Actinomadura syzygii sp. nov., an endophytic actinomycete isolated from the roots of a jambolan plum tree (Syzygium cumini L. Skeels)

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The taxonomic position of an endophytic actinomycete, strain GKU 157T, isolated from the roots of a jambolan plum tree (Syzygium cumini L. Skeels) collected at Khao Khitchakut National Park, Chantaburi province, Thailand, was determined using a polyphasic taxonomic approach. 16S rRNA gene sequence analysis revealed that strain GKU 157T belongs to the genus Actinomadura and formed a distinct phyletic line with Actinomadura chibensis NBRC 106107T (98.6% similarity). Strain GKU 157T formed an extensively branched, non-fragmenting substrate mycelium and aerial hyphae that differentiated into hooked to short spiral chains of about 20 non-motile spores with a warty surface. The cell wall contained meso-diaminopimelic acid and the whole-cell sugars were galactose, glucose, madurose, mannose and ribose. The N-acetyl type of muramic acid was acetyl. Mycolic acids were absent. The phospholipids included phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylinositolmannoside (PIM) and two unknown phospholipids (PLs). The major menaquinone was MK-9(H6) and the predominant fatty acids were C16:0, iso-C16:0, C18:1ω9c, C18:0 and 10-methyl C18:0 (tuberculostearic acid). The genomic DNA G + C content was 73.1 mol%. A combination of DNA–DNA hybridization results and significant differences from related species in cultural, physiological and chemical characteristics indicated that strain GKU 157T represents a novel species of the genus Actinomadura, for which the name Actinomadura syzygii sp. nov. is proposed. The type strain is GKU 157T (=BCC 70456T=NBRC 110399T).

The genus Actinomadura was first described by Lechevalier & Lechevalier (1970a) belonging to the family Thermomonosporaceae, which also includes the genera Actinoallomurus, Actinocorallia, Spirillospora and Thermomonospora (Zhang et al., 2001; Tamura et al., 2009). Members of the genus Actinomadura form an extensively branched, non-fragmenting substrate mycelium and aerial hyphae that differentiate into various spore chain morphologies. These spore chains are short to long, straight, hooked or spiral (one to four turns) with either folded, irregular, smooth, spiny or warty surface ornamentation (Trujillo & Goodfellow, 2012). At the time of writing, the genus Actinomadura comprised 50 recognized species, including the recently described Actinomadura geliboluensis (Sazak et al., 2012), Actinomadura meridiana (Lee, 2012a), Actinomadura rupiterra (Lee, 2012b), Actinomadura sediminis (He et al., 2012) and Actinomadura xylanilytica (Zucchi et al., 2013). A further species, Actinomadura rayonensis has recently been described (Phongsopitanun et al., 2015). The type species of the genus is Actinomadura madurae (Vincent, 1894; Lechevalier & Lechevalier, 1970b).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GKU 157T is KF667496.

One supplementary table and two supplementary figures are available with the online Supplementary Material.
Most species of the genus *Actinomadura* have been isolated from soil (Ara et al., 2008; Tseng et al., 2009; Lee, 2012a, b; Sazak et al., 2012; Zucchi et al., 2013), a few are found in clinical materials, for example *Actinomadura chibensis*, *Actinomadura latina*, *A. madurae*, *Actinomadura pelletieri* and *Actinomadura sp* (Trujillo & Goodfellow, 1997; Hanafy et al., 2006; Yassin et al., 2010), and one strain was reported as an endophyte, *Actinomadura flavala*, from the leaves of a medicinal plant (*Maytenus austroyunanensis*) (Qin et al., 2009). During the course of a study on endophytic actinomycetes from medicinal plants at Khao Kitchakut National Park, Chantaburi province, Thailand, 16 isolates were identified from a jambolan plum tree (*Syzygium cumini* L. Skeels). They were classified to the genera *Streptomyces* (n=12, 75%), *Actinomadura* (n=2, 12.5%), *Nonomuraea* (n=1, 6.25%) and *Streptosporangium* (n=1, 6.25%). A novel species of the genus *Nonomuraea* has been proposed based on this study (Rachniyom et al., 2015). Based on the present polyphasic taxonomic study, we propose another novel species isolated from the same tree, strain GKU 157T, as belonging to the genus *Actinomadura*.

The excised roots of a jambolan plum tree were surface-sterilized and endophytic actinomycetes were isolated as described by Rachniyom et al. (2015). Colonies of endophytic actinomycetes appeared on starch-casein agar (Küster & Williams, 1964) supplemented with ampicillin (100 μg ml⁻¹), penicillin G (2.5 U ml⁻¹), amphotericin B (50 μg ml⁻¹) and cycloheximide (50 μg ml⁻¹) after incubation at 28 °C for 3–4 weeks. Colonies of strain GKU 157T were purified on mannitol-soya agar (Hobbs et al., 1989). The pure culture was maintained in 20 % (v/v) glycerol suspension at −80 °C and as lyophilized cells for long-term preservation.

Genomic DNA was extracted according to the method of Kieser et al. (2000). The 16S rRNA gene was amplified using primers and conditions described by Rachniyom et al. (2015). The PCR product was purified using a Gel/PCR DNA Fragment Extraction kit (Geneaid) and then sent to Macrogen (Korea) for DNA sequencing using the primers described by Rachniyom et al. (2015). The resulting 16S rRNA gene sequence of strain GKU 157T was compared with corresponding sequences of reference type strains available in the EzTaxon-e server (Kim et al., 2012). The sequences of strain GKU 157T and related type strains obtained from the GenBank/EMBL/DDBI database were aligned by using CLUSTAL X version 2.0 (Larkin et al., 2007). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms by MEGA software version 6.0 (Tamura et al., 2013). The topology of the phylogenetic trees was evaluated by bootstrap analysis based on 1000 resampled datasets (Felsenstein, 1985). A distance matrix was generated using Kimura’s two-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset. The values of pairwise 16S rRNA gene sequence similarity among strain GKU 157T and the type strains of related species were determined using the EzTaxon-e server. The almost-complete 16S rRNA gene sequence (1507 nt) of strain GKU 157T was compared with corresponding sequences in public databases via the EzTaxon-e server. The results indicated that strain GKU 157T was a member of the genus *Actinomadura*. It shared highest 16S rRNA gene sequence similarity with *A. chibensis* NBRC 106107T (98.6%; 20 nt differences at 1440 positions), *Actinomadura bangladeshensis* 3-46-b3T (98.5%; 22 nt differences at 1455 positions), *Actinomadura meyerae* A288T (98.2%; 26 nt differences at 1436 positions), *A. geliboluensis* A8036T (98.2%; 27 nt differences at 1464 positions) and *Actinomadura chokoriensis* 3-45-a11T (98%; 29 nt differences at 1459 positions). However, the neighbour-joining and maximum-likelihood trees indicated that strain GKU 157T fell in a lineage with *A. chibensis* NBRC 106107T, *A. pelletieri* NBRC 103052T (97.9 % similarity; 31 nt differences at 444 positions) and *A. meridiana* JCM 17440T (96.9%; 44 nt differences at 1430 positions) (Fig. 1; see also Fig. S1, available in the online Supplementary Material).

The morphological and physiological characteristics of strain GKU 157T were then determined and compared with those of phylogenetically closely related type strains, *A. chibensis* NBRC 106107T, *A. pelletieri* NBRC 103052T and *A. meridiana* JCM 17440T. Cultural characteristics were determined after incubation at 27 °C for 21 days on glucose-yeast extract-malt extract agar (DSMZ medium 65), modified Bennett’s agar (Jones, 1949), oatmeal-nitrate agar (Prauser & Bergholz, 1974) and various International Streptomyces Project (ISP) media (Shirling & Gottlieb, 1966), namely yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7). Mycelium and soluble pigment colours were determined by comparison with colour chips from the Colour Harmony Manual (Jacobson et al., 1958). Spore chain morphology and spore ornamentation of strain GKU 157T were observed by light and scanning electron microscopy (model JSMD-5410; JEOL) using cultures grown on ISP 4 medium at 28 °C for 21 days. The range of temperatures (5–50 °C), pH values (pH 4.0–11.0) and NaCl concentrations (0–7 %, w/v) for growth was examined on nutrient agar (Difco) for 14–21 days. For pH adjustments, the growth medium was amended with 75 mM sodium citrate at the respective pH. Catalase and oxidase activities were observed with 3 % (v/v) hydrogen peroxide solution and 1 % (v/v) tetramethyl-p-phenylenediamine solution, respectively. Acid production from carbohydrates was determined by using the media and methods described by Gordon et al. (1974). Reduction of nitrate was observed using nitrate broth (Difco). Starch hydrolysis was examined on ISP 4 medium. Urease activity was determined based on a colour change in Stuart’s urea agar (Stuart et al., 1945). Gelatin liquefaction was evaluated on gelatin medium (2.0 % glucose, 0.5 % peptone, 20 % similarity among strain GKU 157T and the type strains of related species were determined using the EzTaxon-e server.
Fig. 1. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences (1260 nt) showing the relationship between strain GKU 157^T and all recognized species of the genus *Actinomadura*. *Nonomuraea pusilla* ATCC 27296^T (GenBank accession no. D85491) was used as an outgroup. Asterisks denote branches that were also recovered in the maximum-likelihood tree. Numbers at branch points indicate bootstrap percentages (based on 1000 replications); only values >50% are shown. Bar, 0.005 substitutions per nucleotide position.
Strain GKU 157\textsuperscript{T} grew well on all of the media tested (Table 1; see also Table S1). Substrate mycelia were well developed and the colours were variable depending on the growth medium. Aerial mycelia were observed on ISP 2, ISP 3, ISP 4 and oatmeal-nitrate agar. Pale pink aerial mycelium was found on ISP 2, ISP 3 and ISP 4 media, with abundant sporulation on ISP 4 medium. No soluble pigments were produced on any of the media tested. Strain GKU 157\textsuperscript{T} produced abundantly branched, non-fragmenting substrate mycelium and aerial hyphae, which differentiated into spore chains. Hooked to short spiral (one turn) spore chains consisted of about 20 non-motile spores with a warty surface (Fig. 2). The temperature and pH ranges for growth of strain GKU 157\textsuperscript{T} were 14–40 °C and pH 6–11, with optimum growth at 20–34 °C and

Table 1. Comparison of the phenotypic properties of strain GKU 157\textsuperscript{T} and the type strains of closely related species of the genus \textit{Actinomadura}.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
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</thead>
<tbody>
<tr>
<td>Spore morphology</td>
<td>Hooked to spiral</td>
<td>Hooked to spiral</td>
<td>Hooked to spiral</td>
<td>Hooked</td>
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<tr>
<td>Number of spores (per chain)</td>
<td>About 20</td>
<td>About 20</td>
<td>2–6</td>
<td>About 20</td>
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<td>Colony characteristics on ISP 2 medium:</td>
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<tr>
<td>Growth</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
<td>Moderate</td>
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<tr>
<td>Aerial mycelium</td>
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<td>White</td>
<td>Mustard</td>
<td>None</td>
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<tr>
<td>Substrate mycelium</td>
<td>Light coral red</td>
<td>Amber</td>
<td>Light coral rose</td>
<td>Bamboo</td>
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<tr>
<td>Diffusible pigment</td>
<td>None</td>
<td>+</td>
<td>None</td>
<td>Golden brown</td>
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<tr>
<td>Growth at pH 11</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Growth at 5 % (w/v) NaCl</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Catalase activity</td>
<td>w</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Oxidase activity</td>
<td>+</td>
<td>+</td>
<td>w</td>
<td>+</td>
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<td>Acid production from:</td>
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<tr>
<td>Adonitol</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>Cellobiose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>D-Glucose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>D-Mannose</td>
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<td>+</td>
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<tr>
<td>L-Rhamnose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Gelatin liquefaction</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Milk coagulation</td>
<td>–</td>
<td>w</td>
<td>+</td>
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<td>Milk peptonization</td>
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<td>H\textsubscript{2}S production</td>
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<td>Hypoxanthine decomposition</td>
<td>–</td>
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<td>Utilization of:</td>
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<tr>
<td>Cellobiose</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>D-Mannose</td>
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<td>–</td>
<td>+</td>
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<tr>
<td>D-Sorbitol</td>
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<tr>
<td>Trehalose</td>
<td>+</td>
<td>w</td>
<td>w</td>
<td>+</td>
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<tr>
<td>Dulcitol</td>
<td>–</td>
<td>w</td>
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<tr>
<td>L-Rhamnose</td>
<td>–</td>
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<tr>
<td>Maltose</td>
<td>w</td>
<td>–</td>
<td>–</td>
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<td>Enzyme activities of:</td>
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<tr>
<td>(\alpha)-Glucosidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>(\beta)-Glucosidase</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Strains: 1, GKU 157; 2, \textit{A. chibensis} NBRC 106107\textsuperscript{T}; 3, \textit{A. pelletieri} NBRC 103052\textsuperscript{T}; 4, \textit{A. meridiana} JCM 17440\textsuperscript{T}. All data were obtained in this study. +, Positive; –, negative; w, weakly positive.
pH 8–10. Strain GKU 157T could be differentiated from
the type strains of its phylogenetically closest related species
based on culture characteristics, pH and NaCl tolerance,
acid production from carbohydrates, gelatin liquefaction,
coagulation and peptonization of milk, H2S production,
utilization of carbon sources, and enzyme activities
(Tables 1 and S1). Additional phenotypic properties of
strain GKU 157T are presented in the species description.

For chemotaxonomic studies, freeze-dried cells of strain
GKU 157T were obtained from culture grown in yeast
extract-glucose broth (1 % yeast extract, 1 % glucose;
ph 7.0) on a rotary shaker at 27 °C for 7 days. The
isomer of diaminopimelic acid and reducing sugars in
the cell wall were determined by TLC using whole-cell
hydrolysates according to the methods of Hasegawa
et al. (1983) and Becker et al. (1965), respectively. The N-acyl
type of muramic acid in the peptidoglycan was examined
according to Uchida & Aida (1984). The presence of myco-
lic acids was monitored by TLC following the procedure of
Tomiyasu (1982). Isoprenoid quinones were extracted and
purified by using the method of Collins et al. (1977)
and were determined by LC/MS (JSM-T100LP; JEOL).
Analyses of phospholipids and whole-cell fatty acids
were carried out at the Faculty of Science, King Mongkut’s
Institute of Technology Ladkrabang (KMITL), Thailand.
Phospholipids were extracted and identified by two-
dimensional TLC as described by Minnikin et al. (1984).
Cellular fatty acids were extracted, methylated and analysed
by using the Microbial Identification System (MIDI)
according to the method of Sasser (1990) and the manufac-
turer’s instructions. Methyl esters of cellular fatty acids
were identified by GLC and using the Microbial Identifi-
cation software package (Sherlock version 6.1; MIDI
database RTSBA6). For analysis of the genomic DNA
G+C content, DNA was extracted according to the
method of Marmur (1961) and G+C content was deter-
mined by HPLC according to the procedure of Tamaoka

Chemotaxonomic analyses revealed that strain GKU 157T
exhibited characteristics which were typical of members
of the genus *Actinomadura*. The strain contained *meso-*dia-
mnopimelic acid as the diagnostic diamino acid and
whole-cell sugars were galactose, glucose, malnose, man-
nose and ribose, showing that its possessed cell-wall type III
(Lechevalier & Lechevalier, 1970b) and whole-cell sugar
pattern B (Lechevalier, 1968). The N-acyl type of muramic
acid in the peptidoglycan was acetyl. Mycolic acids were
absent. The predominant menaquinone was MK-9(H4)
(69 %); minor amounts of MK-9(H4) (14 %), MK-9(H4)
(11 %), MK-9(H2) (5 %) and MK-9(H0) (1 %) were also
detected. The diagnostic phospholipids were phosphatidylgly-
cerol (PG), diphosphatidylglycerol (DPG), phospho-
tidylinositol (PI), phosphatidylinositolmannoside (PIM)
and two unknown phospholipids (PLs) (Fig. S2), corre-
sponded to phospholipid type I (Lechevalier et al., 1977).
The major cellular fatty acids were 
C16:0 (29.5 %), iso-C16:0
(19.7 %), C18:0 9c (9.5 %), C18:0
(7.4 %) and 10-methyl
C18:0 (tuberculostearic acid; 6.9 %), corresponding to fatty
acid type 3a (Kroppenstedt et al., 1985); minor amounts of
summed feature 3 (C16:0 7c and/or C16:0 6c; 4.5 %),
C17:0 8c (4.1 %), C17:0
(3.9 %), summed feature 9 (10-
methyl C16:0 and/or iso-C17:0 9c; 2.4 %), iso-C18:0
(2.4 %), summed feature 8 (C18:0 7c and/or C18:0 6c; 1.9 %),
C17:0 6c (1.7 %), C18:0 6c (1.7 %) and C14:0
(1.6 %) were also detected. The genomic DNA G+C content was
73.1 mol %.

The genotypic, morphological and phenotypic characteristics
of strain GKU 157T clearly distinguished it from its closest
phylogenetic neighbours (Tables 1 and S1). Strain GKU
157T possessed long spore chains with warty surface similar
to those of *A. chibensis* NBRC 106107T and *A. meridiana*
JCM 17440T, while *A. pelletieri* NBRC 103052T possessed
short spore chains. However, strain GKU 157T shared lower
16S rRNA gene sequence similarity with *A. meridiana*
JCM 17440T and *A. pelletieri* NBRC 103052T (97.9 and 96.9 %,
respectively) than with *A. chibensis* NBRC 106107T
(98.6 %). Together, 16S rRNA gene sequencing and phylo-
genetic analyses indicated that strain GKU 157T was related
most closely to *A. chibensis* NBRC 106107T with 98 and
95 % bootstrap support with the neighbour-joining
and maximum-likelihood algorithms, respectively (Figs 1
and S1). The validity of a novel species status for strain
GKU 157T was confirmed by DNA–DNA hybridization,
determined fluorometrically using photobiotin-labelled
DNA probes and microplate wells (Ezaki et al., 1989). The
level of DNA–DNA relatedness between strain GKU 157T
and *A. chibensis* NBRC 106107T was examined from two
independent determinations and determined to be
48.8 ± 2.1 %, clearly below the 70 % value considered to be
the threshold for the definition of bacterial species
(Wayne et al., 1987). Based the evidence presented,
strain GKU 157T represents a novel species of the genus

![Fig. 2. Scanning electron micrograph of hooked to short spiral spore chains with warty surface of strain GKU 157T grown on ISP 4 medium at 28 °C for 21 days. Bar, 1 μm.](image-url)
**Actinomadura syzygii sp. nov.**

For which the name *Actinomadura syzygii* sp. nov. is proposed.

**Description of Actinomadura syzygii sp. nov.**

*Actinomadura syzygii* (sy.zy’gi.i. N.L. gen. n. syzygii of Syzygium cumini L. Skeels, the jambolan plum tree from which the type strain was isolated).

Aerobic, Gram-stain-positive, non-acid–alcohol-fast, non-motile actinomycete that forms an extensively branched, non-fragmenting substrate and aerial mycelium. Abundant aerial mycelium is present on ISP 4 medium, which differentiates into hooked to short spiral chains of about 20 non-motile spores with a warty surface. No soluble pigment is produced on any of the media tested. The optimal temperature for good growth is 20–34°C and optimal pH is 8–10. Tolerates up to 4% (w/v) NaCl. Oxidase, DNase and nitrate reduction are positive, while catalase is weakly positive, but urease, citrate utilization, gelatin liquefaction, milk coagulation and peptonization, H₂S and melanin production are negative. Acid is produced from D-glucose, but not from adonitol, cellobiose, D-galactose, D-mannose, D-sorbitol, L-arabinose, L-rhamnose or maltose. Starch, casein and L-tyrosine are degraded, but xanthine and hypoxanthine are not. Cellobiose, D-glucose and trehalose are utilized as sole carbon sources, while maltose is weakly utilized, but D-mannose, raffinose, D-sorbitol, D-xylulose, dulcitol, inulin, L-arabinose, L-rhamnose, myo-inositol and sucrose are not. In the API ZYM system, positive for acid phosphatase, alkaline phosphatase, cysteine arylamidase, esterase (C4), esterase lipase (C8), leucine arylamidase, lipase (C4), N-acetyl-β-glucosaminidase, napthol-AS-BI-phosphohydrodase, trypsin, valine arylamidase, α-chymotrypsin and α-glucosidase, but negative for α-fucosidase, α-galactosidase, α-mannosidase, α-galactosidase, β-glucosidase and β-glucuronidase.

The cell-wall contains meso-diaminopimelic acid and the whole-cell sugars are galactose, glucose, mannoside, mannos and ribose. The N-acetyl type of muramic acid is acetyl. Mycolic acids are absent. The predominant menaquinone is MK-9(H₆); minor amounts of MK-9(H₄), MK-9(H₈), MK-9(H₆) and MK-9(H₄) are present. The phospholipid profile contains phosphatidylglycerol (PG), diphasphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylinositolmannoside (PIM) and two unknown phospholipids (PLs). The major cellular fatty acids are C₁₆:₀ iso-C₁₆:₀, C₁₈:₀ 9c, C₁₈:₀ and 10-methyl C₁₈:₀ (tubercolostearic acid).

The type strain, GKU 157T (=BCC 70456T =NBRC 110399T), was isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels) collected at Khao Khiitchakut National Park, Chantaburi province, Thailand. The genomic DNA G+C content of the type strain is 73.1 mol%.

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**References**


