

Micromonospora rifamycinica sp. nov., a novel actinomycete from mangrove sediment

Huiqin Huang, Jiasen Lv, Yonghua Hu, Zhe Fang, Kaishan Zhang and Shixiang Bao

Correspondence

Shixiang Bao

bsxhhq@yahoo.com.cn

Institute of Tropical Bioscience and Biotechnology/State Key Laboratory of Tropical Crop Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, PR China

An actinomycete strain, AM105^T, that produces rifamycin, was isolated from mangrove sediment samples collected from the South China Sea. The strain showed closest 16S rRNA gene sequence similarity to *Micromonospora matsumotoense* (98.0%). Chemotaxonomic characteristics of the isolate coincided with members of the genus *Micromonospora*. The value of DNA–DNA relatedness to *M. matsumotoense* (53.6%) and phenotypic differences from phylogenetically related *Micromonospora* species indicated that this isolate belongs to a novel species, for which the name *Micromonospora rifamycinica* sp. nov. is proposed. The type strain is AM105^T (=CGMCC 4.2495^T=DSM 44983^T).

Mangroves are a unique woody plant community of intertidal coasts in the tropical and subtropical zones, which are regarded as highly productive ecosystems and abode to large unexplored microbial diversity (Bashan & Holguin, 1997). Strain AM105^T was isolated from mangrove sediment samples from the South China Sea. Isolation was carried out by the standard dilution-plate technique on modified Gause inorganic agar (Gause *et al.*, 1983), which contained 20 g soluble starch, 1 g KNO₃, 0.5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 15 g agar, 1 l old sea water (pH 7.2–7.4). Plates were incubated for 30 days at 28 °C. A pure culture was maintained in a glycerol suspension (20%, w/v) at –70 °C.

Cultural features were observed on oatmeal agar (DSMZ medium 609), glycerol–asparagine agar (Shirling & Gottlieb, 1966), GYM agar (DSMZ medium 65), potato dextrose agar (DSMZ medium 129), Sauton's agar (Mordarska *et al.*, 1972) and Gause inorganic agar after 7, 14 and 21 days incubation at 28 °C. Cell morphology and spore production were observed by light and scanning electron microscopy using 6- and 20-day-old cultures grown on various agar media. The ability to grow on sole carbon sources (1%, w/v) was tested as described by Williams *et al.* (1983). NaCl tolerance (0–4.5%, w/v) and temperatures (4–45 °C) for growth were tested on GYM agar. Methods and media for other physiological tests and the assays for enzymic activities were performed according to Wang (1986).

For chemotaxonomic studies, the strain was grown in GYM broth in a shaking incubator at 200 r.p.m. and 28 °C for 5 days. Biomass was harvested by centrifugation and washed with distilled water. Isomers of diaminopimelic acid (DAP) and sugars were determined in whole-cell hydrolysates by TLC on microcrystalline cellulose (Wang, 1986).

Genomic DNA was isolated as described by Pitcher *et al.* (1989). The DNA G + C content was determined using the thermal melting method (Mandel & Marmur, 1968). PCR amplification of the 16S rRNA gene and sequencing of the purified PCR product were done as described previously (Rivas *et al.*, 2003). The sequence of isolate AM105^T was aligned and compared with representative sequences of members of the genus *Micromonospora* obtained from GenBank using the CLUSTAL_X 1.8 program (Thompson *et al.*, 1997). Phylogenetic analysis was performed with the MEGA version 2.1 program (Kumar *et al.*, 2001). Phylogenetic trees were constructed using three tree-making algorithms, namely, neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Kidd & Sgaramella-Zonta, 1971; Rzhetsky & Nei, 1993) and maximum-parsimony (Fitch, 1972); bootstrap analysis was also conducted. DNA–DNA relatedness experiments were performed by the method of Ezaki *et al.* (1989). DNA–DNA relatedness values were calculated according to Christensen *et al.* (2000).

Strain AM105^T showed good growth and no diffusible pigments on all of the media used. Colonies were orange, but turned dark brown on sporulation after 14–21 days on oatmeal agar and Gause inorganic agar. Spores were smooth rod-shaped, ovoid or spherical. Spores were single or in short chains with two to five spores, which borne on

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AM105^T is AY561829.

short or long sporophores. Some spores were sessile (Fig. 1). Aerial mycelium, at times visible only on Sauton's agar and Gause inorganic agar, was scanty and milk-white. Other cultural and morphological characters are given in the species description.

An almost-complete 16S rRNA gene sequence (1475 bp) was obtained. The 16S rRNA-based phylogenetic analysis showed a close relationship of the isolate to members of the genus *Micromonospora*. The highest 16S rRNA gene sequence similarities were found with *Micromonospora matsumotoense* DSM 44100^T (98.0%, 30 nt differences), *Micromonospora carbonacea* DSM 43168^T (97.8%, 32 nt differences), *Micromonospora mirobrigensis* DSM 44830^T (97.2%, 34 nt differences) and *Micromonospora siamensis* JCM 12729^T (96.9%, 41 nt differences). Various tree-making algorithms yielded very similar tree topologies (only some positions of the branches varied slightly) and the isolate always grouped with *M. matsumotoense* (data not presented). The reduced tree showing the position of strain AM105^T among the closest phylogenetic relatives is given as Fig. 2.

Strain AM105^T contained *meso*-DAP in the cell wall. The diagnostic cell-wall sugars (Lechevalier & Lechevalier, 1970) were arabinose and xylose. The G+C content of the DNA was 71%. The DNA-DNA relatedness between strain AM105^T and *M. matsumotoense* DSM 44100^T was 53.6% (the means of triplicate analyses), which are below the threshold value of 70% for the definition of a bacterial species according to Wayne *et al.* (1987).

Strain AM105^T grew between 20 and 37 °C, but no growth was found at 4, 15 or 45 °C after 3 weeks. Tolerates NaCl up to 3% and no growth occurred at 4.5%. Among the carbohydrates tested, only D-glucose was utilized well. Other physiological and biochemical characters are listed in the species description; characters differentiating the strain from related species are given in Table 1.

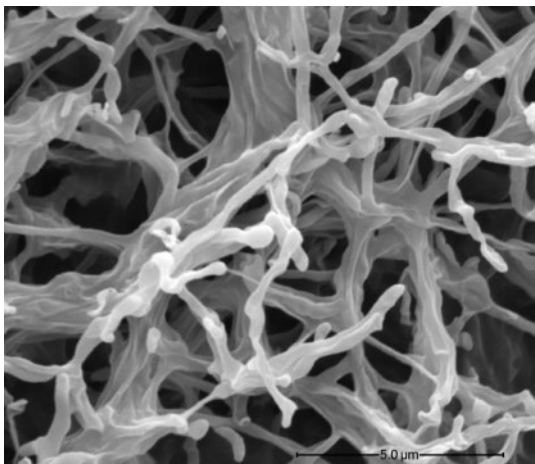


Fig. 1. Scanning electron micrograph of cells of strain AM105^T after growth on oatmeal agar for 21 days at 28 °C. Bar, 5 μm.

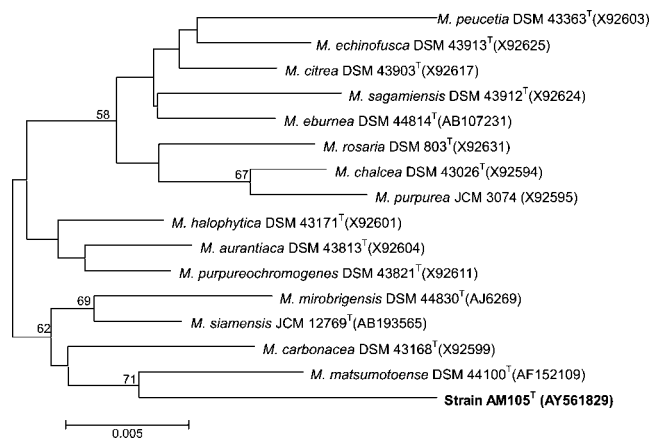


Fig. 2. Phylogenetic neighbour-joining tree based on the 16S rRNA gene sequences of strain AM105^T and other *Micromonospora* species. Bootstrap values (1000 replicates) are shown as percentages at each node for values above 50%. Bar, 0.005 nucleotide substitutions per position.

For analyses of antibiotic production, the strain was cultured in a medium containing 5 g glucose, 5 g yeast extract, 15 g starch, 10 g soy meal, 1 l sea water, pH 7.0, at 200 r.p.m. and 28 °C. After 6 days of cultivation, fermentation broth was centrifuged and the supernatant was assayed for an antimicrobial activity using the paper disc diffusion methods (Isnansetyo & Kamei, 2003). A clear antimicrobial activity was revealed against *Staphylococcus aureus* CGMCC 1.89, *Staphylococcus aureus* OY84 (a methicillin-resistant clinical isolate), *Bacillus subtilis* CGMCC 1.88, but not against *Escherichia coli* CGMCC 1.747, *Candida albicans* CGMCC 2.538 or *Piricularia oryzae* CGMCC 3.3283. The active ethyl acetate extract from the supernatant was separated by silica gel G250 column, and the bioactive fraction was eluted with petroleum ether/ethyl acetate (3:1), concentrated, and further separated by TLC (chloroform/methanol, 98:2). The bioactive compound was then purified through Sephadex LH-20 (chloroform/methanol, 1:1) and analysed by HPLC and various spectroscopic methods (Traxler *et al.*, 1981; Hermann & Zuordnung, 1973; Gallo *et al.*, 1974; Cellai *et al.*, 1982; Arora & Arjunan, 1992). In HPLC analysis, the compound showed one peak but two sets of proton and carbon signals were observed in the ¹H- and ¹³C-NMR spectra. Further identification using ¹H-NMR, ¹³C-NMR, heteronuclear multiple bond correlation, heteronuclear single quantum coherence, rotary overhauser effect spectroscopy, electrospray ionization mass spectrometry and IR spectroscopy showed that the active compound (purple amorphous powder) consisted of rifamycin S and its isomer (Huang *et al.*, 2006), while *M. carbonacea* was reported to produce everninomicin (Luedemann & Brodsky, 1965). No data are available for antibiotic production by other related species, *M. matsumotoense*, *M. mirobrigensis* and *M. siamensis*.

Table 1. Physiological and biochemical characteristics that differentiate strain AM105^T from related *Micromonospora* species

Strains: 1, AM105^T; 2, *M. matsumotoense* DSM 44100^T (data from Lee *et al.*, 1999); 3, *M. carbonacea* DSM 43168^T (Holt *et al.*, 1994); 4, *M. mirobrigensis* DSM 44830^T (Trujillo *et al.*, 2005); 5, *M. siamensis* JCM 12729^T (Thawai *et al.*, 2005). All strains were positive for utilization of D-glucose but negative for mannitol. +, Positive; -, negative; w, weakly positive; v, variable; ND, not determined.

Characteristic	1	2	3	4	5
Growth on sole carbon source (1%, w/v) of:					
L-Arabinose	-	+	+	+	+
D-Xylose	-	+	+	-	+
D-Fructose	-	+	+	ND	w
L-Rhamnose	-	-	-	-	+
Sucrose	-	+	+	+	+
D-Raffinose	-	+	v	+	+
Nitrate reduction	+	+	+	-	-
Cellulose decomposition	+	-	ND	ND	ND
Production of H ₂ S	-	+	-	ND	-
Liquefaction of gelatin	-	+	+	ND	+
Tolerance of 3% NaCl	+	-	+	+	+
Bioactive products	Rifamycin	ND	Everninomicin	ND	ND

Thus, the data obtained in this study clearly show that strain AM105^T merits description as a novel species of the genus *Micromonospora*, for which the name *Micromonospora rifamycinica* sp. nov. is proposed.

Description of *Micromonospora rifamycinica* sp. nov.

Micromonospora rifamycinica (rif.a.my.cin'i.ca. N.L. n. *rifamycinum* -i rifamycin; L. suff. *icus* -a -um related to; N.L. fem. adj. *rifamycinica* referring to the ability to produce rifamycin).

Aerobic, Gram-positive actinomycetes which form an extensively branched fine substrate mycelium. Hard colonies on GYM agar are 2–3 mm in diameter after 2 weeks. Substrate mycelium is orange, turning brown on sporulation on oatmeal agar and Gause inorganic agar. Spores are rod-shaped, ovoid or spherical, smooth, single or in short chains with two to five spores, and borne on short or long sporophores. Some spores are sessile. Aerial mycelium, scanty and milk-white, at times produced on Sauton's agar and Gause inorganic agar. Diffusible pigments are not produced. Growth occurs in 0–3% NaCl and 20–37 °C. D-Glucose is utilized as a sole carbon source; L-arabinose, D-xylose, D-fructose, L-rhamnose, mannitol, sucrose, D-raffinose and inositol are not utilized. Able to degrade starch and cellulose, but not gelatin or casein. Nitrate reductase and tyrosinase are produced but hydrogen sulfide (H₂S) is not. Produces rifamycin S and its isomer. Whole-cell hydrolysates contain *meso*-DAP and the main sugars xylose and arabinose. The DNA G+C content is 71%.

The type strain, AM105^T (=CGMCC 4.2495^T=DSM 44983^T), was isolated from mangrove sediment from the South China Sea.

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