Actibacterium ureilyticum sp. nov., isolated from seawater

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A polyphasic approach was used to characterize a novel marine bacterial strain, designated LS-811^T, isolated from seawater of the South China Sea (Taiwan). Cells of strain LS-811^{T}were Gram-staining negative, aerobic and rod-shaped with polar flagella. The 16S rRNA gene sequence analysis of strain LS-811^T showed highest sequence similarity to *Actibacterium mucosum* (96.5 %) and *Actibacterium atlanticum* (95.6 %), and lower sequence similarity (<96.0 %) to members of all other related genera. Strain LS-811^{T}was able to grow at 15–40 °C and pH 5.0–9.0. The quinone system was ubiquinone (Q-10), and the DNA G+C content was 60.1 mol%. The major fatty acids (>5 %) found in strain LS-811^T were $C_{18:0}$, $C_{10:0}$ 3-OH, $C_{19:0}$ cyclo ω8*c* and $C_{18:1}\omega$7*c*/$C_{18:1}\omega$6*c*. The major polar lipid profile consisted of glycolipids, phosphatidylglycerol and one unidentified aminolipid. Based on the distinct phylogenetic, phenotypic and chemotaxonomic traits together with results of comparative 16S rRNA gene sequence analysis, strain LS-811^T is considered to represent a novel species in the genus *Actibacterium*, for which the name *Actibacterium ureilyticum* sp. nov. is proposed. The type strain is LS-811^T (=BCRC 80823^T=JCM 30681^T).

The genus *Actibacterium* is a member of the class *Alphaproteobacteria*, and the type species was described as *Actibacterium mucosum* (Lucena *et al.*, 2012). At the time of writing, only one species of this genus was described in the LPSN (List of Prokaryotic names with Standing in Nomenclature; http://www.bacterio.net/actibacterium.html) website. Recently, *Actibacterium atlanticum* was proposed as the second species within the genus *Actibacterium* (Li *et al.*, 2014). The members of the genus *Actibacterium* are typically Gram-staining-negative, rod or oval-shaped and non-motile. They do not accumulate poly-β-hydroxybutyrate granules. The major fatty acids are $C_{18:1}\omega$7*c*/$C_{18:1}\omega$6*c*; the major polar lipids are phosphatidylglycerol, unidentified aminolipids and unidentified lipids. The common major respiratory quinone is ubiquinone Q-10.

The present work was undertaken to study the taxonomic status of a newly isolated marine bacterium. While investigating bacterial diversity, seawater (approximate depth 5 m) was collected from the South China Sea (21° 36.2071′ N 118° 11.6022′ E) and used to isolate bacterial strains by the standard serial dilution-plating technique on marine agar 2216 (MA Difco) at 30 °C for 3 days. A presumably novel strain (designated strain LS-811^T) was selected for further phenotypic and phylogenetic characterization. Strain LS-811^T was routinely grown aerobically on marine agar and stored at −80 °C in marine broth (MB; Difco) supplemented with 30 % (v/v) glycerol for long-term preservation.

For taxonomic purposes, the type strains *A. mucosum* KCTC 23349^T (Lucena *et al.*, 2012) and *A. atlanticum* LMG 27158^T (Li *et al.*, 2014) were analyzed under the same conditions. For direct comparative analysis, all these strains were grown on MA at 30 °C for 2 days, unless specified otherwise.

Colony morphology, presence of flagella and morphology of the cells of strain LS-811^T were investigated using colonies/cells grown on MA. Growth of strain LS-811^T was also tested on tryptic soy agar (TSA; Difco) and nutrient agar (NA; HiMedia) to determine its morphological characteristics.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LS-811^T is KP162062.

Two supplementary figures are available with the online Supplementary Material.

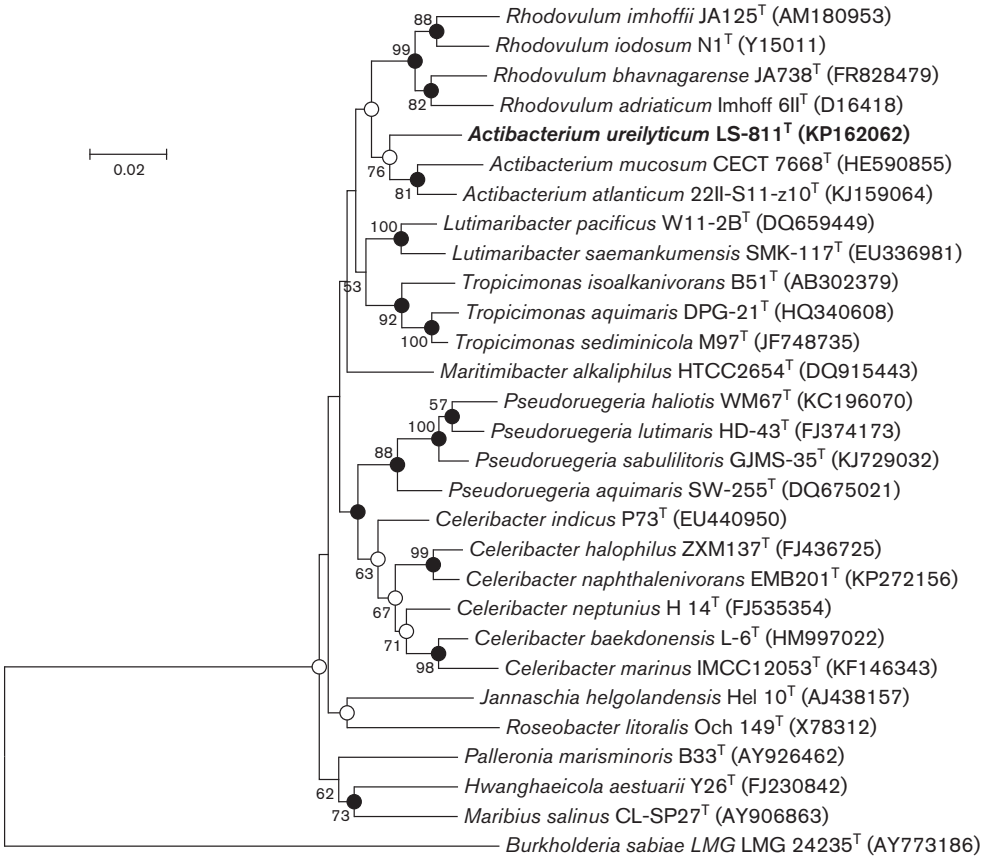

Fig. 1. Phylogenetic analysis of species of the genus *Actibacterium* based on 16S rRNA gene sequences. Distances and clustering were performed by using the neighbour-joining method with the software package MEGA version 6. Open circles indicate that the corresponding nodes were recovered in the tree reconstructed on the basis of the maximum-likelihood algorithm; filled circles indicate that the corresponding nodes were also recovered on the basis of the maximum-likelihood and maximum-parsimony algorithms. Bootstrap values (>50 %) based on 1000 replications are shown as percentages at the branching points. Bar, 0.02 substitutions per nucleotide position.

Anaerobic growth was tested on MA supplemented with potassium nitrate (0.1 %, w/v) using an anaerobic chamber (COY) filled with H_2 : CO_2 : N_2 (5 : 19 : 76) gas. Growth of strain LS-811^T was tested using MB at different temperatures (4, 15, 20, 25, 30, 37, 40, 45 and 50 °C), NaCl concentrations (1–10 % in 1 % increments in MB) and pH (4.0–10.0 at intervals of 1.0 pH unit using the following buffer systems: pH 4.0–5.0, 0.1 M citric acid/0.1 M trisodium citrate; pH 6.0–8.0, 0.2 M Na_2HPO_4/0.2 M NaH_2PO_4; pH 9.0–10.0, 0.1 M $NaHCO_3$/0.1 M Na_2CO_3).

The Gram-staining reaction was performed as described by Murray *et al.* (1994). Catalase activity was determined by assessing bubble production by cells in 3 % (v/v) H_2O_2, and oxidase activity was determined by using 1 % (w/v) *N*,*N*,*N*′,*N*′,-tetramethyl-1,4-phenylenediamine reagent (bioMérieux). Cell morphology was studied by transmission electron microscopy (JEOL JEM-1400) using samples stained with 0.2 % uranyl acetate, as well as by light microscopy (Zeiss model A3000). The following properties were tested in parallel for all strains under the same conditions. Additional enzymic activities, biochemical features and carbon source oxidation abilities of strain LS-811^T and the reference strains were determined by using the API ZYM and API 20NE kits (bioMérieux) and the GN2 MicroPlate system (BioLog), respectively, according to the instructions of the manufacturers.

A commercial DNA extraction kit (MO BIO UltraClean) was used to extract the genomic DNA of LS-811^T for 16S rRNA gene amplification and determination of DNA G+C content. To obtain the almost complete 16S rRNA gene sequence of strain LS-811^T, PCR was performed with bacterial universal primers pA and pH (Edwards *et al.*, 1989). Gene cycle sequencing was performed by using the Bigdye terminator kit (Heiner *et al.*, 1998) and ABI 3730 Genetic Analyzer (Applied Biosystems). For the analysis of DNA G+C content, DNA samples were prepared and degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated

Table 1. Differential characteristics within the genus *Actibacterium*

Strains: 1, LS-811^T; 2, *A. mucosum* KCTC 23349^T; 3, *A. atlanticum* LMG 27158^T. +, Positive; −, negative; w, weakly positive. All the test strains were positive for the utilization of Tween 40, Tween 80, gentiobiose, α-D-glucose, α-lactose and lactulose as carbon sources in the Biolog GN2 system; had positive reactions for acid and alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase in the API ZYM system; and positive reactions for protease (hydrolysis of gelatin) in the API 20NE system.

Characteristic	1	2	3
Carbon source utilization			
Dextrin, glycogen, D-fructose, pyruvic acid methyl ester	+	+	−
D-Mannitol, trehalose	+	−	+
L-Rhamnose, xylitol, citric acid	+	−	−
Maltose	−	+	+
Enzymic activities			
α-Mannosidase	+	−	+
β-Glucosidase	+	−	−
Cystine arylamidase	+	+	−
Reactions in API 20NE			
Reduction of nitrates to nitrites	+	−	−
Urease	+	+	−
β-Glucosidase	−	+	+
Gelatin (protease hydrolysis)	w	+	+
β-Galactosidase	−	+	−
D-Glucose (assimilation)	+	−	−
DNA G+C content (mol%)	60.1	61.3*	59.0*

*Data were obtained from Lucena *et al.* (2012) and Li *et al.* (2014).

via HPLC [Hitachi L-2130 chromatograph equipped with a Hitachi L-2200 autosampler, Hitachi L-2455 Diode array detector, and a reverse-phase C18 column (Phenomenex Synergi 4 μ Fusion-RP80 250×4.60 mm)].

The DNA fragments encoding for the 16S rRNA gene were assembled using the Vector NTI 9.0 software (IBI) and deposited in GenBank using sequin software. The almost complete 16S rRNA gene sequence (1430 nt) of strain LS-811^T was compiled , and compared with all type strains of species with validly published names using the 16S rRNA gene sequence database (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Phylogenetic analysis was performed with MEGA 6 software (version 6.0; Tamura *et al.*, 2013). Closely related 16S rRNA gene sequences were retrieved from EzTaxon-e and GenBank, and aligned by using the CLUSTAL X (1.83) program (Thompson *et al.*, 1997). Phylogenetic trees were reconstructed by using 16S rRNA gene sequences with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods, and evaluated by bootstrap analyses (Felsenstein, 1985) after 1000 replications.

For the investigation of chemotaxonomic characteristics, cells of strain LS-811^T and the reference strains were harvested at a similar physiological age with similar growth kinetics. Isoprenoid quinones were purified by the methods according to Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). Fatty acid methyl esters (FAMEs) were prepared, separated and identified according to the standard protocol (Paisley, 1996) of the Microbial Identification System (MIDI; Sasser, 1990) by using a gas chromatograph (Agilent 7890A) fitted with a flame ionization detector. For the extraction of FAMEs, strain LS-811^T was cultured on MA for 48 h at 30 °C (the same conditions as in our previous studies). Grown culture was harvested from the plate and subjected to saponification, methylation and extraction (Miller, 1982). Identification and comparison were made by using the Aerobe (RTSBA6) database of the MIDI System (Sherlock version 6.0).

The 16S rRNA gene sequence analyses of strain LS-811^T indicated that the strain was affiliated to the genus *Actibacterium* with low similarity to *A. mucosum* (96.5 %) and *A. atlanticum* (95.6 %), and lower sequence similarity to members of other genera. Based on the species delineation described by Stackebrandt & Ebers (2006), the similarity values of the 16S rRNA gene suggested that strain LS-811^T should be considered as a representative of a novel species since sequence divergence was ≥3 %. Phylogenetic trees were reconstructed by using 16S rRNA gene sequences with neighbour-joining, maximum-likelihood and maximum-parsimony methods, which indicated that the novel strain LS-811^T forms a clade with two speceis of the genus *Actibacterium*. Regardless of different evolutionary comparisons, similar topology was obtained in all phylogenetic trees reconstructed by using 16S rRNA gene sequences with neighbour-joining, maximum-likelihood and maximum-parsimony methods (Fig. 1).

Table 2. Comparison of the cellular fatty acid contents (percentages) of strain LS-811^T and closely related species

Strains: 1, LS-811^T; 2, *A. mucosum* KCTC 23349^T; 3, *A. atlanticum* LMG 27158^T. –, Not detected.

Fatty acid	1	2	3
Saturated			
$C_{12:0}$	0.5	0.7	0.6
$C_{16:0}$	1.1	6.4	3.5
$C_{17:0}$	0.6	0.8	0.3
$C_{18:0}$	6.0	3.5	9.9
Unsaturated			
$C_{13:0}$ anteiso	0.1	0.2	0.1
$C_{15:0}$ iso	0.2	–	–
$C_{17:1}$ iso ω10*c*	0.5	0.8	0.5
$C_{17:1}\omega$6*c*	0.5	0.5	0.2
$C_{18:1}\omega$7*c* 11-methyl	1.3	8.6	3.7
$C_{19:0}$ cyclo ω8*c*	11.9	4.2	3.0
$C_{19:0}$ iso	0.2	–	–
$C_{20:1}\omega$7*c*	0.8	1.7	0.5
$C_{20:2}\omega$6,9*c*	0.5	–	–
Hydroxy			
$C_{10:0}$ 2-OH	–	–	1.1
$C_{10:0}$ 3-OH	6.9	6.5	4.8
$C_{11:0}$ iso 3-OH	0.2	–	0.4
$C_{12:0}$ 3-OH	–	3.2	3.1
Summed feature 3*	0.5	0.3	0.3
Summed feature 7*	0.9	0.1	0.2
Summed feature 8*	66.4	61.8	65.3

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains $C_{16:1}\omega$7c/$C_{16:1}\omega$6c; Summed feature 7 contains $C_{19:1}\omega$6c/ω7c/19cy; Summed feature 8 contains $C_{18:1}\omega$7*c*/$C_{18:1}\omega$6*c*.

Colonies of strain LS-811^T are cream–white, circular and raised after 2 days of incubation on MA. Poly-β-hydroxybutyrate granules were not observed by transmission electron microscopy (Fig. S1, available in the online Supplementary Material). Strain LS-811^T was a Gram-staining-negative, aerobic bacterium, able to grow in MB with an additional 1–6 % (w/v) NaCl (optimal 3 %) at 15–40 °C (optimal 30 °C), and in the pH range 5.0–9.0 (optimal pH 7). Additionally, strain LS-811^T showed several distinct physiological and biochemical characteristics; a comparison of the phenotypic properties between strain LS-811^T and the type strains of recognized species in the genus *Actibacterium* is given in Table 1. The detailed phenotypic characteristics of strain LS-811^T are given in the species description. The DNA G+C content analysis of strain LS-811^T showed that the genomic G+C content was 60.1±0.3 mol%, which is similar to the range described for the genus *Actibacterium*. The predominant respiratory quinone was ubiquinone Q-10. The major polar lipid profile consisted of glycolipids, phosphatidylglycerol, unidentified lipids, unidentified aminolipids and phospholipid(Fig. S2). The fatty acid profile of strain LS-811^T was similar to those for recognized species of the genus *Actibacterium*. The major fatty acids (>5 %) were $C_{18:0}$ (6.0 %), $C_{19:0}$ cyclo ω8c (11.9 %), $C_{10:0}$ 3-OH (6.9 %) and $C_{18:1}\omega$7*c*/$C_{18:1}\omega$6*c* (66.4 %) (Table 2).

Based on the distinct phylogenetic, phenotypic, biochemical and chemotaxonomic data provided, characteristics of strain LS-811^{T}were in accordance with the description of the genus *Actibacterium*. The novel strain LS-811^T is therefore proposed to represent a novel species of this genus.

Description of *Actibacterium ureilyticum* sp. nov.

Actibacterium ureilyticum [u.re.i.ly′ti.cum. N.L. fem. n. *urea* urea; N.L. adj. *lyticus -a -um* (from Gr. adj. *lutikos -ê -on*) able to loosen, able to dissolve; N.L. neut. adj. *ureilyticum* urea-dissolving].

Cells are Gram-staining-negative, aerobic, rod-shaped (1.5–1.9 μm in length and 0.6–0.8 μm in diameter), motile with monotrichous flagella, reduce nitrates to nitrites, and are positive for catalase, oxidase, urease and protease activity. Does not accumulate poly-β-hydroxybutyrate granules in cell bodies. Colonies are white, circular and raised after 2 days of incubation on MA; colony size is about 1–2 mm. The temperature for growth ranges from 15 to 40 °C (optimal 30 °C); grows at pH 5.0–9.0 (optimal pH 7) and with 1–6 % (w/v) NaCl (optimal 3 %). Assimilates D-glucose and utilizes numerous compounds as sole carbon source,

including dextrin, glycogen, Tween 40, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, i-erythritol, D-fructose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, α-lactose, lactulose, D-mannitol, methyl β-D-glucoside, L-rhamnose, D-sorbitol, trehalose, turanose, xylitol, pyruvic acid methyl ester, *cis*-aconitic acid, citric acid, formic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-leucine, L-phenylalanine, L-serine and inosine. Acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and α-mannosidase are positive in the API ZYM system. The major fatty acids are $C_{10:0}$ 3-OH, $C_{18:0}$, $C_{19:0}$ cyclo $\omega 8c$ and $C_{18:1}\omega 7c/C_{18:1}\omega 6c$. The predominant quinone system is ubiquinone (Q-10).

The type strain, LS-811^T (=BCRC 80823^T=JCM 30681^T), was isolated from the South China Sea in Taiwan. The DNA G+C content of the type strain is 60.1±0.3 mol%.

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