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

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A Gram-stain-positive, aerobic, coccus-shaped, non-spore-forming actinobacterium, designated strain 2Q3S-4-2T, 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-4-2T 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-4-2T belonged to the genus Nakamurella, sharing highest 16S rRNA gene sequence similarity with Nakamurella flavida DS-52T (96.76%). The DNA G+C content of strain 2Q3S-4-2T 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-C16:0, anteiso-C15:0, anteiso-C17:0 and C16:0. On the basis of the phylogenetic, phenotypic and chemotaxonomic analysis, strain 2Q3S-4-2T represents a novel species of the genus Nakamurella, for which the name Nakamurella endophytica sp. nov. is proposed. The type strain is 2Q3S-4-2T (=DSM 100722T =CGMCC 4.7308T).

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-C15:0, iso-C16:0, anteiso-C15:0 and anteiso-C17:0), and straight-chain saturated (C16:0 and C17:0) or unsaturated (C18:1ω9c) 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-4-2T was isolated from surface-sterilized bark of Kandelia candel. The plant samples were surface-sterilized as described by...
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 Nakamuraella. Polyphasic taxonomic studies showed that strain 2Q3S-4-2^T was distinguishable from species of the genus Nakamuraella 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 VB_1, 0.001 g VB_2, 0.001 g Vpp, 0.001 g VB_1p, 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) H_2O_2. Oxidase activity was assessed by using 1 % (w/v) tetramethyl-p-phenylenediamine (Cappuccino & Sherman, 2002). The hydrolysis of starch, casein, gelatin, Tween 20, 40 and 80, the production of H_2S 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 F_254 plates (Merck) as described by Minnikin et al. (1984); the solvent systems of the first and the second dimensions were chloroform-methanol-water (80 : 18 : 12 : 5, by volume), 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(H_4). 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-4-2ᵀ and its phylogenetic neighbour *N. flavida* DS-52ᵀ

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0.8–1.2</td>
<td>0.6–1.2*</td>
</tr>
<tr>
<td>Colony colour</td>
<td>Vivid orange-yellow</td>
<td>Brilliant yellow</td>
</tr>
<tr>
<td>pH range for growth (optimum)</td>
<td>5.0–9.0 (7.0)</td>
<td>6.0–9.0 (7.0)</td>
</tr>
<tr>
<td>Temperature range for growth (°C)</td>
<td>20–37 (28–30)</td>
<td>4–30 (25)</td>
</tr>
<tr>
<td>NaCl tolerance range (% w/v) (optimum)</td>
<td>0–2 (0)</td>
<td>0–3 (1)</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tween 40</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Carbon source utilization</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>API ZYM results</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Esterase</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Naphthol-AS-BI-phosphohydrolase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cystine arylamidase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Major fatty acids (&gt;10 %)</td>
<td>iso-C₁₆ : ₀, anteiso-C₁₇ : ₀, anteiso-C₁₇ : ₀, C₁₆ : ₀</td>
<td>anteiso-C₁₅ : ₀, iso-C₁₅ : ₀</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>67.8</td>
<td>72.6*</td>
</tr>
</tbody>
</table>

Strains: 1, 2Q3S-4-2ᵀ; 2, *N. flavida* DS-52ᵀ. 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 ZYM 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, β-glucosidase and leucine arylamidase. They were weakly positive for valine arylamidase, but negative for N-acetyl-β-glucosaminidase, β-chymotrypsin, β-fucosidase, x-galactosidase, β-glucuronidase, lipase, z-mannosidase and trypsin. +, Positive; −, negative; w, weakly positive.

contained large amounts of iso-C₁₆ : ₀ (23.6 %), anteiso-C₁₅ : ₀ (19.8 %), anteiso-C₁₇ : ₀ (13.1 %), C₁₆ : ₀ (10.7 %) and small amounts of iso-C₁₅ : ₀ (8.1 %), iso-C₁₇ : ₀ (5.1 %), C₁₇ : ₀ (4.8 %), C₁₈ : ₀ (3.3 %), anteiso-C₁₅ : ₀ 2-OH (2.7 %), iso-C₁₆ : ₀ 2-OH (2.3 %), iso-C₁₅ : ₀ 2-OH (1.5 %), iso-C₁₄ : ₀ (1.3 %), anteiso-C₁₇ : ₀ 2-OH (1.1 %), anteiso-C₁₆ : ₀ (0.9 %), C₁₅ : ₀ (0.8 %), C₁₄ : ₀ (0.5 %) and anteiso-C₁₄ : ₀ (<0.5 %). The cellular fatty acid content of strain 2Q3S-4-2ᵀ and the reference strain are given in Table S1. The DNA G+C content of strain 2Q3S-4-2ᵀ 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-4-2ᵀ 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-4-2ᵀ 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-4-2ᵀ was obtained and a BLAST search showed that strain 2Q3S-4-2ᵀ exhibited the highest levels of 16S rRNA gene sequence similarity with *N. flavida* DS-52ᵀ (96.76 %), followed by *N. panacisegis* P4-7ᵀ (96.31 %), *N. multipartita* DSM 44233ᵀ (95.99 %) and *N. lactea* DLS-10ᵀ (95.77 %). The phylogenetic trees, based on 16S rRNA gene sequences, generated by using all three tree-making methods showed that strain 2Q3S-4-2ᵀ clustered with all species of the genus *Nakamuraella*.
with validly published names and that all members in the cluster formed a separate and stable clade. The position of strain 2Q3S-4-2\textsuperscript{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\textsuperscript{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\textsuperscript{T}, indicated that it represents a novel species.

Important chemotaxonomic characteristics of strain 2Q3S-4-2\textsuperscript{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\textsuperscript{T} was 67.8 mol%, falling within the 67.5–74.3 mol% range of strain 2Q3S-4-2\textsuperscript{T}. The position with validly published names and that all members in the cluster formed a separate and stable clade. The position of strain 2Q3S-4-2\textsuperscript{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\textsuperscript{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\textsuperscript{T}, indicated that it represents a novel species.

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In conclusion, based on phylogenetic analysis, and phenotypic and chemotaxonomic characteristics, strain 2Q3S-4-2\textsuperscript{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 H\textsubscript{2}S, 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
DNA of the type strain is 67.8 mol%. menaquinone is MK-8(H4). The polar lipids comprise diphostatidyglycerol, phosphatidylethanolamine, unidentified aminophospholipid, phosphatidylinositol and unidentified phospholipids. The major fatty acids are iso-C16 : 0, anteiso-C17 : 0 and C16 : 0.

The type strain, 2Q3S-4-2T ( = DSM 100722T = CGMCC 4,7308T) 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%.

Acknowledgements

We are grateful to Environmental Protection Bureau, Government of the Macao SAR for assistance in sampling at Macao’s mangrove wetland. This research was supported by the National Natural Sciences Foundation of China (NSFC, Grant no. 81172963 and Grant no.81373308) and the National Science and Technology Major Project (Grant no. 2012ZX09301-002-001-018) from the Ministry of Science and Technology of China.

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