

Actinoallomurus oryzae sp. nov., an endophytic actinomycete isolated from roots of a Thai jasmine rice plant

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The novel strain GMKU 370^T was isolated from roots of a Thai jasmine rice plant (*Oryza sativa* L. 'KDML 105') collected from Pathum Thani Rice Research Center, Pathum Thani province, Thailand. A phylogenetic analysis based on 16S rRNA gene sequences indicated that strain GMKU 370^T formed a distinct clade within the genus *Actinoallomurus*. Strain GMKU 370^T contained meso-diaminopimelic acid and lysine in the cell-wall peptidoglycan and galactose and madurose as whole-cell sugars. No mycolic acids were detected. The predominant menaquinones were MK-9(H₆) and MK-9(H₈). The polar phospholipids consisted of phosphatidylglycerol and phosphatidylinositol. These chemical properties reveal that strain GMKU 370^T belongs to the genus *Actinoallomurus*. Strain GMKU 370^T is distinct from the phylogenetically closely related type strains *Actinoallomurus iriomotensis* NBRC 103685^T and *Actinoallomurus coprocola* NBRC 103688^T (99.20 % 16S rRNA gene sequence similarity to both) in terms of differences in phenotypic characteristics and DNA–DNA relatedness (51 and 17 %, respectively). It is proposed that strain GMKU 370^T represents a novel species of the genus *Actinoallomurus*, for which the name *Actinoallomurus oryzae* sp. nov. is proposed. The type strain is GMKU 370^T (=BCC 31373^T =NBRC 105246^T).

A programme of isolation and identification of endophytic actinomycetes from Thai plants has been established in order to search for novel strains and bioactive compounds from this under-researched habitat. In the process, novel taxa at the genus/species level have been discovered from strains isolated from medicinal and agricultural plants (Duangmal *et al.*, 2008, 2009; Thamchaipenet *et al.*, 2010), including Thai glutinous rice (Indananda *et al.*, 2010). Thai local rice cultivars are a very promising resource to look for novel endophytic actinomycetes. In this work, an endophytic actinomycete was isolated from roots of a Thai jasmine rice plant and was found to represent a novel species of the genus *Actinoallomurus*.

The genus *Actinoallomurus* is characterized by cell walls that contain meso-diaminopimelic acid and lysine and

contain madurose and galactose as characteristic sugars in whole-cell hydrolysates (Tamura *et al.*, 2009). The acyl type of the muramic acid is *N*-acetyl. Fatty acid profiles include iso-hexadecanoic acid (iso-C_{16:0}) as the major component and the phospholipid pattern is made up of phosphatidylglycerol and diphosphatidylglycerol (phospholipid pattern type PI). Principal menaquinones are MK-9(H₆) and MK-9(H₈). At the time of writing, the genus contains 10 species with validly published names (Tamura *et al.*, 2009; Thamchaipenet *et al.*, 2010).

Strain GMKU 370^T was isolated from roots of a Thai jasmine rice plant (*Oryza sativa* L. 'KDML 105') collected from Pathum Thani Rice Research Center, Pathum Thani province, Thailand. Excised roots were surface-sterilized using serial treatments of 95 % (v/v) ethanol for 10 min, 1 % (w/v) sodium hypochlorite for 15 min and 10 % (w/v) NaHCO₃ for 10 min. The roots were then ground and spread onto starch casein agar (SCA; Küster & Williams, 1964) supplemented with 2.5 U penicillin G ml⁻¹ and 50 mg cycloheximide ml⁻¹. Colonies of endophytic actinomycetes appeared on the medium after incubation

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A supplementary table is available with the online version of this paper.

at 30 °C for 4–5 weeks. Colonies were isolated and purified on mannitol soya agar (MS agar; Hobbs *et al.*, 1989). Pure cultures were maintained as 20% (v/v) glycerol suspensions at –80 °C or as lyophilized hyphae for long-term preservation.

Cultural characteristics of strain GMKU 370^T were examined by growing the strain on yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), nutrient agar (NA), oatmeal-nitrate agar (JCM medium 52), potato dextrose agar (PDA) and 1/10 yeast extract-starch (YS) agar. The colour of mycelium was determined by comparing its colour with colour chips from the *Color Harmony Manual* (Jacobson *et al.*, 1958). After incubation for 2 weeks at 27 °C, moderate growth was observed on ISP 3, oatmeal-nitrate agar and YS agar. Poor growth was detected on ISP 2 and ISP 5. No growth was found on ISP 4. Strain GMKU 370^T grew well on PDA and NA but did not produce spores. No soluble pigment was produced on any of the media tested. Whitish aerial mycelium was produced on ISP 3 and oatmeal-nitrate agar after 3 weeks of growth. Spore morphology of strain GMKU 370^T was examined using scanning electron microscopy (JSM5600; JEOL), showing short spiral chains of smooth-surfaced spores (Fig. 1).

Sequencing of the 16S rRNA gene was performed to identify strain GMKU 370^T at the genus level. Total DNA was extracted and purified by the method of Kieser *et al.* (2000). The 16S rRNA gene was amplified in a TaKaRa thermal cycler using primers described by Tajima *et al.* (2001). The PCR product was purified using a QIAquick gel extraction kit (Qiagen) and 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

370^T (1468 bp) was preliminarily compared with 16S rRNA gene sequences in the GenBank database and this comparison indicated a close relationship with members of the genus *Actinoallomurus* (Tamura *et al.*, 2009). Multiple alignment of sequences obtained from the 10 *Actinoallomurus* species with validly published names (Tamura *et al.*, 2009; Thamchaipenet *et al.*, 2010) and strain GMKU 370^T (using the sequence of *Actinomadura madurae* NBRC 14623^T as an outgroup) was performed using CLUSTAL_X, version 2 (Larkin *et al.*, 2007). Phylogenetic trees were deduced using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Eck & Dayhoff, 1966) methods and trees were reconstructed by using NJPlot (Perrière & Gouy, 1996), PHYLIP 3.68 and MEGA 4 (Tamura *et al.*, 2007), respectively. The resultant neighbour-joining tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. Evolutionary distances were computed by using Kimura's two-parameter method (Kimura, 1980). The result of the phylogenetic analysis indicated that strain GMKU 370^T formed a clade with the members of the genus *Actinoallomurus* (Fig. 2). The closest phylogenetic neighbours were *Actinoallomurus coprocola* TT04-09^T and *Actinoallomurus iriomotensis* TT02-47^T, with 99.20% 16S rRNA gene sequence similarity to both type strains, corresponding to 11 nucleotide substitutions.

In order to clarify the taxonomic relationships between strain GMKU 370^T and closely related species, genomic DNA of strain GMKU 370^T and the type strains *A. iriomotensis* NBRC 103685^T and *A. coprocola* NBRC 103688^T was extracted and purified according to the method of Saito & Miura (1963) for use in DNA–DNA hybridization. DNA–DNA hybridization was performed by the photobiotin-labelling method of Ezaki *et al.* (1989) at 54 °C using a Cytofluor multiwell plate-reader series 4000.

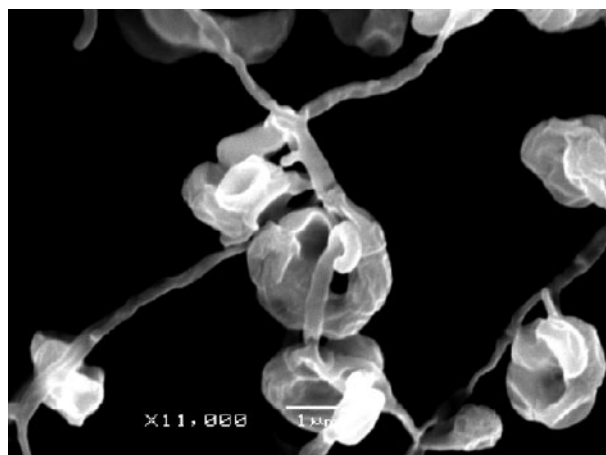


Fig. 1. Scanning electron micrograph of spores of *Actinoallomurus oryzae* sp. nov. GMKU 370^T grown on oatmeal-nitrate agar at 27 °C for 5 weeks. Bar, 1 μm.

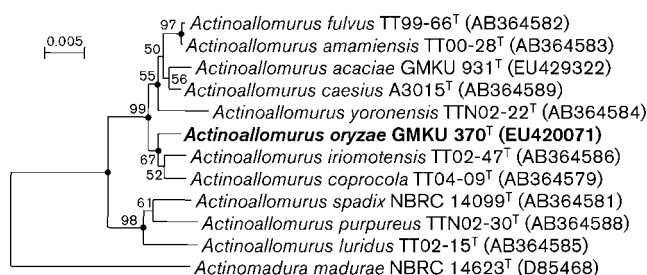


Fig. 2. Phylogenetic tree constructed by using the neighbour-joining method based on almost-complete 16S rRNA gene sequences, showing relationships between strain GMKU 370^T and all members of the genus *Actinoallomurus*. The sequence of *Actinomadura madurae* NBRC 14623^T was used as an outgroup. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. Solid circles indicate corresponding nodes that were also recovered from maximum-likelihood and maximum-parsimony trees. Bar, 0.005 substitutions per site.

DNA–DNA relatedness between *A. coprocola* NBRC 103688^T and *A. iriomotensis* NBRC 103685^T and strain GMKU 370^T was 17 and 51% (means of duplicate determinations), respectively. Thus, the results clearly indicated that strain GMKU 370^T does not belong to either *A. coprocola* or *A. iriomotensis*, as these values are well below the threshold value of 70% suggested for the definition of bacterial species according to Wayne *et al.* (1987).

To analyse the chemical characteristics of strain GMKU 370^T, biomass was prepared by growing the strain in 1% (w/v) potato dextrose broth containing 1% (w/v) yeast extract in an orbital shaker (200 r.p.m.) at 27 °C for 14 days and then cells were freeze-dried. Isomers of diaminopimelic acid in the cell wall were determined by TLC according to the method of Hasegawa *et al.* (1983). The acyl type of muramic acids in the cell wall was analysed by using the method of Uchida & Aida (1984). Phospholipids were extracted and determined by the method of Minnikin *et al.* (1984). Whole-cell sugars were analysed according to the method of Becker *et al.* (1965). Menaquinones were extracted and purified by 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 (Tamaoka *et al.*, 1983). Mycolic acids were determined by TLC, using the method of Tomiyasu (1982). The cellular fatty acid composition was analysed by TechnoSuruga Co., Ltd according to the instructions of the Microbial Identification System (MIDI) by using a gas chromatograph (model HP6890; Hewlett Packard) (Sasser, 1990). DNA G+C content was determined by HPLC as described by Tamaoka & Komagata (1984). The results of chemical analysis indicated that strain GMKU 370^T has chemotaxonomic markers typical of members of the genus *Actinoallomurus* (Tamura *et al.*, 2009). The diagnostic amino acids of the peptidoglycan layer of strain GMKU 370^T were *meso*-diaminopimelic acid and lysine. The sugars present in whole-cell hydrolysates 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 the peptidoglycan was of the acetyl type. The phospholipids included phosphatidylglycerol and phosphatidylinositol, representing type PI (Lechevalier *et al.*, 1977). The major menaquinones were MK-9(H₆) and MK-9(H₈); minor components were MK-9(H₂) and MK-9(H₄), while a small amount of MK-9(H₀) was also detected. The predominant fatty acid was iso-C_{16:0} (Supplementary Table S1, available in IJSEM Online). No mycolic acids were detected. The DNA G+C content of strain GMKU 370^T was 65.4 mol%.

The range of temperature for growth was determined on PDA in a temperature gradient incubator. NaCl tolerance was studied on PDA containing NaCl at final concentrations of 1–7% (w/v). The range of pH was studied on PDA at pH 4–10. Reduction of nitrate and production of melanin pigments were determined by the method of Shirling &

Gottlieb (1966). Catalase and oxidase activities were determined with a 3% (v/v) hydrogen peroxide solution and 1% (v/v) tetramethyl-*p*-phenylenediamine solution, respectively. Hydrolysis of starch was determined as described by Gordon *et al.* (1974). Utilization of carbohydrates as sole carbon sources was tested on carbon utilization medium (ISP 9) (Shirling & Gottlieb, 1966). Utilization of casein, gelatin, hypoxanthine and xanthine, peptonization of milk and acid production from carbohydrates were assessed according to the method of Gordon *et al.* (1974). Strain GMKU 370^T was able to grow at 21–45 °C, with optimal growth at 32–39 °C, at pH 5.0–9.0 and in 1–4% NaCl. Strain GMKU 370^T did not reduce nitrate or peptonize milk. No production of melanin pigment was determined on tryptone-yeast extract broth (ISP 1) or tyrosine agar (ISP 7). The strain did not utilize hypoxanthine, xanthine or starch but degraded casein and gelatin poorly. The strain showed catalase and oxidase activities. Strain GMKU 370^T was able to use dulcitol, D-galactose, *myo*-inositol, lactose, maltose, D-mannose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose and D-xylose. Acids were produced from D-galactose, D-glucose and raffinose.

Phenotypic differences between strain GMKU 370^T and its phylogenetically closest relatives, *A. coprocola* NBRC 103688^T and *A. iriomotensis* NBRC 103685^T, are shown in Table 1. Based on the chemotaxonomic and phylogenetic analysis, it is confirmed that strain GMKU370^T belongs to the genus *Actinoallomurus*. It is also evident from physiological characteristics and levels of DNA–DNA relatedness that strain GMKU 370^T is distinct from the type strains of phylogenetically closely related species. It is therefore proposed that strain GMKU 370^T represents a novel species of the genus *Actinoallomurus*, for which the name *Actinoallomurus oryzae* sp. nov. is proposed.

Description of *Actinoallomurus oryzae* sp. nov.

Actinoallomurus oryzae (o.ry'za.e. L. gen. n. *oryzae* of rice, referring to the isolation of the type strain from roots of Thai jasmine rice).

Aerobic and Gram-stain-positive. Grows well on PDA and NA and shows moderate growth on ISP 3, oatmeal-nitrate agar and 1/10 yeast extract-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. The optimal temperature and pH for growth are 32–39 °C and pH 5.0–9.0. Grows in 0–4% NaCl. Catalase- and oxidase-positive. Nitrate reduction is negative. Hydrolysis of casein and gelatin is weakly positive. Degradation of hypoxanthine, starch and xanthine is negative. No peptonization of milk. Dulcitol, D-galactose, *myo*-inositol, lactose, maltose, D-mannose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose and D-xylose are utilized as sole carbon sources but L-arabinose, D-fructose, D-glucose and D-mannitol are not. Acid is produced from D-galactose, D-glucose and raffinose but not from L-arabinose, dulcitol, D-fructose, *myo*-

Table 1. Phenotypic characteristics that differentiate strain GMKU 370^T from the type strains of phylogenetically closely related *Actinoallomurus* species

Data for reference strains were obtained in this study or taken from Tamura *et al.* (2009). +, Positive; –, negative; w, weakly positive; NA, not applicable.

Characteristic	GMKU 370 ^T	<i>A. coprocola</i> NBRC 103688 ^T	<i>A. iriomotensis</i> NBRC 103685 ^T
Growth on ISP 2			
Growth	Poor	Absent	Good
Colour of substrate mycelium	Pale yellow–brown	NA	Moderate orange–yellow
Soluble pigment	None	NA	None
Growth on ISP 3			
Growth	Moderate	Good	Moderate
Colour of substrate mycelium	White	Grey purplish red	Light yellow to moderate orange
Soluble pigment	None	Moderate to strong yellow	None
Growth on ISP 4			
Growth	Absent	Moderate	Poor
Colour of substrate mycelium	NA	Pale yellow	Light yellow
Soluble pigment	NA	None	None
Growth on YS agar			
Growth	Moderate	Moderate	Poor
Colour of substrate mycelium	White	Pale yellow	White
Soluble pigment	None	Pale green	None
Temperature for growth (°C)	21–45	20–37	20–37
Growth at 4% NaCl	w	–	w
Growth at pH 8–9	+	w	+
Utilization of:			
L-Arabinose	–	+	+
D-Fructose	–	+	+
D-Glucose	–	+	+
D-Mannitol	–	+	+
Hydrolysis of:			
Starch	–	+	+
Gelatin	w	+	+

inositol, lactose, D-mannitol or L-rhamnose. Contains *meso*-diaminopimelic acid and lysine in the cell-wall peptidoglycan. Whole-cell sugars include galactose, glucose, madurose, mannose and ribose. The glycan moiety of the murein is acetylated. Predominant menaquinones are MK-9(H₆) and MK-9(H₈); minor menaquinones are MK-9(H₄), MK-9(H₂) and MK-9(H₀). No mycolic acids. The major fatty acid is iso-C_{16:0}. Main phospholipids are phosphatidylglycerol and phosphatidylinositol. The DNA G + C content of the type strain is 65.4 mol%.

The type strain, strain GMKU 370^T (=BCC 31373^T =NBRC 105246^T), was isolated from roots of a Thai jasmine rice plant (*Oryza sativa* L. 'KDML 105') collected from Pathum Thani Rice Research Center, Pathum Thani province, Thailand.

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