Pseudonocardia thailandensis sp. nov., an actinomycete isolated from a subterranean termite nest

Kanaporn Sujarit, 1 Nikhom Sujada, 1 Takuji Kudo, 2 Moriya Ohkuma, 2 Wasu Pathom-Aree 1 and Saisamorn Lumyong 1,*

Abstract

A novel Gram-stain-positive bacterium designated CMU-NKS-70 T was isolated from a subterranean termite nest and characterized using a polyphasic approach. The strain exhibited branching, pinkish-cream aerial mycelium and cream-brown substrate mycelium, and formed chains of rod-like spores. The 16S rRNA gene sequence analyses indicated that strain CMU-NKS-70 T belonged to the genus Pseudonocardia, showing high similarity with Pseudonocardia oxyxii D10 T (98.9 % 16S rRNA gene sequence similarity), Pseudonocardia xishanensis YIM 63638 T (98.9 %) and Pseudonocardia kujensis A4038 T (98.5 %). However, DNA–DNA relatedness values between strains CMU-NKS-70 T and the closest phylogenetically related species ranged from 40.5±2.9 to 48.6±0.7 %. Whole-cell hydrolysates of strain CMU-NKS-70 T consisted of meso-diaminopimelic acid, glucose, galactose, arabinose, mannanose, ribose and rhamnose. The predominant menaquinone was MK-8(H 4). The major cellular fatty acids (>10 %) were iso-C16 : 0, C16 : 0, C16 : 1ω7c and/or iso-C15 : 0 2-OH and 10-methyl C16 : 0. The polar lipids detected were phosphatidylethanolamine, phosphatidylmethylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, three unidentified glycolipids and two unidentified phospholipids. The G+C content of genomic DNA was 71.9 mol%. The physiological and biochemical properties also supported the phenotypic distinction of strain CMU-NKS-70 T from its closely related species. On the basis of evidence from this study using a polyphasic approach, strain CMU-NKS-70 T represents a novel species of the genus Pseudonocardia for which the name Pseudonocardia thailandensis sp. nov. is proposed. The type strain is CMU-NKS-70 T (=JCM 31292 T=TBRC 2000 T).

The genus Pseudonocardia was first described by Henssen [1] and is classified in the family Pseudonocardiaceae. Members of the genus are aerobic, Gram-stain-positive, non-acid-fast actinomycetes with high DNA G+C content (68–79 mol%) in their genomes and form branched aerial and substrate hyphae. The vegetative mycelium may fragment into chains of two or more spores, or into chains of oval or rod-shaped elements together with a tendency to form apical or intercalary swellings. Spores are normally smooth and may be formed on substrate and/or aerial mycelium by acropetal budding or basipetal septation [2]. They have a cell-wall chemotype IV which contains meso-diaminopimelic acid, arabinose and galactose, but lacks mycolic acids [3]. The predominant menaquinone is MK-8 (H 4) and the major fatty acid is iso-branched hexadecanoic acid. The polar lipids may comprise phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmethylethanolamine, and glucosamine-containing phospholipids, which varies between species [2]. A combination of chemotaxonomic and morphological criteria can be used to distinguish members of the genus Pseudonocardia from the other genera classified in the family Pseudonocardiaceae [4]. Discrimination of species within the genus Pseudonocardia must be based on clearly different phenotypic and genotypic features [2, 5].

Strains classified in novel species of the genus Pseudonocardia have been isolated from a variety of sources such as soils [6–8], plant materials [9–15], coastal and marine sediment [16, 17], and sludge [18], although their roles in natural habitats are virtually unknown. During a study on the bioactivity of the actinomycetes from a termite nest, an actinomycete strain (CMU-NKS-70 T) showing antimicrobial activity was isolated. On the basis of mycelium fragmentation and rod-shaped spore formation as well as meso-diaminopimelic acid in cell-wall composition, the strain was presumptively assigned to the genus Pseudonocardia. The present study aimed to clarify the taxonomic position of strain CMU-NKS-70 T using a polyphasic approach.

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Abbreviation: ISP, International Streptomycetes Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CMU-NKS-70 T is LC073313.

Three supplementary figures and two supplementary tables are available with the online Supplementary Material.

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Strain CMU-NKS-70T was obtained from a subterranean termite nest collected from a rubber tree plantation in Phayao Province, Thailand. The sample was collected inside the termite nest at a depth of approximately 20 cm underground. Two hundred grams of nest sample were ground into fine particles and air-dried for 7 days. Ten grams of dried sample were suspended in sterile distilled water by 10-fold dilution and then subsequently pretreated by moist heating at 50°C in a water bath for 1 h, before being spread using the serial dilution technique. Strain CMU-NKS-70T was isolated on humic acid-vitamin agar [19] supplemented with cycloheximide (50 µg ml−1) and nalidixic acid (25 µg ml−1), and then sub-cultivated on International Streptomyces Project (ISP) 2 medium [20]. The pure culture was maintained on ISP 2 slants at 28°C and in 15% (v/v) glycerol at −80°C.

Genomic DNA of strain CMU-NKS-70T was extracted using the FastPrep FP100A instrument (MP Biomedical) and the MonoFas DNA purification kit 1 (GL Sciences). The 16S rRNA gene was amplified using two PCR primers, 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACCTTGTTACGACTT-3′). The amplified gene fragment was purified and subjected to sequencing following the method of Nakajima et al. [21]. The obtained sequence was compared with corresponding sequences of type strains available from the EzTaxon-e server [22]. The 16S rRNA gene sequence of strain CMU-NKS-70T and sequences of its closest relative species retrieved from the GenBank database were aligned using MAFFT multiple sequence alignment software version 7 [23]. Phylogenetic trees were reconstructed using neighbour-joining [24], maximum-parsimony [25] and maximum-likelihood [26] tree-making algorithms in the Molecular Evolutionary Genetics Analysis (MEGA) software package version 6.06 [27]. Evolutionary distances of neighbour-joining and maximum-likelihood trees were inferred based on Kimura’s two-parameter method [28]. The statistical reliability of the tree topology was evaluated using bootstrap analysis with 1000 replicates [29].

Strain CMU-NKS-70T was subjected to chemotaxonomic analyses. Biomass for chemotaxonomic characterization studies was obtained from strain CMU-NKS-70T grown in ISP 2 broth at 28°C for 7 days on a rotary shaking incubator. The cells were harvested by centrifugation at 6500 r.p.m. for 10 min and then washed twice with sterile distilled water before being freeze-dried using a lyophilizer. The isolomers of daminopimelic acid were detected by TLC on a cellulose sheet (Merck) following the method of Hasegawa et al. [30]. Whole-cell sugars were determined using HPLC according to the procedures reported by Mikami and Ishida [31]. Cellular fatty acids were extracted, methylated and analysed by GC (model 6890; Hewlett Packard) using the Microbial Identification software package Sherlock version 6.2B (MIDI, TSBA40 database) according to the method of Sasser [32]. Menaquinones were extracted and purified as described by Collins et al. [33] and identified by HPLC [34]. Polar lipids were extracted and determined by two-dimensional TLC [35].

Gram staining was performed using the standard Gram reaction [36]. Morphological characteristics of spores and mycelia of strain CMU-NKS-70T grown on ISP 2 medium for 14 days at 28°C were observed using both light microscopy and scanning electron microscopy (ISM-5910LV; JEOL). Cultural properties of the isolate were determined on various agar media according to the guidelines of the ISP [20]. The production of melanoid pigments was detected on ISP 6 and ISP 7 media incubated at 28°C for 7–14 days. Aerial and substrate mycelia color was recorded using Colour Harmony Manual chips [37].

The physiological characteristics of strain CMU-NKS-70T and its closest phylogenetic neighbours were determined using several standard methods. Growth was tested on ISP 2 medium for 14 days at temperatures of 10, 15, 20, 28, 30, 35, 40, 45 and 50°C. Growth at different NaCl concentrations and pH values was determined on modified ISP 2 as the basal medium after incubation for 14 days at 28°C. The medium for the NaCl tolerance test was supplemented with various concentrations of NaCl (0–10%, at intervals of 1%, w/v), and the pH was adjusted after autoclaving to the desired value (pH 4.0–10.0, at intervals of 1.0 pH unit) with sterile KH2PO4/HCl, KH2PO4/K2HPO4 and K2HPO4/NaOH. Catalase and oxidase activities, nitrate reduction and degradation of cellulose, urea and gelatin were determined as described by Smibert and Krieg [38]. Carbon sources utilization was tested on the modified basal medium [39] following the described method of Shirling and Gottlieb [40]. Other physiological characteristics and acid production from carbohydrates were determined according to the procedures of Gordon et al. [40]. Enzyme activities were determined using the API ZYM system (bioMérieux) according to the manufacturer’s instructions.

Determination of G+C content of the genomic DNA was carried out using freeze-dried cells for chemotaxonomic assays which were stored at 4°C. Genomic DNA was extracted, purified following the standard protocol of Reader and Broda [41], and analysed by HPLC [42]. DNA–DNA hybridization between strain CMU-NKS-70T and the closest phylogenetic neighbours was performed under an optimal condition using the fluorescence-based microplate method [43] with five replications for each sample. One of the two DNAs for hybridization was labelled while the other was immobilized. A reciprocal experiment was performed. The highest and lowest DNA–DNA relatedness values in each sample were excluded and the mean (±SD) of the three remaining values was calculated.

The almost-complete 16S rRNA gene sequence (1439 bp) of strain CMU-NKS-70T was closely related to those of members of the genus Pseudonocardia. Strain CMU-NKS-70T showed high 16S rRNA gene sequence similarity to Pseudonocardia oroxyli D10T (98.9% 16S rRNA gene sequence similarity; 15 nt differences at 1412 sites), Pseudonocardia
xishanensis YIM 63638<sup>T</sup> (98.9 %; 16/1438), *Pseudonocardia kujensis* A4038<sup>T</sup> (98.5 %; 21/1438), *Pseudonocardia halophobica* IMSNU 21327<sup>T</sup> (98.4 %; 23/1417), *Pseudonocardia yuanmenensis* YIM 75926<sup>T</sup> (98.1 %; 28/1438) and *Pseudonocardia ailaonensis* YIM 45505<sup>T</sup> (97.6 %; 34/1438). Sequence similarities among other members of the genus *Pseudonocardia* were less than 97.6 %. The phylogenetic tree based on the neighbour-joining algorithm showed that strain CMU-NKS-70<sup>T</sup> formed a distinct phyletic line in the cluster containing *P. oroxyli* D10<sup>T</sup>, *P. xishanensis* YIM 63638<sup>T</sup> and *P. ailaonensis* YIM 45505<sup>T</sup> (Fig. 1), an association which was also recovered by the two other tree-making methods: maximum-likelihood and maximum-parsimony (Figs S1 and S2, available in the online Supplementary Material). The phylogenetic analysis indicated that strain CMU-NKS-70<sup>T</sup> belongs to the genus *Pseudonocardia* and is most closely related to *P. oroxyli* and *P. xishanensis*. The genomic DNA G+C content of strain CMU-NKS-70<sup>T</sup> was 71.9 mol%, a value in the range typical of members of the genus *Pseudonocardia* [2].

Whole-cell hydrolysates of strain CMU-NKS-70<sup>T</sup> consisted of *meso*-diaminopimelic acid, glucose, galactose, arabinose, mannose, ribose and rhamnose, indicating cell-wall chemotype IV [3]. The cellular fatty acids of strain CMU-NKS-70<sup>T</sup> were iso-C<sub>16:0</sub> (24.7 %), C<sub>16:0</sub> (16.2 %), C<sub>16:1ω7c</sub> and/or iso-C<sub>15:0</sub> 2-OH (14.0 %) and 10-methyl C<sub>16:0</sub> (12.9 %), and minor amounts of fatty acids iso-C<sub>15:0</sub> (6.0 %), C<sub>18:2ω6c</sub> and/or anteiso-C<sub>18:0</sub> (3.9 %), iso-C<sub>17:0</sub> (3.6 %), anteiso-C<sub>17:0</sub> (3.0 %), C<sub>17:0ω9c</sub> (2.9 %), iso-C<sub>16:1H</sub> (2.4 %), C<sub>18:1ω9c</sub> (2.0 %), C<sub>14:0</sub> (1.0 %) and C<sub>15:0</sub> (1.0 %) (Table S1). The respiratory quinone was MK-8(H<sub>4</sub>) (82.7 %) with a minor amount of MK-9(H<sub>2</sub>) (1.4 %). The phospholipids comprised phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, hydroxyphosphatidylethanolamine, phosphatidylinositol, three unidentified glycolipids and two unidentified phospholipids (Fig. S3). All chemotaxonomic data supported that strain CMU-NKS-70<sup>T</sup> was consistent with members of the genus *Pseudonocardia* [2].

Strain CMU-NKS-70<sup>T</sup> was aerobic and Gram-stain-positive. The strain was able to grow on all tested media, but grew well and developed abundant aerial mycelium on ISP 2. Pinkish-cream aerial mycelium and cream to brown substrate mycelium were variously produced on different ISP media (Table S2). No diffusible pigment was produced on any media tested. Melanin pigment was not produced on either ISP 6 (peptone-yeast extract iron agar) or ISP 7 (tyrosine agar). The

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**Fig. 1.** Neighbour-joining tree based on 16S rRNA gene sequences (1439 nt) showing the phylogenetic position of strain CMU-NKS-70<sup>T</sup> relative to the type strains of other species of the genus *Pseudonocardia*. *Amycolatopsis coloradensis* NRRL 3218<sup>T</sup> was used as an outgroup. Asterisks indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. Number given at each node are the bootstrap support value (%) based on 1000 replicates; only values >50 % are shown. Bar, 0.01 substitutions per nucleotide position.
substrate hyphae branched and fragmented into rod-shaped elements. The aerial hyphae fragmented into spore chains which consisted of two or more smooth, rod-shaped spores (Fig. 2). Growth occurred at 15–40 °C (optimum 28–35 °C), with 0–7 % (w/v) NaCl (optimum 1–3 %) and at pH 5.0–10.0 (optimum pH 7–9). More detailed physiological and biochemical characteristics of strain CMU-NKS-70T are in the species description. Differences in the cultural, physiological and biochemical characteristics of strain CMU-NKS-70T and closely related strains are given in Table 1, i.e. colour of aerial and substrate mycelia, pigment production, growth in the presence of sodium chloride, utilization of sole carbon sources and acid production from carbohydrates. The different cellular fatty acid components of strain CMU-NKS-70T, *P. oroxyli* D10T, *P. xishanensis* YIM 63638T and *P. kujensis* A4038T are shown in Table S1. These data clearly indicate that strain CMU-NKS-70T differed from those of close phylogenetically related strains.

DNA–DNA hybridizations between strain CMU-NKS-70T and members of the genus *Pseudonocardia* which shared 16S rRNA gene sequence similarity less than 98.7 % were not carried out following the suggestion of Stackebrandt and Ebers [44]. Furthermore, it has been shown that 16S rRNA gene sequence similarity between two strains in the range of 97.1–99.2 % shared DNA–DNA relatedness lower than the species boundary threshold value of 70 % [8, 10, 15, 17]. Thus, DNA–DNA hybridization was performed between strain CMU-NKS-70T and the closest related strains *P. oroxyli* JCM 13909T and *P. xishanensis* JCM 17906T. Strain CMU-NKS-70T showed low DNA–DNA relatedness values, less than the species cut-off value of 70 %, with both *P. oroxyli* JCM 13909T (40.5±2.9 %) and *P. xishanensis* JCM 17906T (48.6±0.7 %), thus confirming that strain CMU-NKS-70T should be differentiated from the closely related species of the genus *Pseudonocardia* and considered a novel species.

Morphological and chemotaxonomic characteristics, as well as phylogenetic data, show that strain CMU-NKS-70T is a member of the genus *Pseudonocardia*. Differences in 16S rRNA gene sequence, cultural and biochemical characteristics could be used to distinguish strain CMU-NKS-70T from its closest relatives. This evidence together with the low level of DNA–DNA relatedness strongly shows that strain CMU-NKS-70T represents a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia thailandensis* sp. nov. is proposed.

**DESCRIPTION OF PSEUDONOCARDIA THAILANDENSIS SP. NOV.**

*Pseudonocardia thailandensis* (thai.land.en’sis. N.L. fem. adj. *thailandensis* pertaining to Thailand, where the termite nest was collected and the type strain was isolated).

Aerobic, Gram-stain-positive actinomycete, which forms extensively branched substrate and aerial mycelia. Substrate mycelium is fragmented into rod-shaped elements and rod-like spore chains formed on aerial mycelium. Spore chains consist of two or more smooth spores. Grows well and forms abundant aerial mycelia on ISP 2, but grows poorly on ISP 3–5. Diffusible pigments are not produced in all media and melanin is not formed on ISP 6 or ISP 7. The aerial mycelia are pinkish-cream and the substrate mycelia are cream to brown. On ISP 2, growth occurs at 15–40 °C (optimum 28–35 °C), with 0–7 % (w/v) NaCl (optimum 1–3 %) and at pH 5.0–10.0 (optimum pH 7.0–9.0). The cell-wall hydrolysates contain *meso*-diaminopimelic acid, glucose, galactose, arabinose, mannose, ribose and rhamnose. Major cellular fatty acids are iso-C16:0, C16:1ω7c and/or iso-C15:0 2-OH, and 10-methyl C16:0. The menaquinone is MK-8(H4). The polar lipid profile contains phosphatidylethanolamine, phosphatidylethylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, three unidentified glycolipids and two unidentified phospholipids. Utilizes D-glucose, D-mannitol, L-arabinose, *myo*-inositol and sucrose as carbon sources for growth, but not citrate, D-fructose, D-xylene, raffinose or rhamnose. Positive for catalase, oxidase and urease activity, but negative for milk coagulation, nitrate reduction, gelatin liquefaction, cellulose hydrolysis and H2S production. Produces acid from D-glucose, D-mannitol, D-ribose, D-sorbitol, L-arabinose, mannose, rhamnose, sorbitol and sucrose, but not from lactose, maltose, D-xylene or inositol. Utilizes L-arginine, L-lysine and L-ornithine as sole nitrogen sources. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase activity, but negative for tryptophan, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activity.

![Fig. 2. Scanning electron micrograph of strain CMU-NKS-70T grown on ISP 2 medium at 28°C for 14 days showing mycelia and spores morphology. Bar, 2 μm.](Image)
The type strain, CMU-NKS-70^T (=JCM 31292^T=TBRC 2000^T), was isolated from a subterranean termite nest collected from a rubber tree plantation in Phayao Province, Thailand. The G+C content of the genomic DNA of the type strain is 71.9 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

Table 1. Differential cultural and physiological characteristics of strain CMU-NKS-70^T and its closest phylogenetic neighbours in the genus Pseudonocardia

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<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>b</td>
</tr>
</tbody>
</table>

*Data from: a, Huang and Goodfellow [2]; b, Zhao et al. [15].


