

Nakamurella endophytica sp. nov., a novel endophytic actinobacterium isolated from the bark of *Kandelia candel*

Li Tuo,^{1,2} Fei-Na Li,² Zhen Pan,^{1,2} Inchio Lou,³ Min Guo,⁴ Simon Ming-Yuen Lee,⁴ Li Chen,⁵ Lin Hu⁵ and Cheng-Hang Sun²

Correspondence

Cheng-Hang Sun
chenghangsun@hotmail.com or
sunchenghang@imb.pumc.edu.cn

¹Research Center for Medicine & Biology, Zunyi Medical University, Zunyi 563003, PR China

²Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, PR China

³Faculty of Science and Technology, Department of Civil and Environmental Engineering, University of Macau, Macao, PR China

⁴State Key Laboratory of Quality Research of Chinese Medicine and Institute of Chinese Medical Sciences, University of Macau, Macao, PR China

⁵Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China

A Gram-stain-positive, aerobic, coccus-shaped, non-spore-forming actinobacterium, designated strain 2Q3S-4-2^T, was isolated from the surface-sterilized bark of *Kandelia candel*, collected from Cotai Ecological Zones in Macao, PR China. It was tested using a polyphasic approach to determine its taxonomic position. Strain 2Q3S-4-2^T grew optimally without NaCl at 28–30 °C and at pH 7.0. Substrate mycelia and aerial mycelia were not formed and no diffusible pigments were observed on the media tested. Phylogenetic analysis, based on 16S rRNA gene sequences, suggested that strain 2Q3S-4-2^T belonged to the genus *Nakamurella*, sharing highest 16S rRNA gene sequence similarity with *Nakamurella flavida* DS-52^T (96.76 %). The DNA G + C content of strain 2Q3S-4-2^T was 67.8 mol%. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid and MK-8(H₄) was the predominant menaquinone. The predominant polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, unidentified aminophospholipids and phosphatidylinositol. The major fatty acids were iso-C_{16:0}, anteiso-C_{15:0}, anteiso-C_{17:0} and C_{16:0}. On the basis of the phylogenetic, phenotypic and chemotaxonomic analysis, strain 2Q3S-4-2^T represents a novel species of the genus *Nakamurella*, for which the name *Nakamurella endophytica* sp. nov. is proposed. The type strain is 2Q3S-4-2^T (=DSM 100722^T=CGMCC 4.7308^T).

Microsphaera, as an illegitimate genus name, was replaced by *Nakamurella*, consequently, *Nakamurella multipartita* instead of *Microsphaera multipartita* became the type species of the genus *Nakamurella* (Yoshimi *et al.*, 1996; Tao *et al.*, 2004). At the time of writing, the genus contains four species with validly published names: *N. multipartita* (Yoshimi *et al.*, 1996; Tao *et al.*, 2004) from activated sludge, *Nakamurella flavida* (Yoon *et al.*, 2007; Kim *et al.*, 2012) from soil, *Nakamurella lactea* (Lee *et al.*, 2008;

Kim *et al.*, 2012) from rock, and *Nakamurella panacisegetis* (Kim *et al.*, 2012) from the soil of a ginseng field. All these species are characterized chemotaxonomically by the presence of *meso*-diaminopimelic acid in the cell-wall, MK-8(H₄) as the predominant menaquinone, branched-chain saturated (iso-C_{15:0}, iso-C_{16:0}, anteiso-C_{15:0} and anteiso-C_{17:0}), and straight-chain saturated (C_{16:0} and C_{17:0}) or unsaturated (C_{18:1}ω9c) compounds as the major fatty acids, and diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and unidentified aminophospholipid (APL) as the major polar lipids (Kim *et al.*, 2012).

During a study on the cultivable actinobacterial diversity of mangrove in Cotai Ecological Zones (22° 08' 28" N 113° 33' 90" E), Macao, PR China, strain 2Q3S-4-2^T was isolated from surface-sterilized bark of *Kandelia candel*. The plant samples were surface-sterilized as described by

Abbreviations: APL, aminophospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 2Q3S-4-2^T is KT971019.

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

Qin *et al.* (2008). After being dried up in a hood, the surface-sterilized bark was ground into a powder by using a micromill and distributed on different media plates before being incubated at 28 °C for 4 weeks. Based on phylogenetic analysis, strain 2Q3S-4-2^T showed high levels of 16S rRNA gene sequence similarities to members of the genus *Nakamurella*. Polyphasic taxonomic studies showed that strain 2Q3S-4-2^T was distinguishable from species of the genus *Nakamurella* with validly published names and represented a novel species. The aim of the present study was to clarify the exact taxonomic position of strain 2Q3S-4-2^T.

Strain 2Q3S-4-2^T was isolated on modified Gauze's Medium No.2 [containing, 1⁻¹ distilled water: 1.0 g glucose, 0.5 g propeptone, 0.3 g tryptone, 0.5 g NaCl, vitamin mixture (0.001 g VB₁, 0.001 g VB₂, 0.001 g Vpp, 0.001 g VB₆, 0.001 g phenylalanine, 0.001 g alanine, 0.0005 g biotin), 20.0 g agar, pH 7.2] after 4 weeks of incubation at 28 °C. Colonies were transferred and streaked onto the ISP 2 agar (Shirling & Gottlieb, 1966) until pure strains were obtained. The strain was cultivated, maintained on ISP 2 agar slants at 4 °C and stored as glycerol suspensions (20 %, v/v) at -80 °C.

Cultural characteristics, plus the physiological and biochemical characteristics of strain 2Q3S-4-2^T and the reference strain, *N. flavida* DS-52^T (=JCM 15652^T) [obtained from Japan Collection of Microorganisms (JCM), Saitama, Japan] 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 media (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. The cell morphology and dimensions were observed and recorded by transmission electron microscopy (JEOL; JEM-1400) after incubation on ISP 2 agar at 28 °C for 3 days. The Gram-staining test was performed as described by Magee *et al.* (1975). The temperature range for growth was determined by incubation of the strain on R2A 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 concentrations of 0, 1, 2, 3, 4, and 5 % (w/v) NaCl for 14 days. Catalase activity was determined by bubble production in 3 % (v/v) H₂O₂. Oxidase activity was assessed by using 1 % (w/v) tetramethyl-*p*-phenylenediamine (Cappuccino & Sherman, 2002). The hydrolysis of starch, casein, gelatin, Tweens 20, 40 and 80, the production of H₂S and the methyl red test, were examined as described by Gonzalez *et al.* (1978). Acid production from carbon sources was tested using the API 50CH system (bioMérieux). Oxidation of the carbon sources and sensitivity to antimicrobial compounds were tested using Biolog GEN III MicroPlates. Other biochemical characteristics and enzyme activities were tested by using

API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions.

Strain 2Q3S-4-2^T was Gram-stain-positive, non-spore-forming, aerobic and coccus-shaped (0.8–1.2 µm in diameter; Fig. S1, available in the online Supplementary Material). The colonies of strain 2Q3S-4-2^T on ISP 2 agar were circular, smooth and entire, and vivid orange-yellow. Substrate mycelia and aerial mycelia were not observed and no diffusible pigments were produced on the media tested. Strain 2Q3S-4-2^T grew well on ISP 2 agar, ISP 3 agar and R2A agar. Poor growth occurred on nutrient agar, TSA and ISP 7 agar. No growth occurred on ISP 4 agar and ISP 5 agar. The temperature range for growth of strain 2Q3S-4-2^T was 20–37 °C, the pH range for growth was pH 5.0–9.0 and the concentration range of NaCl for growth was 0–2 %. No growth occurred at 8 °C, 45 °C, pH 4.0, pH 11.0 or in the presence of 3 % NaCl. The best growth occurred at pH 7.0, 28–30 °C and without NaCl. Detailed physiological and biochemical characteristics of strain 2Q3S-4-2^T are given in Table 1 and in the species description.

For molecular systematic studies and chemotaxonomic analyses of menaquinones and polar lipids, strain 2Q3S-4-2^T, together with the reference strain, were grown in ISP 2 broth for 7 days at 28 °C and at 180 r.p.m. The isomers of diaminopimelic acid in the whole-cell hydrolysates of strain 2Q3S-4-2^T were identified by TLC as described by Schleifer & Kandler (1972). The polar lipids were extracted and analysed by two-dimensional TLC on silica gel 60 F₂₅₄ plates (Merck) as described by Minnikin *et al.* (1984); the solvent systems of the first and the second dimensions were chloroform-methanol-water (64 : 27 : 5, by vol.) and chloroform-methanol-acetic acid-water (80 : 18 : 12 : 5, by vol.), respectively. Menaquinones were isolated and purified according to the method of Collins *et al.* (1977), then analysed and confirmed by HPLC and a single quadrupole mass spectrometer (Guo *et al.*, 2015). For the analysis of whole-cell fatty acids, the cell mass of strain 2Q3S-4-2^T and reference strains were harvested from ISP 2 agar at 30 °C when the bacterial communities had 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 the determination of the G+C content, the genomic DNA of strain 2Q3S-4-2^T was prepared according to the method described by Marmur (1961) and was determined by reversed-phase HPLC as described by Mesbah *et al.* (1989).

The whole-cell hydrolysate of strain 2Q3S-4-2^T contained *meso*-diaminopimelic acid and the predominant menaquinone was MK-8(H₄). The polar lipids comprised DPG, PE, APL, PI and unidentified phospholipids. The polar lipid profiles are shown in Fig. S2. The whole-cell fatty acids

Table 1. Comparison of the characteristics of strain 2Q3S-4-2^T and its phylogenetic neighbour *N. flavida* DS-52^T

Strains: 1, 2Q3S-4-2^T; 2, *N. flavida* DS-52^T. All data shown were obtained in this study, except for that marked with an asterisk, which was from Yoon *et al.* (2007). All strains were Gram-stain-positive, positive for catalase and for the hydrolysis of Tween 20, but negative for oxidase and negative for the hydrolysis of Tween 80, urease, the methyl red test, nitrate reduction and the production of H₂S. In API 20NE strips, all strains were negative for the assimilation of *N*-acetyl-D-glucosamine, potassium gluconate, capric acid, malic acid, trisodium citrate, adipate, D-mannitol, maltose and phenylacetate. In API ZYM strips, all strains were positive for acid phosphatase, esterase lipase, α -glucosidase, β -glucosidase and leucine arylamidase. They were weakly positive for valine arylamidase, but negative for *N*-acetyl- β -glucosaminidase, α -chymotrypsin, α -fucosidase, α -galactosidase, β -glucuronidase, lipase, α -mannosidase and trypsin. +, Positive; -, negative; w, weakly positive.

Characteristic	1	2
Cell size(μ m)	0.8–1.2	0.6–1.2*
Colony colour	Vivid orange-yellow	Brilliant yellow
pH range for growth (optimum)	5.0–9.0 (7.0)	6.0–9.0 (7.0)
Temperature range for growth (optimum) (°C)	20–37 (28–30)	4–30 (25)
NaCl tolerance range (% w/v) (optimum)	0–2 (0)	0–3 (1)
Hydrolysis of:		
Tween 40	–	+
Gelatin	+	–
Casein	+	–
Starch	–	+
Carbon source utilization		
D-Glucose	–	+
L-Arabinose	–	+
D-Mannose	–	+
API ZYM results		
β -Galactosidase	–	w
Esterase	–	+
Naphthol-AS-BI-phosphohydrolase	+	w
Alkaline phosphatase	+	–
Cystine arylamidase	–	w
Major fatty acids (>10 %)	iso-C _{16:0} , anteiso-C _{15:0} , anteiso-C _{17:0} , C _{16:0}	anteiso-C _{15:0} , iso-C _{15:0}
DNA G+C content (mol%)	67.8	72.6*

contained large amounts of iso-C_{16:0} (23.6 %), anteiso-C_{15:0} (19.8 %), anteiso-C_{17:0} (13.1 %), C_{16:0} (10.7 %) and small amounts of iso-C_{15:0} (8.1 %), iso-C_{17:0} (5.1 %), C_{17:0} (4.8 %), C_{18:0} (3.3 %), anteiso-C_{15:0} 2-OH (2.7 %), iso-C_{16:0} 2-OH (2.3 %), iso-C_{15:0} 2-OH (1.5 %), iso-C_{14:0} (1.3 %), anteiso-C_{17:0} 2-OH (1.1 %), anteiso-C_{16:0} (0.9 %), C_{15:0} (0.8 %), C_{14:0} (0.5 %) and anteiso-C_{14:0} (<0.5 %). The cellular fatty acid content of strain 2Q3S-4-2^T and the reference strain are given in Table. S1. The DNA G+C content of strain 2Q3S-4-2^T was 67.8 mol%. The major components of fatty acids and the polar lipids of the reference strain were similar to those previously reported (Yoon *et al.*, 2007; Kim *et al.*, 2012). The differences in the proportions of fatty acids and the slight differences in the types of polar lipids may be due to the different experimental conditions used.

The extraction of genomic DNA from strain 2Q3S-4-2^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), according to the manufacturer's instructions, and sequenced at the Sangon Biotech Company (<http://www.sangon.com>). The 16S rRNA gene

sequence similarity values between strain 2Q3S-4-2^T and related species were achieved by using the EzTaxon server (<http://eztaxon-e.ezbiocloud.net/>; Chun *et al.*, 2007). Multiple alignments were made using CLUSTAL X (Thompson *et al.*, 1997). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods with MEGA version 5.0 software (Tamura *et al.*, 2011). The topologies of the phylogenetic trees were evaluated by using the bootstrap method with 1000 replications (Felsenstein, 1985).

The almost complete sequence of the 16S rRNA gene (1475 bp) of strain 2Q3S-4-2^T was obtained and a BLAST search showed that strain 2Q3S-4-2^T exhibited the highest levels of 16S rRNA gene sequence similarity with *N. flavida* DS-52^T (96.76 %), followed by *N. panacisegetis* P4-7^T (96.31 %), *N. multipartita* DSM 44233^T (95.99 %) and *N. lactea* DLS-10^T (95.77 %). The phylogenetic trees, based on 16S rRNA gene sequences, generated by using all three tree-making methods showed that strain 2Q3S-4-2^T clustered with all species of the genus *Nakamurella*

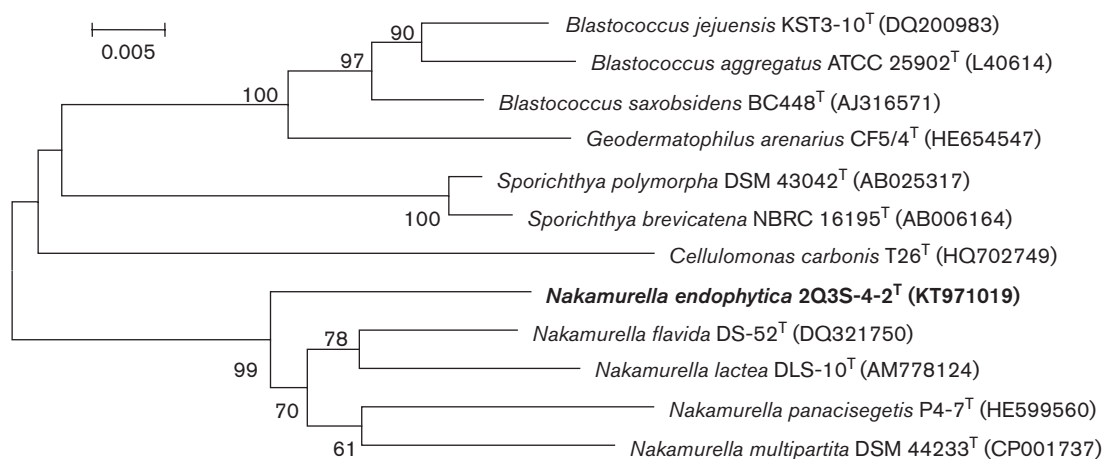


Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences of strain 2Q3S-4-2^T and related strains of species of the genus *Nakamurella*. Numbers at the nodes indicate the level of bootstrap values (>50 %) based on 1000 replications. Bar, 5 nt substitutions per 1000 nt.

with validly published names and that all members in the cluster formed a separate and stable clade. The position of strain 2Q3S-4-2^T did not vary with the method of tree reconstruction used and it was supported by high bootstrap values (Figs 1, S3 & S4). It was clear that strain 2Q3S-4-2^T was phylogenetically affiliated to the genus *Nakamurella*. However, the low levels of sequence similarity with species of the genus *Nakamurella* with validly published names, and the phylogenetic position of strain 2Q3S-4-2^T, indicated that it represents a novel species.

Important chemotaxonomic characteristics of strain 2Q3S-4-2^T, such as *meso*-diaminopimelic acid as the diagnostic diamino acid of the cell-wall peptidoglycan and MK-8(H₄) as the predominant menaquinone, were consistent with those of members of the genus *Nakamurella* (Kim *et al.*, 2012). The DNA G+C content of strain 2Q3S-4-2^T was 67.8 mol%, falling within the 67.5–74.3 mol% range reported for members of the genus *Nakamurella* (Kim *et al.*, 2012). Phylogenetic analysis, based on 16S rRNA gene sequences, revealed that strain 2Q3S-4-2^T could be assigned to the genus *Nakamurella*, but could also be differentiated from the most closely related phylogenetic species of the genus *Nakamurella* based on its phenotypic and chemotaxonomic properties, such as fatty acids and the temperature range for growth. The minimum temperature for growth of *N. flavida* DS-52^T was 4 °C, but strain 2Q3S-4-2^T was unable to grow at this temperature. The dominant fatty acids (>10 %) of strain 2Q3S-4-2^T were iso-C_{16:0} (23.6 %), anteiso-C_{15:0} (19.8 %), anteiso-C_{17:0} (13.1 %) and C_{16:0} (10.7 %), but the major fatty acids of *N. flavida* DS-52^T were anteiso-C_{15:0} (41.4 %) and iso-C_{15:0} (14.0 %). Further phenotypic characteristics also differentiate strain 2Q3S-4-2^T from its most closely related phylogenetic species of the genus *Nakamurella* such as certain enzyme activities, acid production from

some carbohydrates and the utilization of carbon sources (Table 1).

In conclusion, based on phylogenetic analysis, and phenotypic and chemotaxonomic characteristics, strain 2Q3S-4-2^T represents a novel species of the genus *Nakamurella*, for which the name *Nakamurella endophytica* sp. nov. is proposed.

Description of *Nakamurella endophytica* sp. nov.

Nakamurella 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 Gram-stain-positive, aerobic, coccus-shaped and 0.8–1.2 µm in diameter. Substrate mycelia and aerial mycelia are not observed, no diffusible pigments are produced on any tested media. Colonies on ISP 2 agar are circular, smooth and entire, and vivid orange-yellow. Grows well on ISP 2 agar, ISP 3 agar and R2A agar; poor growth occurs on TSA, ISP 7 agar and nutrient agar. No growth occurs on ISP 4 agar or ISP 5 agar. Growth occurs at 20–37 °C (optimum, 28–30 °C), pH 5.0–9.0 (optimum, pH 7.0) and with NaCl concentrations of 0–2 % [w/v (optimum, 0 %)]. No growth occurs at 8 °C and 45 °C. No growth occurs at pH 4.0 and pH 10.0. Cells are positive for catalase activity and the hydrolysis of casein and Tween 20. The methyl red test, nitrate reduction, urease production, production of H₂S, activity of oxidase and the hydrolysis of Tweens 40, 80 and starch are negative. According to the API ZYM test, positive for acid phosphatase, alkaline phosphatase, esterase lipase (C8), α-glucosidase, β-glucosidase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase, weakly positive

for valine arylamidase, negative for *N*-acetyl- β -glucosaminidase, α -chymotrypsin, cystine arylamidase, esterase (C4), α -fucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, lipase (C14), α -mannosidase and trypsin. In API 50CH strips, produces acid from aesculin and weakly produces acid from 5-ketogluconate, D-lyxose, D-ribose, L-rhamnose, salicin, D-tagatose, D-xylose. In API 20NE strips, negative for the assimilation of *N*-acetyl-D-glucosamine, potassium gluconate, capric acid, malic acid, trisodium citrate, adipate, D-mannitol, maltose, D-glucose, L-arabinose, D-mannose and phenylacetate. In the Biolog system, positive for the oxidation of acetoacetic acid, *N*-acetyl-D-glucosamine, *N*-acetyl- β -D-mannosamine, *N*-acetylneuraminic acid, D-arabitol, cellobiose, D-fructose, D-fucose, D-fructose-6-phosphate, D-galactonic acid lactone, D-galactose, galacturonic acid, gelatin, gentiobiose, D-gluconic acid, α -D-glucose, D-glucose-6-phosphate, glucuronamide, D-glucuronic acid, glycerol, L-lactic acid, α -lactose, maltose, D-mannitol, D-mannose, methyl pyruvate, pectin, L-rhamnose, salicin, D-sorbitol, sucrose, trehalose, turanose and Tween 40, but negative for acetic acid, *N*-acetyl-D-galactosamine, D-alanine, γ -amino-butyric acid, L-arginine, D-aspartic acid, L-aspartic acid, bromosuccinic acid, citric acid, dextrin, formic acid, L-fucose, L-glutamic acid, glycyl-L-proline, L-histidine, α -hydroxy-butyric acid, β -hydroxy-DL-butyric acid, inosine, *p*-hydroxy-phenylacetic acid, α -keto-butyric acid, α -keto-glutaric acid, D-lactic acid methyl ester, D-malic acid, L-malic acid, melibiose, 3-methyl-D-glucose, β -methyl-D-glucoside, mucic acid, myo-inositol, propionic acid, L-pyroglutamic acid, quinic acid, raffinose, D-saccharic acid, D-serine, L-serine and stachyose. Cells are sensitive to fusidic acid, lithium chloride, minocycline, niaproof 4, rifamycin SV, sodium bromate, tetrazolium blue, troleandomycin and tolerant to aztreonam, guanidine HCl, lincomycin, nalidixic acid, potassium tellurite, sodium butyrate, 1% (w/v) sodium lactate, tetrazolium violet and vancomycin. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid as the diagnostic diamino acid. The predominant menaquinone is MK-8(H₄). The polar lipids comprise diphosphatidylglycerol, phosphatidylethanolamine, unidentified aminophospholipid, phosphatidylinositol and unidentified phospholipids. The major fatty acids are iso-C_{16:0}, anteiso-C_{15:0}, anteiso-C_{17:0} and C_{16:0}.

The type strain, 2Q3S-4-2^T (= DSM 100722^T = CGMCC 4.7308^T) was isolated from surface-sterilized bark of *Kandelia candel* collected from Cotai Ecological Zones in Macao, PR China. The G+C content of the genomic DNA of the type strain is 67.8 mol%.

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