Nocardia speluncae sp. nov., isolated from a cave

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The taxonomic status of a mycolic acid-containing actinomycete, isolated from a natural cave on Jeju Island, Republic of Korea, was investigated by means of a polyphasic approach. The isolate, designated strain N2-11^T, produced yellow- to orange-coloured vegetative hyphae and white- to pinkish white-coloured aerial mycelia, both of which fragmented into irregular rod-shaped elements. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the organism belonged to the family *Nocardiaceae*, occupying a distinct position between *Nocardia harenae* and a *Nocardia carnea* cluster. The results of chemotaxonomic analyses were consistent with the affiliation of the organism with the genus *Nocardia*. On the basis of 16S rRNA gene sequence similarities, the closest phylogenetic neighbours were the type strains of *N. carnea* (98.3%), *Nocardia flavorosea* (98.0%), *Nocardia sienata* (97.9%) and *Nocardia testacea* (97.8%), but the organism could be clearly distinguished from its phylogenetic relatives with reference to a broad range of physiological markers. On the basis of phenotypic and molecular genetic data presented in this study, strain N2-11^T represents a novel species of the genus *Nocardia*, for which the name *Nocardia speluncae* sp. nov. is proposed. The type strain is N2-11^T (=JBRI 2006^T =KCTC 19223^T =DSM 45078^T).

The genus *Nocardia* Trevisan 1889 is a member of the family *Nocardioidaceae*, suborder *Corynebacterineae* (Stackebrandt *et al.*, 1997), and includes mycolic acid-containing actinomycetes that have the major menaquinone MK-8(H₄, ω-cycl.), in which the two terminal isoprene moieties are cyclized. At the time of writing, the genus encompasses 63 species with validly published names, including the recently described species *Nocardia jejuensis* (Lee, 2006), *Nocardia harenae* (Seo & Lee, 2006) and *Nocardia exalbida* (Iida *et al.*, 2006). *N. jejuensis* was isolated from a natural cave, as reported in our recent study (Lee, 2006); in this work, we describe another novel species of the genus *Nocardia* isolated from a cave.

Strain N2-11^T was isolated from a soil sample collected at a natural cave in Jeju, Republic of Korea. The treatment of the soil sample and the procedure used for bacterial isolation were as described previously (Lee, 2006). The isolate was maintained on yeast extract-malt extract agar (ISP 2 medium; Shirling & Gottlieb, 1966) or as mycelial fragments in 20% (v/v) glycerol at –20 and –80 °C. The cultural characteristics of strain N2-11^T were investigated on ISP 2, ISP 3 and ISP 4 media (Difco; Shirling &

Gottlieb, 1966), trypticase soy agar (TSA; Difco) and nutrient agar (NA; Difco). The results were recorded after 14 days incubation at 30 °C. Cell morphology was observed by using a light microscope, with a 14-day culture grown on ISP 3 medium at 30 °C. Strain N2-11^T showed good growth on most of the media tested, but showed only moderate growth on ISP 4 medium. Abundant production of aerial and substrate mycelia, both of which fragmented into irregular rod-shaped elements, was observed on all of the media tested. Substrate mycelium was yellow (ISP 3 and ISP 4) to orange (ISP 2, TSA and NA) in colour, while aerial mycelium was white (ISP 3, ISP 4, TSA and NA) or pinkish white (ISP 2). Diffusible pigments were not produced on any of the media tested.

For the comparison of phenotypic characteristics, *Nocardia carnea* KCTC 9957^T, *Nocardia flavorosea* KCTC 9371^T, *Nocardia sienata* DSM 44766^T and *Nocardia testacea* DSM 44765^T were obtained from the Korean Collection for Type Cultures (KCTC) (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). All reference strains were grown on ISP 2 medium at 30 °C. Strain N2-11^T and the four reference strains of the genus *Nocardia* were tested for carbohydrate utilization, degradation of various compounds and growth at various temperatures and pH values by using the methods described by Lee (2006). Characteristics that serve to

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain N2-11^T is AM422449.

An extended neighbour-joining phylogenetic tree for strain N2-11^T and a table showing the cellular fatty acid profiles of N2-11^T and related strains are available as supplementary material with the online version of this paper.

differentiate strain N2-11^T from four type strains of the genus *Nocardia* are shown in Table 1.

PCR-amplification of the 16S rRNA gene, gene sequencing and phylogenetic analyses were performed as described elsewhere (Lee, 2006; Seo & Lee, 2006). An almost-complete 16S rRNA gene sequence (1407 nt) of strain N2-11^T was compared with the corresponding sequences from the 63 type strains of *Nocardia* species with validly published names. A neighbour-joining (Saitou & Nei, 1987) phylogenetic tree (Fig. 1; the full version of this tree is available as Supplementary Fig. S1 in IJSEM Online) based on 16S rRNA gene sequences revealed that the

Table 1. Differential characteristics for strain N2-11^T with respect to its closest phylogenetic neighbours within the genus *Nocardia*

Strains: 1, N2-11^T; 2, *N. carnea* KCTC 9957^T (data from this study); 3, *N. flavorosea* KCTC 9371^T (this study); 4, *N. harenae* WS-26^T (Seo & Lee, 2006); 5, *N. pigrifrangens* JCM 11884^T (Wang *et al.*, 2004); 6, *N. sienata* DSM 44766^T (this study); 7, *N. testacea* DSM 44765^T (this study). +, Positive; –, negative; w, weak; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Utilization of:							
Acetate	+	+	+	–	+	+	+
D-Arabinose	–	–	–	–	+	+	–
Adonitol	–	–	+	–	–	–	–
Benzoate	–	–	–	–	+	–	–
2,3-Butanediol	+	–	+	–	–	+	+
Citrate	–	–	–	–	–	+	+
Dextrin	+	+	–	+	ND	+	+
D-Dulcitol	–	–	–	w	+	–	–
meso-Erythritol	–	–	+	–	+	–	–
Glycerol	–	+	w	w	+	+	–
myo-Inositol	–	+	+	–	ND	–	+
Inulin	–	–	–	–	+	–	–
Maltose	+	+	–	w	–	+	+
D-Mannitol	+	+	+	w	–	–	+
D-Mannose	–	–	–	–	+	–	–
Melibiose	–	+	–	–	–	+	–
L-Rhamnose	–	–	+	–	–	+	+
D-Ribose	–	+	–	–	+	–	+
Salicin	–	+	–	–	+	+	–
D-Sorbitol	–	+	–	–	–	–	–
Succinate	+	+	+	+	+	+	–
D-Xylitol	–	–	+	–	–	–	–
D-Xylose	–	+	–	+	+	+	+
Degradation of:							
Aesculin	w	+	+	+	–	+	+
DNA	–	–	+	ND	ND	–	–
Hypoxanthine	–	+	–	–	–	–	–
DL-Tyrosine	–	+	–	–	–	–	–
Urea	–	+	–	+	+	–	–
Growth at:							
45 °C	–	–	+	–	–	–	+
pH 4.1	–	–	–	+	ND	–	–

Fig. 1. Neighbour-joining phylogenetic tree showing the position of strain N2-11^T within the genus *Nocardia* (the full version of this tree is shown in Supplementary Fig. S1). The model of Jukes & Cantor (1969) was used to generate evolutionary distance matrices. Asterisks indicate branches that were also found in both the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) trees. Bootstrap support percentages are shown at nodes (where greater than 50 %). Bar, 0.01 substitutions per nucleotide position.

organism formed a distinct lineage between *N. harenae* and the *N. carnea* cluster, which includes *N. carnea*, *N. flavorosea*, *Nocardia pigrifrangens*, *N. sienata* and *N. testacea*. On the basis of 16S rRNA gene sequence similarities, the closest relatives of strain N2-11^T were the type strains of *N. carnea* (98.3 %), *N. flavorosea* (98.0 %), *N. sienata* (97.9 %) and *N. testacea* (97.8 %). Other closely related type strains, those of *N. harenae* and *N. pigrifrangens*, showed 16S rRNA gene sequence identities with our isolate of 97.3 and 96.8 %, respectively. Strain N2-11^T shows relatively low values for 16S rRNA gene sequence similarity (<98.3 %) with respect to the closest phylogenetic relatives in comparison with values for pairs within the *N. carnea* cluster: *N. carnea*–*N. flavorosea*, 99.2 % similarity and 2–5 % DNA–DNA relatedness (Chun *et al.*, 1998); *N. sienata*–*N. testacea*, 99.4 % similarity and 51 % relatedness (Kageyama *et al.*, 2004). Therefore DNA–DNA hybridization experiments were not performed to prove assignment of the isolate to a separate genomic species.

Chemotaxonomic characteristics of strain N2-11^T were examined using cells grown in shake flasks containing YMG broth (0.4 % yeast extract, 1 % malt extract and 0.4 % glucose; pH 7.0) at 30 °C for 3 days. The chemotaxonomic features and the G+C content of the DNA were determined as described previously (Seo & Lee, 2006). The chemotaxonomic characteristics determined were found to be typical of members of the genus *Nocardia*. Strain N2-11^T contained *meso*-diaminopimelic acid as the

diagnostic diamino acid and arabinose and galactose as the diagnostic cell-wall sugars. The glycan moiety of the murein was *N*-acetylated. The polar lipid profile contained phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol. The major lipoquinone was menaquinone MK-8(H₄, ω -cycl.), in which the two terminal isoprene moieties were cyclized. The G+C content of the DNA was 66.3 ± 0.4 mol% (as determined by HPLC).

The cellular fatty acid methyl esters were extracted from freeze-dried biomass as described previously (Minnikin, 1988) and then analysed using one-dimensional TLC (Minnikin *et al.*, 1980). Strain N2-11^T, together with *N. carnea* KCTC 9957^T, *N. flavorosea* KCTC 9371^T, *N. sienata* DSM 44766^T and *N. testacea* DSM 44765^T, was cultivated in trypticase soy broth (Difco) for 3 days at 30 °C. Two spots were identified on the chromatogram: the lower one corresponded to mycolic acids (relative *R_F* values of 0.47) and the higher one corresponded to non-hydroxylated fatty acids. Fatty acid methyl esters were purified and analysed by GC (model 6850 gas chromatograph; Agilent) as described previously (Lee *et al.*, 2000). The cellular fatty acid profile of strain N2-11^T was characterized by the presence of saturated, unsaturated and 10-methyl-branched fatty acids (typical of the genus *Nocardia*), together with additional cyclo fatty acids (see Supplementary Table S1, available in IJSEM Online). The major fatty acids were C_{18:1} *cis* (21.7%), C_{16:0} (12.0%) and cyclo-C_{19:0} (9.1%). Strain N2-11^T could be differentiated from its phylogenetic neighbours *N. carnea*, *N. flavorosea*, *N. sienata* and *N. testacea* in having relatively small amounts of C_{16:0} and 10-methyl C_{18:0} and a large amount of cyclo-C_{19:0}.

On the basis of the phenotypic and molecular genetic data from this study, strain N2-11^T represents a novel species of the genus *Nocardia*, for which the name *Nocardia speluncae* sp. nov. is proposed.

Description of *Nocardia speluncae* sp. nov.

Nocardia speluncae (spe.lun'cae. L. gen. n. *speluncae* of a cave, grotto or hole, referring to the site of isolation of the type strain).

Gram-positive, catalase-positive, urease-negative and non-motile. Good growth occurs on ISP 2, ISP 3, TSA and NA media, but growth is only moderate on ISP 4 medium. Substrate mycelium is yellow to orange in colour; aerial mycelium is white or pinkish white. Abundant aerial and substrate mycelia fragment into irregular rod-shaped elements. The temperature range for growth is 10–37 °C. Good growth is observed at 30 °C. Initial pH for growth ranges from 6.1 to 12.1. Good growth occurs in the presence of 0–4% NaCl, but growth is weak at 5% NaCl. Nitrate is reduced to nitrite. Gelatin is not liquefied. Aesculin, casein, chitin, DNA and starch are hydrolysed. Decomposition of elastin, hypoxanthine, DL-tyrosine and xanthine is not observed. Dextran, D-glucose, maltose,

sucrose, trehalose, 2,3-butanediol, D-mannitol, acetate, malate and succinate are utilized as sole carbon and energy sources. The following compounds are not utilized: D-arabinose, L-arabinose, D-cellobiose, D-fructose, D-galactose, inulin, D-lactose, D-mannose, D-melezitose, melibiose, methyl α -D-glucoside, methyl α -D-mannoside, D-raffinose, L-rhamnose, D-ribose, salicin, L-sorbose, D-xylose, adonitol, D-dulcitol, *meso*-erythritol, glycerol, *myo*-inositol, 1,2-propanediol, D-sorbitol, D-xylitol, benzoate, citrate, formate and tartrate. The major fatty acids are C_{18:1} *cis* (21.7%), C_{16:0} (12.0%) and cyclo-C_{19:0} (9.1%). The DNA G+C content is 66.3 ± 0.4 mol%.

The type strain, N2-11^T (=JBRI 2006^T =KCTC 19223^T =DSM 45078^T), was isolated from a soil sample from a natural cave on Jeju Island, Republic of Korea.

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