Nocardia jejuensis sp. nov., a novel actinomycete isolated from a natural cave on Jeju Island, Republic of Korea

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A novel actinomycete, strain N3-2^T, was isolated from a natural cave on Jeju Island, Republic of Korea, using a dilution method and was subjected to polyphasic taxonomy. The almost complete 16S rRNA gene sequence was determined by direct sequencing of the purified PCR product and was compared with those of representatives of the genus *Nocardia*. It was revealed from the phylogenetic analysis that the organism forms a distinct clade between the *Nocardia salmonicida* cluster and the *Nocardia alba* branch within the evolutionary radius occupied by the genus *Nocardia* of the family *Nocardiaceae*. The organism showed 16S rRNA gene sequence similarity of 97.4% with its nearest phylogenetic neighbours, namely *N. salmonicida* and *N. alba*. The chemotaxonomic properties, such as the principal amino acid of peptidoglycan, predominant menaquinone and polar lipids, supported the classification in the genus *Nocardia*. The organism was readily differentiated from *Nocardia* species with validly published names by a broad set of phenotypic properties and its unique phylogenetic position; the name *Nocardia jejuensis* sp. nov. is proposed, with N3-2^T (=JCM 13281^T=NRRL B-24430^T) as the type strain.

Recently, improved classification based on a polyphasic approach has provided an invaluable framework for the recognition of additional species from diverse substrates, and has led to a rapid increase in the number of described species. Most of the recently described *Nocardia* species were isolated from clinical materials (Yassin *et al.*, 2001; Kageyama *et al.*, 2004) or environmental samples, such as soil (Zhang *et al.*, 2003; Kämpfer *et al.*, 2004), rivers (Maldonado *et al.*, 2000) and activated sludge (Lemmer & Kroppenstedt, 1984).

During the investigation of the biodiversity of cave bacteria, a novel actinomycete, strain N3-2^T, was isolated from a soil sample inside a natural cave on Jeju Island, Republic of Korea, by using starch casein agar [SCA; 1% soluble starch, 0.03% casein, 0.2% KNO₃, 0.2% NaCl, 0.002% CaCO₃, 0.005% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O and 1.8% agar (pH 7.2)] and maintained as mycelial fragments or spores in 20% (v/v) glycerol or as lyophilized cells. For bacterial isolation, a soil sample (1 g) was placed into a sterile plastic tube containing 9 ml sterile distilled water. After mixing,

aliquots (100 µl) of the serial diluent of the samples were transferred onto SCA and the agar plates were incubated at 30 °C for 14 days. The organism was subjected to phenotypic and genotypic characterization in order to unravel its taxonomic status. The resultant data supported the conclusion that the organism be classified as a novel species of the genus *Nocardia*.

Cultural and morphological characteristics were observed by using the various culture media, yeast extract/malt extract agar (ISP 2 medium), oatmeal agar (ISP 3 medium), ISP 4 medium (Shirling & Gottlieb, 1966), trypticase soy agar (Difco) and nutrient agar (Difco). The degree of growth, the colour of aerial and vegetative mycelium and the presence of diffusible pigments of the organism were recorded on all tested media, incubated for 14 days at 30 °C, after which cell morphology was observed by using a light microscope. For electron microscopy, agar blocks with growth were fixed with 1% osmium tetroxide, dehydrated through a graded series of ethanol and isoamyl acetate and critical-point-dried. The gold-coated specimen was observed using a Hitachi S-2460 scanning electron microscope.

Carbohydrate utilization was tested using ISP 9 medium (Shirling & Gottlieb, 1966) including each filter-sterilized carbon source at a final concentration of 1% (w/v). The growth temperature and pH were tested at 10–45 °C and 4.1–10.1, respectively. Gram and acid-fast staining were performed as previously described (Collins *et al.*, 1995).

Published online ahead of print on 28 October 2005 as DOI 10.1099/ijs.0.63866-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain N3-2^T is AY964666.

Extended version of the neighbour-joining tree showing the relationship between strain N3-2^T and representatives of the genus *Nocardia* is available as supplementary material in IJSEM Online.

Oxidase activity was determined by monitoring the oxidation of tetramethyl-*p*-phenylenediamine on filter-paper discs. Catalase activity was determined with a 3% (v/v) hydrogen peroxide solution. Urease activity was determined by a colour change in Bacto urea broth (Difco). The production of hydrogen sulfide was detected on peptone iron agar (Difco). DNA hydrolysis was observed by using DNase test agar with methyl green (Difco). Nitrate reduction, gelatin liquefaction and hydrolysis of aesculin and starch were examined by using previously described methods (MacFaddin, 1980). Decomposition of adenine, elastin, hypoxanthine, DL-tyrosine and xanthine was examined using the method described by Gordon *et al.* (1974). Sodium chloride tolerance was determined at concentrations of 0–7% (w/v) on yeast extract/malt extract agar.

For chemical analysis, the strain was cultivated in YMG broth (0.4% yeast extract, 1% malt extract and 0.4% glucose; pH 7.0) for 3 days at 30 °C with shaking. The isomer of diaminopimelic acid, the acyl type of cell wall and the sugar composition of whole-cell walls were analysed according to previously described methods (Staneck & Roberts, 1974; Saddler *et al.*, 1991; Uchida & Aida, 1984). Purified menaquinones were identified by HPLC (Kroppenstedt, 1985). The phospholipid composition was determined by a previously described method (Minnikin *et al.*, 1977). Analysis of mycolic acids was performed using a previously described method by Minnikin *et al.* (1980). Cellular fatty acid methyl esters were prepared by a previously described method (Minnikin, 1988) and analysed by gas chromatography with a Hewlett Packard model 6850 gas chromatograph as previously described (Lee *et al.*, 2000).

Chromosomal DNA was extracted using a previously described method (Hopwood *et al.*, 1985). The 16S rRNA gene of strain N3-2^T was amplified by PCR and directly sequenced using an ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730xl; Applied Biosystems). The sequence determined in this study was aligned with reference sequences of the genus *Nocardia* by using CLUSTAL X program (Thompson *et al.*, 1997) and the alignment was manually optimized by comparison with the secondary structure of the *Escherichia coli* sequence (Brosius *et al.*, 1978). Phylogenetic analyses were performed using three tree-making algorithms, namely neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was reconstructed from evolutionary distances calculated by the method described by Jukes & Cantor (1969) using the neighbour-joining method. The tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining dataset, using 1000 replications. The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989).

It was revealed from chemotaxonomic analyses that strain N3-2^T contained chemical characters that were consistent with those of the genus *Nocardia*. Whole-cell hydrolysates

of the isolate were rich in *meso*-diaminopimelic acid, arabinose and galactose [wall chemotype IV (Lechevalier & Lechevalier, 1970)]. The polar-lipid profile contained phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol as characteristic phospholipids [phospholipid type PII pattern (Lechevalier *et al.*, 1977)]. The predominant menaquinone was a tetrahydrogenated menaquinone with eight isoprene units, in which the two terminal isoprene moieties were cyclized [MK-8(H₄, ω -cycl)]. It was shown from one-dimensional TLC of whole-cell methanolsates that two lipid spots were identified; the lower one corresponded to mycolic acids (relative R_f value of 0.47) and the higher one to non-hydroxylated fatty acids. The fatty-acid profile contained a mixture of straight-chain saturated, unsaturated and branched fatty acids, including the major components (as a proportion of the total fatty acids) hexadecanoic acid (C_{16:0}; 31.3%), 10-methyloctadecanoic acid (tuberculos-tearic acid, 10-methyl-C_{18:0}; 31.1%), octadecanoic acid (C_{18:0}; 12.7%), *cis*-9-octadecenoic acid (*cis*-9-C_{18:1}; 12.1%) and hexadecenoic acid (C_{16:1}; 7.0%). The mixture of fatty acid methyl esters also contained tetradecanoic acid (C_{14:0}; 2.8%), pentadecanoic acid (C_{15:0}; 1.7%) and heptadecanoic acid (C_{17:0}; 1.3%) as minor components. The DNA base composition of strain N3-2^T was 69.6 mol% G+C as determined by HPLC.

The 16S rRNA gene sequence of strain N3-2^T was determined by direct sequencing of the purified PCR product. The nearly complete sequence, consisting of a continuous stretch of 1392 nt, was aligned with the sequences of 41 type strains of *Nocardia* species with validly published names. A phylogenetic tree (Fig. 1), constructed by the

Fig. 1. Abbreviated neighbour-joining tree based on 16S rRNA gene sequence showing the relationship between strain N3-2^T and representatives of the genus *Nocardia* (the full version is available as Supplementary Fig. S1 in IJSEM Online). Double asterisks indicate branches of the tree that were recovered using both maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) tree-making algorithms. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets (only values greater than 60% are indicated). Bar, 1 nt substitution per 100 nt.

neighbour-joining method, showed that the organism is a member of the genus *Nocardia* but forms a distinct clade between the *Nocardia salmonicida* cluster (Maldonado *et al.*, 2000; Yassin *et al.*, 2001), which contains *Nocardia cummidelens*, *N. fluminea*, *N. salmonicida*, *N. ignorata* and *N. soli*, and the *N. alba* branch (Li *et al.*, 2004). The robustness of this clade was supported by a moderately high bootstrap value (74 %). The levels of 16S rRNA gene sequence similarity to the phylogenetic neighbours were 96.6–97.4 %, whereas the sequence similarity to the other type strains of *Nocardia* species ranged from 94.4 to 97.0 %. Strain N3-2^T shared a relatively low value of 16S rRNA gene sequence similarity (97.4 %) with its nearest relatives, *N. cummidelens* and *N. alba*, indicating that the organism merits separate species status within the genus *Nocardia*.

Strain N3-2^T showed good growth on all the media tested and formed a well-developed, branched substrate mycelium that fragmented into irregular rod-shaped elements. The reverse side of colonies was orange-coloured. The well-developed, white to pinkish aerial mycelium fragmented into irregular rod-shaped elements. Diffusible pigments were not produced on any media tested. Strain N3-2^T utilized a broad range of carbohydrates and alcohols as sole carbon sources. Growth occurred between 10 and 37 °C. The pH range for growth was 4.1–10.1. Growth occurred in the presence of 0–4 % NaCl but not in 5 % NaCl. Strain N3-2^T can be readily distinguished from its phylogenetic neighbours within the genus *Nocardia* using a range of phenotypic properties (Table 1).

Table 1. Phenotypic characteristics that differentiate strain *Nocardia jejuensis* N3-2^T from its phylogenetic neighbours

Strains: 1, *N. jejuensis* N3-2^T; 2, *N. alba* YIM 30243^T; 3, *N. cummidelens* DMS 44490^T; 4, *N. fluminea* DSM 44489^T; 5, *N. ignorata* DSM 44496^T; 6, *N. salmonicida* JCM 4826^T; 7, *N. soli* DSM 44488^T. Data were taken from Isik *et al.* (1999), Li *et al.* (2004), Maldonado *et al.* (2000) and Yassin *et al.* (2001). +, Positive; –, negative; W, weak; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Growth on sole carbon sources							
L-Rhamnose	–	+	–	+	–	–	+
myo-Inositol	+	+	–	–	–	–	–
D-Mannitol	–	+	–	–	+	+	–
D-Sorbitol	+	–	–	–	W	+	–
L-Arabinose	+	–	ND	ND	–	–	ND
Maltose	–	+	ND	ND	+	–	ND
Decomposition of:							
Casein	+	ND	–	–	–	–	–
Elastin	–	ND	–	–	–	–	–
Hypoxanthine	+	ND	–	–	–	–	–
DL-Tyrosine	+	ND	–	+	–	+	–
Xanthine	–	ND	–	–	–	+	–
Growth at pH 4.1	+	ND	–	–	ND	ND	–

On the basis of phenotypic and genotypic data, the isolate merits novel species status in the genus *Nocardia*, for which we propose the name *Nocardia jejuensis* sp. nov.

Description of *Nocardia jejuensis* sp. nov.

Nocardia jejuensis (je.ju.en'sis. N.L. fem. adj. *jejuensis* of Jeju Island, Republic of Korea, from which the type strain was isolated).

Aerobic, Gram-positive, catalase-positive, slightly acid–alcohol-fast, non-motile. Forms a well-developed and branched substrate mycelium that fragments into irregular rod-shaped elements. Colour of the substrate mycelium is orange. The white to pinkish aerial mycelium fragments into rod-shaped elements. Urease-positive. Nitrate is reduced to nitrite. Gelatin liquefaction does not occur. Aesculin, casein and DNA are hydrolysed but not starch. Growth occurs at 10–37 °C. The pH range for growth is 4.1–10.1. Growth occurs in the presence of 0–4 % NaCl but not in 5 % NaCl. Hypoxanthine and DL-tyrosine are degraded but not elastin or xanthine. L-Arabinose, D-fructose, D-glucose, inulin, D-lactose, D-melezitose, melibiose, L-ribose, salicin, sucrose, glycerol, myo-inositol and D-sorbitol are used as sole carbon and energy sources, but not D-arabinose, D-cellobiose, D-galactose, maltose, D-mannose, methyl α-D-glucoside, methyl α-D-mannoside, D-raffinose, L-rhamnose, L-sorbose, D-trehalose, D-xylose, adonitol, 2,3-butanediol, dulcitol, meso-erythritol, D-mannitol, 1,2-propanediol or D-xylitol. Type IV cell wall (meso-diaminopimelic acid, arabinose and galactose). Predominant menaquinone is MK-8(H₄, ω-cycl). Phospholipid profile contains phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol (phospholipid type PII pattern). Mycolic acids are present. Predominant cellular fatty acids are C_{16:0} (31.3 %), 10-methyl-C_{18:0} (31.1 %), C_{18:0} (12.7 %), cis-9-C_{18:1} (12.1 %) and C_{16:1} (7.0 %). The G+C content of the DNA is 69.6 mol%.

The type strain, N3-2^T (= JCM 13281^T = NRRL B-24430^T), was isolated from a natural cave on Jeju Island, Republic of Korea.

Acknowledgements

This work was supported by Korean Research Foundation Grant (KRF-2003-041-C00316). The author is indebted to H. L. Yang for the analysis of cellular fatty acids.

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