Amnibacterium endophyticum sp. nov., an endophytic actinobacterium isolated from Aegiceras corniculatum

Fei-Na Li,1 Li Tuo,2 Simon Ming-Yuen Lee,3 Tao Jin,4,5 Shuilin Liao,4,5,6 Wenlian Li,7 Xinyu Yan7 and Cheng-Hang Sun1,*

Abstract

A Gram-stain-positive, aerobic, non-motile, non-spore-forming and short-rod-shaped actinobacterium, designated strain 1T4Z-3T, was isolated from a piece of surface-sterilized branch of Amnibacterium endophyticum which had L-2,4-diaminobutyric acid as the diagnostic cell-wall diamino acid. The diagnostic diamino acid of the cell-wall peptidoglycan is L-2,4-diaminobutyric acid.

During a study on the diversity of cultivable endophytic bacteria from mangrove plants in the Cotai Ecological Zones (22° 08’ 23º N, 113° 33’ 07º E), Macao, China, a brilliant yellow strain, designated strain 1T4Z-3T, was isolated from a piece of surface-sterilized branch of Aegiceras corniculatum. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain 1T4Z-3T should be assigned to the genus Amnibacterium, and a polyphasic taxonomic study showed that strain 1T4Z-3T differed from all existing species within the genus Amnibacterium and represented a novel species of the genus Amnibacterium.

The branch of Aegiceras corniculatum was surface-sterilized as described by Qin et al. [4]. After drying in the hood for 2 days, the surface-sterilized branch was ground into a powder using a micromill and distributed on modified International Streptomyces Project (ISP) 2 agar medium [containing, 1−1 distilled water: 4.0 g glucose, 4.0 g yeast extract powder, 5.0 g malt extract powder, vitamin mixture (0.001 g vitamin B6, 0.001 g vitamin B12, 0.001 g vitamin B3, 0.001 g vitamin B2, 0.001 g phenylalanine, 0.001 g alanine, 0.0005 g biotin), trace salt mixture (0.2 g FeSO4·7H2O, 0.1 g ZnSO4·7H2O, 0.1 g MnCl2·4H2O), 18.0 g agar, pH 7.2] supplemented with 1 % (v/v) plant tissue extract. After incubation at 28 °C for 2 weeks, the colony was picked up and

The genus Amnibacterium, affiliated to the family Microbacteriaceae [1] of the order Micrococcales, was proposed by Kim and Lee [2] with Amnibacterium kyonggiense as the type species. At the time of writing, the genus Amnibacterium contains two validly named species. A. kyonggiense KSL51201-037T (=JCM 16463T), isolated from a stream [2], and Amnibacterium soli PB243T (=JCM 19015T), isolated from grass soil [3]. The predominant menaquinones of species in the genus Amnibacterium are MK-11 and MK-12. The major fatty acids are anteiso-C15:0 and iso-C16:0. The genetic similarity of the 16S rRNA gene sequences showed that strain 1T4Z-3T should be assigned to the genus Amnibacterium, and a polyphasic taxonomic study showed that strain 1T4Z-3T differed from all existing species within the genus Amnibacterium and represented a novel species of the genus Amnibacterium.

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Abbreviations: DPG, diphostydiglycerol; GL, unidentified glycolipid; HPLC, high-performance liquid chromatography; ISP, International Streptomyces Project; L, unidentified lipid; LB, Luria–Bertani; NA, nutrient agar; PG, phosphatidylglycerol; PL, unidentified phospholipid; R2A, Reasoner’s ZA; TSA, tryptic soy agar.

The GenBank accession number for the 16S rRNA gene sequence of strain 1T4Z-3T is MG656998. Four supplementary figures and one supplementary table are available with the online version of this article.
streaked on ISP 2 agar [5] to obtain the pure isolate. A brilliant-yellow-coloured isolate, designated strain 1T4Z-3T, was obtained. After subculturing on ISP 2 agar at 28 °C, the strain was preserved in ISP 2 broth supplemented with 20% (v/v) autoclaved glycerol at −80 °C. To verify the evolutionary position of strain 1T4Z-3T, *A. kyonggiense* JCM 16463T and *A. soli* JCM 19015T (both from the Japan Collection of Microorganisms) were chosen as reference strains for phenotypic characterization and fatty acid analysis under the same experimental conditions.

The Gram-staining test was performed as described by Magee *et al.* [6]. Cell morphology and motility of strain 1T4Z-3T were observed with a light microscope (model BH 2; Olympus) and a transmission electron microscope (JEM-1400; JEOL) after incubation on ISP 2 [5] at 30 °C for 2–3 days. Cultural characteristics were determined by observing the growth of the strain at 30 °C on ISP 2, ISP 4, ISP 5 agars [5], nutrient agar (NA; BD), Reasoner’s 2A (R2A; BD) agar, Luria–Bertani (LB) agar and tryptic soy agar (TSA; Oxoid). Colours of colonies were observed according to colour chips from the ISCC-NBS colour charts standard [7]. Growth was investigated on R2A agar at different temperatures (4, 10, 15, 20, 25, 28, 30, 32, 35, 37, 40 and 45 °C). The pH range for growth was determined at 30 °C in R2A broth adjusted to pH 4.0–12.0 at intervals of 1 pH unit using the buffer systems described by Xu *et al.* [8]. NaCl tolerance for growth was tested at 30 °C on R2A agar with different NaCl concentrations (0, 1, 2, 3, 4, 5, 6 and 7%, w/v). Oxidase activity was tested by using 1% (w/v) tetramethyl-p-phenylenediamine [9]. Catalase activity was tested by using 3% (v/v) H2O2. Hydrolysis of Tweens 20, 40 and 80, production of H2S, gelatin liquefaction, milk coagulation and peptonization were examined as described by Gonzalez *et al.* [10]. Hydrolysis of starch and cellulose was tested using R2A medium (BD) as a basal medium, supplemented with a final concentration of 1% (w/v) starch and cellulose, respectively. The enzyme activities were tested by using the API ZYM kits (bioMérieux) by following the manufacturer’s instructions. Carbon source utilization and acid production from carbon sources were tested using the API ID 32GN and API 50CH kits (bioMérieux), respectively. Other biochemical characteristics were tested by using the API 20NE kits (bioMérieux) according to the manufacturer’s recommendations.

Strain 1T4Z-3T was Gram-stain-positive, aerobic, short-rod-shaped (approximately 0.5–0.7 × 1.0–1.5 μm in size), non-motile and non-spore-forming (Fig. S1, available in the online version of this article). The colonies after 3 days of incubation on ISP 2 medium were 0.2–0.3 mm in diameter, circular with wavy edges, rough and brilliant yellow in colour. Strain 1T4Z-3T displayed good growth on ISP 2 agar, TSA, R2A agar and NA. No growth occurred on LB agar, ISP 4 agar and ISP 5 agar. Strain 1T4Z-3T was able to grow on R2A medium at 10–35 °C (optimum 28–30 °C), at pH 6.0–8.0 (optimum pH 7.0) and in the presence of 0–5% (w/v) NaCl (optimum 0–1% (w/v) NaCl). The detailed physiological and biochemical characteristics of strain 1T4Z-3T are given in Table 1 and the species description.

For phylogenetic analysis, the genomic DNA was extracted from strain 1T4Z-3T using the method described by Li *et al.* [11], and used as the template to amplify the 16S rRNA gene by PCR with primers 27F (5’-AGAGTTTGATCCTGCGCTGAG-3’) and 1492R (5’-GTTACCTTGTACGAC-3’) [12]. The cloning and sequencing of the 16S rRNA gene were performed according to Li *et al.* [13]. The nearly complete sequence of the 16S rRNA gene was compiled with SeqMan (www.ezbiocloud.net/) [15] and NCBI for a BLAST search to obtain the 16S rRNA gene sequence similarity values between strain 1T4Z-3T and the related species. Multiple alignments were made by using the CLUSTAL_X tool in MEGA version 5.0 [16]. Phylogenetic trees were reconstructed using neighbour-joining [17], maximum-likelihood [18] and maximum-parsimony [19] methods with MEGA version 5.0. Evolutionary distances were calculated by using Kimura’s two-parameter model [20]. The topologies of the phylogenetic trees were evaluated by using the bootstrap method with 1000 replications [21].

The almost full-length 16S rRNA gene sequence of strain 1T4Z-3T determined in this study comprised 1480 nucleotides. The results of the BLAST search in EzBioCloud showed that strain 1T4Z-3T showed the high 16S rRNA gene sequence similarities to *A. kyonggiense* JCM 16463T (97.9%) and *A. soli* JCM 19015T (97.3%). The 16S rRNA gene sequence similarities between strain 1T4Z-3T and other members of the family *Microbacteriaceae* were less than 96.4%. The phylogenetic trees based on 16S rRNA gene sequences, generated by using the neighbour-joining algorithm (Fig. 1) showed that strain 1T4Z-3T fell within the lineage of the genus *Amnibacterium* and formed a distinct phyletic line, clustered with *A. kyonggiense* JCM 16463T and *A. soli* JCM 19015T. The position of strain 1T4Z-3T did not vary with the methods of tree reconstruction used and it was supported by high bootstrap values (Figs S2 and S3). It was clear that strain 1T4Z-3T was phylogenetically affiliated to the genus *Amnibacterium*. However, the 16S rRNA similarity values between strain 1T4Z-3T and its closest phylogenetic neighbours were lower than the 98.65%, which is used as the threshold for differentiating two species [22], and the phylogenetic position showed that strain 1T4Z-3T should be assigned to a novel species in the genus *Amnibacterium*.

For whole-cell fatty acid analysis, biomass of both strain 1T4Z-3T and the reference strains were harvested from ISP 2 agar at 30 °C, when the bacterial growth had reached the late-exponential stage [23]. Fatty acid methyl esters were prepared according to the standard protocol described by Sasser [23], analysed by using a gas chromatograph (Agilent 7890B) according to the instructions of the Sherlock Microbial Identiﬁcations System (version 6.2; MIDI), and identiﬁed by the Rtsba 6 database of the Microbial Identiﬁcation System. Biomass for the chemotaxonomic studies except fatty acid analysis was obtained from cultures grown in ISP 2
Table 1. Differential characteristics between strain 1T4Z-3T and members of the genus Amnibacterium
Strains: 1, Amnibacterium kyonggiense JCM 16463T; 2, Amnibacterium soli JCM 19015T. All data shown were obtained in this study unless indicated otherwise noted. All strains were Gram-stain-positive, non-motile, short-rod-shaped and aerobic. All strains displayed good growth on ISP 2 agar, TSA, R2A agar and NA. No growth occurred on ISP 4 agar and ISP 5 agar. Hydrolysis of Tween 40 and starch, and catalase activity were positive. Hydrolysis of cellulose was weakly positive. Hydrolysis of Tween 20 and Tween 80, coagulation and peptonization of milk, liquefaction of gelatin, oxidase activity and H₂S production were negative. In the API 20NE kits, all strains were positive for acetic acid production from glycerol, D-mannopyranoside, inulin, D-glucose, D-xylose, D-fructose, D-mannose, D-mannitol, D-sorbitol, methyl D-D-glucopyranoside, D-glucuronate, gluconate, caprate, adipate, malate, citrate and phenylacetate. In the API ZYM kits, all strains were positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase, but negative for lipase (C14), trypsin, α-chymotrypsin, β-glucoronidase, N-acetyl-β-glucosaminidase and α-fucosidase. In the API 50CH kits, all strains were positive for acid production from glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, methyl D-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, starch, gentiobiose, turanose and D-lyxose, weakly positive for acid production from dulcitol, potassium gluconate and potassium 5-ketogluconate, but negative for acid production from erythritol, D-adenitol, L-sorbose, inositol, methyl α-D-mannopyranoside, inulin, D-fucose, D-arabitol, L-arabitol and potassium 2-ketogluconate. +, Positive; −, negative; w, weakly positive; NA, no data available.

<table>
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<tr>
<th>Characteristic</th>
<th>Source</th>
<th>Stream*</th>
<th>Soil†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
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<td>0.15–0.20×0.25–0.30*</td>
<td>NA</td>
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<tr>
<td>Colony colour</td>
<td>Brilliant yellow</td>
<td>Greenish yellow</td>
<td>Orange yellow</td>
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<td>Growth temperature (°C)</td>
<td>10–35</td>
<td>10–37</td>
<td>10–32</td>
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<td>NaCl tolerance range (% w/v)</td>
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<td>0–2</td>
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<td>Growth on LB medium</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>Enzymic activities (API ZYM):</td>
<td></td>
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<tr>
<td>Alkaline phosphatase</td>
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<td>–</td>
<td>w</td>
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<tr>
<td>α-Mannosidase</td>
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<td>–</td>
<td>–</td>
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<td>API 20NE</td>
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<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Assimilation of (API 20NE):</td>
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<tr>
<td>D-Glucose</td>
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<td>–</td>
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<td>L-Arabinose</td>
<td>w</td>
<td>+</td>
<td>–</td>
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<td>D-Mannose</td>
<td>w</td>
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<tr>
<td>D-Mannitol</td>
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<td>–</td>
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<tr>
<td>Maltose</td>
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<td>–</td>
<td>–</td>
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<td>Acid production from carbon sources (API 50CH):</td>
<td></td>
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<tr>
<td>L-Rhamnose</td>
<td>–</td>
<td>+</td>
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<tr>
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<tr>
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<td>—</td>
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<tr>
<td>D-Tagatose</td>
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<td>w</td>
<td>–</td>
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<tr>
<td>L-Fucose</td>
<td>—</td>
<td>+</td>
<td>–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>71.4</td>
<td>72.7*</td>
<td>71.5*</td>
</tr>
</tbody>
</table>

*Data from other studies indicated as: a, Kim and Lee [2]; b, Jin et al. [3].

broth on a rotary shaker at 28 °C for 4 days. Menaquinones were isolated and purified as described by Collins et al. [24], and then analysed and identified by high-performance liquid chromatography–mass spectrometry according to the method of Guo et al. [25]. The polar lipids were extracted and analysed by two-dimensional thin-layer chromatography on silica gel 60 F₂₅₄ plates (Merck) by using the procedures of Minnikin et al. [26]. The solvent systems of the first and second dimension were chloroform–methanol–water (64:27:5, by vol) and chloroform–methanol–acetic acid–water (80:18:12:5, by vol), respectively. Purified cell walls were prepared using the modified Marfey method of Nozawa et al. [27], and then cell walls were hydrolysed at 110 °C for 10 h with 200 µl of 6 M HCl. The derivatization with L-FDLA was basically performed according to the method described by Fujii et al. [28]. The separation and identification of amino acids in the cell wall were performed using high-performance liquid chromatography (HPLC; Shimadzu) coupled with a diode array detector and a single quadrupole mass spectrometer (LCMS-2020; Shimadzu).
For calculation of the G+C content, the genomic DNA of strain 1T4Z-3T was L-2,4-diaminobutyric acid. The DNA G+C content of strain 1T4Z-3T was 71.4 mol%, which is close to the DNA G+C contents of the reference strains (72.7 % and 71.5 %) [2, 3].

The conclusion drawn from phylogenetic analysis that strain 1T4Z-3T represents a novel species of the genus *Amnibacterium* is also supported by its chemotaxonomic characteristics. The predominant menaquinones, the major fatty acids and the diagnostic cell-wall diamino acid are all in line with the description of the genus *Amnibacterium*. However, distinct differences have been observed between strain 1T4Z-3T and the reference strains. It is notable that strain 1T4Z-3T contained a larger proportion of iso-C\(_{16}\), while the amount of anteiso-C\(_{15}\) was lower than that of the reference strains A. *kyonggiense* JCM 16463\(^T\) (37.4 %) and A. *soli* JCM 19015\(^T\) (52.7 %). The details of the fatty acid profiles of strain 1T4Z-3T and the reference strains are given in Table S1. In the polar lipid profiles obtained from our study, all the strains contained DPG, PG, six GLs and two PLs, this differs from the reports of Kim and Lee and Jin et al. [2, 3], both of which did not report the presence of DPG and GL. Moreover, the L1 and L2 detected in 1T4Z-3T and A. *kyonggiense* JCM 16463\(^T\) were absent in A. *soli* JCM 19015\(^T\). The PL3 detected in 1T4Z-3T was absent in A. *kyonggiense* JCM 16463\(^T\) and A. *soli* JCM 19015\(^T\). The PL4 detected in 1T4Z-3T and A. *soli* JCM 19015\(^T\) was absent in A. *kyonggiense* JCM 16463\(^T\) (Fig. S4).

Furthermore, the conclusion drawn from phylogenetic and chemotaxonomic analysis that strain 1T4Z-3T represents a novel species of the genus *Amnibacterium* is also supported by physiological characteristics including: (i) the high tolerance of NaCl for growth; (ii) the rough surface of the colony. Other characteristics that differentiate strain 1T4Z-3T from members of the genus *Amnibacterium* are shown in Table 1. The novel strain 1T4Z-3T is proposed to represent...
a novel species of the genus *Amnibacterium*, for which the name *Amnibacterium endophyticum* sp. nov. is proposed.

**DESCRIPTION OF AMNIBACTERIUM ENDOPHYTICUM SP. NOV.**

*Amnibacterium endophyticum* (en.do.phy’ti.cum. Gr. pref. *endo* within; Gr. n. *phyton* plant; L. neut. suff. *-icum* adjective suffix used with the sense of belonging to; N.L. neut. adj. *endophyticum* within plant, endophytic, pertaining to the original isolation from plant tissues).

Cells are Gram-stain-positive, non-spore-forming, aerobic, non-motile, short-rod-shaped (0.5–0.7 µm wide and 1.0–1.5 µm long) after incubation for 2 days at 28 °C on ISP 2. Colonies on ISP 2 for 3 days are 0.2–0.3 mm in diameter, circular with wavy edges, rough and brilliant yellow in colour. Good growth occurs on ISP 2 agar, TSA, R2A agar and NA. No growth occurred on LB agar, ISP 4 agar and ISP 5 agar. The growth temperature is between 10 and 35 °C on R2A agar, pH range is from 6.0 to 8.0 on R2A broth and the species can tolerate less than 5 % (w/v) NaCl on R2A agar. The optimum growth on R2A medium occurs at 28–30 °C, pH 7.0 and 0–1 % (w/v) NaCl. Hydrolysis of Tween 40 and starch, catalase activity are positive. Hydrolysis of cellulose is weakly positive. Hydrolysis of Tween 20 and Tween 80, coagulation and peptonization of milk, liquefaction of gelatin, oxidase activity and H₂S production are negative. According to API ZYM strips test results, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-mannosidase (weakly), α-glucosidase and β-glucosidase. Negative for lipase (C14), trypsin, α-chymotrypsin, β-gluconoridase, N-acetyl-β-glucosaminidase and α-fucosidase. In the API 50CH strips, acid production is positive for glycerol, D-arabinose L-arabinose, D-ribose, D-xylene, L-xylene, methyl β-D-xylpyranoside, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, N-acetylgalactosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, melezitose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, potassium gluconate and potassium 5-ketogluconate. Weakly positive for L-sorbose, dulcitol, melibiose and raffinose. Other substrates are negative. In the API ID 32GN test strips, positive results are observed for d-sorbitol. Weakly positive results for L-ribose, N-acetylglucosamine, D-ribose, sucrose, maltose, D-mannitol, D-glucose, salicin, melibiose and L-arabinose. Other substrates are negative. The diagnostic cell-wall diamino acid is L-2,4-diaminobutyric acid. The predominant fatty acids are anteiso-C₁₅:₀ and iso-C₁₆:₀. The predominant menaquinones are MK-11 and MK-12. The major polar lipids contain DPG, PG, six unidentified glycolipids, four unidentified phospholipids and two unidentified lipids.

The type strain, 1T4Z-3T (=KCTC 62053T=CGMCC 1.16279T) was isolated from a piece of branch of *Aegiceras corniculatum* collected from the Cotai Ecological Zones in Macao, China. The G+C content of the genomic DNA of the type strain is 71.4 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


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