Friedmanniella endophytica sp. nov., an endophytic actinobacterium isolated from bark of *Kandelia candel*

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A coccus-shaped, non-spore-forming actinobacterium, designated strain 4Q3S-3^T, was isolated from surface-sterilized bark of the mangrove plant *Kandelia candel* collected from Cotai Ecological Zones in Macao, China, and tested by a polyphasic approach to clarify its taxonomic position. This actinobacterium was Gram-stain-positive and aerobic. Neither substrate nor aerial mycelia were formed, and no diffusible pigments were observed on the media tested. Strain 4Q3S-3^T grew optimally without NaCl at 28–30 °C, pH 7.0–8.0. Phylogenetic analysis based on 16S rRNA gene sequence showed that strain 4Q3S-3^T belonged to the genus *Friedmanniella* and shared the highest 16S rRNA gene sequence similarity with *Friedmanniella flava* W6^T (96.57 %). The DNA G+C content of strain 4Q3S-3^T was 69.5 mol%. The cell-wall peptidoglycan contained LL-2,6-diaminopimelic acid, and MK-9(H_4) was the predominant menaquinone. The polar lipids comprised phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, unidentified glycolipid, amino lipids and two unidentified phospholipids. The predominant fatty acids were anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$. Based on phylogenetic, phenotypic and chemotaxonomic data, strain 4Q3S-3^T represents a novel species of the genus *Friedmanniella*, for which the name *Friedmanniella endophytica* sp. nov. is proposed. The type strain is 4Q3S-3^T (=DSM 100723^T=CGMCC 4.7307^T).

The genus *Friedmanniella* with *Friedmanniella antarctica* as the type strain was first proposed by Schumann *et al.* (1997). At the time of preparing this manuscript, the genus contains nine species with validly published names (http://www.bacterio.net/friedmanniella.html#flava; Euzéby, 1997). These species were isolated from different habitats: *F. antarctica* was isolated from antarctic sandstone (Schumann *et al.*, 1997), *Friedmanniella capsulata* and *Friedmanniella spumicola* were reported from activated sludge foam (Maszenan *et al.*, 1999), *Friedmanniella lacustris* was isolated from a hypersaline, heliothermal and meromictic antarctic lake (Lawson *et al.*, 2000), *Friedmanniella luteola*, *Friedmanniella lucida*, *Friedmanniella okinawensis* and *Friedmanniella sagamiharensis* were isolated from spiders and their webs (Iwai *et al.*, 2010), and *Friedmanniella flava* was reported from soil (Zhang *et al.*, 2013).

During a study on cultivable actinobacterial diversity of mangrove in Cotai Ecological Zones (22° 08′ 28″ N 113° 33′ 90″ E), Macao, China, strain 4Q3S-3^T was isolated from surface-sterilized bark of the mangrove plant *Kandelia candel*. Based on phylogenetic analysis, strain 4Q3S-3^T showed high levels of 16S rRNA gene sequence similarities to members of the genus *Friedmanniella*. A polyphasic taxonomic study showed that strain 4Q3S-3^T differs from previously described species of the

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Four supplementary figures and one supplementary table are available with the online Supplementary Material.

genus *Friedmanniella* and represents a novel species. The taxonomic position of this strain is herein reported.

The bark of *K candel* was surface-sterilized as described by Qin *et al*. (2008). After drying in a laminar flow hood, the surface-sterilized bark was ground into powder by using a micromill and spread on fucose-proline agar [containing, per litre distilled water: 5.0 g fucose, 1.0 g proline, 1.0 g $(NH_4)_2SO_4$, 1 g NaCl, 2.0 g $CaCl_2$, 1.0 g $MgSO_4.7H_2O$, 1.0 g K_2HPO_4, vitamin mixture (0.001 g VB_1, 0.001 g VB_2, 0.001 g Vpp, 0.001 g VB_6, 0.001 g phenylalanine, 0.001 g alanine, 0.0005 g biotin), 18.0 g agar, pH 7.2] before being incubated at 28 °C for 4 weeks. Colonies were transferred and streaked onto ISP 2 agar (Shirling & Gottlieb, 1966) until pure strains were obtained. Strain 4Q3S-3^T was cultivated, maintained on ISP 2 agar slant at 4 °C and stored as aqueous glycerol suspensions (20 %, v/v) at −80 °C.

The cultural physiological and biochemical characteristics of strain 4Q3S-3^T and reference strain *F. flava* CGMCC 4.6856^T (=W6^T) [obtained from China General Microbiological Culture Collection Center (CGMCC), Beijing, China] were tested under the same conditions. Cultural characteristics were determined by observing the growth of the strain at 28 °C for 3–4 weeks on ISP 2, ISP 3, ISP 4, ISP 5 and ISP 7 agars (Shirling & Gottlieb, 1966), nutrient agar (Waksman, 1961), R2A agar (Difco) and tryptic soy agar (TSA; Bacto). ISCC-NBS colour charts (Kelly, 1964) were used to assess the colony colours. Cell morphology and motility were observed and recorded by transmission electron microscopy (JEM-1400; JEOL) after incubation on ISP 2 agar at 28 °C for 2 days. The temperature range for growth was determined by incubation of the strain on ISP 2 agar at 4, 8, 20, 25, 28, 30, 37 and 45 °C for 14 days. The pH range (pH 4.0–13.0, at intervals of 1 pH unit) for growth was measured in R2A broth for 4 weeks using the buffer system described by Xu *et al*. (2005). Salt tolerance was tested in R2A agar supplemented with a concentration of 0, 1, 2, 3, 4, 5, 7 and 10 % (w/v) NaCl for 14 days. The Gram-staining test was performed as described by Magee *et al*. (1975). Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2. Oxidase activity was assessed by using 1 % (w/v) tetramethyl-*p*-phenylenediamine (Cappuccino & Sherman, 2002). Hydrolysis of casein, starch, gelatin and Tweens 20, 40 and 80, the methyl red test and production of H_2S, were examined as described by Gonzalez *et al*. (1978). Enzyme activities were tested by using the API ZYM kit (bioMérieux) according to the manufacturer's instructions. Acid production from carbon sources was examined using the API 50CH (bioMérieux) system. Oxidation of carbon sources and sensitivity to antimicrobial compounds were tested using Biolog GEN III MicroPlates. Other biochemical characteristics were tested by using API 20NE (bioMérieux) according to the manufacturer's instructions.

Strain 4Q3S-3^T was Gram-staining-positive, aerobic, non-spore-forming and coccus-shaped (0.6–1.0 μm in diameter) (Fig. S1, available in the online Supplementary Material). The colonies of strain 4Q3S-3^T on ISP 2 agar after 7 days were circular, smooth and entire, vivid yellow in colour. Substrate and aerial mycelia were not observed, and no diffusible pigments were produced on the media tested. Strain 4Q3S-3^T grew well on TSA, ISP 2 agar, R2A agar and nutrient agar. Poor growth occurred on ISP 3 agar and ISP 5 agar. No growth occurred on ISP 4 agar or ISP 7 agar. The temperature range for growth of strain 4Q3S-3^{T}was 20–37 °C, the pH range was pH 5.0–9.0 and the concentration range of NaCl was 0–5 % (w/v). No growth occurred at 8 °C, 45 °C, pH 4.0, pH 10.0 or in the presence of 7 % (w/v) NaCl. The best growth occurred at 28–30 °C, pH 7.0–8.0 and without NaCl. The detailed physiological and biochemical characteristics of strain 4Q3S-3^{T}are given in Table 1 and the species description.

For the chemotaxonomic analyses of menaquinones and polar lipids, strain 4Q3S-3^T together with the reference strain *F. flava* CGMCC 4.6856^T was grown in ISP 2 broth at 28 °C with shaking at 180 r.p.m. The isomers of diaminopimelic acid in whole-cell hydrolysates of strain 4Q3S-3^T were identified by TLC as described by Schleifer & Kandler (1972). Menaquinones were isolated and purified as described by Collins *et al*. (1977), and then analysed and confirmed according to the method of Guo *et al*. (2015). The polar lipids were extracted and analysed by two-dimensional TLC on silica gel 60 F_{254} plates (Merck) according to the method of Minnikin *et al*. (1984); the solvent systems of the first and second dimensions were chloroform/methanol/water (64 : 27 : 5, by vol.) and chloroform/methanol/acetic acid/water (80 : 18 : 12 : 5, by vol.), respectively. For the analysis of whole-cell fatty acids, cell mass of strain 4Q3S-3^T and the reference strain was harvested from ISP 2 agar at 28 °C, when the bacterial communities reached the late-exponential stage of growth. The whole-cell fatty acids were saponified, methylated and extracted according to the standard protocol described by Sasser (1990), and analysed by using an Agilent 7890A gas chromatograph coupled with an Agilent 5975C single quadrupole mass spectrometer equipped with the Nist08 Library software database (Tuo *et al*., 2015).

For calculation of DNA G+C content, the genomic DNA of strain 4Q3S-3^T was prepared as described by Marmur (1961) and G+C content was determined by reversed-phase HPLC as described by Mesbah *et al*. (1989).

The whole-cell hydrolysate of strain 4Q3S-3^T contained LL-2,6-diaminopimelic acid, and the predominant menaquinone was MK-9(H_4). The polar lipids comprised phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, an unidentified glycolipid, two unidentified amino lipids and two unidentified phospholipids. The polar lipid profiles of strain 4Q3S-3^T and the reference strain are shown in Fig. S2. The whole-cell fatty acids contained large amounts of anteiso-$C_{15:0}$ (28.4 %), iso-$C_{16:0}$ (28.2 %) and iso-$C_{15:0}$ (19.1 %) and small amounts of anteiso-$C_{17:0}$ (9.7 %), $C_{16:0}$ (7.4 %), iso-$C_{17:0}$ (2.5 %), $C_{18:0}$ (2.0 %), iso-$C_{14:0}$ (1.9 %), $C_{14:0}$ (0.5 %) and $C_{15:0}$ (<0.5 %). The cellular fatty acid contents of strain 4Q3S-3^T and the reference strain are given

Table 1. Comparison of the characteristics of strain 4Q3S-3^T and related species of the genera *Friedmanniella* and *Microlunatus*

Strains: 1, 4Q3S-3^T; 2, *Friedmanniella flava* W6^T (data from this study); 3, *Microlunatus panaciterrae* Gsoil 954^T (data from An *et al.*, 2008; Lee & Kim, 2012). All strains were Gram-staining-positive, non-motile, and negative for production of H_2S, methyl red test and urease. In API 20NE kits, all strains were negative for assimilation of potassium gluconate, caprate, malic acid, trisodium citrate, adipate and phenylacetate. In API ZYM kits, all strains were positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, β-galactosidase, α-glucosidase, leucine arylamidase and β-glucosidase, but negative for cystine arylamidase and lipase. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GL, unidentified glycolipid; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; AL, unidentified amino lipid; PL, unidentified phospholipid; +, positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3
Cell size (μm)	0.6–1.0	0.6–0.7*	0.3–0.7
Growth pH range (optimum)	5.0–9.0 (7.0–8.0)	5.0–9.0 (6.0–7.0)	5.0–9.0 (ND)
Temperature range for growth (optimum) (°C)	20–37 (28–30)	8–30 (28–30)	20–30 (ND)
NaCl tolerance range (optimum) (%, w/v)	0–5 (0)	0–1 (0)	0–5 (ND)
Oxidase	−	−	+
Catalase	W	+	+
Nitrate reduction	−	−	+
Hydrolysis of:			
Tween 80	+	W	ND
Tween 40	+	W	ND
Tween 20	+	+	ND
Starch	−	−	+
Gelatin	+	−	−
Casein	+	W	−
Carbon source utilization:			
D-Glucose	+	−	W
L-Arabinose	+	−	W
D-Mannose	+	−	W
D-Mannitol	+	−	−
Maltose	+	−	W
N-Acetyl-D-glucosamine	+	−	−
API ZYM:			
Valine arylamidase	+	W	−
α-Chymotrypsin	+	−	−
α-Mannosidase	+	W	+
Esterase lipase	W	−	W
Esterase	+	W	W
β-Glucuronidase	W	−	−
α-Fucosidase	+	−	+
Trypsin	+	−	+
α-Galactosidase	+	W	+
Alkaline phosphatase	+	+	−
Naphthol-AS-BI-phosphohydrolase	+	+	W
Polar lipids	DPG, PG, PI, GL, AL, PL	PG, PC, PI, AL, PL, DPG	PG, DPG, PE
DNA G+C content (mol%)	69.5	72*	65.1

*Data from Zhang *et al.* (2013).

in Table S1. The DNA G+C content of strain 4Q3S-3^T was 69.5 mol%. The major menaquinones, polar lipids and fatty acids of the reference strain were similar to those reported previously (Zhang *et al.*, 2013). The difference in the proportion of fatty acids and slight difference in the types of polar lipids may be due to the different experimental conditions used.

The extraction of genomic DNA from strain 4Q3S-3^T and PCR amplification of the 16S rRNA gene were performed as described by Li *et al.* (2007). The purified PCR products were cloned using the pEASY-T1 Cloning kit (TransGen Biotech), and sequenced by Sangon Biotech (Shanghai) (http://www. sangon. com). 16S rRNA gene sequence similarity values between strain 4Q3S-3^T and related species

were obtained from the EzTaxon server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Multiple alignmene using clustal x (Thompson et al., 1997). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980). Phylogenetic trees were reconstructed using neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods with mega version 5.0 (Tamura et al., 2011). The topologies of the phylogenetic trees were evaluated by using the bootstrap method with 1000 repeats (Felsenstein, 1985).

An almost full-length 16S rRNA gene (1479 bp) sequence of strain 4Q3S-3^T was obtained. EzTaxon-e-based BLAST analysis of the 16S rRNA gene sequence showed that strain 4Q3S-3^T exhibited the highest similarity with *F. flava* W6^T (96.57 %), followed by *F. lucida* FA2^T (96.54 %), *F. okinawensiss* FB1^T (96.40 %), *Microlunatus panaciterrae* Gsoil 954^T (96.35 %), *F. lacustris* EL-17A^T (96.06 %), *F. antarctica* DSM 11053^T (95.95 %), *F. sagamiharensis* FB2^T (95.81 %), *F. spumicola* ACM 5121^T (95.80 %), *F. capsulata* Ben 108^T (95.65 %) and *F. luteola* FA1^T (95.46 %). The phylogenetic trees based on 16S rRNA gene sequence generated by using all three tree-making methods showed that strain 4Q3S-3^T clustered with all species of genus *Friedmanniella* with validly published names, and all members in the cluster formed a separate clade. Although *M. panaciterrae* Gsoil 954^T shared relatively high similarity with strain 4Q3S-3^T, it clustered with all species of the genus *Microlunatus,* and all members in the cluster formed another distinct branch (Figs 1, S3 and S4). It was obvious that strain 4Q3S-3^T was phylogenetically affiliated to the genus *Friedmanniella*. However, the relatively low levels of sequence similarity with recognized species of the genus *Friedmanniella* and the phylogenetic position showed that strain 4Q3S-3^T represents a novel species.

The results of morphological, physiological and biochemical characteristics for strain 4Q3S-3^{T}are given in the species description and Table 1. The diagnostic diamino acid of the cell-wall peptidoglycan and the predominant menaquinone of strain 4Q3S-3^T were LL-2,6-diaminopimelic acid and MK-9(H_4), respectively, which was a consistent result in the genus *Friedmanniella*. The fatty acid profile was dominated by anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$. Previous studies have shown that anteiso-$C_{15:0}$ and iso-$C_{15:0}$are predominant in members of the genus *Friedmanniella* (Schumann & Pukall, 2012). Although strain 4Q3S-3^T shared relatively high levels of 16S rRNA gene sequence similarity with *M. panaciterrae* Gsoil 954^T, a member of the genus *Microlunatus*, strain 4Q3S-3^T formed a distinct cluster within the genus *Friedmanniella* whilst *M. panaciterrae* Gsoil 954^T formed another distinct branch within the genus *Microlunatus* in the three phylogenetic trees. It was obvious that strain 4Q3S-3^T could not be assigned to the genus *Microlunatus*. In addition, strain 4Q3S-3^T could be differentiated from *M. panaciterrae* Gsoil 954^T based on phenotypic and chemotaxonomic properties, such as polar lipids, nitrate reduction, certain enzyme activities, acid production from some carbohydrates and utilization of carbon sources (Table 1). Distinct differences between strain 4Q3S-3^T and its closest phylogenetic neighbour *F. flava* W6^{T}were also

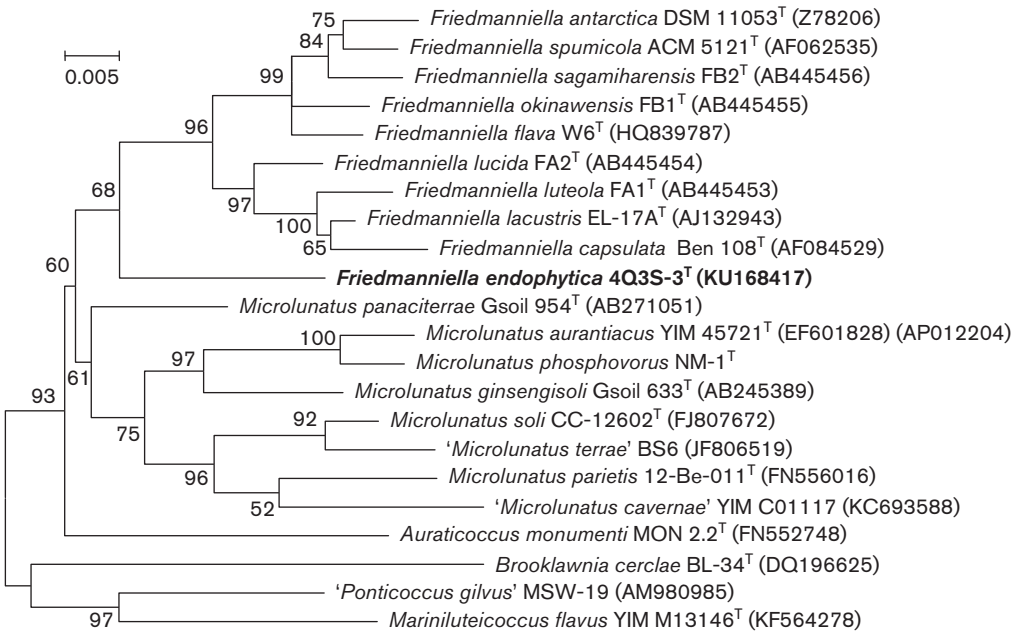

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences of strain 4Q3S-3^T and related strains in the genus *Friedmanniella*. Numbers at nodes refer to bootstrap values (based on 1000 replicates; only values >50 % are shown). Bar, 5 substitutions per 1000 nt.

observed. It was notable that the amounts of fatty acids iso-$C_{16:0}$ and anteiso-$C_{17:0}$ of strain 4Q3S-3^T (28.2 %, 9.7 %) were significantly higher than those of *F. flava* W6^T (0.9 %, 0.6 %), and the amount of fatty acid anteiso-$C_{15:0}$ of strain 4Q3S-3^T (28.4 %) was significantly lower than that of *F. flava* W6^T (66.9 %) (Table S1). In the polar lipid profiles, an unidentified glycolipid and unidentified amino lipid (AL1) detected in strain 4Q3S-3^T were absent in *F. flava* W6^T, while phosphatidylcholine and an unidentified phospholipid (PL3) detected in *F. flava* W6^T were not found in strain 4Q3S-3^T (Fig. S2). Furthermore, other phenotypic characteristics could obviously differentiate strain 4Q3S-3^T from phylogenetically related species of the genus *Friedmanniella* by the range of salinity and temperature required for growth, certain enzyme activities and utilization of carbon sources (Table 1).

In conclusion, based on the phylogenetic analysis, and phenotypic and chemotaxonomic characteristics, strain 4Q3S-1^T represents a novel species of the genus *Friedmanniella*, for which the name *Friedmanniella endophytica* sp. nov. is proposed.

Description of *Friedmanniella* endophytica sp. nov.

Friedmanniella endophytica (en.do.phy′ti.ca. Gr. pref. *endo* within; Gr. n. *phyton* plant; L. fem. suff. *-ica* adjectival suffix used with the sense of belonging to; N.L. fem. adj. *endophytica* within plant, endophytic, pertaining to the original isolation from plant tissues).

Cells are aerobic, Gram-stain-positive, non-spore-forming, non-motile, coccus-shaped and 0.6–1.0 μm in diameter after incubation for 2 days at 28 °C on ISP 2 agar. Neither substrate nor aerial mycelium is formed, and no diffusible pigments are produced on any media tested. Cells are weakly positive for catalase but negative for oxidase. Colonies on ISP 2 agar for 7 days are smooth, circular and entire, vivid yellow in colour. Good growth occurs on ISP 2 agar, R2A agar, TSA and nutrient agar, poor growth occurs on ISP 3 agar and ISP 5 agar, no growth occurs on ISP 4 agar or ISP 7 agar. Growth occurs at 20–37 °C (optimum, 28–30 °C), pH 5.0–9.0 (optimum, pH 7.0–8.0) and with NaCl concentrations of 0–5 % (w/v) (optimum, 0 %). Cells are positive for hydrolysis of casein, gelatin, Tween 20, Tween 40 and Tween 80. Hydrolysis of starch, urease, nitrate reduction, methyl red test and H_2S production are negative. According to API ZYM strips test results, positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase (C4), α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase, weakly positive for esterase lipase (C8) and β-glucuronidase, negative for cystine arylamidase and lipase (C14). In API 50CH strips, acid is produced from aesculin, amygdalin, L-arabinose, arbutin, cellobiose, gentiobiose, D-glucose, lactose, maltose, melezitose, melibiose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, raffinose, D-ribose, sucrose, salicin, starch, trehalose, turanose and D-xylose. In the BIOLOG system, positive for oxidation of acetic acid, acetoacetic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetylneuraminic acid, D-alanine, γ-aminobutyric acid, D-arabitol, L-arginine, D-aspartic acid, L-aspartic acid, bromosuccinic acid, cellobiose, citric acid, dextrin, formic acid, D-fructose, D-fructose 6-phosphate, D-fucose, L-fucose, D-galactose, galacturonic acid, D-galactonic acid lactone, gelatin, gentiobiose, D-gluconic acid, α-D-glucose, D-glucose 6-phosphate, glucuronamide, D-glucuronic acid, L-glutamic acid, glycerol, glycyl-L-proline, L-histidine, α-hydroxybutyric acid, β-hydroxy-D,L-butyric acid, *p*-hydroxyphenylacetic acid, inosine, α-ketobutyric acid, α-ketoglutaric acid, L-lactic acid, D-lactic acid methyl ester, α-lactose, D-malic acid, L-malic acid, maltose, D-mannitol, D-mannose, melibiose, 3-methyl-D-glucose, methyl β-D-glucoside, methyl pyruvate, mucic acid, *myo*-inositol, pectin, propionic acid, L-pyroglutamic acid, quinic acid, raffinose, L-rhamnose, D-saccharic acid, salicin, D-serine, L-serine, D-sorbitol, stachyose, sucrose, trehalose, turanose and Tween 40. Cells are sensitive to fusidic acid, lincomycin, minocycline, niaproof 4, tetrazolium blue and vancomycin but can tolerate aztreonam, guanidine hydrochloride, lithium chloride, nalidixic acid, potassium tellurite, rifamycin SV, sodium bromate, sodium butyrate, tetrazolium violet, troleandomycin and 1 % (w/v) sodium lactate. The cell-wall peptidoglycan contains LL-2,6-diaminopimelic acid as the diagnostic diamino acid. The predominant menaquinone is MK-9(H_4). The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, an unidentified glycolipid, two unidentified amino lipids and two unidentified phospholipids. The major fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$.

The type strain, 4Q3S-3^T (=DSM 100723^T=CGMCC 4.7307^T), was isolated from bark of *Kandelia candel* collected from Cotai Ecological Zones in Macao, China. The G+C content of the genomic DNA of the type strain is 69.5 mol%.

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