	5–13	ND¶
iso-C _{17:0} 3-OH	4	8–15	ND¶
Summed feature 3#	1	18–24	2–9
Carbon utilization:			
Citrate	+	V	–
Glycerol	–	–**	V
L-Leucine	+	V	–
Tartrate	+	NA	–
Pyruvic acid	+	–**	–
Succinate	+	–**	–
Sucrose	–	V	V
L-Proline	–	V	–
L-Glutamate	–	V	V

*All species except *Tenacibaculum skagerrakense* are positive for this characteristic.

†Data not available for *Tenacibaculum skagerrakense*.

‡All species except *Polaribacter glomeratus* are negative for this characteristic.

§Data for *Tenacibaculum* are from *Tenacibaculum lutimaris*, *Tenacibaculum skagerrakense*, *Tenacibaculum mesophilum* and *Tenacibaculum maritimum*; data for *Polaribacter* are from *Polaribacter filamentus*, *Polaribacter irgensii* and *Polaribacter franzmannii*. CL-TF09^T and *Tenacibaculum* species were grown on marine agar 2216 at 30 °C for 1–2 days, whereas *Polaribacter* species were grown on sea water–cytrophaga media at 5 °C for 3–4 weeks.

||Not detected or <1%.

¶Not detected or <5%.

#C_{16:1ω7cl}/iso-C_{15:0} 2-OH.

**Data not available for *T. lutimaris*.

family *Flavobacteriaceae*. The type species is *Lutibacter litoralis*.

Description of *Lutibacter litoralis* sp. nov.

Lutibacter litoralis (li.to.ra'lis. L. masc. adj. *litoralis* of the shore).

Exhibits the following properties in addition to those given in the genus description. Cells are approximately 0.3–0.8 µm wide and 1.0–5.7 µm long. Spherical cells appear in ageing culture. Cells are non-motile. On MA medium, colonies are circular, entire, convex, shining, opaque and yellow. Absorption spectral peaks of the pigments are observed at 450 and 475 nm. Growth occurs at 5–30 °C (optimum 25–30 °C) and at pH values of between 7 and 8. Growth occurs at sea salt concentrations of 1–5 % (w/v). Catalase, amylase, gelatinase and DNase activities are positive. Cytochrome oxidase, nitrate reductase and Tween 80 hydrolysis activities are negative. According to API 20NE tests, aesculin hydrolysis and gelatinase activities are positive, whereas nitrate reductase, indole production, acid production from glucose, arginine dihydrolase and urease are negative. Growth occurs on acetone, citrate, D-fructose, D-raffinose, D-salicin, D-sorbitol, glycine, glycogen, *myo*-inositol, L-arginine, L-lysine, L-ornithine, pyruvic acid, succinate, tartrate, urea, Casamino acids, L-leucine, peptone, tryptone and yeast extract. No growth occurs on acetate, acetamide, α -ketobutyric acid, benzoate, DL-cysteine, D-cellobiose, D-galactose, D-glucose, D-mannitol, D-mannose, D-ribose, D-trehalose, D-xylose, ethanol, formic acid, glycerol, inulin, 2-propanol, lactose, L-arabinose, L-ascorbate, L-asparagine, L-rhamnose, maleic acid, *N*-acetylglucosamine, polyethylene glycol, salicylate, sucrose, thiamine, DL-aspartate, L-proline or L-glutamate. The DNA G+C content is 33.9 mol%.

The type strain, CL-TF09^T (=KCCM 42118^T=JCM 13034^T), was isolated from a tidal flat sediment in Ganghwa, Korea.

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