Agromyces tropicus sp. nov., isolated from soil

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Two actinomycete strains, CM9-9^T and AK2-48, which produced straight rod-shaped, non-motile cells, were isolated from soils in Chiang Mai and Phuket provinces, respectively, Thailand. The morphological and chemotaxonomic characteristics of the isolates coincided with those of the genus *Agromyces*. Phylogenetic analysis using 16S rRNA gene sequences also indicated that the isolates were clearly separated from their closest relative, *Agromyces aurantiacus* YIM 21741^T, and should be classified in the genus *Agromyces*. Furthermore, a combination of DNA–DNA hybridization results and physiological and biochemical properties indicated that the isolates could be distinguished from all recognized members of the genus *Agromyces*. The isolates therefore represent a novel species, for which the name *Agromyces tropicus* sp. nov. is proposed. The type strain is CM9-9^T (=JCM 15672^T =BCC 34764^T).

The genus *Agromyces* Gledhill and Casida 1969 belongs to the family *Microbacteriaceae* in the order *Actinomycetales* (Zhi *et al.*, 2009). The genus is well defined on a chemotaxonomic basis (Zgurskaya *et al.*, 1992) as well as on morphological grounds. Reclassification of *Agromyces fucosus* subsp. *hippuratus* as *Agromyces hippuratus* and emendation of the description of *A. fucosus* by Ortiz-Martinez *et al.* (2004) were based on 16S rRNA gene sequence analyses, DNA–DNA hybridization experiments and phenotypic traits. At the time of writing, the genus *Agromyces* is considered to accommodate 21 species: *Agromyces ramosus* (the type species; Gledhill & Casida, 1969), *A. cerinus* (Zgurskaya *et al.*, 1992), *A. mediolanus* (Suzuki *et al.*, 1996), *A. luteolus*, *A. rhizosphaerae* and *A. brachium* (Takeuchi & Hatano, 2001), *A. albus* (Dorofeeva *et al.*, 2003), *A. aurantiacus* (Li *et al.*, 2003), *A. fucosus* and

A. hippuratus (Zgurskaya *et al.*, 1992; Ortiz-Martinez *et al.*, 2004), *A. ulmi* (Rivas *et al.*, 2004), *A. neolithicus* and *A. salentinus* (Jurado *et al.*, 2005a), *A. humatus*, *A. italicus* and *A. lapidis* (Jurado *et al.*, 2005b), *A. subbeticus* (Jurado *et al.*, 2005c), *A. allii* (Jung *et al.*, 2007), *A. terreus* (Yoon *et al.*, 2008), *A. atrinae* (Park *et al.*, 2010) and *A. bauzanensis* (Zhang *et al.*, 2010). Here, we report on the taxonomic characterization and classification of strains CM9-9^T and AK2-48, which were isolated from soils in Chiang Mai and Phuket provinces of Thailand, respectively.

Strains CM9-9^T and AK2-48 were isolated on humic acid-salts vitamin (HV) agar supplemented with (1⁻¹) 25 mg nalidixic acid and 50 mg cycloheximide after a pre-treatment step as described by Thawai *et al.* (2004) at 30 °C for 21 days. The pure cultures were kept at 4–10 °C on yeast extract-malt extract [International *Streptomyces* Project (ISP) 2 medium; Shirling & Gottlieb, 1966] agar slants.

Strains CM9-9^T and AK2-48 were grown for 21 days at 30 °C on HV agar and observed using light microscopy and scanning electron microscopy (JSM-5410 LV; JEOL).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CM9-9^T and AK2-48 are AB454378 and AB515329, respectively.

A supplementary figure and two supplementary tables are available with the online version of this paper.

Samples for scanning electron microscopy were prepared as described by Itoh *et al.* (1989).

Phenotypic characteristics were examined by using several standard methods; cultural characteristics were tested using 14-day cultures grown on various agar media at 30 °C. The Jacal Colour Card L2200 (Japan Colour Research Institute) was used for determining colony colour designations. The hydrolysis of various compounds and acid production from carbon sources were examined using the basal medium recommended by Gordon *et al.* (1974). Conditions for growth were determined on ISP 2 medium. Gelatin liquefaction, peptonization of milk, nitrate reduction and starch hydrolysis were determined on various media as described by Arai (1975) and Williams & Cross (1971).

For chemotaxonomic analysis, freeze-dried cells were obtained from cultures grown in ISP 2 broth on a rotary shaker at 30 °C for 4 days. The cell-wall peptidoglycan was prepared and hydrolysed by the methods of Kawamoto *et al.* (1981) and the amino acid composition was analysed by TLC (Lechevalier & Lechevalier, 1980). The isomer of diaminopimelic acid (A₂pm) in the cell wall was determined by the method of Stanek & Roberts (1974). The acyl group of muramic acid in the peptidoglycan was determined by the method of Uchida & Aida (1984). The reducing sugars from whole-cell hydrolysates were analysed by the cellulose TLC method of Komagata & Suzuki (1987). Phospholipids in cells were extracted and analysed by the method of Minnikin *et al.* (1984). Fatty acid methyl ester analysis was performed by GLC according to the instructions of the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted by the method of Collins *et al.* (1977) and analysed by HPLC equipped with a Cosmosil 5C₁₈ column (4.6 × 150 mm; Nacalai Tesque). The elution solvent was methanol/2-propanol (2:1, v/v).

Chromosomal DNA was isolated from cells grown in ISP 2 broth according to the method of Tamaoka (1994). The G + C content of the DNA was determined using the HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides (Yamasa Shoyu) was used as a quantitative standard. DNA–DNA hybridization was conducted in microdilution-well plates, as reported by Ezaki *et al.* (1989). DNA–DNA relatedness was determined using the colorimetric method (Verlander, 1992). PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Suriyachadkun *et al.* (2009). The 16S rRNA gene sequence was aligned with selected sequences obtained from public databases using CLUSTAL W version 1.81 (Thompson *et al.*, 1994). The alignment was verified and adjusted manually prior to the construction of phylogenetic trees with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) methods using MEGA version 2.1 (Kumar *et al.*, 2001). Confidence values of nodes were determined using bootstrap analysis (Felsenstein, 1985)

based on 1000 resamplings. 16S rRNA gene sequence similarities between the isolates and recognized members of the genus *Agromyces* were determined first using the EzTaxon server (Chun *et al.*, 2007) and then calculated manually after pairwise alignment with the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

Strains CM9-9^T and AK2-48 produced branching hyphae that broke up into diphtheroid- and rod-like, irregular, non-motile fragments that had morphological characteristics typical of the genus *Agromyces* (Supplementary Fig. S1, available in IJSEM Online). The isolates produced well-developed substrate mycelium on ISP 2 agar, oatmeal agar (ISP 3), peptone-yeast extract-iron agar (ISP 6) and nutrient agar. Aerial mycelium was absent on these media. Colonies of strains CM9-9^T and AK2-48 were greenish yellow to yellow. Soluble pigments were not produced on most of the media tested. The physiological and biochemical characteristics of the isolates are presented in Table 1 and the species description.

Strains CM9-9^T and AK2-48 had identical chemotaxonomic characteristics and these were similar to those of members of the genus *Agromyces*. Both strains contained 2,4-diaminobutyric acid, glycine, alanine and glutamic acid in their cell wall. The acyl type of the cell-wall muramic acid was acetyl. Glucose, galactose, mannose and ribose were found as whole-cell sugars. The characteristic phospholipids were phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid. The major cellular fatty acids were anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0} and smaller amounts (<10%) of iso-C_{15:0}, iso-C_{14:0}, C_{16:0}, C_{18:1ω9c}, iso-C_{17:0}, C_{17:1ω9c}, C_{14:0}, C_{15:0}, iso-C_{18:0}, C_{17:0} and anteiso-C_{13:0} were also found (Supplementary Table S1). The predominant menaquinones were MK-12 (32.9%) and MK-11 (11.8%). The DNA G + C contents of strains CM9-9^T and AK2-48 were 72.7 and 72.6 mol%, respectively.

Almost-complete 16S rRNA gene sequences (1481 and 1490 nt) were determined for strains CM9-9^T and AK2-48, respectively, and a 1409 nt fragment was used for phylogenetic analysis and comparison against 16S rRNA gene sequences of members of the family *Microbacteriaceae*. Phylogenetic analysis based on this large dataset placed strains CM9-9^T and AK2-48 within the clade of the genus *Agromyces* (not shown). When the 16S rRNA gene sequences of strains CM9-9^T and AK2-48 were analysed with corresponding sequences of the type strains of all recognized members of the genus *Agromyces* and selected sequences of the genera *Rathayibacter* and *Clavibacter*, strains CM9-9^T and AK2-48 formed a separate lineage in a clade containing *A. aurantiacus* YIM 21741^T (Fig. 1). The relationship between the isolates was supported with both the neighbour-joining and maximum-parsimony methods. 16S rRNA gene sequence similarity between strain CM9-9^T and type strains of the genus *Agromyces* ranged from 94.8% (*A. hippuratus* JCM

Table 1. Differential characteristics of strains CM9-9^T and AK2-48 and the type strains of the most closely related *Agromyces* species

Strains: 1, *Agromyces tropicus* sp. nov. CM9-9^T (strain AK2-48 gave identical results); 2, *A. aurantiacus* JCM 12113^T; 3, *A. mediolanus* JCM 3346^T; 4, *A. ulmi* JCM 13315^T; 5, *A. brachium* JCM 11433^T; 6, *A. luteolus* JCM 11431^T. All data were obtained in this study. All strains produced acid from glycerol and L-rhamnose. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5	6
Gelatin liquefaction	w	+	+	–	–	–
Peptonization of milk	–	+	–	–	–	–
Hydrolysis of starch	w	+	–	–	+	+
Growth at 37 °C	–	+	+	+	–	–
Maximum NaCl concentration (%)	6	4	6	2	2	4
Minimum pH	6	5	6	5	6	6
Acid production from:						
D-Fructose	–	+	+	–	+	+
D-Galactose	+	+	+	–	–	–
D-Mannitol	+	+	–	–	+	w
Melibiose	+	+	–	+	+	+
Raffinose	+	+	–	+	w	w
D-Ribose	+	+	+	w	+	+
D-Xylose	+	+	–	+	+	+
Lactose	+	+	–	–	–	–
L-Arabinose	+	+	–	–	+	+
Sucrose	+	+	–	+	–	–

9086^T) to 97.7 % (*A. aurantiacus* YIM 21741^T). Strain CM9-9^T showed 100 % 16S rRNA gene sequence similarity to strain AK2-48. On the basis of morphological, chemotaxonomic and phylogenetic data, the isolates should be classified in the genus *Agromyces*.

Strain CM9-9^T showed physiological and biochemical properties identical to those of strain AK2-48. The phenotypic characteristics (Table 1) clearly indicate that strains CM9-9^T and AK2-48 possess some distinct characteristics that discriminate them from their closest

Fig. 1. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences (1409 nt) showing relationships between strains CM9-9^T and AK2-48, members of the genus *Agromyces* and representative members of the genera *Rathayibacter* and *Clavibacter*. Bootstrap values (>50 %) based on 1000 resamplings are shown at branch nodes. Asterisks indicate that the corresponding nodes were recovered in the tree generated with the maximum-parsimony method. *Streptomyces ambofaciens* ATCC 23877^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

phylogenetic neighbour, *A. aurantiacus* JCM 12113^T, in particular the peptonization of milk, minimum pH tolerance, growth at 37 °C and acid production from D-fructose. Strains CM9-9^T and AK2-48 could be distinguished from *A. aurantiacus* JCM 12113^T by the lack of rhamnose in their whole-cell hydrolysates. Furthermore, DNA–DNA relatedness between strains CM9-9^T and AK2-48 was $93.2 \pm 0.99\%$ (reciprocal hybridization $98.4 \pm 1.00\%$), whereas relatedness between the isolates and their closest phylogenetic neighbours ranged from 16.7 ± 0.25 to $28.7 \pm 0.65\%$ (Supplementary Table S2). DNA–DNA relatedness between strains CM9-9^T and AK2-48 and *A. aurantiacus* JCM 12113^T was $28.7 \pm 0.65\%$ and $27.4 \pm 1.02\%$, respectively.

The phenotypic and genotypic data indicated that strains CM9-9^T and AK2-48 belong to the same species and merit classification in a novel species of the genus *Agromyces*, for which we propose the name *Agromyces tropicus* sp. nov.

Description of *Agromyces tropicus* sp. nov.

Agromyces tropicus [tro'pi.cus. L. masc. adj. *tropicus* tropical, of or pertaining to the tropic(s), relating to isolation from a tropical forest].

Gram-positive, mesophilic, aerobic actinomycete that forms branching hyphae which break up into irregular diphtheroid- and rod-like, non-motile fragments. Colonies are convex and yellow on ISP 2 agar after 14 days at 30 °C. On all media tested, aerial mycelium is absent and no soluble pigment is produced. Nitrate is not reduced to nitrite. Produces acid from cellobiose, D-galactose, D-mannitol, melibiose, raffinose, D-ribose, D-xylose, glucose, glycerol, lactose, L-arabinose, L-rhamnose, salicin and sucrose, but not D-fructose. Hydrolysis of starch and gelatin liquefaction are weakly positive, but peptonization of milk is negative. Optimal temperature for growth is 25–30 °C; no growth occurs at 37 °C. Growth occurs at pH 6–12 (optimum pH 8). The maximum NaCl concentration for growth is 6% (w/v) (optimum 4%, w/v). The cell wall contains 2,4-diaminobutyric acid, glycine, glutamic acid and alanine. The acyl type of the cell wall is the acetyl type. The predominant menaquinone is MK-12. The characteristic whole-cell sugars are glucose, galactose, mannose and ribose. The phospholipid profile contains phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid. The fatty acid pattern consists of anteiso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:0}, iso-C_{15:0}, iso-C_{14:0}, C_{16:0}, C_{18:1}ω9c, iso-C_{17:0}, C_{17:1}ω9c, C_{14:0}, C_{15:0}, iso-C_{18:0}, C_{17:0} and anteiso-C_{13:0}. The DNA G+C content of the type strain is 72.7 mol%.

The type strain is strain CM9-9^T (=JCM 15672^T =BCC 34764^T), which was isolated from a soil sample collected from Chiang Mai Province, Thailand. Strain AK2-48 is a second strain of the species.

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