**Micromonospora oryzae** sp. nov., isolated from roots of upland rice

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An actinomycete strain, designated CP2R9-1ᵀ, was isolated from root internal tissues of upland rice (*Oryza sativa*). Based on a polyphasic approach, strain CP2R9-1ᵀ was characterized as a member of the genus *Micromonospora*. meso-Diaminopimelic acid and 3-OH-diaminopimelic acid were present in the cell-wall peptidoglycan. The polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, two unidentified phospholipids and four unidentified polar lipids. Predominant menaquinones were MK-9(H₄), MK-9(H₆) and MK-10(H₉). Whole-cell sugars consisted of ribose, xylose, arabinose and glucose. Phylogenetic analysis of the nearly complete 16S rRNA gene sequence suggested that strain CP2R9-1ᵀ was closely related to *Micromonospora haikouensis* 232617ᵀ (99.32 % similarity), *Micromonospora carbonacea* DSM 43168ᵀ (99.18 %) and *Micromonospora krabiensis* MA-2ᵀ (99.16 %). Strain CP2R9-1ᵀ was distinct from its closest relatives based on low levels of DNA–DNA relatedness (21.3 ± 0.1–41.7 ± 0.7 %) and phenotypic differences. The results presented in this study showed that strain CP2R9-1ᵀ represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora oryzae* sp. nov. is proposed. The type strain is CP2R9-1ᵀ (= BCC 67266ᵀ = NBRC 110007ᵀ).

The genus *Micromonospora* was first proposed by Ørskov (1923). It is classified as a member of the family *Micromonosporaceae* that belongs to the order *Actinomycetales* (Stackebrandt et al., 1997). Effort has been made to identify novel species in the genus *Micromonospora*. This is largely due to their ability to produce secondary metabolites that have antibiotic properties (Supong et al., 2013). Additionally, several strains have been proposed as potential candidates for plant growth-promoting bacteria through nitrogen fixation and antagonistic activities against phytopathogens (Martínez-Hidalgo et al., 2014; Raaijmakers & Mazzola, 2012). The typical characteristic of members in this genus is the formation of single, non-motile spores on sporophores of the substrate mycelium and the absence of aerial mycelium (Nimaichand et al., 2013; Ren et al., 2013). Recently, two new species of the genus *Micromonospora*, *Micromonospora palomenae* and *Micromonospora harpali*, were reported (Fang et al., 2015). In the present study, we report on the isolation and characterization of actinomycete strain CP2R9-1ᵀ. The results obtained from the polyphasic study indicate that strain CP2R9-1ᵀ represents a novel species of the genus *Micromonospora*.

Upland rice plants were collected from Chumporn province, Thailand. Dirt was removed from root samples by rinsing with water several times. Surface sterilization was carried out by submerging roots in 70 % ethanol for 5 min and absolute ethanol for 1 min. Samples were air-dried and placed in 6 % (v/v) sodium hypochlorite solution for 2 min. Subsequently, excessive sodium hypochlorite solution was removed by rinsing the samples five times in sterilized distilled water. To obtain bacterial suspension from root internal tissues, surface-sterilized roots were homogenized in sterilized distilled water.

The GenBank/EMBL/DDBJ accession number for the partial 16S rRNA gene sequence of strain CP2R9-1ᵀ is AB981052.

Three supplementary tables and five supplementary figures are available with the online Supplementary Material.
using a mortar and pestle. Two hundred microlitres of the suspension was plated on starch casein nitrate agar (SCA) (per litre: 0.3 g casein, 10 g soluble starch, 2 g KNO₃, 2 g NaCl, 2 g K₂HPO₄, 0.05 g MgSO₄, 7H₂O, 0.02 g CaCO₃, 0.01 g FeSO₄.7H₂O, 15 g agar; pH 7.0–7.5) that was supplemented with 25 mg nalidixic acid l⁻¹ and 100 mg nystatin l⁻¹. Plates were incubated at 30 °C for 21 days. The final rinsing water was plated on SCA and used as a control plate. An actinomycite isolate, designated strain CP2R9-1ᵀ, was obtained on the isolation plate and purified on International Streptomyces Project (ISP)2 medium (Shirling and Gottlieb, 1966).

Strain CP2R9-1ᵀ was grown on ISP2–7 agar medium including glucose-asparagine agar, Czapek’s sucrose agar and nutrient agar for 14 days at 30 °C and for determination of its cultural characteristics as described by Shirling & Gottlieb (1966). The colour designation of strain CP2R9-1ᵀ was made according to the National Bureau of Standards/Inter Society Colour Council (NBS/IBCC) colour system (Kelly, 1964). Cell morphology was examined using scanning electron microscopy (JSM-5410 LV; JEOL). Preparation of samples for scanning electron microscopy was done according to Itoh et al. (1989). Utilization of various compounds and acid production from different carbon sources were determined on the basal medium (Gordon et al., 1974). NaCl tolerance (0–7 % NaCl, w/v) and the pH (pH 4–11) and temperature (10–50 °C) ranges for growth were examined on ISP2 agar. Starch hydrolysis, gelatin liquefaction, nitrate reduction and peptonization of milk were studied on media described by Arai (1975) and Williams & Cross (1971).

Freeze-dried cells of strain CP2R9-1ᵀ were obtained from the bacterial culture that was grown in ISP2 broth on a rotary shaker (200 r.p.m.) at 30 °C for 4 days. Determination of cell-wall peptidoglycan was performed as described by Kawamoto et al. (1981). The cell-wall isomer of diaminopimelic acid was analysed using the method described by Stanec & Roberts (1974). The acyl group of muramic acid in the peptidoglycan was analysed using the method of Uchida & Aida (1984). Analysis of whole-cell hydrolysate sugars was performed using the TLC method (Komagata & Suzuki, 1987). Polar lipids were extracted and determined according to the method described by Minnikin et al. (1984). Cellular fatty acid profile analysis was carried out using GLC according to the instructions of the Microbial Identification System (MIDI) Sherlock version 6.0 (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Cellular fatty acids were identified using the ACTIN1 MIDI database. Isoprenoid quinones were prepared according to the method of Collins et al. (1977) and identified by HPLC. The HPLC solvent system was methanol/2-propanol (2:1, v/v) at a flow rate of 0.6 ml min⁻¹.

Strain CP2R9-1ᵀ was grown in ISP2 broth, and cells were harvested for genomic DNA extraction according to a previously described method (Tamaoka, 1994). Amplification of the nearly complete 16S rRNA gene was performed using the universal primers 20F (5’-GAGTTTGTACCTGGCTCAG-3’, positions 9–27) and 1541R (5’-GTACCTGTAGCACGTGGTT-3’) (Weisburg et al., 1991). The temperature profile was as follows: initial denaturation at 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s; and final extension at 72 °C for 5 min. Sequencing of the PCR product was carried out using the 27F, 1492R, 350F (5’-TACGGAGGAGCACGAG-3’), 780F (5’-GATTAGATACCTGTTAG-3’), 1100F (5’-GCAAACGGACCAACCC-3’), 350R (5’-CTGCTGGCCTC-CCGTAG-3’) and 780R (5’-CTACAGGGATATCTA-ATCC-3’) primers (Lane, 1991). Subsequently, pairwise alignment analysis of the 16S rRNA gene sequence of strain CP2R9-1ᵀ was performed on the EzTaxon server (Kim et al., 2012).

The CLUSTAL W program, version 1.81 (Thompson et al., 1994), was used for multiple alignment analysis of the nearly complete 16S rRNA gene sequences of strain CP2R9-1ᵀ and members of the genus Micromonospora. Ambiguous nucleotides and gaps were manually corrected. Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods in the program MEGA version 6.0 (Tamura et al., 2013). Evolutionary distances were determined using Kimura’s two-parameter model (Kimura, 1980). The confidence levels of clusters were determined based on bootstrap analysis (Felsenstein, 1985) with 1000 resamplings. Amplification and sequencing of the gyrB gene was performed according to the method described by Garcia et al. (2010). Phylogenetic relationships based on the gyrB gene sequence were analysed as described for the 16S RNA gene.

Analysis of the DNA G+C content of strain CP2R9-1ᵀ was carried out according to the HPLC method described by Mesbah et al. (1989). Lambda DNA (Invitrogen) was included in the experiment as a standard. DNA–DNA relatedness between strain CP2R9-1ᵀ and its phylogenetically closest relatives was studied in microdilution well plates (Ezaki et al., 1989) and determined using the colorimetric method described by Verlander (1992).

Strain CP2R9-1ᵀ grew well and formed well-developed substrate mycelium on ISP2, ISP3 and ISP4 media. Moderate growth was observed on ISP5, ISP6, ISP7, glucose-asparagine agar and nutrient agar media. It grew poorly on Czapek's sucrose agar medium. Aerial mycelium was absent in all tested media. Greyish to yellowish brown soluble pigments were observed when strain CP2R9-1ᵀ was grown on ISP3 medium. Further cultural characteristics are provided in Table S1 (available in the online Supplementary Material). Spherical spores were borne singly on substrate mycelium (Fig. 1). The surface of spores was warty.

meso-Diaminopimelic acid and 3-OH-meso-diaminopimelic acid were present in the cell wall of strain CP2R9-1ᵀ. The presence of meso-diaminopimelic acid is characteristic of peptidoglycan type A1γ, which is found in the genus "Micromonospora oryzae sp. nov."
**Micromonospora** (Schleifer & Kandler, 1972; Garcia et al., 2010). Whole-cell sugars were ribose, xylene, arabinose and glucose. Analysis of polar lipids showed that dihydrophatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylglysinolinositol (PIMs), two unidentified phospholipids and four unidentified polar lipids were present in strain CP2R9-1T (Fig. S1). Significant cellular fatty acids of strain CP2R9-1T included iso-C_{16:0} (24.3 %), iso-C_{15:0} (17.6 %), anteiso-C_{15:0} (9.0 %), C_{16:0} (8.7 %), C_{17:1 iso} (5.6 %), C_{17:0} (5.2 %), C_{18:1iso} (4.9 %) and anteiso-C_{17:0} (4.2 %). The full fatty acid profile of strain CP2R9-1T is given in Table S2. The menaquinones observed in strain CP2R9-1T were MK-9(H4) (76.3 %), MK-9(H6) (12.1 %), MK-10(H4) (7.9 %), MK-10(H6) (1.4 %), MK-10(H2) (1.7 %), MK-9(H2) (0.4 %) and MK-10(H2) (0.2 %).

Pairwise alignment of the nearly complete 16S rRNA gene sequence (1502 nt) of strain CP2R9-1T indicated that the strain belonged to the genus Micromonospora. Based on 16S rRNA gene sequence similarity analysis, the closest relatives of strain CP2R9-1T were *Micromonospora haikouensis* 232617^T^ (99.32 %), *Micromonospora carbonacea* DSM 43168^T^ (99.18 %) and *Micromonospora krabensis* MA-2^T^ (99.16 %). The phylogenetic tree reconstructed from the nearly complete 16S rRNA gene sequences using the neighbor-joining method showed that strain CP2R9-1T formed a cluster with *M. carbonacea* DSM 43168^T^ and *M. krabensis* MA-2^T^. The phylogenetic trees obtained with the maximum-likelihood and maximum-parsimony methods (Figs S3 and S4). To verify the phylogenetic relationship between strain CP2R9-1T and its closest relatives based on 16S rRNA gene sequences, phylogenetic analysis of the gyrB gene was carried out (Fig. S5). The highest levels of gyrB gene sequence similarity were observed between strain CP2R9-1T and *M. haikouensis* 232617^T^ (98.8 %) and *M. carbonacea* IFO 14304^T^. This result was consistent with that obtained from analysis of the 16S rRNA gene. Additionally, the phylogenetic tree obtained from the neighbour-joining method indicated that strain CP2R9-1T was placed in the same clade as *M. haikouensis* 232617^T^ and *M. carbonacea* IFO 14304^T^. This was significantly supported by the bootstrap level of 100 %. The same cluster was also observed in the phylogenetic trees reconstructed with the maximum-likelihood and maximum-parsimony methods (Figs S3 and S4). These results indicated that *M. carbonacea* DSM 43168^T^ and *M. krabensis* MA-2^T^ were the phylogenetically closest relatives of strain CP2R9-1T.

Analysis of the DNA base composition revealed that the DNA G+C content of strain CP2R9-1T was 73.8 mol% (Tm). DNA–DNA relatedness between strain CP2R9-1T and its closest relative *M. haikouensis* 232617^T^, *M. carbonacea* DSM 43168^T^ and *M. krabensis* MA-2^T^ ranged from 21.3 ± 0.1 to 41.7 ± 0.7 % (Table S3). These values were well below the 70 % cut-off level recommended for assigning two bacterial strains to the same species (Wayne et al., 1987). Additionally, phenotypic and chemotaxonomic differences were observed between strain CP2R9-1T and its close relatives (Table 1). For example, strain CP2R9-1T contained phosphatidylglycerol, had MK-9(H4) as the major menaquinone and was able to tolerate 4 % NaCl. Strain CP2R9-1T and *M. haikouensis* 232617^T^ were able to utilize L-rhamnose as the carbon source. In contrast, only strain CP2R9-1T, *M. carbonacea* DSM 43168^T^ and *M. krabensis* MA-2^T^ were able to grow in medium supplemented with D-fructose as the carbon source. These results indicated that strain CP2R9-1T was readily distinguishable from its phylogenetically close relatives and represented a novel species of the genus *Micromonospora*, for which the name *Micromonospora oryzae* sp. nov. is proposed. In this study, we found that the menaquinone compositions of *M. haikouensis* 232617^T^, *M. carbonacea* DSM 43168^T^ and *M. krabensis* MA-2^T^ were consistent with previous analyses (Jongrungruangchok et al., 2008; Xie et al., 2012).

**Description of *Micromonospora oryzae* sp. nov.**

*Micromonospora oryzae* (o.ry’zæ. L. gen. n. oryzae of rice). An aerobic, Gram-stain-positive, mesophilic actinomycete that produces extensively branched substrate hyphae. Aerial mycelium is absent. The colour of the substrate mycelium on ISP2 medium is moderate orange yellow. Pale yellowish brown soluble pigments are produced on ISP3 medium. Non-motile, spherical, single spores are borne on the substrate mycelium. The surface of spores is warty. Starch is hydrolysed. Negative for gelatin liquefaction and nitrate reduction. Positive for peptonization. Utilizes L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, D-mannose, melibiose, raffinose, L-rhamnose, D-ribose, D-salicin, sucrose and D-xylose as sole carbon sources, but not glycerol, mannitol or myo-inositol.
Produces acid from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, melibiose, raffinose, L-rhamnose, D-ribose and D-xylose. Grows at 20–45 °C and at pH 5–10. The optimum temperature for growth is 30 °C. The optimum pH for growth is 7.0. Tolerates 1–4% (w/v) NaCl. The optimum NaCl concentration for growth is 2% (w/v). The cell-wall peptidoglycan contains meso-diaminopimelic acid and 3-OH-meso-diaminopimelic acid. Glycolyl is the acyl type of the cell-wall muramic acid. The polar lipids consist of diphosphatidylglycerol, phosphatidyethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, two unidentified phospholipids and four unidentified polar lipids. Major menaquinones are MK-9(H4), MK-9(H6) and MK-10(H4). Whole-cell sugars are arabinose, glucose, ribose and xylose. Major fatty acids are iso-C16:0, iso-C15:0 anteiso-C15:0, C16:0, C17:1ω8c, C17:0ω9c and anteiso-C17:0.

The type strain, CP2R9-1T (=BCC 67266T=NBRC 110007T), was isolated from root tissues of upland rice plants grown in Chumphorn province, Thailand. The DNA G+C content of the type strain is 73.8 mol%.

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Table 1. Differential characteristics between strain CP2R9-1T and the type strains of related species of the genus Micromonospora

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>2</th>
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<tr>
<td>Cell-wall amino acid(s)</td>
<td>meso-Diaminopimelic acid and 3-OH-meso-diaminopimelic acid</td>
<td>meso-Diaminopimelic acid</td>
<td>meso-Diaminopimelic acid and 3-OH-meso-diaminopimelic acid</td>
<td>meso-Diaminopimelic acid</td>
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<tr>
<td>Whole-cell sugars*</td>
<td>Ara, Glu, Rib, Xyl</td>
<td>Ara, Glu, Xyl</td>
<td>Ara, Glu, Xyl</td>
<td>Ara, Glu, Man, Rib, Xyl, Gal</td>
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<tr>
<td>Polar lipids†</td>
<td>DPG, PE, PG, PI, PIMs</td>
<td>DPG, PE, PIM</td>
<td>DPG, PE, PIM</td>
<td>DPG, PE, PI</td>
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<tr>
<td>Major menaquinones</td>
<td>MK-9(H4) (76.3 %), MK-10(H6) (60.4 %), MK-10(H4) (29.8 %) and MK-9(H4) (8.2 %)</td>
<td>MK-10(H4) (5.7 %)</td>
<td>MK-9(H4) (2.2 %)</td>
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<td>Carbon utilization:</td>
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<tr>
<td>D-Arabinose</td>
<td>W</td>
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<td>D-Fructose</td>
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<td>D-Mannitol</td>
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<td>D-Mannose</td>
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<td>Melibiose</td>
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<td>Raffinose</td>
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<td>L-Rhamnose</td>
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<td>D-Xylose</td>
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<td>Starch hydrolysis</td>
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<td>Maximum NaCl tolerance (%)</td>
<td>4</td>
<td>3</td>
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* Ara, arabinose; Gal, galactose; Glu, glucose; Man, mannose; Rib, ribose; Xyl, xylose.
† DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides.

References


