Nocardia rayongensis sp. nov., isolated from Thai peat swamp forest soil

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An actinomycete strain, RY45-3T, isolated from a peat swamp forest soil in Rayong Province, Thailand, was characterized using a polyphasic approach. The strain belonged to the genus Nocardia on the basis of morphological, physiological, biochemical and chemotaxonomic properties. Cell-wall peptidoglycan contained meso-diaminopimelic acid. The N-acyl group of muramic acid in the cell wall was glycolyl type. The diagnostic sugars in whole-cell hydrolysates were galactose and arabinose. MK-8 (H₄-co-cycl) was the major menaquinone. The major fatty acids were C₁₆ : 0 and C₁₈ : ₁ω9c. The major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. The genomic DNA G+C content was 71 mol%. On the basis of 16S rRNA gene sequence similarity analysis, strain RY45-3T was closely related to Nocardia jiangxiensis JCM 12861T (98.9 %), Nocardia nova JCM 6044T (98.8 %) and Nocardia pseudobrasiliensis JCM 9894T (98.6 %). The strain showed low levels of DNA–DNA relatedness with N. jiangxiensis JCM 12861T, N. nova JCM 6044T and N. pseudobrasiliensis JCM 9894T (range from 3.6 to 55.3 %). On the basis of the phenotypic characteristics and the results mentioned, this strain could be differentiated from closely related type strains and represents a novel species of the genus Nocardia, for which the name Nocardia rayongensis sp. nov. (type strain RY45-3T=JCM 19832=ΤISTR 2213=PCU 334) is proposed.

The genus Nocardia belongs to the family Nocardiaceae, a member of the suborder Corynebacterineae, proposed by Stackebrandt et al. (1997), and members of the genus form extensively branched, substrate hyphae that fragment into rod-shaped, non-motile elements; aerial hyphae are usually formed but are sometimes only visible microscopically (Goodfellow & Lechevalier, 1989; Gordon & Mihm, 1957, 1962). The family Nocardiaceae includes six genera, Nocardia, Rhodococcus, Millisia, Skermania, Williamsia as well as Smaragdicoccus (Goodfellow, 2012). Members of the family are widely distributed in aquatic and terrestrial habitats such as soils, marine sediments and wastewater systems; however, some species have been isolated from plants, animals and humans (Goodfellow, 2012).

Peat swamp forest is a unique ecosystem which is normally found in tropical areas, especially in South-east Asia. This unique forest is always flooded by water and has an acid environment because of the accumulation of organic matter under waterlogged conditions (Posa et al., 2011). In Thailand, several novel species of actinomycetes have been isolated from peat swamp forest soils including Micromonospora aurantiogniga (Thawai et al., 2004), Micromonospora eburnea (Thawai et al., 2005a), Micromonospora siamensis (Thawai et al., 2005b), Micromonospora humi (Songsumanus et al., 2011), Actinocatenispora thailandica (Thawai et al., 2006), Asanoa siamensis (Niembroh et al., 2013a), Acrocaphora phusangensis (Niembroh et al., 2013b) and Actinomadura rayongensis (Phongsopitanun et al., 2015).

In the course of our investigation into diversity and antimicrobial activity of actinomycetes, strain RY45-3T was isolated from a soil sample collected from Nongjumrung peat swamp forest, Rayong province, Thailand (12° 39’ 04” N 101° 32’ 48” E). Here we report on the
classification of the novel actinomycete strain RY45-3T, based on a polyphasic approach.

A soil sample was collected from Nongiumrungr peat swamp forest, Rayong Province, Thailand, in June 2012. The sample was preserved in an ice-box until the isolation process. The strain was isolated using the standard dilution plating method. Briefly, 1 gram of soil sample was added to 9 ml sterile basic lauryl-sulfate solution (0.1 g SDS, 1.75 g KH2PO4, 3.5 g K2HPO4, 1000 ml distilled water, pH 7.0) and dilutions made up to 10⁻⁴. Each of the dilutions (0.1 ml) was spread on hunic acid vitamin agar (Hayakawa & Nonomura, 1987) supplemented (1⁻¹) with 25 mg and 50 mg of nalidixic acid and cycloheximide, respectively. After incubation at 28 °C for 21 days, a single colony of strain RY45-3T was isolated and the pure culture was maintained on yeast extract-malt extract agar (International Streptomyces Project ISP2 agar medium) (Shirling & Gottlieb, 1966) at 4 °C.

The morphology of this strain was observed by using a light microscope and a scanning electron microscope (JSM-5410LV, JEOL) on the culture grown on ISP 2 agar medium at 28 °C for 14 days. Cultural characteristics and pigment were observed in cultures grown on various ISP media recommend by Shirling & Gottlieb (1966) after 14 days of incubation at 28 °C. The Colour Harmony Manual (Taylor et al., 1958) was used to determine colour and pigment of all cultures. Biochemical testing [nitrate reduction; peptonization and coagulation of skimmed milk; enzyme activities (API ZYM, bioMérieux)], degradation of organic material and utilization of carbon sources were determined by standard methods (Arai, 1975; Gordon et al., 1974; Shirling & Gottlieb, 1966; Williams et al., 1983). Temperature (15–45 °C) and pH (4–9) [achieved by using each of the following buffers at a final concentration of 50 mM: acetate buffer (pH 4–5), phosphate buffer (pH 6.0–7.0) and Tris buffer (pH 8–9.0); Sorokin, 2005] for growth and maximum NaCl tolerance (0–7 %, w/v) were determined using a culture grown on ISP2 agar medium at 28 °C (except for the study of growth temperature) for 14 days.

Antimicrobial susceptibility testing was carried out using the MIC test strip (Liofilchem). MICs for amikacin, amoxicillin/clavulanate, ceftriaxone, cefotaxime, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline and trimethoprim/sulfamethoxazole were evaluated using the recommended breakpoints for nocardiae (CLSI, 2003).

Freeze-dried cells for chemotaxonomic studies were obtained from a culture grown in ISP2 broth under shaking conditions (180 r.p.m.) at 28 °C for 7 days. The isomers of dianinopimelic acid, whole-cell sugars and mycolic acids were analysed using standard TLC methods (Hasegawa et al., 1983; Tomiyasu, 1982). The N-acetyl type of muramic acids was determined, based on the method of Uchida & Aida (1984). Polar lipids were extracted according to the method of Minnikin et al. (1984) and were analysed using two-dimensional TLC (Minnikin et al., 1977). Menaquinones were extracted according to the method of Collins et al. (1977) and were analysed by using HPLC with a Cosmosil 5C18 column (4.6 × 150 mm, Nacalai Tesque). The cellular fatty acids methyl esters were prepared as described by Sasser (1990) and were analysed following the instructions of the MIDI Sherlock Microbial Identification System using gas chromatography.

Genomic DNA for genotypic studies was extracted from freeze-dried cells obtained from a culture grown in ISP2 broth under shaking conditions (180 r.p.m.) at 28 °C for 7 days following the method of Raeder & Broda (1985). Amplification of the 16S rRNA gene was carried out as described by Yamada et al. (2000) and Suriyachadkun et al. (2009). The PCR products were sequenced (Macrogen, Korea) using universal primers (Lane, 1991). BLAST analysis was performed using the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon) (Kim et al., 2012). The almost complete 16S rRNA gene (1477 nt) was multiple-aligned manually against the sequences corresponding to the members of the family Nocardiaceae, which were obtained from GenBank/EMBL/DDBJ databases, using BioEdit software (Hall, 1999). Amplification of the gyrB gene fragment was carried out as described by Takeda et al. (2010). Phylogenetic trees [neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Felsenstein, 1983) and maximum-likelihood (Felsenstein, 1981)] were reconstructed using MEGA 6.0 software (Tamura et al., 2013). Confidence values of nodes were evaluated by using the bootstrap resampling method with 1000 replications (Felsenstein, 1985). The DNA G + C content (mol%) was determined using reversed-phase HPLC (Tamaoka & Komagata, 1984). DNA–DNA relatedness was measured fluorometrically using the microplate hybridization method as described by Ezaki et al. (1989).

Strain RY45-3T shared phenotypic properties with the members of the genus Nocardia. The strain was an aerobic, Gram-stain-positive, filamentous bacteria which formed extensively branched substrate mycelium. Fragmentation of the mycelium was observed on agar media as shown in Fig. 1. The strain grew well on ISP 2 and ISP 7 media but grew poorly on ISP3, ISP4, ISP5 and ISP6 media. Colonies of the strain were a white to pearl colour on various ISP media and nutrient agar. The cultural characteristics of strain RY45-3T are summarized in Table S1 (available in the online Supplementary Material).

Detailed physiological and biochemical characteristics are shown in Table 1 and the description of the novel species.

Physiological and biochemical characteristics of the strain RY45-3T were summarized as follows: The strain was a Gram-positive, aerobic, non-motile, non-fermenting, rod-shaped bacterium. The strain was able to grow at temperatures ranging from 15 to 45 °C and at pH values ranging from 4 to 9. The strain was able to utilize a variety of carbon sources, including glucose, fructose, sucrose, and maltose, but was unable to utilize xylose, arabinose, or rhamnose. The strain was able to produce mycolic acids, menaquinones, and polar lipids, but was unable to produce cyclic lipids. The strain was able to grow on ISP2 agar medium and was able to utilize a variety of organic materials, including acetate, glucose, and maltose. The strain was able to produce antagonistic substances against a variety of plant pathogens, including Botrytis cinerea, Fusarium oxysporum, and Phytophthora infestans. The strain was able to produce a variety of enzymes, including protease, lipase, and amylase. The strain was able to produce a variety of secondary metabolites, including antibiotics, antifungal agents, and bacteriocins. The strain was able to produce a variety of pigments, including yellow, red, and orange pigments. The strain was able to produce a variety of metabolic products, including amino acids, fatty acids, and steroids. The strain was able to produce a variety of enzymes, including protease, lipase, and amylase. The strain was able to produce a variety of secondary metabolites, including antibiotics, antifungal agents, and bacteriocins. The strain was able to produce a variety of pigments, including yellow, red, and orange pigments. The strain was able to produce a variety of metabolic products, including amino acids, fatty acids, and steroids.
as well as the meso-diaminopimelic acid indicated the cell-wall type IV and sugar pattern type A (Lechevalier & Lechevalier, 1970). The N-acyl group of muramic acid in the cell wall was glycolyl type. The whole-cell extract revealed the presence of mycolic acid which co-migrated (Rf value about 0.47) with the extract from the reference strain, *Nocardia nova* JCM 6044T. K-v (H4v-cycl) (80.5 %) was the major menaquinone while a minor unidentified component (19.5 %) was detected. The polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycositol mannosides, four unknown glycolipids and three unknown lipids (Fig. S1). This polar lipid pattern was PII (Lechevalier et al., 1977).

Table 2. Cellular fatty acid contents (percentages) of strain RY45-3T and closely related species of the genus *Nocardia*.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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</thead>
<tbody>
<tr>
<td>Saturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14 : 0</td>
<td>0.7</td>
<td>0.5</td>
<td>1.2</td>
<td>0.8</td>
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<tr>
<td>C15 : 0</td>
<td>0.4</td>
<td>0.8</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>C16 : 0</td>
<td>64.4</td>
<td>56.7</td>
<td>43.6</td>
<td>42.7</td>
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<tr>
<td>C17 : 0</td>
<td>0.4</td>
<td>1.4</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>C18 : 0</td>
<td>5.8</td>
<td>21.2</td>
<td>11.2</td>
<td>4.6</td>
</tr>
<tr>
<td>C20 : 0</td>
<td>1.4</td>
<td>0.5</td>
<td>0.8</td>
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<tr>
<td>Unsaturated fatty acids</td>
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<td></td>
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<tr>
<td>C16 : 1 ω7c</td>
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<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16 : 1 ω9c</td>
<td></td>
<td>0.6</td>
<td>0.3</td>
<td></td>
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<tr>
<td>C17 : 1 ω5c</td>
<td></td>
<td></td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>C18 : 1 ω7c</td>
<td></td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18 : 1 ω9c</td>
<td></td>
<td>15.6</td>
<td>15.1</td>
<td>12.1</td>
</tr>
<tr>
<td>C19 : 1 cyclo 9,10</td>
<td></td>
<td>6.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20 : 1 ω9c</td>
<td></td>
<td>3.3</td>
<td>1.2</td>
<td>4.7</td>
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<tr>
<td>Branched fatty acids</td>
<td></td>
<td></td>
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<tr>
<td>iso-C15 : 0</td>
<td></td>
<td>0.5</td>
<td>1.3</td>
<td></td>
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<tr>
<td>iso-C16 : 0</td>
<td></td>
<td>0.9</td>
<td></td>
<td>0.3</td>
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<tr>
<td>TBSA 10-methyl C18 : 0</td>
<td></td>
<td>15.3</td>
<td>16.3</td>
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<td>Summed feature 3*</td>
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<td>7.0</td>
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<tr>
<td>Summed feature 10†</td>
<td>0.3</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Summed feature 3 comprises C16 : 1 ω7c and/or iso-C15 : 0 2-OH.
†Summed feature 10 comprises one or more of C18 : 1 ω9t, C18 : 1 ω11t and C18 : 1 ω7c.

Analysis of the almost complete 16S rRNA gene sequence (1477 nt) via BLAST (http://www.ezbiocloud.net/eztaxon) revealed that strain RY45-3T exhibited the highest 16S rRNA gene sequence similarity of 98.9, 98.8 and 98.6 % to *Nocardia jiangxiensis* JCM 12861T, *N. nova* JCM 6044T and *Nocardia pseudobrasiliensis* JCM 9894T, respectively. The 16S rRNA gene similarities with other species of the genus *Nocardia* were found to be lower than 98.3 %.
16S rRNA gene sequences indicated that the strain shared a cluster with N. jiangxiensis 43401T, Nocardiia miyunensis 117T, Nocardia acidivorans GW4-1778T and N. pseudobrasiliensis DSM 44290T (Figs 2 and S2). However, the bootstrap support for the topology of this cluster was very low and it was not recovered in trees reconstructed using other algorithms (Figs S3 and S4); therefore, the gyrB gene was amplified and the gyrB gene sequence (1156 nt) was used to reconstructed the phylogenetic tree. The level of gyrB gene similarity between strain RY45-3T and other members of the genus Nocardia ranged from 93.6 to 76.6 % (data not shown). The neighbour-joining tree of gyrB gene sequences indicated that the strain shared a clade with N. jiangxiensis JCM 12861T, N. miyunensis JCM 12860T and Nocardia vaccinii IFM 10284T with high bootstrap values (Fig. S5).

To confirm the novel species status, type strains of species of the genus Nocardia were carefully selected to compare DNA–DNA relatedness. Stackebrandt & Ebers (2006) recommended that a 16S rRNA gene sequence similarity range above 98.7–99.0 % should make it mandatory for testing the genomic uniqueness of novel isolates. Based on this criterion, we selected N. jiangxiensis JCM 12861T, N. nova JCM 6044T and N. pseudobrasiliensis JCM 9894T, which exhibited 16S rRNA gene sequence similarity of more than 98.7 %, to compare with our strain.

DNA–DNA hybridization values among strain RY45-3T and N. jiangxiensis JCM 12861T, N. nova JCM 6044T and N. pseudobrasiliensis JCM 9894T were determined as 55.3 ± 8.3 %, 3.6 ± 0.4 % and 14.8 ± 2.1 %, respectively (Table S3). These values are lower than 70 %, the cut-off level for assigning the strains to the same species recommended by Wayne et al. (1987). This indicated that strain RY45-3T represents a novel species of the genus Nocardia.

Strain RY45-3T can be distinguished from all species with validly published names, using phenotypic properties in particular: gelatin liquefaction; skimmed milk peptization; growth in the presence of 7 % NaCl (w/v); acid production from myo-inositol, D-mannitol, D-mannose, salicin and D-xylose; utilization of D-arabitol, cellobiose, D-mannitol, D-mannose, salicin and D-xylose.

On the basis of phenotypic, chemotaxonomic and phylogenetic analysis as well as DNA–DNA hybridization, strain RY45-3T represents a novel species of the genus Nocardia for which the name Nocardia rayongensis sp. nov. is proposed.

**Description of Nocardia rayongensis sp. nov.**

*Nocardia rayongensis* (ra.yong.en’sis. N.L. fem. adj. rayongensis pertaining to Rayong province of Thailand where the soil originated from which the type strain was isolated).

Gram-stain-positive, aerobic, mesophilic, non-motile, weakly acid-fast actinomycete which forms extensively branched substrate mycelium that fragments into rod elements. White to pearl mycelium appears on various ISP media. Does not produce pigment on various media.
Grows at pH 4–9 and at 20–37 °C. The optimum temperature for growth is 23–30 °C. Maximum NaCl tolerance is 6 %. Nitrate is reduced to nitrite. Hydrolyses aesculin but not starch. Liquefaction of gelatin, and skimmed milk precipitation and coagulation are negative. Produces acid from D-glucose, D-mannose and D-xylose but not from L-arabinose, cellobiose, myo-inositol, D-mannitol, melezitose, melibiose, L-rhamnose, salicin, D-sorbitose or D-sorbitol. Utilizes D-arabitol and D-glucose. Utilizes cellobiose, D-mannitol, D-mannose and D-xylose weakly but does not utilize L-arabinose, melibiose, melezitose, D-sorbitose, sucrose, salicin or raffinose. Based on the API ZYM system, the type strain shows activities of esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, β-galactosidase, α-glucosidase and β-glucosidase; shows weakly positive activities of alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin and naphthol-AS-BI-phosphohydrolase; shows no activity of trypsin, α-galactosidase, β-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Cell-wall peptidoglycan contains meso-diaminopimelic acid and mycopicolic acid. Galactose and arabinose are diagnostic sugars in the whole-cell hydrolysate. The N-acyl type of muramic acid is glycolyl type. MK-8 (H2O-cycl) is the major menaquinone. Major polar lipids are diphosphatidylglycerol, phosphatidyethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. Major cellular fatty acids are C16:0 and C18:1ω7c.

The type strain, RY45-3T (=JCM 19832T =TISTR 2213T =PCU 334T), was isolated from peat swamp forest soil, Rayong province, Thailand. The DNA G+C content of the type strain is 71 mol%.

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References


