Nonomuraea monospora sp. nov., an actinomycete isolated from cave soil in Thailand, and emended description of the genus Nonomuraea

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A novel actinomycete, designated strain PT708T, was isolated from cave soil collected in Pha Tup Cave Forest Park, Nan province, Thailand. It produced compounds with antimicrobial and anticancer activities. Its chemotaxonomic properties were consistent with those of members of the genus Nonomuraea. The major menaquinone was MK-9(H4), with minor amounts of MK-9(H6), MK-9(H2), MK-10(H2) and MK-8(H4). The polar lipid profile contained phosphatidylmonomethyl-ethanolamine, diphosphatidylglycerol, hydroxy-phosphatidylmonomethylethanolamine, hydroxy-phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositolmannoside and phosphatidylinositol. The major fatty acids were iso-C16:0, 10-methyl C17:0, C16:0 and C17:1ω6c. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain PT708T belonged to the genus Nonomuraea and was most closely related to Nonomuraea rhizophila YIM 67092T (98.50 % sequence similarity) and Nonomuraea rosea GW 12687T (98.30 %). The genomic DNA G+C content of strain PT708T was 73.3 mol%. Unlike the recognized members of the genus Nonomuraea, the novel strain formed single spores at the tips of aerial hyphae. Based on the phenotypic, phylogenetic and genotypic evidence, strain PT708T represents a novel species of the genus Nonomuraea, for which the name Nonomuraea monospora sp. nov. is proposed. The type strain is PT708T (=TISTR 1910T=JCM 16114T).

The genus Nonomura was established by Zhang et al. (1998) but Chiba et al. (1999) subsequently corrected the spelling of the genus to Nonomuraea. The genus was created, on the basis of data on spore formation and 16S rRNA gene sequences, to hold some spore-forming species that had initially been placed in the genera Actinomadura (Fischer et al., 1983; Athalye et al., 1985; Poschner et al., 1985) and Microtetraspora (Kroppenstedt et al., 1990). At the time of writing, the genus comprises 27 species (Gyobu & Miyadoh, 2001; Stackebrandt et al., 2001; Quintana et al., 2003; Ara et al., 2007a, b; Le Roes & Meyers, 2008; Kämpfer et al., 2010; Li et al., 2011; Wang et al., 2011; Xi et al., 2011; Zhao et al., 2011); Nonomuraea pusilla is the type species. Members of the genus have been isolated from diverse natural habitats, including soil, marine and river sediments, caves and plants. In this report, we describe the identification, classification and nomenclature of a novel actinomycete that was isolated from soil collected in a Thai cave. The novel strain showed a close phylogenetic relationship to the genus Nonomuraea and produced compounds with antimicrobial and anticancer activities.

Soil samples, collected from caves in the Pha Tup Cave Forest Park, Nan province, northern Thailand, were held in an oven at 120 °C for 1 h and then treated with phenol to isolate rare actinomycetes (Hayakawa et al., 1995). Each resultant soil suspension was spread onto humic acid-nystatin (50 μg ml⁻¹) and cycloheximide (50 μg ml⁻¹). A pure isolate from one of these cultures, designated strain...
PT708T was routinely maintained on Hickey-Tresner (HT) agar (Hickey & Tresner, 1952) at 4 °C and held in long-term storage, at −20 °C, as a suspension containing 20% (v/v) glycerol.

The capacity of strain PT708T to produce one or more antibiotic compounds was investigated in disk diffusion assays, after incubation of the strain in AMHU-5 medium and extraction of the cell-free supernatant with ethyl acetate (Nakaew et al., 2009). The crude extract was used to determine minimum inhibitory concentrations (MICs) against several bacteria (Bacillus cereus TISTR 687, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 9027, Paenibacillus larvae LMG 9820T, Staphylococcus aureus TISTR 517 and methicillin-resistant Staphylococcus aureus), a yeast (Candida albicans) and several filamentous fungi (Fusarium oxysporum, Didymella sp., Colletotrichum sp. and Sclerotium solani). The test organisms were provided by the Faculty of Associated Medical Sciences (bacteria) or the Sustainable Development of Biodiversity Resources Centre (yeast and fungi), both of Chiang Mai University, Chiang Mai, Thailand. The crude ethyl acetate extract was also used, in sulphorhodamine B assays (Skehan et al., 1990), to determine the activity of strain PT708T against three lines of cancer cells [human breast cancer (MCF7), human oral cavity cancer (KB), and human small cell lung cancer (NCI-H187)]. Doxorubicin and ellipticine were used as the positive controls in the anticancer assays and DMSO was used as the negative control. For each cell line, the concentration of the crude extract that inhibited 50% of growth (IC50) was evaluated.

Growth on various compounds (at 1%, w/v) as sole carbon sources (Pridham & Gottlieb, 1985; Stackebrandt et al., 1989) and several filamentous fungi (Aspergillus, Penicillium, Trichoderma, Sclerotium, TISTR 687, TISTR 687), a yeast and a number of bacteria showed 100% 16S rRNA gene sequence similarity but a DNA–DNA relatedness value of only 31.0% (Stackebrandt & Kroppenstedt, 1996).

Genomic DNA was extracted, from biomass obtained from shaking incubation in ISP2 broth at 28 °C for 14 days, using the method described by Hopwood et al. (1985). The G+C content of this DNA was quantified by HPLC, following the protocol described by Mesbah et al. (1989). The universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTATTCACGARCC-3') were used in a PCR to amplify the 16S rRNA gene (Lane, 1991). The almost-complete 16S rRNA gene sequence of the novel strain (1453 nt) was determined and compared with the corresponding sequences in the GenBank database by using the BLAST program. A multiple sequence alignment was generated before a phylogenetic tree was constructed, within version 4 of the MEGA program (Tamura et al., 2007), by the neighbour-joining method of Saitou & Nei (1987). In pairwise comparisons, sequence similarities were computed using version 1.0 of the PHYDIT program (http://plaza.snu.ac.kr/~jchun/phydhit/index.php).

The genomic DNA G+C content of strain PT708T was 73.3 mol%. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain PT708T was clustered with members of the genus Nonomuraea and formed a subclade, with Nonomuraea rhizophila YIM 67092T and Nonomuraea rosea GW 12687T, that was supported by a bootstrap value of 97% (Fig. 1). Similarly, in the pairwise comparisons, strain PT708T appeared most closely related to N. rhizophila YIM 67092T (98.50% sequence similarity) and N. rosea GW 12687T (98.30%). Strains belonging to the same species generally have sequence similarities of >98.7% (Stackebrandt & Ebers, 2006). However, 16S rRNA gene sequence similarities of 97.1–100% have been reported between several Nonomuraea species that, in the corresponding hybridization experiments, showed low DNA–DNA relatedness values (Fischer et al., 1983; Poschner et al., 1985; Stackebrandt et al., 2001). For example, Nonomuraea kuesteri GW 14-1925T and Nonomuraea turkeniaca DSM 43926T were found to have a 16S rRNA gene sequence similarity of 98.9% but a DNA–DNA relatedness value of only 40.5% (Kämpfer et al., 2005). Similarly, Nonomuraea dietzia DSM 44320T and Nonomuraea roseola DSM 43767T showed 100% 16S rRNA gene sequence similarity but a DNA–DNA relatedness value of only 31.0% (Stackebrandt et al., 2001).

Whole-cell hydrolysates of strain PT708T contained meso-diaminopimelic acid, madurose, galactose and arabinoarse, indicating that the cell wall was of type IIIB (Lechevalier & Lechevalier, 1970). The major menaquinone of strain PT708T was MK-9(H4) (73%), with minor amounts of MK-9(H8) (10%), MK-9(H1) (9%), MK-10(H3) (3%) and MK-8(H4) (3%). Established members of the genus Nonomuraea have been found to have similar menaquinone

2 mm x 25 m Ultra 2 capillary column (Hewlett-Packard). All of the peaks generated were automatically identified by the Microbial Identification software (MIDI), by comparison with the ACTINO database (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).
profiles, with MK-9(H₄) or MK-9(H₄) predominant (Kroppenstedt & Goodfellow, 1991; Stackebrandt et al., 2001; Quintana et al., 2003). The novel strain’s polar lipids comprised diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, hydroxyphosphatidylmonomethylethanolamine, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylinositol mannoside, phosphatidylinositol and an unidentified aminophosphoglycolipid that possibly contained N-acetyld-g-glucosamine (Fig. S1, available in IJSEM Online). This polar lipid profile is generally similar to those of recognized species of the genus Nonomuraea. Thermopolyspora flexuosa DSM 43186T was used as an outgroup. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bar, 0.005 substitutions per nucleotide position.

Fig. 1. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between strain PT708T and recognized members of the genus Nonomuraea. Thermopolyspora flexuosa DSM 43186T was used as an outgroup. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bar, 0.005 substitutions per nucleotide position.

The major fatty acids of strain PT708T were iso-C₁₆:0 (19.6%), 10-methyl C₁₇:0 (14.8%), C₁₆:0 (7.6%), C₁₇:0 10:6c (6.8%), iso-C₁₅:0 (6.1%), iso-C₁₆:1G (6.0%), 10-methyl C₁₆:0 (5.1%) and C₁₇:10:8c (5.0%). The minor fatty acids were a summed feature (C₁₆:1 9c7c and/or iso-C₁₅:0 2:OH; 4.8%), C₁₅:0 (3.6%), 10-methyl C₁₈:0 (3.6%), C₁₄:0 (3.2%), C₁₆:0 2:OH (2.8%), C₁₈:0 (1.9%), C₁₇:0 1 (1.8%), iso-C₁₇:0 (1.5%), iso-C₁₄:0 (1.4%), C₁₈:1 10:9c (1.4%) and anteiso-C₁₇:0 (1.3%). Although this fatty acid profile is generally similar to those of recognized members of the genus Nonomuraea, it is markedly different from the fatty acid profile reported for N. rhizophila YIM 67092T, which had major amounts of 10-methyl C₁₇:0 (26.66%), iso-C₁₆:0 (24.00%), iso-C₁₆:1G (14.11%), C₁₇:10:6c (5.63%) and iso-C₁₅:0 (4.57%) and no detectable C₁₆:1 9c7c or iso-C₁₅:0 2:OH (Zhao et al., 2011). In terms of its content of 2-hydroxy fatty acids (needed for the production of hydroxyphosphatidyimonomethylethanolamine and hydroxy-phosphatidylethanolamine), strain PT708T grown in TSB (present study) appeared similar to N. rosea GW 12687T grown in DSMZ medium 65 (Kämpfer et al., 2010).

The novel strain was Gram-staining positive and produced single spores on the ends of the branched hyphae (Fig. 2a). All recognized members of the genus Nonomuraea produce chains of spores. Colony morphology, soluble pigment production and amount of growth after cultivation on ISP 2, ISP 3, ISP 4, Czapek and nutrient agars at 30 °C for 15 days are shown, and compared with the corresponding data for N. rhizophila YIM 67092T, in Table S1; the cultural characteristics of the two strains are distinct. After cultivation on ISP 3, the spore characteristics of strain PT708T are clearly different from those of its closest phylogenetic relatives (Table 1). The substrate mycelium, aerial mycelium and single spores of strain PT708T observed, by scanning electron microscopy, after cultivation for different periods of time are illustrated in Fig. 2. The diameters of mature single spores (aged 1 month) varied between 1.5 and 1.7 μm. The results of biochemical tests on strain PT708T are presented and compared with the often contrasting, corresponding results for the novel strain’s closest phylogenetic relative (N. rhizophila YIM 67092T), either in Table 1 or in the species description. When grown in AMH-Y-5 medium, the novel strain produced one or more
compounds that had antimicrobial activity when tested, in vitro, against B. cereus TISTR 687, methicillin-resistant S. aureus and P. larvae LMG 9820T, with MIC values of 80, 80 and 175 µg crude extract ml⁻¹, respectively. The crude ethyl acetate extract that was tested also showed anticancer activity when tested, in vitro, against human small lung cancer (NCI-H187) cells and oral cavity cancer (KB) cells, with IC₅₀ values of 3.48 and 16.11 µg ml⁻¹, respectively. However, no inhibition was observed when the extract was tested against breast cancer (MCF7) cells at concentrations up to 50 µg ml⁻¹.

Based on the chemotaxonomic, morphological, biochemical and phylogenetic evidence, strain PT708T represents a novel species of the genus Nonomuraea, for which the name Nonomuraea monospora sp. nov. is proposed.

**Description of Nonomuraea monospora sp. nov.**

*Nonomuraea monospora* [nɔ.nɔ.'spɔ'ra. Gr. adj. monos-single; N.L. fem. n. *spora* (from Gr. fem. n. *spora* seed) spore; N.L. fem. n. *monospora*, single spore].

Gram-staining positive. The colours of the substrate mycelium vary depending upon the medium used: deep red (ISP 2 and HT agar), red (ISP 3), vivid yellow pink (ISP 4), vivid reddish orange (NA) and brilliant orange–yellow (Czapek agar). White aerial mycelium is observed after culture on ISP 3, ISP 4, HT or Czapek agar. Production of a soluble pigment occurs on ISP 2, ISP 3 and HT agar. Single spores are observed after culture on ISP 4 for 16 days at 30 °C. Sporangia are not found. The diameters of the mature spores produced on ISP 4 vary between 1.5 and 1.7 µm. Citrate, L-arabinose, cellobiose, D-fructose, *myo*-inositol, mannitol, D-mannose, L-rhamnose, sucrose, D-xylose and lactose are utilized as sole carbon sources but raffinose is not. Gelatin, starch, casein and L-tyrosine are degraded. Produces one or more antimicrobial substances that inhibit the growth of Bacillus cereus TISTR 687, methicillin-resistant Staphylococcus aureus and Paenibacillus larvae LMG 9820T in vitro. Also produces one or more anticancer substances that inhibit the growth of human small lung cancer (NCI-H187) cells and oral cavity cancer (KB) cells in vitro. The diagnostic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid.

**Fig. 2.** Photographs of the growth of strain PT708T seen on ISP 4 after incubation at 30 °C. Light micrograph of the Gram-staining-positive hyphae and single spores at the hyphal tips seen after 16 days as shown by the arrow in the close-up view of a single spore (a) and scanning electron micrographs showing the single spores on the tips of branched mycelium after 16 (b) and 30 days (c and d). Bars, 2 µm (a and b), 1 µm (c) and 0.5 µm (d).
The description of the genus is as given by Zhang et al. (1998) with the following changes. Aerial hyphae generally bear chains of spores that are hooked, spiral or straight, but single spores may be produced. Genomic DNA G+C contents range from 64 to 74 mol%.

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**References**


**Table 1.** Phenotypic characteristics of strain PT708T and its closest phylogenetic neighbour, *Nonomuraea rhizophila* YIM 67092T, after cultivation at 30 °C for 15 days

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PT708T</th>
<th><em>N. rhizophila</em> YIM 67092T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore morphology</td>
<td>Single spores at the tips of aerial hyphae</td>
<td>Spirals of one or two turns, each of 7–10 spores</td>
</tr>
<tr>
<td>Ornamentation</td>
<td>Smooth</td>
<td>Rough</td>
</tr>
<tr>
<td>Growth on ISP 3 medium</td>
<td>Vivid red</td>
<td>Brown–yellow</td>
</tr>
<tr>
<td>Substrate mycelium</td>
<td>Vivid red</td>
<td>None</td>
</tr>
<tr>
<td>Soluble pigment</td>
<td>Vivid red</td>
<td>None</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Arabinose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Raffinose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Degradation of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>–</td>
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</table>
| **acid.** Cell hydrolysates contain madurose, galactose and arabino. The predominant menaquinone is MK-9(H4), with minor amounts of MK-9(H2), MK-9(H3), MK-10(H2) and MK-8(H4). The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol, phosphatidylinositol-mannoside and phosphatidylinositol. The major fatty acids are iso-C16:0, 10-methyl C17:0, C16:0, C17:1ω6c, iso-C15:0, iso-C16:1ω6c, 10-methyl C16:0 and C17:1ω8c. The type strain, PT708T (=TISTR 1910T=JCM 16114T), was isolated from a sample of soil collected from a cave in Pha Tup Cave Forest Park, Nan province, Thailand. The genomic DNA G+C content of the type strain is 73.3 mol%.**


