Lutibacter maritimus sp. nov., isolated from a tidal flat sediment

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A Gram-staining-negative, aerobic, non-motile, non-gliding, yellow-pigmented and rod-shaped bacterial strain, designated S7-2^T, was isolated from a tidal flat sediment at Saemankum on the west coast of Korea and investigated using a polyphasic taxonomic approach. Strain S7-2^T grew optimally at pH 7.0–8.0, at 25–30 °C and in the presence of 2% (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain S7-2^T clustered with *Lutibacter littoralis* CF-TF09^T, a member of the family *Flavobacteriaceae*, with which it showed 95.8% 16S rRNA gene sequence similarity. It contained MK-6 as the predominant menaquinone and iso-C_{15:0} and C_{15:1ω6c} as the major fatty acids. The major polar lipids of strain S7-2^T and *L. littoralis* JCM 13034^T were phosphatidylethanolamine and three unidentified lipids. The DNA G + C content was 34.6 mol%. Differential phenotypic properties and phylogenetic distinctiveness suggested that strain S7-2^T represents a novel species of the genus *Lutibacter*, for which the name *Lutibacter maritimus* sp. nov. is proposed. The type strain is S7-2^T (=KCTC 22635^T=CCUG 57524^T).

The genus *Lutibacter*, a member of the family *Flavobacteriaceae* (Bernardet *et al.*, 1996, 2002; Bernardet & Nakagawa, 2006) of the phylum *Bacteroidetes*, comprises a single recognized species, *Lutibacter littoralis* (Choi & Cho, 2006). In this study, we describe a bacterial strain, designated S7-2^T, which was isolated from a tidal flat sediment on the west coast of Korea. Comparative 16S rRNA gene sequence analysis indicated that strain S7-2^T was phylogenetically most closely affiliated to the genus *Lutibacter*. The aim of the work was to determine the exact taxonomic position of strain S7-2^T by using a polyphasic taxonomic approach.

Strain S7-2^T was isolated by the dilution-plating technique on marine agar 2216 (MA; Difco) at 25 °C. *L. littoralis* JCM 13034^T was used as a reference strain for phenotypic characterization and polar lipid analysis. The morphological, physiological and biochemical characteristics of strain S7-2^T were investigated by using routine cultivation on MA at 30 °C. Growth at 4, 15, 20, 25, 30, 37, 40 and 45 °C was measured on MA. Growth with various NaCl concentrations (0.5%, w/v, and 1.0–12.0%, w/v, in increments of 1.0%) was measured in marine broth 2216 (MB; Difco) and trypticase soy broth (TSB; Difco). Growth in the absence of NaCl was investigated by using TSB prepared according to the formula of the Difco medium except that NaCl was excluded and supplemented with 0.45% (w/v) MgCl₂·6H₂O or 0.06% (w/v) KCl. The pH

range for growth was determined in MB adjusted to pH 4.5–9.5 (in increments of 0.5 pH unit) by the addition of HCl or Na₂CO₃. The pH values were verified after autoclaving. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and on MA supplemented with 0.1% (w/v) potassium nitrate, both of which had been prepared anaerobically under a nitrogen atmosphere. Cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips). Flagellation was determined by using transmission electron microscopy with cells from an exponentially growing culture. For this purpose, the cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being air-dried. Gliding motility was investigated as described by Bowman (2000).

The Gram reaction was determined by using the bioMérieux Gram-stain kit according to the manufacturer's instructions. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, starch, Tween 80 and tyrosine were performed on MA, using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate were investigated as described by Lányi (1987) with the modification that artificial seawater (containing per litre distilled water: 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O, 1.3 g CaCl₂·2H₂O; Bruns *et al.*, 2001) was used for the preparation of media. Cellulase and xylanase activities were investigated on MA containing

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0.5 % CM-cellulose (Sigma) and xylan (Sigma), respectively, and detected according to the method of Teather & Wood (1982). DNase activity was examined by using DNase test agar with methyl green (Difco) with the modification that artificial seawater was used for the preparation of the medium. Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of substrates as sole carbon and energy sources was tested as described by Baumann & Baumann (1981), using supplementation with 2 % (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and 1 % (v/v) vitamin solution (Staley, 1968). H₂S production was tested as described by Bruns *et al.* (2001).

Susceptibility to antibiotics was investigated on MA plates by using antibiotic discs containing the following (µg per disc unless otherwise stated): polymyxin B (100 U), streptomycin (50), penicillin G (20 U), chloramphenicol (100), ampicillin (10), cephalothin (30), gentamicin (30), novobiocin (5), tetracycline (30), kanamycin (30), lincomycin (15), oleandomycin (15), neomycin (30) and carbenicillin (100). Enzyme activities were determined by using the API ZYM system (bioMérieux) incubated for 4 h at 30 °C. Additional biochemical tests were performed using the API 20E system (bioMérieux); strips were inoculated with cells suspended in artificial seawater. The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). For pigment-absorption spectrum analysis, strain S7-2^T was cultivated aerobically in the dark at 30 °C in MB. The culture was centrifuged, washed twice using a MOPS buffer (0.01 M MOPS/NaOH, 0.1 M KCl, 0.001 M MgCl₂; pH 7.5) and disrupted by sonication with a Branson Sonifier 450. After removal of cell debris by centrifugation, the absorption spectrum of the supernatant was examined on a Beckman Coulter DU800 spectrophotometer.

Strain S7-2^T was cultivated for 3 days in MB at 30 °C to obtain the cell biomass required for DNA extraction and for the analysis of isoprenoid quinones and polar lipids. *L. litoralis* JCM 13034^T was cultivated under the same conditions for polar lipid analysis. Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). For cellular fatty acid analysis, cell mass of strain S7-2^T and *L. litoralis* JCM 13034^T was harvested from MA plates after cultivation for 2 days at 30 °C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined using the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by a reversed-phase HPLC

Table 1. Differential phenotypic characteristics of strain S7-2^T and *L. litoralis* JCM 13034^T

Data are taken from this study and Choi & Cho (2006) unless otherwise stated. Both strains are Gram-staining-negative, non-motile, non-gliding and rod-shaped. Both strains are positive for catalase, hydrolysis of aesculin, DNA and gelatin, for acid production from D-galactose, D-glucose, D-mannose, cellobiose and lactose, for activity of alkaline phosphatase, esterase lipase (C8) and leucine arylamidase and for susceptibility to carbenicillin, chloramphenicol, lincomycin and oleandomycin. Both strains are negative for anaerobic growth, for oxidase, for production of indole and H₂S (this study), for nitrate reduction, for hydrolysis of CM-cellulose (this study), tyrosine (this study), urea and xylan (this study), for acid production from myo-inositol, D-mannitol, D-sorbitol, L-arabinose, melezitose, melibiose, raffinose, L-rhamnose, trehalose and D-xylose, for activity of arginine dihydrolase, lipase (C14), trypsin, α-chymotrypsin, β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase and α-fucosidase and for susceptibility to ampicillin, novobiocin, neomycin, kanamycin, gentamicin and polymyxin B. +, Positive; w, weakly positive; –, negative.

Characteristic	S7-2 ^T	<i>L. litoralis</i> JCM 13034 ^T
Growth with 0.5 % (w/v) NaCl	+*	–
Hydrolysis of:		
Casein	+	–†
Starch	–	+
Tween 80	+	–
Utilization of:		
Cellobiose	+	–
Citrate	–	+
D-Fructose	–	+
D-Galactose	+	–
D-Glucose	+	–
Pyruvate	–	+
Succinate	–	+
Acid production from:		
D-Fructose	+	–†
Maltose	+	–†
D-Ribose	–	w†
Sucrose	+	–†
Enzyme activity (API ZYM)†		
N-Acetyl-β-glucosaminidase	–	+
α-Galactosidase	–	+
β-Glucosidase	+	–
Esterase (C4)	w	–
Cystine arylamidase	w	–
Valine arylamidase	w	–
Acid phosphatase	+	–
Naphthol-AS-BI-phosphohydrolase	+	–
Susceptibility to antibiotics		
Cephalothin	–	+†
Streptomycin	–	+†
Penicillin G	–	+†
Tetracycline	–	+†
DNA G+C content (mol%)	34.6	33.9

*Positive when Mg²⁺ ions are added.

†Data from this study.

equipped with a YMC ODS-A (250 × 4.6 mm) column. The nucleotides were eluted by a mixture of 0.55 M $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 4.0) and acetonitrile (40:1, v/v), using flow rate of 1 ml min^{-1} at room temperature and detected by UV absorbance at 270 nm.

Morphological, cultural, physiological and biochemical characteristics of strain S7-2^T are given in the species description and in Table 1. The sonicated cell extracts of strain S7-2^T showed absorption peak maxima at 450 and 475 nm, indicating the presence of carotenoids (Asker *et al.*, 2007). Flexirubin-type pigments were not produced, as shown by the negative KOH test. Chromosomal DNA was extracted and purified by the procedure described by Yoon *et al.* (1996), with the modification that RNase T1 was used in combination with RNase A to minimize contamination by RNA. Amplification of the 16S rRNA gene sequence was performed using two universal primers as described previously (Yoon *et al.*, 1998) and the PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Sequencing of the PCR products and phylogenetic analysis were performed as described by Yoon *et al.* (2003). The almost-complete 16S rRNA gene sequence of strain S7-2^T comprised 1436 bp, representing approximately 96% of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree constructed using the neighbour-joining algorithm, strain S7-2^T joined *L. littoralis* CF-TF09^T with a bootstrap resampling value of 100% (Fig. 1). The two strains exhibited 95.8% 16S rRNA gene sequence similarity. The relationship between strain S7-2^T and *L. littoralis* CF-TF09^T was also maintained in the trees constructed using the maximum-likelihood and max-

imum-parsimony algorithms (Fig. 1). Strain S7-2^T exhibited 16S rRNA gene sequence similarity values of less than 91.5% with the other strains used in the phylogenetic analysis.

The predominant isoprenoid quinone detected in strain S7-2^T was menaquinone-6 (MK-6) in line with *L. littoralis* (Choi & Cho, 2006) and all other members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006). The cellular fatty acid profile of strain S7-2^T is shown in Table 2, together with that of *L. littoralis* JCM 13034^T, which was also analysed in this study. The fatty acid profiles of the two strains were essentially similar, although there were differences in the proportions of some fatty acids. The major fatty acids (>10% of total fatty acids) of strain S7-2^T were iso-C_{15:0} (29.6%) and C_{15:1ω6c} (10.3%). The major polar lipids found in strain S7-2^T and *L. littoralis* JCM 13034^T were phosphatidylethanolamine and three unidentified lipids. Hence, the absence of distinct chemotaxonomic properties between strain S7-2^T and *L. littoralis* supports the classification of the novel strain into the genus *Lutibacter*. The DNA G+C content of strain S7-2^T was 34.6 mol%, a value similar to that reported for *L. littoralis* JCM 13034^T.

Strain S7-2^T was clearly distinguished from *L. littoralis* by differences in several phenotypic characteristics, including hydrolysis of, utilization of and acid production from several substrates, activity of some enzymes and susceptibility to antibiotics (Table 1). The phylogenetic distinctiveness and the differential phenotypic properties of strain S7-2^T were sufficient to demonstrate that this strain is separate from *L. littoralis* (Stackebrandt & Goebel, 1994).

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the positions of *Lutibacter maritimus* sp. nov. S7-2^T, *L. littoralis* CL-TF09^T and related members of the family *Flavobacteriaceae*. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Flexibacter flexilis* ATCC 23079^T (GenBank accession no. M62794) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

Table 2. Cellular fatty acid compositions of strain S7-2^T and *L. litoralis* JCM 13034^T

Data from this study. Fatty acids that represented <0.5% in both strains are omitted. tr, Trace (<0.5%); –, not detected; ECL, equivalent chain-length.

Fatty acid (%)	S7-2 ^T	<i>L. litoralis</i> JCM 13034 ^T
Straight-chain fatty acid		
C _{15:0}	7.2	3.3
Branched fatty acids		
iso-C _{13:0}	tr	1.0
iso-C _{14:0}	3.9	6.8
iso-C _{15:0}	29.6	17.6
iso-C _{16:0}	2.1	1.1
iso-C _{15:1}	3.8	6.9
iso-C _{16:1}	3.0	3.0
anteiso-C _{13:0}	–	1.1
anteiso-C _{15:0}	6.4	17.0
anteiso-C _{15:1}	tr	1.3
Unsaturated fatty acids		
C _{15:1} ω6c	10.3	4.6
C _{17:1} ω6c	0.8	0.9
Hydroxy fatty acids		
C _{17:0} 2-OH	0.6	1.0
C _{15:0} 3-OH	2.3	1.1
iso-C _{14:0} 3-OH	0.6	0.9
iso-C _{15:0} 3-OH	8.4	11.6
iso-C _{16:0} 3-OH	7.1	9.6
iso-C _{17:0} 3-OH	3.9	2.0
Summed features*		
1	3.1	1.8
3	0.6	1.3
Unknown fatty acids		
ECL 11.543	1.5	3.3
ECL 13.565	1.4	0.9

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 1 consisted of iso-C_{15:1} and/or C_{13:0} 3-OH. Summed feature 3 consisted of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

Therefore, on the basis of the phenotypic and phylogenetic data, strain S7-2^T is considered to represent a novel species of the genus *Lutibacter*, for which the name *Lutibacter maritimus* sp. nov. is proposed.

Description of *Lutibacter maritimus* sp. nov.

Lutibacter maritimus (ma.ri'ti.mus. L. masc. adj. *maritimus* of the sea, marine, maritime).

Cells are Gram-staining-negative, aerobic, non-flagellated, non-gliding and rod-shaped, 0.3–0.8 µm in diameter and 1.0–5.0 µm in length. Spherical cells are not found. Colonies on MA are circular with entire margins, convex and yellow in colour. Carotenoid pigments are produced, but flexirubin-type pigments are not produced. Growth occurs at 4 and 37 °C (optimum 25–30 °C), but not at

40 °C. Growth occurs at pH 5.0 (optimum pH 7.0–8.0), but not at pH 4.5. Growth occurs in the presence of 0.5–5.0% (w/v) NaCl (optimum approximately 2.0% (w/v) NaCl). Mg²⁺ ions are required for growth. Maltose is utilized as sole carbon and energy source, but L-arabinose, L-glutamate, D-mannose, sucrose, trehalose, D-xylose, acetate, benzoate, L-malate, salicin and formate are not. The predominant menaquinone is MK-6. The major polar lipids are phosphatidylethanolamine and three unidentified lipids. The major cellular fatty acids (>10% of total fatty acids) are iso-C_{15:0} and C_{15:1}ω6c. Other phenotypic characteristics are given in Table 1.

The type strain, S7-2^T (=KCTC 22635^T=CCUG 57524^T), was isolated from a tidal flat sediment at Saemankum on the west coast of Korea. The DNA G+C content of the type strain is 34.6 mol% (determined by HPLC).

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