Actinoallomurus acaciae sp. nov., an endophytic actinomycete isolated from Acacia auriculiformis A. Cunn. ex Benth.

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A novel endophytic actinomycete, strain GMKU 931T, was isolated from the root of a wattle tree, Acacia auriculiformis A. Cunn. ex Benth., collected at Kasetsart University, Bangkok, Thailand. Strain GMKU 931T produced short spiral chains of smooth-surfaced spores on the aerial mycelium. Lysine and meso-diaminopimelic acid were present in the cell-wall peptidoglycan. Whole-cell hydrolysates contained galactose, madurose and mannose. The predominant menaquinones were MK-9(H6) and MK-9(H8). The major fatty acids were iso-C16 : 0 and iso-C16 : 1. The major phospholipids were phosphatidylinositol and phosphatidylglycerol. A phylogenetic analysis based on 16S rRNA gene sequences suggested that strain GMKU 931T forms a distinct phyletic line within the recently proposed genus Actinoallomurus. The significant differences in phenotypic and genotypic data indicate that strain GMKU 931T represents a novel species of the genus Actinoallomurus, for which the name Actinoallomurus acaciae sp. nov. is proposed. The type strain is GMKU 931T (=BCC 28622T =NBRC 104354T =NRRL B-24610T).

Endophytic actinomycetes have recently attracted a lot of attention. Their mutual association with plants may play important roles in protecting the plant from pathogenic infection, promoting plant growth or assisting plant survival during environmental stress (Kunoh, 2002; Hasegawa et al., 2006). The isolation of novel endophytic actinomycetes is expected to lead to the identification of novel bioactive compounds and/or growth-regulating agents as well as novel actinomycete genera and species. Thailand is a natural-resource-rich country and there is a wide range of plant diversity. Therefore, we have established a programme for the isolation and identification of endophytic actinomycetes from plants, including those of important agricultural and medicinal species. In this work, endophytic actinobacteria were isolated from the wattle tree (Acacia auriculiformis A. Cunn. ex Benth.), of which one is described here.

The genus Actinoallomurus (Tamura et al., 2009) is the most recently described genus in the family Thermomonosporaceae (Kroppenstedt & Goodfellow, 1991; Zhang et al., 1998, 2001), which includes Actinocorallia, Actinomadura (Nonomura & Ohara, 1971), Excellospora, Spirillospora and Thermomonospora. Members of the genus Actinoallomurus were found to be phylogenetically related to Actinomadura spadix, which exhibited low 16S rRNA gene sequence similarity with other Actinomadura species (Tamura et al., 2009). Polyphasic investigation also revealed that Actinomadura spadix could be clearly distinguished at the genus level from other Actinomadura species and, therefore, the species was assigned to the genus Actinoallomurus as Actinoallomurus spadix comb. nov. (Tamura et al., 2009). At the same time, other isolates from soil and dung samples, collected from various places in Japan, were chemotaxonomically and phylogenetically characterized and eight more novel species belonging to the genus Actinoallomurus were proposed (Tamura et al., 2009). Actinoallomurus spadix was designated the type species of the genus.

Members of the genus Actinoallomurus contain meso-diaminopimelic acid in the cell wall and madurose as a
Strain GMKU 931T was isolated from a root of *Lechevalier & Lechevalier, 1970; Goodfellow, 1989*. Additionally, the cell-wall peptidoglycan encloses D- and L-lysine, which is an important characteristic to differentiate *Actinoallomurus* species from *Actinomadura* species (Tamura *et al*., 2009). The acyl type of the muramic acid is N-acetyl. The fatty acid profiles include iso-hexadecanoic acid (iso-C<sub>16:0</sub>) as the major component and the phospholipid patterns contain phosphatidylinositol mannoside. The main menaquinones are MK-9(H<sub>6</sub>) and MK-9(H<sub>8</sub>).

Strain GMKU 931T was isolated from a root of *Acacia auriculiformis* A. Cunn. ex Benth. collected at Kasetsart University, Bangkok, Thailand. The root was surface sterilized with 95 % ethanol and 1 % sodium hypochlorite before being ground and spread onto starch-casein agar (Küster & Williams, 1964) supplemented with (ml–1) 100 µg ampicillin, 2.5 U penicillin G and 20 µg ketoconazole. After incubation at 30 °C for 21 days, abundant small, white colonies were observed. The strain was isolated and purified on mannitol soya (MS) agar (Hobbs *et al*., 1989). The pure culture was maintained as a suspension in 20 % glycerol at –80 °C and as lyophilized cells for long-term preservation.

Strain GMKU 931T grew well on International Streptomyces Project (ISP) medium 2 (Shirling & Gottlieb, 1966) and MS agar at 30 °C and it started to produce whitish spores after 14 days. Moderate growth was observed on ISP 3, oatmeal-nitrate agar (1 °C; 3.0 g Quaker white oat, 0.2 g KNO<sub>3</sub>, 0.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 15.0 g agar; pH 7.0) and 1/10 yeast extract-starch agar, poor growth was observed on ISP 5 and there was no growth on ISP 4. No soluble pigment was produced on any of the media tested. No production of melanin pigment was observed on ISP 1 or ISP 7. Morphological characteristics were examined by subculturing the strain on oatmeal-nitrate agar and observing under a light microscope and a scanning electron microscope (JSM 5600 LV; JEOL). Strain GMKU 931T formed short spiral chains of smooth-surfaced spores (Fig. 1). Growth of strain GMKU 931T was determined over the temperature range 5–50 °C in a temperature-gradient incubator over 14 days on ISP 2. Growth was observed at 12–41 °C, with the optimal temperature for good growth being 28–30 °C. The strain was able to grow at pH 5.0–8.0, with optimal growth at pH 6.0–7.0. On ISP 2, the strain was able to tolerate NaCl up to 3 % (w/v); no growth was observed at 4 % NaCl.

Urease activity was determined by a colour change in urea broth (Gordon *et al*., 1974). Hydrolysis of casein and gelatin was evaluated using the media of Gordon *et al*. (1974). Reduction of nitrate was determined using ISP 8 by the method of the ISP (Shirling & Gottlieb, 1966). Catalase and oxidase activities were determined with 3 % (v/v) hydrogen peroxide solution and 1 % tetramethyl-p-phenylenediamine solution, respectively. Strain GMKU 931T showed activity for catalase and urease but not for oxidase. Degradation of gelatin and casein and reduction of nitrate were negative.

Genomic DNA of strain GMKU 931T was extracted from mycelium material scraped from a well-grown culture on ISP 2 according to the protocol described by Kieser *et al*. (2000). The 16S rRNA gene sequence was amplified using primers described by Tajima *et al*. (2001). Amplification was carried out in a thermal cycler (TaKaRa), with an initial incubation step at 94 °C for 1 min, 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1–5 min and a final extension step at 72 °C for 2 min. The PCR product was purified using a QIAquick Gel Extraction kit (Qiagen) and was sequenced directly on an ABI model 3130 automatic DNA sequencer using a BigDye Terminator cycle sequencing kit (Applied Biosystems). An almost-complete 16S rRNA gene sequence of strain GMKU 931T (1468 bp) was preliminarily compared with 16S rRNA gene sequences in the GenBank database, which indicated a close relationship with members of the genus *Actinoallomurus* (Tamura *et al*., 2009). Multiple alignment of the sequences obtained from strain GMKU 931T and the type strains of the nine *Actinoallomurus* species with validly published names, using *Actinomadura madurae* NBRC 14623<sup>T</sup> as an outgroup, was performed using CLUSTAL X version 2 (Larkin *et al*., 2007). A phylogenetic tree was constructed with MEGA version 4.0 (Tamura *et al*., 2007) using the neighbour-joining method (Saitou & Nei, 1987) and the reliability of the tree topology was evaluated by bootstrap analysis with 1000 resamplings (Felsenstein, 1985). The result of the phylogenetic analysis indicated that strain GMKU 931T formed a distinct clade within the genus *Actinoallomurus* (Fig. 2). The closest phylogenetic
neighbours were Actinoallomurus caesius NBRC 103678\textsuperscript{T},
Actinoallomurus amamiensis NBRC 103682\textsuperscript{T} and
Actinoallomurus fulvus NBRC 103680\textsuperscript{T}, with 16S rRNA gene
sequence similarity values of 99.30, 99.20 and 99.11 \%, respectively.

Strain GMKU 931\textsuperscript{T} was analysed chemically using the
methodology for the genus Actinoallomurus (Tamura et al.,
2009). The biomass was obtained after incubation at 27 \textdegree C
for 5 days in ISP 2 broth. Whole-cell amino acids and
sugars were analysed using the method of Hasegawa et al.
(1983) and Becker et al. (1965), respectively. The acyl type
of the cell wall was analysed according to the method of
Uchida & Aida (1984). Phospholipids were extracted and
determined by the method of Minnikin et al. (1984).
Menaquinones were extracted and purified by using the
method of Collins et al. (1977) and isoprene units were
analysed by HPLC using a Jasco 802-SC chromatograph
equipped with a Shiseido CAPCELL PAK C18 column as
described by Tamaoka et al. (1983). Analysis of the fatty
acids was performed according to the procedures for the
Sherlock Microbial Identification System (Microbial ID).
Mycolic acids were analysed by TLC according to the
method of Tomiyasu (1982). The G + C content (mol\%)
of DNA, which was isolated according to the method of
Marmur (1961), was determined by HPLC according to the
amino acids of the peptidoglycan layer of strain GMKU
931\textsuperscript{T} were meso-diaminopimelic acid, lysine, alanine
and glutamic acid. The sugars presented in whole-cell hydro-
ylates were galactose, glucose, madurose, mannose and
ribose. Madurose was the characteristic sugar, indicating
type-B whole-cell sugars (Lechevalier & Lechevalier, 1970).
The N-acyl group of the muramic acid in peptidoglycan
was of the acetyl type. Phosphatidylinositol and phospha-
tidyglycerol were detected as the major phospholipids. The
acyl group of the muramic acid in peptidoglycan
was of the acetyl type. Phosphatidylinositol and phospha-
tidyglycerol were detected as the major phospholipids. The
major menaquinones were MK-9(H\textsubscript{6}) and MK-9(H\textsubscript{8}),
while a small amount of MK-9(H\textsubscript{4}) was also detected.
The predominant fatty acids were iso-C\textsubscript{16:0} (40.9 \%) and
iso-C\textsubscript{16:1} (16 \%). The minor fatty acids were C\textsubscript{16:0 (4.3 \%),
10-methyl C\textsubscript{16:0 (3.4 \%), C\textsubscript{17:0 (3.8 \%), antioiso-C\textsubscript{17:0 (5 \%),
10-methyl C\textsubscript{17:0 (6.9 \%), C\textsubscript{18:0 (4.6 \%) and 10-
methyl C\textsubscript{18:1} (tuberculostearic acid; 3.3 \%). No mycolic
acids were detected. The G + C content of the DNA of
strain GMKU 931\textsuperscript{T} was 70.6 mol\%.

There are a number of phenotypic differences between
strain GMKU 931\textsuperscript{T} and its closest phylogenetic neighbours,
Actinoallomurus caesius NBRC 103678\textsuperscript{T}, Actinoallomurus
amamiensis NBRC 103682\textsuperscript{T} and Actinoallomurus fulvus
NBRC 103680\textsuperscript{T}, including differences in morphological
characteristics, optimal temperature for growth, utilization
of sole carbon sources, degradation abilities and enzymic
activities (Table 1). The significant characteristics that
distinguish strain GMKU 931\textsuperscript{T} from the other three strains
are that strain GMKU 931\textsuperscript{T} does not produce diffusible
pigment on ISP 2, ISP 3 or yeast-starch agar, does not grow
on ISP 4, grows well at 28–30 \textdegree C, does not utilize maltose,
raffinose or gelatin, hydrolyses urea and exhibits x-
glucosidase activity when examined with the API ZYM
enzyme assay.

To examine the finer taxonomic relationships between
strain GMKU 931\textsuperscript{T} and its three closest phylogenetic
neighbours, DNA–DNA hybridization relatedness values
(means of duplicate measurements) were determined
fluorometrically by the method of Ezaki et al. (1989).
The results supported the phenotypic and genotypic data
and confirmed that strain GMKU 931\textsuperscript{T} belongs to a
different species: low DNA–DNA relatedness values were
found between strain GMKU 931\textsuperscript{T} and Actinoallomurus
casu s NBRC 103678\textsuperscript{T} (44 \%), Actinoallomurus amamiensis
NBRC 103682\textsuperscript{T} (43 \%) and Actinoallomurus fulvus
NBRC 103680\textsuperscript{T} (43 \%).

On the basis of the data presented in this study, it is evident
that strain GMKU 931\textsuperscript{T} represents a distinct novel genomic
species belonging to the genus Actinoallomurus. Strain
GMKU 931\textsuperscript{T} is readily distinguished from its closest
phylogenetic neighbours Actinoallomurus caesius,
Actinoallomurus amamiensis and Actinoallomurus fulvus
on the basis of distinct phyletic lines, differences in
phenotypic data and low levels of DNA–DNA relatedness.
The name Actinoallomurus acaciae sp. nov. is proposed.

Description of Actinoallomurus acaciae sp. nov.

Actinoallomurus acaciae (a.ca.ci.ae. L. n. acacia the acacia
tree and also the name of a botanical genus; L. gen. n.
acaciae of Acacia, referring to the isolation of the type
strain from a root of Acacia auriculiformis A. Cunn. ex
Benth.).
Aerobic and Gram-positive. Cells grow well on ISP 2 and MS agar and show moderate growth on ISP 3, oatmeal-nitrate agar and 1/10 yeast-starch agar, forming a well-developed white aerial mycelium that differentiates into short spiral spore chains with smooth surfaces. Neither diffusible pigment nor melanin is produced on any of the media tested. The optimal temperature for growth is 28–30 °C and optimal pH is pH 6.0–7.0. Tolerates up to 3% (w/v) NaCl. Catalase- and urease-positive, oxidase-negative. Nitrate reduction is negative. Hydrolysis of casein, milk and gelatin is negative and degradation of starch is weakly positive. D-Mannitol, L-rhamnose and trehalose are utilized as sole carbon sources but L-arabinose, dulcitol, D-fructose, D-galactose, D-glucose, β-lactose, malate, D-mannitol, raffinose, D-sorbitol, sucrose and D-xylose are not utilized. With the API ZYM enzyme assay,

<table>
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<td>Strong brown</td>
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<td>Good</td>
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<tr>
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<td>Pale yellow to light olive</td>
<td>Pale yellow to strong yellow</td>
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<tr>
<td>Soluble pigment</td>
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<td>None</td>
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<td>Growth on yeast-starch agar</td>
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<td>Moderate</td>
<td>Moderate</td>
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<tr>
<td>Growth</td>
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<td>Aerial mycelium</td>
<td>Pale yellow</td>
<td>Pale yellow</td>
<td>Pale yellow to light olive</td>
<td>Pale yellow to strong yellow</td>
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<tr>
<td>Soluble pigment</td>
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<td>D-Sorbitol</td>
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<td>Urea</td>
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<td>v</td>
<td>–</td>
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<tr>
<td>β-Glucosidase</td>
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acid phosphatase, N-acetyl-β-glucosaminidase, alkaline phosphatase, esterase (C4), 3′- and 2′-glucosidases, β-glucuronidase, leucine aminopeptidase, lipase (C8), α-mannosidase and phosphoamidate are detected; chymotrypsin, cystine aminopeptidase, α-fucosidase, 3′- and β-galactosidases, lipase (C14), trypsin and valine aminopeptidase are not detected. The diagnostic diamino acids of the peptidoglycan are meso-diaminopimelic acid, lysine, alanine and glutamic acid. Whole-cell sugars include galactose, maluctose and mannose. The glycans moieties of the murein is acetylated. The predominant menaquinones are MK-9(H8) and MK-9(H6), with MK-9(H4) as a minor component. The major fatty acids are iso-C16:0 and iso-C16:1ω5c and the minor fatty acids are C16:0, 10-methyl C16:0, C17:0, anteiso-C17:0, 10-methyl C17:0, C18:0 and 10-methyl C18:0. The phospholipid pattern comprises phosphatidylinositol and phosphatidylglycerol. The G+C content of the DNA of the type strain is 70.6 mol%. The type strain, GMKU 931T (=BCC 28622T =NBRC 104354T =NRRL B-24610T), was isolated from a root of Acacia auriculiformis A. Cunn. ex Benth. collected in Kasetsart University, Bangkok, Thailand.

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References


