Actinophytocola oryzae gen. nov., sp. nov., isolated from the roots of Thai glutinous rice plants, a new member of the family *Pseudonocardiaceae*

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A novel endophytic actinomycete, strain GMKU 367^T, was isolated from roots of Thai glutinous rice plants (*Oryza sativa* L. 'RD6') collected from Pathum Thani Rice Research Center, Pathum Thani province, Thailand. Strain GMKU 367^T formed cylindrical spores on aerial mycelium, but sporangium-like structures and fragmentation of substrate mycelium were not observed. The cell-wall amino acids contained meso-diaminopimelic acid, alanine, glutamic acid and acetylated muramic acid. The whole-cell sugars were arabinose, galactose, mannose, rhamnose and ribose. Major fatty acids were iso-C_{15:0}, iso-C_{16:0} and C_{16:0}. The diagnostic menaquinone was MK-9(H₄). The polar phospholipids were phosphatidylethanolamine and hydroxyphosphatidylethanolamine. The G+C content of the genomic DNA was 71.1 mol%. Phylogenetic analyses based on 16S rRNA gene sequence data indicated that strain GMKU 367^T differed from members of the family *Pseudonocardiaceae*. On the basis of the evidence presented in this polyphasic study, it is proposed that strain GMKU 367^T represents a novel species in a new genus in the family *Pseudonocardiaceae*, with the name *Actinophytocola oryzae* gen. nov., sp. nov.; the type strain of *Actinophytocola oryzae* is GMKU 367^T (=BCC 31372^T =NBRC 105245^T).

The family *Pseudonocardiaceae* was firstly described by Embley *et al.* (1988) and its description was emended by Stackebrandt *et al.* (1997) on the basis of 16S rRNA gene sequence analysis. The family currently comprises 16 genera with validly published names, including *Actinoalloteichus* (Tamura *et al.*, 2000), *Actinomycetospora* (Jiang *et al.*, 2008), *Allokutzneria* (Labeda & Kroppenstedt, 2008), *Amycolatopsis* (Lechevalier *et al.*, 1986), *Crossiella* (Labeda, 2001), *Goodfellowiella* (Labeda *et al.*, 2008), *Kibdelosporangium* (Shearer *et al.*, 1986), *Kutzneria* (Stackebrandt *et al.*, 1994), *Prauserella* (Kim & Goodfellow, 1999), *Pseudonocardia* (Henssen, 1957), *Saccharomonospora* (Nonomura & Ohara, 1971), *Saccharopolyspora* (Lacey & Goodfellow, 1975), *Sciscionella* (Tian *et al.*, 2009), *Streptoalloteichus* (Tomita *et al.*, 1987), *Thermobispora* (Wang *et al.*, 1996) and

Thermocrispum (Korn-Wendisch *et al.*, 1995). During the search for novel actinomycetes from varieties of local Thai rice plant cultivars, an endophytic actinomycete, strain GMKU 367^T, was isolated. Identification and characterization of strain GMKU 367^T and its placement in the family *Pseudonocardiaceae* as a representative of a novel species in a new genus are reported here.

Strain GMKU 367^T was isolated from roots of Thai glutinous rice plants (*Oryza sativa* L. 'RD6') collected from Pathum Thani Rice Research Center, Pathum Thani province, Thailand. The excised roots were surface-sterilized using serial treatments of 95% ethanol for 10 min, 1% sodium hypochlorite for 15 min and 10% NaHCO₃ for 10 min. The roots were then ground and spread onto starch-casein agar (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 at 30 °C for 4–5 weeks. Cells of strain GMKU 367^T were isolated and purified on mannitol-soy agar (Hobbs *et al.*, 1989). The

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pure culture was maintained as a 20 % glycerol suspension at -80°C or as lyophilized cells for long-term preservation.

To study cultural characteristics, strain GMKU 367^T was grown on several media, namely yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), oatmeal-nitrate agar (JCM medium 52) and 1/10 yeast extract-starch agar. Mycelium and soluble pigment colours were determined by comparison with colour chips from the *Color Harmony Manual* (Jacobson *et al.*, 1958). In order to examine morphological characteristics, strain GMKU 367^T was grown on humic acid-vitamin agar (HV medium; Hayakawa & Nonomura, 1987), tap water agar and sucrose-nitrate agar for 8 weeks at 27°C ; spore morphology was then observed using light and scanning electron microscopy (JSM 5600; JEOL).

Physiological characteristics of strain GMKU 367^T were determined as follows. The temperature range for growth was determined on ISP 2 in a temperature gradient incubator. Reduction of nitrate and production of melanin pigments were determined according to Shirling & Gottlieb (1966). Catalase and oxidase activities were determined with a 3 % (v/v) hydrogen peroxide solution and 1 % 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 by using ISP 4 without soluble starch, since strain GMKU 367^T did not grow on carbon utilization medium (ISP 9) (Shirling & Gottlieb, 1966). Utilization of benzoate, citrate, xanthine and hypoxanthine and acid production from carbohydrates were assessed according to the methods of Gordon *et al.* (1974).

To analyse chemotaxonomic characteristics, biomass samples were prepared by growing the strain in yeast extract-glucose broth at 27°C for 7 days and then freeze-drying the cells. Isomers of diaminopimelic acid in the cell wall were determined by TLC according to the method of Hasegawa *et al.* (1983). Peptidoglycan of cell walls was isolated and purified according to the method of Schleifer & Kandler (1972). Purified samples were hydrolysed (Becker *et al.*, 1965) and analysed by TLC (Hasegawa *et al.*, 1983) and the Pico Tag method using HPLC (Waters). The cell-wall acyl type was analysed by using the method of Uchida & Aida (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 detected by TLC using the method of Tomiyasu (1982). The cellular fatty acid composition was analysed by TechnoSuruga Laboratory (Japan) according to the instructions of the Microbial Identification System (MIDI) by GC (model HP6890; Hewlett Packard) (Sasser, 1990). DNA G+C content was

determined by HPLC as described by Tamaoka & Komagata (1984).

Morphological analysis of cells of strain GMKU 367^T revealed cylindrical spores on aerial mycelium, but no sporangium-like structures or fragmentation of substrate mycelium (Fig. 1). Good growth was observed on oatmeal agar and oatmeal-nitrate agar. The cell-wall amino acids of strain GMKU 367^T contained *meso*-diaminopimelic acid, alanine and glutamic acid in a molar ratio of 1.0:1.2:1.0. The peptidoglycan was of the acetyl type. No mycolic acids were detected. Whole-cell sugars were arabinose, galactose, mannose, rhamnose and ribose. The major fatty acids (above 10 %) were iso-C_{16:0} (40.24 %), iso-C_{15:0} (12.17 %) and C_{16:0} (10.39 %); minor fatty acids (3–10 %) were iso-C_{16:0} 2-OH (7.67 %), C_{15:0} (3.39 %), iso-C_{16:1} (3.27 %) and iso-C_{17:0} (3.15 %). MK-9(H₄) was the only diagnostic menaquinone. The polar phospholipids were phosphatidylethanolamine and hydroxyphosphatidylethanolamine. The DNA G+C content of strain GMKU 367^T was 71.1 mol%.

The 16S rRNA gene from strain GMKU 367^T was amplified and sequenced. Total DNA of strain GMKU 367^T was extracted and purified according to Kieser *et al.* (2000). PCR amplification of the 16S rRNA gene was carried out using primers and PCR conditions described previously (Kataoka *et al.*, 1997). The almost-complete 16S rRNA gene sequence of strain GMKU 367^T was determined by direct sequencing of the PCR product (1444 bp). Initially, the sequence was compared with those available in GenBank; comparisons revealed that strain GMKU 367^T belonged to the suborder *Pseudonocardineae*. The nearly complete 16S rRNA gene sequence of strain GMKU 367^T was aligned with those of representative type strains of members of the other 23 genera of the suborder *Pseudonocardineae* using CLUSTAL_X version 2 (Larkin *et al.*, 2007). Phylogenetic trees were deduced using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood

Fig. 1. Scanning electron micrograph of growth of strain GMKU 367^T on HV medium after incubation at 27°C for 8 weeks. Bar, 2 μm .

(Felsenstein, 1981) and maximum-parsimony (Eck & Dayhoff, 1966) methods and constructed by NJPLOT (Perrière & Gouy, 1996), PHYLIP 3.68 and MEGA4 (Tamura *et al.*, 2007), respectively. The resultant neighbour-joining tree topology was evaluated by levels of bootstrap support based on analysis of 1000 resampled datasets (Felsenstein, 1985). Evolutionary distances were computed by using Kimura's two-parameter method (Kimura, 1980).

On the basis of 16S rRNA gene sequence analysis, strain GMKU 367^T showed the highest sequence similarity to *Kibdelosporangium aridum* subsp. *aridum* DSM 43828^T (95.5%), *Streptoalloteichus hindustanus* IFO 15115^T (95.4%), *Actinokineospora enzanensis* IFO 16517^T (95.4%) and *Amycolatopsis nigrescens* CSC17Ta-90^T (95.3%), belonging to the suborder *Pseudonocardineae*. Sequence similarities higher than 95% were not observed between strain GMKU 367^T and any other type strains of species in the suborder *Pseudonocardineae*. Although high sequence similarity was found between strain GMKU 367^T and members of the genera *Actinokineospora* and *Streptoalloteichus*, which belong to the family *Actinosynnemataceae*, strain GMKU 367^T was phylogenetically separate from both genera (Fig. 2). It is evident from the phylogenetic tree that strain GMKU 367^T

formed a distinct subclade within members of the family *Pseudonocardaceae* and was recovered as a sister group of the genus *Kibdelosporangium* (Fig. 2). Although the bootstrap value of the neighbour-joining tree at the corresponding node was moderate (43%), the close relationship was solidly supported by maximum-likelihood and maximum-parsimony trees (Fig. 2).

The morphological and chemotaxonomic characteristics of strain GMKU 367^T that distinguish it from closely related members in the family *Pseudonocardaceae* are shown in Table 1. Scanning electron microscopic observations of strain GMKU 367^T showed cylindrical spores on aerial mycelium, but did not reveal sporangium-like structures or fragmentation of substrate mycelium (Fig. 1), which enables it to be differentiated from members of the genus *Kibdelosporangium*, the nearest neighbouring genus, and phylogenetically closely related genera. The phospholipid profile of strain GMKU 367^T was clearly distinct from those of members of closely related genera as it contained both phosphatidylethanolamine and hydroxyphosphatidylethanolamine, but lacked phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol and phosphatidyl-inositol. The fatty acid profile of strain GMKU 367^T was

Fig. 2. Phylogenetic tree for taxa of the suborder *Pseudonocardineae* constructed using the neighbour-joining method based on almost complete 16S rRNA sequences to display the taxonomic position of the strain GMKU 367^T. *Streptomyces ambofaciens* ATCC 23877^T was used as the root organism. Numbers at nodes indicate levels of bootstrap support (%) based on neighbour-joining analysis of 1000 resampled datasets; only values above 50% are shown. Solid circles indicate nodes that were also recovered from maximum-likelihood and maximum-parsimony trees; asterisks indicate nodes that were recovered from maximum-parsimony trees. Bar, 0.02 substitutions per site.

Table 1. Morphological and chemotaxonomic profiles of *Actinophytocola* gen. nov. and phylogenetically related genera

Genera: 1, *Actinophytocola* gen. nov. (data from this study); 2, *Amycolatopsis* (Lechevalier *et al.*, 1986; Lee & Hah, 2001); 3, *Kibdelosporangium* (Shearer *et al.*, 1986; Labeda & Kroppenstedt, 2008); 4, *Pseudonocardia* (Warwick *et al.*, 1994; Reichert *et al.*, 1998; Huang *et al.*, 2002; Park *et al.*, 2008); 5, *Saccharopolyspora* (Korn-Wendisch *et al.*, 1989); 6, *Thermocrisum* (Korn-Wendisch *et al.*, 1995). –, Absent; +, present; v, variable.

Character	1	2	3	4	5	6
Morphology						
Aerial hyphae	+	v	+	v	+	+
Fragmented mycelium	–	+	+	v	+	–
Sporangium-like structures	–	–	+	–	–	+
Whole-cell sugars*	Ara, Gal, Man, Rha, Rib	Ara, Gal	Ara, Gal, (Mad)	Ara, Gal	Ara, Gal	Ara, (Gal), Glc, Man
Phospholipids†	<u>PE</u> , OH-PE	<u>PE</u> , DPG, PI, PIM	<u>PE</u> , PME, PG, PI, PIM, DPG	<u>PC</u> or <u>PE</u> , PME	<u>PC</u> , PE, <i>lyso</i> -PE, PME, DPG, PI, PG	<u>PE</u> , OH-PE, PI
Major menaquinone(s)	MK-9(H ₄)	MK-9(H ₂), MK-9(H ₄)	MK-9(H ₄)	MK-8(H ₄) or MK-9(H ₀)	MK-9(H ₄)	MK-9(H ₄)
Major fatty acids	i-C _{15:0} , i-C _{16:0} , C _{16:0}	i-C _{15:0} , i-C _{16:0} , ai-C _{17:0} , C _{17:0}	ai-C _{15:0} , i-C _{16:0} , ai-C _{17:0}	i-C _{16:0} , C _{16:0}	i-C _{15:0} , i-C _{16:0} , i-C _{17:0} , ai-C _{17:0}	i-C _{16:0}
DNA G + C content (mol%)	71.1	66–69	66	68–79	70.4–71.5	69–73

*Ara, Arabinose; Gal, galactose; Glc, glucose; Mad, madurose; Man, mannose; Rha, rhamnose; Rib, ribose. Sugars given in parentheses are present in trace amounts.

†DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; OH-PE, PE with hydroxyl fatty acid; *lyso*-PE, PE with one fatty acid chain missing from the glycerol backbone; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PME, phosphatidylmonomethylethanolamine. Underlined components represent the diagnostic phospholipids according to Lechevalier *et al.* (1977).

also dissimilar from those of members of phylogenetically closely related genera.

On the basis of significant differences in phylogenetic data, chemotaxonomic properties and morphological analyses, strain GMKU 367^T represents a novel species in a new genus in the family *Pseudonocardiaceae*, for which the name *Actinophytocola oryzae* gen. nov., sp. nov. is proposed.

Description of *Actinophytocola* gen. nov.

Actinophytocola [Ac.ti.no.phy.to'co.la. Gr. n. *actis*, *actinos* a ray, beam; Gr. n. *phuton* a plant; L. masc. suff. *-cola* (from L. n. *incola*) a dweller, inhabitant; N.L. masc. n. *Actinophytocola* actinobacterial dweller inside a plant].

Aerobic, Gram-stain-positive, non-acid-fast, non-motile actinomycetes. Have non-fragmented substrate mycelium and, on some media, aerial mycelium is produced. Cylindrical spores are produced on aerial mycelium, but no sporangium-like structures are observed. Good growth occurs at 18–28 °C and on oatmeal agar and oatmeal-nitrate agar. The cell wall contains *meso*-diaminopimelic acid, alanine, glutamic acid and acetylated muramic acid. The whole-cell sugars are arabinose, galactose, mannose, rhamnose and ribose. Mycolic acids are absent. The major fatty acids are iso-C_{15:0}, iso-C_{16:0} and C_{16:0}. The diagnostic menaquinone is MK-9(H₄). The polar phospholipids are phosphatidylethanolamine and hydroxyphos-

phatidylethanolamine. The type species is *Actinophytocola oryzae*.

Description of *Actinophytocola oryzae* sp. nov.

Actinophytocola oryzae (o.ry'zae. L. n. *oryza* rice and also the name of a botanical genus; L. gen. n. *oryzae* of rice, denoting the isolation of the type strain from roots of Thai glutinous rice plants).

In addition to the characteristics given in the genus description, has the following properties. Grows well on ISP 3 and oatmeal-nitrate agar and grows moderately on ISP 2 and 1/10 yeast extract-starch agar, but grows poorly on ISP 4 and ISP 5. Pale peach (5ca) aerial mycelium is produced on ISP 3 and substrate mycelium is light melon yellow (3ea); no soluble pigment is produced. A trace of rust tan (5le) soluble pigment is produced on oatmeal-nitrate agar. No aerial mycelium is observed on ISP 2 or 1/10 yeast extract starch agar. Does not grow on ISP 9 or ISP 6. Temperature range for growth is 12–30 °C, with optimal growth at 18–28 °C. Grows at pH 5.0–10.0, with optimal growth at pH 6.0–7.0. Tolerates up to 2 % NaCl. Negative for reduction of nitrate and production of melanin pigments and H₂S. Catalase-positive and oxidase-negative. Degrades starch, but negative for decomposition of benzoate, hypoxanthine, xanthine and milk. Liquefaction of gelatin is weakly positive. Degradation of

citrate is positive. D-Fructose, D-glucose and L-rhamnose can be utilized as sole carbon sources, but L-arabinose, dulcitol, D-galactose, *myo*-inositol, lactose, maltose, D-mannitol, D-mannose, raffinose, D-sorbitol, sucrose, trehalose and D-xylose are not utilized. Produces acid from L-arabinose, D-fructose and D-glucose, but not from adonitol, dulcitol, erythritol, D-galactose, *myo*-inositol, lactose, D-mannitol, raffinose or L-rhamnose.

The type strain is GMKU 367^T (=BCC 31372^T =NBRC 105245^T), isolated from roots of Thai glutinous rice plants (*Oryza sativa* L. 'RD6') collected from Pathum Thani Rice Research Center, Pathum Thani province, Thailand.

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