Nocardia araoensis sp. nov. and *Nocardia pneumoniae* sp. nov., isolated from patients in Japan

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Two actinomycete strains isolated from two patients with lung nocardiosis between 1995 and 1997 in Japan were assigned to novel species of the genus *Nocardia* based on morphological and chemical criteria. Comparative 16S rRNA gene sequence analysis of the two strains revealed that they belong to the genus *Nocardia* and are most closely related to the species *Nocardia beijingensis*. Determination of DNA–DNA relatedness indicated that these strains could be assigned to two novel species. Based on their phenotypic and phylogenetic characters, two novel species of the genus *Nocardia* are proposed: *Nocardia araoensis* sp. nov. for IFM 0575^T (=NBRC 100135^T=JCM 12118^T=DSM 44729^T) and *Nocardia pneumoniae* sp. nov. for IFM 0784^T (=NBRC 100136^T=JCM 12119^T=DSM 44730^T).

The genus *Nocardia* belongs to the family *Nocardiaceae* of the suborder *Corynebacterineae* within the order *Actinomycetales* (Stackebrandt *et al.*, 1997) and has undergone a revolution of its taxonomy in recent years (Kiska *et al.*, 2002; Roth *et al.*, 2003). The genus *Nocardia* encompasses 31 recognized species at the time of writing, 20 of which have been described in the last 5 years.

Most *Nocardia* species are associated with human and animal infections that are difficult to diagnose because of their non-specific clinical manifestation and histological evidence (Gordon *et al.*, 1974; Goodfellow, 1998; Boiron *et al.*, 1992). Nocardiosis is a potentially life-threatening infection caused by several species of the genus *Nocardia*: *Nocardia asteroides*, *Nocardia farcinica*, *Nocardia nova*, *Nocardia brasiliensis*, *Nocardia pseudobrasiliensis*, *Nocardia transvalensis* and *Nocardia otitidiscaviarum*.

The two strains examined here (IFM 0575^T and IFM 0784^T), isolated from clinical specimens, were initially identified as *N. asteroides* based on morphological and biochemical

characteristics. Strains IFM 0575^T and IFM 0784^T were respectively isolated from the sputum as well as broncho-alveolar lavage (BAL) of a 75-year-old Japanese male patient with non-tuberculous mycobacterial infection and the sputum of a 69-year-old Japanese male patient with a history of lung cancer, diabetes, emphysema and radiation pneumonitis. Based on phylogenetic criteria, the taxonomic position of the two strains was determined and revealed that both organisms are most closely related to *Nocardia beijingensis*. From phenotypic and phylogenetic characteristics, together with data regarding genomic DNA–DNA relatedness, we propose the inclusion of these strains within two novel *Nocardia* species, *Nocardia araoensis* sp. nov. and *Nocardia pneumoniae* sp. nov.

Strains IFM 0575^T and IFM 0784^T were initially isolated using Ogawa agar (Eiken). Strains IFM 0575^T, IFM 0784^T and *Nocardia beijingensis* IFM 10174^T were then cultured on Mueller–Hinton II (MH II; Difco) agar slants with 1 % glucose and 1 % glycerol for 1 week at 30 °C. Strains IFM 0575^T and IFM 0784^T were cultured on MH II medium with 0.2 % glucose for 1 week at 30 °C for colonization. The isolated strains were cultured on brain heart infusion (BHI; Difco) broth with 0.1 % glucose and 0.1 % glycerol for 5 days at 30 °C for DNA sequencing. The bacterial strains were cultured on BHI broth with 2 % glucose and 2 % glycine for 3 days at 30 °C for DNA–DNA hybridization analysis. Strains were stained using a modified Kinyoun method (Chapin & Murray, 1999) using 0.5 % sulfuric acid

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Nocardia araoensis* IFM 0575^T and *Nocardia pneumoniae* IFM 0784^T are AB108779 and AB108780, respectively.

Additional phenotypic characteristics and an expanded phylogenetic tree are available as supplementary material in IJSEM Online.

for determination of acid–alcohol-fastness. Morphological observations under a scanning electron microscope (model S-5200; Hitachi) were made on cultures grown on MH II agar with 0.2 % glucose (Suzuki *et al.*, 2000) at 30 °C for 7 days. For scanning electron microscope inspections, cultures were fixed in 2 % osmium tetroxide vapour *in situ* at 25 °C for 24 h, dehydrated with ethanol and *t*-butanol and then freeze-dried.

Decomposition of adenine, casein, hypoxanthine, tyrosine, urea and xanthine was examined using the methods of Gordon *et al.* (1974). Acid production from adonitol, arabinose, erythritol, galactose, glucose, inositol, maltose, mannose, rhamnose and sorbitol, utilization of citrate, adipic acid, acetamide and gluconate and growth at 37 and 45 °C were determined by the modified method of Poonwan *et al.* (1995). Decomposition of arbutin, elastin, aesculin and testosterone was examined using the methods of Goodfellow & Pirouz (1982). Utilization of nitrogen sources was tested using an N-Buiyon set (Eiken). Aryl-sulfatase activity was determined by the method of Kubica & Beam (1961). Isolated strains were tested for their ability to grow in MH II agar with 0.2 % glucose with each antibiotic TRIDISK (Eiken) at 32 °C for 2 or 3 days (Mikami & Yazawa, 1989).

Whole-cell hydrolysates were analysed for diaminopimelic acid (DAP) isomers using TLC (Staneck & Roberts, 1974). Whole-cell sugars were prepared as described by Lechevalier & Lechevalier (1980) and analysed by TLC (Miyadoh, 2001). Mycolic acids were prepared as reported by Minnikin *et al.* (1980). Menaquinones were extracted from freeze-dried biomass (500 mg) and analysed as described by Chun & Goodfellow (1995). Strains were grown for fatty acid analysis for 7 days at 28 °C in Petri dishes on trypticase soy broth agar (DSMZ medium 535). Three to four inoculation loops of cell material were scraped from the plates and used for analysis. Fatty acid methyl esters were obtained from cells after saponification, methylation and extraction as described by Miller (1982). The fatty acid methyl ester mixtures were separated using a 5 % phenyl-methyl silicone capillary column (0.2 mm × 25 m) and a gas chromatograph (model 5898A; Hewlett Packard) controlled by MIS software (Microbial ID). Peaks were automatically integrated and fatty acid names and percentages were determined using the MIS software package (Sasser, 1990). The following conditions were applied: carrier gas, ultrahigh-purity hydrogen; column head pressure, 60 kPa; injection volume, 2 µl; column split ratio, 100:1; septum purge, 5 ml min⁻¹; column temperature, 170–270 °C at 5 °C min⁻¹; injection port temperature, 250 °C; detector temperature, 300 °C.

Preparation of genomic DNA samples for sequencing was performed using the guanidine thiocyanate method of Kageyama *et al.* (2002).

16S rRNA genes were amplified and sequenced by PCR employing six prokaryotic 16S rRNA gene universal

primers. PCR was performed with a DNA thermal cycler (TaKaRa) using 35 cycles consisting of denaturation at 94 °C for 60 s, primer annealing at 60 °C for 60 s and primer extension at 72 °C for 120 s. DNA sequences were determined with an automatic sequence analyser (ABI PRISM 3100; PE Applied Biosystems) using a dye terminator cycle sequencing kit (PE Applied Biosystems).

Species related to the new isolates were identified by performing a nucleotide sequence database search using BLAST. Sequence data of related species were retrieved from GenBank. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura & Ohta, 1972) and phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987). The topology of the trees was evaluated by a bootstrap analysis of the sequence data using CLUSTAL W software (Thompson *et al.*, 1994). Sequence similarity values were determined by visual comparison and manual calculation.

Preparation of genomic DNA samples for DNA–DNA hybridization was performed using a modified version of the method described by Saito & Miura (1983). DNA G+C content was estimated by HPLC (Tamaoka & Komagata, 1984) using the same DNA as for DNA–DNA hybridization. Levels of DNA–DNA relatedness were determined by the method of Ezaki *et al.* (1989) using photobiotin and microplates.

Comparison of nearly complete 16S rRNA gene sequences of isolated strains with the corresponding sequences of other *Nocardia* species shows that the two strains form a monophyletic clade with *N. beijingensis* in the neighbour-joining tree. Fig. 1 shows a phylogenetic tree of the two isolated strains and related *Nocardia* species (an extended

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences. The tree was created using the neighbour-joining method and K_{nuc} values. Numbers on the tree indicate bootstrap percentages for the branch points. Only values above 50 % are indicated.

tree including a larger selection of reference sequences is available as supplementary material in IJSEM Online). Sequence similarity values among the two strains and *N. beijingensis* were between 98.1 and 99.1 %, and a high bootstrap value (99 %) was obtained in the neighbour-joining analysis. However, DNA–DNA relatedness between the two isolated strains and *N. beijingensis* is <46 %, and that between strains IFM 0575^T and IFM 0784^T is 35 %. These values were well below the 70 % cut-off point for species classification as recommended by Wayne *et al.* (1987).

Chemotaxonomic and morphological characteristics of strains IFM 0575^T and IFM 0784^T were consistent with their assignment to the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999), i.e. they contained galactose and arabinose as characteristic whole-cell sugars in addition to meso-DAP as the dominant cell wall diamino acid. In addition, these strains contained mycolic acid that co-migrated (*R_f* value of about 0.47) with that extracted from *Nocardia* type strains. The major menaquinone was MK-8(H_{4ω-cycl}). Strains IFM 0575^T and IFM 0784^T were partially acid-fast. These two strains were also examined for phenotypic characterization. IFM 0575^T decomposes urea and aesculin and reduces nitrate. *Nocardia paucivorans* and *Nocardia veterana* have the same characteristics, but IFM 0575^T could be distinguished from these two species on the basis of utilization of glucose and rhamnose. IFM 0784^T decomposes casein and utilizes citrate. *N. brasiliensis* and *N. pseudobrasiliensis* have the same characteristics, but IFM 0784^T could be distinguished from these two species on the basis of decomposition of hypoxanthine. Table 1 shows phenotypic properties of the two isolated strains, *N. asteroides* and *N. beijingensis*. A comparison with further species of *Nocardia* is available as supplementary material in IJSEM Online. Fatty acid analysis by GLC revealed the expected pattern diagnostic for members of the genus *Nocardia* and related taxa, i.e. straight-chain saturated and unsaturated fatty acids together with a diagnostic percentage of tuberculostearic acid (10-methyl-octadecanoic acid). The major fatty acid for the two isolated strains, *N. asteroides* and *N. beijingensis* is 16:0. However, the second most abundant fatty acid varies: IFM 0575^T, 18:1 *cis*-9; IFM 0784^T, 16:1 *cis*-7; *N. beijingensis*, 18:0. Strains IFM 0575^T and IFM 0784^T could be separated from the closely related *N. beijingensis* IFM 10174^T by quantitative differences in their fatty acid profiles (Table 2). Fig. 2 shows scanning electron micrographs (Hitachi) of strains IFM 0575^T and IFM 0784^T.

It is apparent from both phenotypic and genotypic data that these two isolated strains represent novel species within the genus *Nocardia*, for which we propose the names *Nocardia araoensis* sp. nov. and *Nocardia pneumoniae* sp. nov.

Description of *Nocardia araoensis* sp. nov.

Nocardia araoensis (a.ra.o.en'sis. N.L. fem. adj. *araoensis* referring to Arao city, where the bacterium was isolated).

Table 1. Phenotypic properties of strains IFM 0575^T and IFM 0784^T, *N. asteroides* and *N. beijingensis*

Strains: 1, IFM 0575^T; 2, IFM 0784^T; 3, *N. asteroides* ATCC 19247^T; 4, *N. beijingensis* JCM 10666^T. Data were taken from Kinoshita *et al.* (2001), Kudo *et al.* (1988), Maldonado *et al.* (2000), Yassin *et al.* (2001), Wang *et al.* (2001) and this study. +, Positive; –, negative, w, weak; ND, no data available. All strains are negative for tyrosine decomposition.

Property	1	2	3	4
Nitrate reduction	–	+	+	+
Decomposition of:				
Casein	–	+	–	–
Urea	+	–	–	–
Xanthine	–	–	–	+
Aesculin	–	+	+	+
Arbutin	–	+	+	ND
Utilization of:				
Arabinose	–	–	–	+
Erythritol	–	–	–	+
Galactose	–	–	–	+
Maltose	–	–	–	+
Mannose	–	–	–	+
Rhamnose	–	–	–	+
Citrate	+	+	+	+
Acetamide	–	+	–	ND
Gluconate	–	–	+	ND
Arylsulfatase activity	–	w	ND	ND
Growth at 37 °C	+	+	+	–
Growth at 45 °C	+	+	–	+
Susceptibility test:*				
Imipenem	1+	3+	3+	3+
Kanamycin	1+	2+	1+	3+

*Scored as follows. Imipenem: 3+, highly susceptible, growth inhibition at 2.5 µg per disc; 1+, slightly susceptible, growth inhibition at 10.0 µg per disc. Kanamycin: 3+, highly susceptible, growth inhibition at 5.0 µg per disc; 2+, moderately susceptible, growth inhibition at 10 µg per disc; 1+, slightly susceptible, growth inhibition at 30 µg per disc.

Aerobic, Gram-positive, partially acid-fast, non-motile actinomycetes that form a branched substrate mycelium that fragments into rod-shaped elements (0.3–0.5 × 1.0–1.7 µm). Faint yellow substrate mycelium, and cotton-white to greyish white aerial mycelium. No soluble pigment is produced. Colony size is small; dimensions are 0.5–1.4 mm for 7 days at 30 °C on MH II medium with 0.2 % glucose. Acetate, citrate and glucose are utilized, but adonitol, arabinose, erythritol, galactose, inositol, maltose, mannose, rhamnose, sorbitol, adipic acid, acetamide and gluconate are not. Produces acid from glucose only. Adenine, casein, hypoxanthine, tyrosine, xanthine, aesculin, arbutin and elastin are not decomposed. Urea and testosterone are decomposed. Nitrogen sources are not utilized.

Table 2. Comparison of fatty acid composition of *N. araoensis* sp. nov. IFM 0575^T, *N. pneumoniae* sp. nov. IFM 0784^T and *N. beijingensis* IFM 10174^T

Values are percentages of total fatty acids. —, Not detected. Abbreviations are exemplified as follows: 16:1 *cis*-7, *cis*-7 hexadecanoic acid (palmitoleic acid); 18:0 10-methyl, 10-methyl octadecanoic acid (tuberculostearic acid).

Fatty acid	IFM 0575 ^T	IFM 0784 ^T	IFM 10174 ^T
13:1	—	—	0·16
14:0	2·87	2·22	1·79
15:1	—	—	0·42
15:0	0·75	0·69	1·90
16:1 <i>cis</i> -9	0·47	0·66	0·25
16:1 <i>cis</i> -8	0·97	0·72	0·75
16:1 <i>cis</i> -7	10·91	21·96	10·98
16:0	41·45	35·77	28·56
16:0 10-methyl	—	0·74	0·43
17:1 <i>cis</i> -9	—	0·74	1·27
17:0	—	—	—
17:0 10-methyl	—	0·40	0·50
18:1 <i>cis</i> -9	20·03	15·63	13·41
18:1 <i>cis</i> -11	—	—	1·14
18:0	8·60	5·26	19·28
18:0 10-methyl	9·28	10·85	14·01
19:1 <i>cis</i> -9	1·95	1·98	1·50
19:1 <i>cis</i> -11	0·66	0·29	0·76
19:0	0·59	0·93	1·01
20:4 <i>cis</i> 5,8,11,14	0·70	1·19	0·79
Unknown	—	—	0·30
20:1 <i>cis</i> -11	0·75	—	0·61
20:0	—	—	0·17

No arylsulfatase activity. Grows at 45 °C. The DNA G + C content of the type strain is 69 mol%.

The type strain, IFM 0575^T (=NBRC 100135^T=JCM 12118^T=DSM 44729^T), was isolated from BAL of a male Japanese patient with non-tuberculosis mycobacterium infection.

Description of *Nocardia pneumoniae* sp. nov.

Nocardia pneumoniae (pneu.mo'ni.ae. N.L. gen. n. *pneumoniae* referring to the disease pneumonia).

Aerobic, Gram-positive, partially acid-fast, non-motile actinomycetes that form a branched substrate mycelium that fragments into rod-shaped elements (0·3–0·5 × 0·6–1·1 µm). Faint yellow substrate mycelium with white to greyish white aerial hyphae. No soluble pigment is produced. Dimensions of colonies are 2·0–3·0 mm for 7 days at 30 °C on MH II medium with 0·2 % glucose. Acetate, glucose, citrate and acetamide are utilized, but adonitol, arabinose, erythritol, galactose, inositol, maltose, mannose, rhamnose, sorbitol, adipic acid or gluconate are not.

Fig. 2. Scanning electron micrographs of strains IFM 0575^T (a) and IFM 0784^T (b) grown on MH II agar with 0·2 % glucose at 30 °C for 7 days. Bars, 2·0 µm.

Produces acid from glucose only. Adenine, hypoxanthine, tyrosine, urea, xanthine and elastin are not decomposed. Casein, aesculin, arbutin and testosterone are decomposed. Nitrogen sources are utilized. Weak arylsulfatase activity. Grows at 45 °C. The DNA G + C content of the type strain is 68 mol%.

The type strain, IFM 0784^T (=NBRC 100136^T=JCM 12119^T=DSM 44730^T), was isolated from sputum of a male Japanese patient with a history of lung cancer, diabetes, emphysema and radiation pneumonia.

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