Leeuwenhoekiella nanhaiensis sp. nov., isolated from deep-sea water

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A novel heterotrophic, aerobic, Gram-stain-negative, rod-shaped and yellow bacterium, designated strain G18^T, was isolated from a water sample collected from the deep South China Sea. Strain G18^T grew at 4–40 °C (optimum 28–32 °C), at pH 6.0–8.0 (optimum pH 6.5–7.5) and with 0–12 % (w/v) NaCl (optimum 3–4 %). The organism was mesophilic and piezotolerant, its optimal growth pressure was 0.1 MPa, which was lower than that at the depth from which it was isolated. Its optimal growth temperature was higher than that at the depth of its isolation. The predominant cellular fatty acids were $C_{15:0}$iso, $C_{17:0}$iso 3-OH and $C_{15:1}$iso. The major polar lipids were composed of phosphatidylethanolamine, one unknown aminolipid and one unknown polar lipid. The major respiratory quinone was menaquinone 6. The G+C content of the genomic DNA was 35 mol%. Phylogenetic analysis, based on 16S rRNA gene sequences, revealed that strain G18^T clustered with species of the genus *Leeuwenhoekiella* with validly published names within the family *Flavobacteriaceae* with 95.9–98.2 % sequence similarity. DNA–DNA reassociation values ranged from 9 to 42 %. Differential phenotypic properties, together with the phylogenetic distinctiveness, suggest that strain G18^T differs from species of the genus *Leeuwenhoekiella* with validly published names. On the basis of the polyphasic evidence, strain G18^T represents a novel species, isolated from deep-sea, of the genus *Leeuwenhoekiella* for which the name *Leeuwenhoekiella nanhaiensis* sp. nov. is proposed. The type strain is G18^T (=CCTCC AB 2015204^T=KCTC 42729^T).

The family *Flavobacteriaceae* was validated by Reichenbach (1992) and amended by Bernardet *et al.* (1996, 2002). Its genera have diverse physiological characteristics and ecological niches (soil, freshwater environments, marine environments, food and dairy products and their processing environments, specimens from humans and so on). Even within some genera, such as *Flavobacterium*, individual species may show contrasting characteristics, with some species identifiable by their very typical protein profiles, whereas others exhibit intraspecific heterogeneity (Bernardet *et al.*, 1996). Functionally, members of this family play important roles in the marine carbon cycle (Kirchman, 2002) and in the degradation of dissolved and particulate organic matter (Davey *et al.*, 2001). The genus *Leeuwenhoekiella,* which belongs to the family *Flavobacteriaceae*, comprises five recognized species: *Leeuwenhoekiella aequorea*, *Leeuwenhoekiella marinoflava*, *Leeuwenhoekiella blandensis*, *Leeuwenhoekiella palythoae* and *Leeuwenhoekiella polynyae* (Reichenbach, 1989; Nedashkovskaya *et al.*, 2005, 2009; Pinhassi *et al.*, 2006; Si *et al.*, 2015). However, these species of this genus were isolated either from surface seawater or from soft coral.

The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain G18^T is KR809379.

Four supplementary figures are available with the online Supplementary Material.

In searching for pizeophilic bacteria from the deep water of the South China Sea, we isolated a *Leeuwenhoekiella*-like bacterial strain, designated G18^T. In this study, the new isolate was taxonomically characterized further using a polyphasic approach; based on the results it is proposed that the isolate represents a novel species ofenhoekiella-blandensis KCTC the genus *Leeuwenhoekiella*.

Strain G18^T was isolated from a seawater sample collected at a depth of 2000 m from the South China Sea (13° 60′ N 114° 00′ E) on July 12, 2014. The sample was serially diluted ten-fold with sterile saline solution (containing 2.75 % NaCl, 0.5 % $MgCl_2$, 0.2 % $MgSO_4$, 0.05 % $CaCl_2$, 0.1 % KCl, 0.0001 % $FeSO_4$ (all w/v) in distilled water; Smibert & Krieg, 1994) to a 10^{-6} dilution, and aliquots of 100 µl diluted deep-sea water samples were spread on marine agar 2216 (MA; Difco) plates. The plates were then incubated at 15 °C for 3–7 days and individual colonies were picked and purified by subcultivation on MA. After primary isolation and purification, strain G18^T was routinely cultivated at 28 °C on MA and stored at −80 °C in marine broth 2216 medium (MB; Difco) supplemented with 20 % (v/v) glycerol. *L. blandensis* KCTC 22103^T, *L. palythoae* KCTC 22020^T, *L. marinoflava* LMG 1345^T and *L. aequorea* LMG 22550^T were used as reference strains and were all routinely cultivated on MA or in MB at 28 °C.

Genomic DNA of strain G18^T was extracted using a bacterial genomic DNA isolation kit (Bioteke). The 16S rRNA gene sequence of the strain was amplified from the genomic DNA by PCR with the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′) (Lane, 1991) and sequenced using an automated DNA sequencer (model 3730; Applied Biosystems). The 16S rRNA gene sequence of strain G18^T was aligned with those of representative members of the family *Flavobacteriaceae* retrieved from GenBank, and the phylogenetic trees were generated using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. Bootstrap analysis, based on 1000 replications, was used to estimate the confidence levels of the tree topologies. Evolutionary distances for the neighbour-joining method were calculated using the Jukes & Cantor (1969) model. A nearly full-length 16S rRNA gene sequence of strain G18^T (1495 bp) was obtained and phylogenetic analysis revealed that strain G18^T formed a distinct lineage within the genus *Leeuwenhoekiella* (Fig. 1). 16S rRNA gene sequence comparisons revealed that strain G18^T had the highest sequence similarity to *L. blandensis* KCTC 22103^T (98.2 %), followed by *L. palythoae* KCTC 22020^T (97.9 %) and *L. marinoflava* LMG 1345^T (97.2 %) with 95.9–96.5 % sequence similarity to other species of the genus *Leeuwenhoekiella* with validly published names. This topology was confirmed with a maximum-parsimony tree (Fig. S1, available in the online Supplementary Material).

The DNA G+C content of strain G18^T was determined by the thermal denaturation method (Marmur & Doty, 1962), using *Escherichia coli* K-12 ATCC 10798 (51 mol%) as the reference strain. The DNA G+C content of strain G18^T is 35 mol%, within the range reported for the genus *Leeuwenhoekiella* (35–43 mol%) (Table 1).

DNA–DNA hybridization was carried out using the spectrophotometric renaturation kinetics approach (Huss *et al.*, 1983). The DNA–DNA reassociation values between strain G18^T and the type strains of the species of the genus *Leeuwenhoekiella* with validly published names, including *L. blandensis* KCTC 22103^T, *L. palythoae* KCTC 22020^T and *L. marinoflava* LMG 1345^T, were 42 %, 14 % and 9 %, respectively, which were all far below the threshold value of 70 % recommended for the delineation of bacterial species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), suggesting that strain G18^T represents a novel species of the genus *Leeuwenhoekiella*.

For cellular fatty acid analysis, strain G18^T and the reference strains were incubated in MB at 28 °C for 24 h. Cellular fatty acids of each strain were extracted and analysed according to the instructions of the Sherlock Microbial Identification System (version 4.5 and the TSBA40 database). The major cellular fatty acids of strain G18^T were $C_{15:0}$ iso (26.5 %),

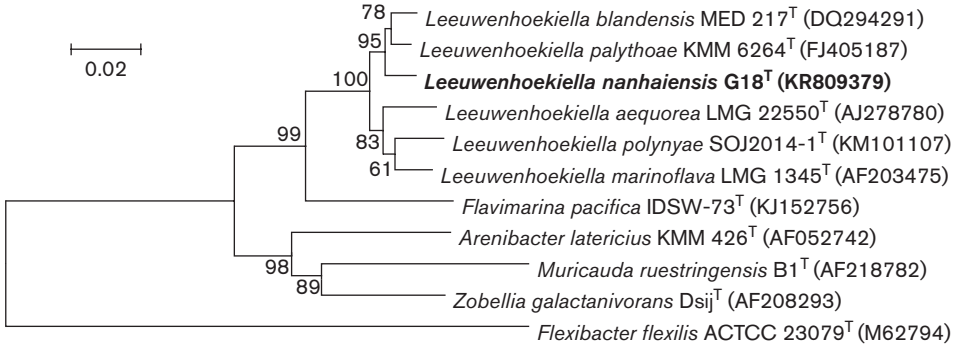

Fig. 1. Phylogenetic tree of representative members of the family *Flavobacteriaceae* based on 16S rRNA gene sequences, showing the phylogenetic position of strain G18^T. The tree is based on the neighbour-joining algorithm. *Flexibacter flexilis* ATCC 23079^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differential phenotypic characteristics of strain G18^T and type strains of related species of the genus *Leeuwenhoekiella*

Strains: 1, strain G18^T; 2, *L. blandensis* KCTC 22103^T (Pinhassi *et al.*, 2006); 3, *L. palythoae* KCTC 22020^T (Nedashkovskaya *et al.*, 2009); 4, *L. marinoflava* LMG 1345^T (Nedashkovskaya *et al.*, 2005, 2014); 5, *L. aequorea* LMG 22550^T (Nedashkovskaya *et al.*, 2005, 2014); 6, *L. polynyae* SOJ2014-1^T (Si *et al.*, 2015). The data from the API 20NE, API 20E, API ZYM and antibiotic susceptibility tests for strains 1, 2, 3, 4 and 5 were determined in this study. +, Positive; −, negative.

Characteristic	1	2	3	4	5	6
Growth conditions						
Salinity range (% NaCl, w/v)	0–12	0–17	0–12	0–15	0–15	0–14
Temperature range (°C)	4–40	10–41	4–38	4–37	4–37	0–35
Optimum Temperature (°C)	28–32	28–30	23–25	21–23	23–25	23–25
Hydrolysis of:						
Casein	−	+	−	+	+	−
Tween 20	+	+	−	+	+	+
Acid formation from:						
D-Maltose, L-rhamnose	+	+	+	−	−	+
L-Arabinose	+	−	+	−	−	−
D-Glucose	+	+	+	−	−	+
D-Lactose	−	−	+	−	−	+
D-Sucrose	−	−	+	−	+	−
D-Galactose	+	−	+	+	+	+
DL-Xylose	−	+	−	−	−	+
Mannitol	+	−	−	−	+	−
Glycerol	−	+	+	+	+	+
Utilizition of mannitol	−	−	−	−	+	−
Enzyme activity (API ZYM)						
Esterase (C4)	−	+	−	+	+	+
Lipase (C14)	−	+	−	+	−	−
Cystine arylamidase	+	+	−	+	−	+
Trypsin	+	+	+	+	−	+
α-Chymotrypsin	+	+	−	−	−	−
Naphthol-AS-BI-glucuronidase	+	−	+	+	+	+
α-Galactosidase	−	+	+	+	+	+
β-Glucuronidase	−	+	−	−	−	−
Susceptibility to:						
Erythromycin, Tetracyclin	+	+	+	−	+	+
Chloramphenicol	−	+	+	+	+	+
Penicillin	+	+	+	+	+	−
DNA G+C content (mol%)	35	42	41	38	35–36	39

$C_{17:0}$iso 3-OH (13.9 %), $C_{15:1}$iso (10.4 %), $C_{17:1}$ isoω9*c* (5.9 %) and co-eluted fatty acids (composed of $C_{16:1}\omega$7*c* and/or $C_{15:0}$iso 2-OH) (10.2 %). The whole-cell fatty acid composition of strain G18^T was similar to that of other species of the genus *Leeuwenhoekiella*, but with a higher proportion of $C_{15:0}$ iso (Table 2).

Polar lipid analysis of strain G18^T was performed by two–dimensional TLC. Polar lipids were extracted according to Komagata & Suzuki (1987) and separated on Kieselgel 60 F_{254} plates (10 × 10; Merck) using chloroform/methanol/water (65 : 25 : 4, by vol.) in the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.) in the second dimension (Collins & Jones, 1980). Lipid spots were visualized by spraying with 10 % (v/v) molybdophosphoric acid in ethanol followed by heating at 110 °C for 15 min and further characterized by spraying with specific reagents, including ninhydrin (aminolipids), zinzadze reagent (phospholipids) and anisaldehyde/sulfuric acid (glycolipids) (Zhang *et al.*, 2012). The polar lipids of strain G18^T were composed of phosphatidylethanolamine, one unknown aminolipid and one unknown polar lipid (Fig. S2). Quinones were extracted according to the method of Komagata & Suzuki (1987) and further analysed using an LC-MS system consisting of a Dionex Ultimate 3000 HPLC coupled to a Bruker Impact HD mass spectrometer. The major respiratory quinone of the isolate was menaquinone 6 (MK-6), which is a characteristic lipoquinone for members of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002).

High pressure cultivation and characterization was carried out as described by Fang *et al.* (2006). The isolate was grown in a pressure vessel under a range of hydrostatic

Table 2. Cellular fatty acid composition of strain G18^T and type strains of related species of the genus *Leeuwenhoekiella*

Strains: 1, strain G18^T; 2, *L. blandensis* KCTC 22103^T; 3, *L. palythoae* KCTC 22020^T; 4, *L. marinoflava* LMG 1345^T; 5, *L. aequorea* LMG 22550^T; 6, *L. polynyae* SOJ2014-1^T (Si *et al.*, 2015). Values are percentages of total fatty acids; fatty acids for which the amount (for all taxa) is less than 1 % are not given. The data for strains 1, 2, 3, 4 and 5 were determined in this study. –, Not detected; tr, trace amounts (<1 %).

Fatty acid	1	2	3	4	5	6
$C_{15:0}$	1.1	1.5	1.5	3.8	3.3	–
$C_{16:0}$	TR	1.1	1.1	2.2	1.3	2.4
$C_{15:1}$anteiso A	TR	TR	TR	1.0	1.1	TR
$C_{15:0}$anteiso	4.7	2.6	1.6	4.9	7.9	3.5
$C_{15:1}$iso	10.4	9.2	21.6	3.1	12.1	11.2
$C_{15:0}$iso	26.5	10.7	17.3	12.5	13.0	12.4
$C_{15:0}$iso 3-OH	2.4	2.4	2.6	2.5	1.3	3.7
$C_{15:0}$ 2-OH	TR	TR	TR	1.7	1.5	1.4
$C_{16:0}$iso	TR	1.8	1.2	2.0	4.0	1.4
$C_{16:0}$iso 3-OH	1.2	2.3	1.7	1.2	1.4	1.4
$C_{17:1}$iso$\omega 9c$	5.9	6.5	5.1	6.4	5.6	–
$C_{17:0}$iso	1.7	1.8	2.0	1.7	1.9	2.2
$C_{17:0}$iso 3-OH	13.9	15.5	14.1	10.8	10.0	17.3
$C_{17:0}$ 2-OH	3.0	3.3	TR	3.7	6.3	3.4
$C_{19:0}$ iso	TR	TR	TR	TR	TR	1.2
Co-eluted fatty acids*	10.2	23.7	11.9	17.6	18.3	–

*Represents two or three fatty acids ($C_{16:1}\omega 7c$ and/or $C_{15:0}$iso 2-OH) that cannot be separated by the Microbial Identification System.

pressures (0.1–40 MPa) at an optimal temperature (28 °C). MB (filtered through 0.22 μm polycarbonate filter and autoclaved) was used as the medium in this test. To supply oxygen to the cultures, Fluorinert (3M) was added to cultures (25 % of the total volume). Prior to use, Fluorinert was filtered through a 0.22 μm polycarbonate filter and then saturated with oxygen by bubbling with high-purity oxygen at 4 °C for 10 h. The bacterial densities were determined by the absorbance measurement at 600 nm (OD_{600}) using a Shimadzu UV-2600 spectrophotometer. Growth rates were calculated from 3–5 points along the logarithmic portion of the resulting growth curves using linear regression analysis (Kato *et al.*, 1995). The optimal growth pressure for strain G18^T was 0.1 MPa and the growth rate decreased steadily with increasing pressure from 0.1 MPa to 40 MPa except for at 20 MPa, under which the growth rate was higher relative to other high pressures (Fig. S3). This results show that G18^T is piezotolerant (Fang *et al.*, 2010).

For the observation of cellular morphology, cells were grown at 28 °C for 48 h in MB. After negatively staining with 20 % (v/v) phophotungstic acid, samples were observed by transmission electron microscopy (JEOL; JEM-100CXII) (Fig. S4). Colony morphology was observed after incubation for 3–4 days on MA at 28 °C. Gram-staining was performed following the procedure of Murray *et al.* (1994). Growth at pH 5.0–9.0 (in increments of 0.5 pH units) was tested at 28 °C in TYS broth [0.5 % tryptone (Oxoid), 0.1 % yeast extract (Oxoid), artificial seawater (containing 2.75 % NaCl, 0.1 % KCl, 0.54 % $MgCl_2 . 6H_2O$, 0.68 % $MgSO_4 . 7H_2O$, 0.14 % $CaCl_2 . 2H_2O$, 0.02 % $NaHCO_3$, all w/v in distilled water; Miyazaki *et al.*, 2008)] with the pH adjusted with MES (pH 5.0–6.0, 50 mM), MOPS (pH 6.5–7.0, 50 mM), Tris (pH 7.5–8.5, 50 mM) or CHES (pH 9.0, 50 mM). Growth at 4, 10, 15, 20, 25, 28, 30, 32, 35, 40, 45 and 50 °C was determined in MB. Growth with 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 15 % (w/v) NaCl was examined in broth consisting of 0.5 % trypton, 0.1 % yeast extract, 0.5 % $MgCl_2$, 0.2 % $MgSO_4$, 0.05 % $CaCl_2$, 0.1 % KCl, 0.0001 % $FeSO_4$ (all w/v) in distilled water. Acid production from carbohydrates, hydrolysis of casein, gelatin, starch, chitin, Tweens 20, 40, 60, 80, agar (1 %, w/v), DNA, urea and cellulose (CM-cellulose and filter paper), oxidase and catalase activities were carried out as described by Zhang *et al.* (2010). Nitrate reduction, production of hydrogen sulfide, indole and acetoin (Voges–Proskauer reaction) were performed by using API ZYM, API 20E and API 20NE strips (bioMérieux) according to the manufacturer's instructions, except that the strips were incubated at 28 °C. Susceptibility to antibiotics was examined by the disc-diffusion plate method, following the method of Nedashkovskaya *et al.* (2003). Discs (Cypress Diagnostics) were impregnated with the following antibiotics: carbenicillin (100 μg); lincomycin (15 μg); doxycycline (30 μg); erythromycin (15 μg); chloramphenicol (30 μg); ampicillin (10 μg); oleandomycin (15 μg); streptomycin (10 μg); gentamicin (10 μg); kanamycin (30 μg); neomycin (30 μg); polymyxin b (300 μg); tetracyclin (30 μg); vancomycin (30 μg); rifampicin (5 μg); penicillin (10 μg); amikacin (30 μg); cephalexin (30 μg) and novobiocin (5 μg).

Strain G18^T exhibited many phenotypic characteristics in common with species of the genus *Leeuwenhoekiella* with validly published names, such as the presence of oxidase and catalase activities, the ability to hydrolyse starch and the inability to produce indole and H_2S. However, strain G18^T differed due to the presence of aginine dehydrolase activity. In addition, strain G18^T also exhibited distinct phenotypic differences from the five species of the genus *Leeuwenhoekiella* with validly published names, such as the absence of α-galactosidase activity and the inability to produce acid from glycerol (Table 1). Thus, phylogenetic, phenotypic and chemotaxonomic characterization together with the results from the DNA–DNA hybridization experiments indicate that strain G18^T represents a novel species of the genus *Leeuwenhoekiella*, for which the name *Leeuwenhoekiella nanhaiensis* sp. nov. is proposed.

Description of *Leeuwenhoekiella nanhaiensis* sp. nov.

Leeuwenhoekiella nanhaiensis [nan.hai.en'sis. L. fem. adj. *nanhaiensis* of or pertaining to Nanhai (the Chinese name for the South China Sea), from where the type strain was isolated].

Cells are Gram-stain-negative rods, aerobic, motile by gliding and ranging from 0.4–0.7 μm in width and 1.4–4.1 μm in length. On MA, colonies are round, 2–3 mm in diameter and yellow. Oxidase- and catalase-positive. Aginine dehydrolase is positive. Growth is observed at 4–40 °C, with an optimum of 28–32 °C (mesophilic) and at pH 6.0–8.0 with an optimum of pH 6.5–7.5. Piezotolerant, its optimal growth pressure is 0.1 MPa, which is lower than that at the depth of isolation. Grows with 0–12 % (w/v) NaCl (optimum 3–4 %, w/v). Nitrate is not reduced. Indole, H_2S and acetoin (Voges–Proskauer reaction) are not produced. Decomposes starch, gelatin and Tweens 20, 40, 60 and 80. Does not hydrolyse casein, urea, DNA, agar, cellulose (CM-cellulose and filter paper) or chitin. Produces acid from D-glucose and L-arabinose, but not mannitol, inostiol, sorbitol, malonate or D-galactose, D-maltose, L-fucose, L-raffinose, fructose, L-rhamnose, mannitol, sorbitol, *N*-acetylglucosamine, D-lactose, D-mannose, D-sucrose, inostiol, D-ribose, glycerol or DL-xylose. Utilizes D-glucose, L-arabinose, D-lactose, D-mannose, D-sucrose and D-maltose, citrate. According to the API ZYM gallery, the type strain is positive for alkaline and acid phosphatases, leucine and valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-glucuronidase, β-galactosidase, α- and β-glucosidases, *N*-acetyl-β-glucosaminidase and α-mannosidase; weakly positive for esterase (C4) and esterase lipase (C8); negative for lipase (C14), α-galactosidase, β-glucuronidase and α-fucosidase. The major cellular fatty acids (>10 %) are $C_{15:0}$iso, $C_{17:0}$iso3-OH, $C_{15:1}$iso and co-eluted fatty acids (composed of $C_{16:1}\omega7c$ and/or $C_{15:0}$iso2-OH).

The type strain is G18^T (=CCTCC AB 2015204^T=KCTC 42729^T), which was isolated from a deep-sea sample from the South China Sea, PR China. The G+C content of the DNA of the type strain is 35 mol%.

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