# *Nocardia asiatica* sp. nov., isolated from patients with nocardiosis in Japan and clinical specimens from Thailand

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Five strains isolated from two patients with nocardiosis in Japan and three clinical samples from Thailand were found to have morphological, biochemical and chemotaxonomic properties consistent with their classification in the genus *Nocardia*. DNA–DNA hybridization, coupled with sequence analysis of 16S rDNA, indicated that these strains belong to a novel species of the genus *Nocardia*, named *Nocardia asiatica* sp. nov. because the isolation sites were in Asian countries; the type strain is IFM 0245$^T$ (=NBRC 100129$^T$=JCM 11892$^T$=DSM 44668$^T$).

The genus *Nocardia* Trevisan 1889 contains several species, such as *Nocardia asteroides*, *Nocardia farcinica*, *Nocardia nova*, *Nocardia brasiliensis*, *Nocardia pseudobrasiliensis*, *Nocardia transvalensis* and *Nocardia otitidiscaviarum*, that are major pathogens that cause clinical disease in humans. Nocardiosis, which occurs most frequently in immunocompromised patients, is usually caused by the *N. asteroides* group, *N. asteroides sensu stricto*, *N. farcinica* and *N. nova* (Poonwan *et al.*, 1995; Javaly *et al.*, 1992). Cutaneous nocardiosis is usually caused by *N. brasiliensis* and *N. otitidiscaviarum*. Recently, several novel pathogenic species of *Nocardia* have been described (Wang *et al.*, 2001; Yassin *et al.*, 2001a). *Nocardia africana* was also described in 2001 (Hamid *et al.*, 2001), having been isolated from sputum samples taken from patients with pulmonary diseases in Sudan. To date, it has been isolated only in that country. The five strains were also isolated in restricted areas. The strains described here have been isolated only in Japan and Thailand, i.e. in Asia. In these cases, it appears that some agents of nocardiosis may be restricted in their geographical distribution.

The genus *Nocardia* has been redefined (Goodfellow, 1992, 1998), and current methods of identification of *Nocardia* species now include not only analysis of microscopic and colony morphology and biological and chemotaxonomic characteristics, but also phylogenetic analyses. In recent years, many novel species of the genus, for example *Nocardia beijingensis* (Wang *et al.*, 2001), *Nocardia cyriacigeorgica* (Yassin *et al.*, 2001a), *Nocardia ignorata* (Yassin *et al.*, 2001b) and *Nocardia vinacea* (Kinoshita *et al.*, 2001), have been proposed.

In this paper, we report on the morphological, physiological and biochemical characteristics, analysis of cell compositions, DNA–DNA hybridization and the 16S rRNA gene sequence of five *Nocardia* isolates in comparison with those of reference strains of *Nocardia*. On the basis of the characteristics presented, these isolates represent a novel taxon within the genus *Nocardia*, for which we propose the name *Nocardia asiatica* sp. nov.

Strain IFM 0245$^T$ was isolated in 1985 from the sputum of a 41-year-old female patient with nocardiosis. Strain IFM 0263 was isolated in 1986 from a granuloma from a 68-year-old male patient with nocardiosis. These two strains were isolated in Japan. Strain IFM 0425 was isolated in 1994 from trans-tracheal aspirate, and strains IFM 0731 and IFM 0860 were respectively isolated in 1997 and 1999 from sputum; these three strains were isolated in Thailand. Clinical histories of the Thai patients were not publicly available. Strains IFM 0245$^T$, IFM 0263, IFM 0425, IFM 0731, IFM 0860, *N. beijingensis* and *N. brasiliensis* were cultured on Mueller–Hinton II agar slants with 1 % glucose and 1 % glycerol for 1 week at 27 °C. For extraction of DNA and sequencing, bacterial strains were cultured on brain heart infusion (BHI; Difco) broth for 4 days at 32 °C. Bacterial strains were cultured on BHI broth with 2 % glucose and 2 % glycine for 3 days at 32 °C for DNA–DNA hybridization. Morphological observations under a scanning

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The DDBJ accession numbers for the 16S rDNA sequences of *Nocardia asiatica* IFM 0245$^T$, IFM 0263, IFM 0425, IFM 0731 and IFM 0860 are respectively AB092566–AB092570.

electron microscope (model S-5200; Hitachi) were made on cultures grown on humic acid/MOPS/gellan gum medium (HMG; Suzuki *et al.*, 2000) at 30 °C for 10 days. For inspections using a scanning electron microscope (Hitachi), the cultures were fixed in 2 % osmium tetroxide vapour *in situ* at 25 °C for 24 h, dehydrated with ethanol and tert-butanol and then freeze-dried. Each specimen was coated with osmium using an osmium plasma coater OPC 80N (NLE Nippon Laser & Electronics Laboratory).

Decomposition of adenine, casein, hypoxanthine, tyrosine, urea and xanthine was examined by using the methods of Gordon *et al.* (1974). Acid production from carbohydrates, utilization of organic acids and growth temperature were determined by using the modified method of Poonwan *et al.* (1995). Decomposition of arbutin, elastin, aesculin and testosterone were examined by using the methods of Goodfellow & Pirouz (1982). Utilization of nitrogen sources was tested using an N-Buiyon set (Eiken). Arylsulfatase activity was determined by the method of Kubica & Beam (1961), Kubica & Rigdon (1961) and Kubica & Vestal (1961). Isolated strains were tested for their ability to grow in Mueller–Hinton II agar supplied 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 isomers using TLC (Staneck & Roberts, 1974). Whole-cell sugars were prepared as reported by Lechevalier & Lechevalier (1980) and analysed by using the TLC method (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).

A 1 ml volume of broth culture was centrifuged at 12 000 r.p.m. for 10 min. The pellet was resuspended in 200 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8·0), 250 µl GPT reagent (6 M guanidine thiocyanate dissolved in 50 mM Tris/HCl, pH 8·3) and 450 µl Tris-buffered phenol (pH 8·0). The tube was placed in a boiling-water bath for 15 min and extracted with 250 µl chloroform/isoamyl alcohol (24 : 1, v/v). After 10 min centrifugation at 12 000 r.p.m., the aqueous phase ( ~ 500 µl) was transferred to a fresh tube, mixed with 500 µl 100 % 2-propanol and 50 µl 3 M sodium acetate and then centrifuged at 12 000 r.p.m. for 15 min at 4 °C before the supernatant was decanted. Traces of GPT reagent were removed by the addition of 500 µl ice-cold 70 % ethanol to the nucleic acid pellet and a further centrifugation at 12 000 r.p.m. for 5 min at 4 °C. The ethanol was then removed and the pellet dried under a vacuum for 20 min. The pellet was finally resuspended in 50 µl TE buffer.

For sequencing of 16S rRNA genes, 16S rDNA was amplified and sequenced using a PCR and the following universal prokaryotic 16S rDNA primers: 8F (5′-AGAGTTTGATC-CTGGCTCAG-3′) and 691R (5′-ACCGCTACACCAGGA-3′), 520F (5′-CAGCAGCCGCGGTAATAC-3′) and 1100R (5′-GGGTTGCGCTGTTG-3′) and 926F (5′-AAACTCAA-AGGAATTGACGG-3′) and 1542R (5′-ACAAAGGAGGT-GATC-3′). PCR was performed with a DNA thermal cycler (TaKaRa) using 35 cycles 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. The PCR products were purified with a Centri-Sep column (Princeton Separations). DNA sequences were determined with an automatic sequence analyser (ABI PRISM 3100; PE Applied Biosystems) using the BigDye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems).

A BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) against various sequence databases was used to identify strains related to strain IFM 0245$^T$. Sequence data for related species were retrieved from GenBank. Nucleotide substitution rates ($K_{nuc}$ values) were calculated (Kimura & Ota, 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 (Thompson *et al.*, 1994). Sequence similarity values were calculated manually.

DNA was isolated as described by Saito & Miura (1983), with modifications. DNA base compositions were estimated by HPLC (Tamaoka & Komagata, 1984) and levels of DNA–DNA relatedness were determined by the method of Ezaki *et al.* (1989), using photobiotin and microplates.

Almost complete 16S rDNA sequences were determined for the five isolated strains; these sequences have been deposited in the DDBJ database. A database search demonstrated that the isolated strains belong to the suborder *Corynebacterineae* and the family *Nocardiaceae* (Stackebrandt *et al.*, 1997). It is clear from the phylogenetic tree (Fig. 1) that the five isolates belong to the same cluster and are loosely associated with *N. beijingensis*. *Nocardia* sp. DSM 43301 and *Nocardia* sp. DSM 43396 are unnamed strains of the genus *Nocardia* (Roth *et al.*, 2003); the BLAST search showed that these two strains are also closely related to the five isolated strains. Sequence similarity values among the five strains were 99·24–99·93 %. This is a high degree of similarity, but strain IFM 0245$^T$ has a 16S rDNA sequence that is slightly different from that of the other four isolates. On the other hand, sequence similarity values between these five strains and *N. beijingensis* were 98·49–98·66 %.

The chemotaxonomic and morphological characteristics of these five strains are consistent with their assignment to the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999). All of them contain galactose and arabinose as characteristic whole-cell sugars in addition to *meso*-diaminopimelic acid as the wall diamino acid; all contain two kinds of mycolic acid that co-migrated ($R_f$ values of approximately 0·47 and 0·91) with those extracted from marker *Nocardia* strains. These types of mycolic acid were seen only in *Nocardia abscessus*, *N. asiatica* and *N. beijingensis*. More than 90 % of the menaquinones were MK-8($H_{4\omega\text{-cycl}}$).

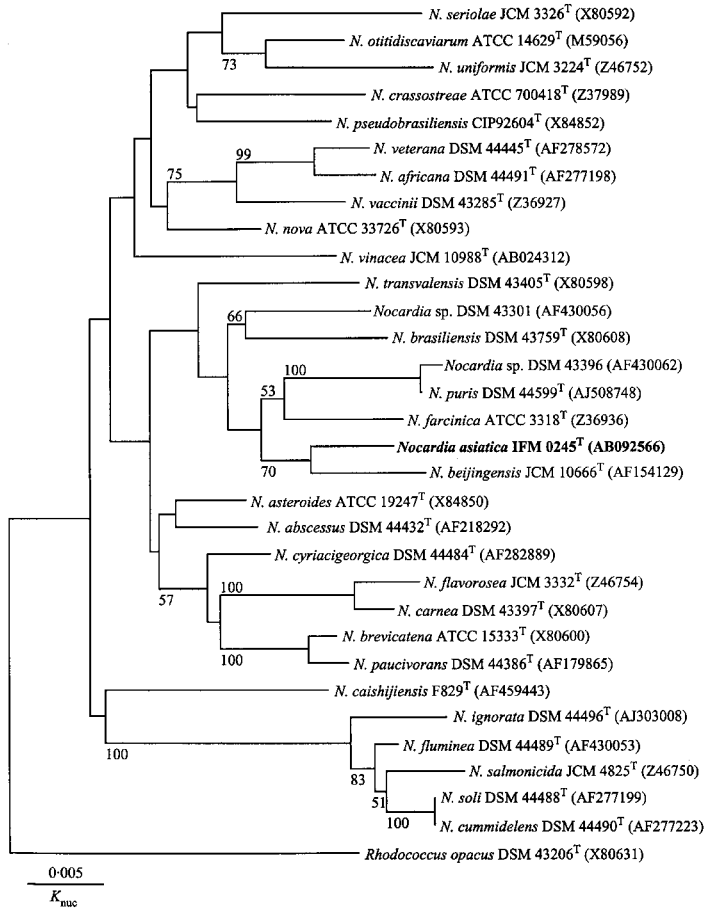


**Fig. 1.** Phylogenetic tree derived from 16S rDNA sequences. The tree was created using the neighbour-joining method and $K_{nuc}$ values. Numbers on the tree indicate bootstrap values for branch-points. Only values above 50 % are indicated. The tree was rooted by using *Rhodococcus opacus* an outgroup.

In contrast to the chemotaxonomic similarities between these five strains and other species of the genus *Nocardia*, the results of physiological tests showed clear differences between them (Table 1). Table 1 lists all *Nocardia* species with the phenotypic properties that distinguish the present five strains from the type strains of *Nocardia* species. Fig. 2 shows scanning electron micrographs (Hitachi) of strain IFM 0245$^T$.

On the basis of these phenotypic characteristics and the phylogenetic analysis, the five strains merit recognition as members of a novel *Nocardia* species. The G+C contents of IFM 0245$^T$, IFM 0263, IFM 0425, IFM 0731 and IFM 0860 were 68·4–69·9 mol% with the HPLC nucleoside method. To confirm that these strains belong to a novel species of *Nocardia*, DNA–DNA hybridization relatedness was determined among the five isolated strains and with respect to *N. beijingensis*. The values among the five isolated strains were 79–93 %; the values between the five strains and *N. beijingensis* were less than 49 % (Table 2). Therefore, these five strains were confirmed as belonging to the same novel *Nocardia* species.

On the basis of the phylogenetic and phenotypic data described above, we have concluded that IFM 0245$^T$, IFM 0263, IFM 0425, IFM 0731 and IFM 0860 represent a novel species of the genus *Nocardia*, *Nocardia asiatica* sp. nov.

## Description of *Nocardia asiatica* sp. nov.

*Nocardia asiatica* (a.si.a′ti.ca. L. gen. masc. n. *asiatica* of Asia, the source of the isolates).

Aerobic, Gram-positive, partially acid-fast, non-motile actinomycetes forming a beige substrate. Substrate mycelia are fragmented. Aerial mycelia are straight to flexuous with a mean spore number of 2–20. Spores are cylindrical (0·3–0·5 × 1·5–1·7 μm). Colonies on BHI agar and on most other commonly used media, such as Sabouraud's dextrose agar, are very small compared with those of *N. brasiliensis* and *N. asteroides* type strains. The reverse side of colonies is yellowish. Colonies are 0·3–1·0 mm in diameter after 7 days at 30 °C on Mueller–Hinton II medium with 0·2 % glucose. Glucose, acetate, rhamnose and citrate are utilized, but adipic acid, adonitol, arabinose, arbutin, aesculin, erythritol, galactose, inositol, mannose and sorbitol are not. Adenine, casein, hypoxanthine, testosterone and xanthine are not hydrolysed. The type strain is a clinical isolate. It grows at 37 °C, but not at 45 °C; nitrate is reduced to nitrite. It does not have arylsulfatase activity. The G+C content of the DNA is 68·4–69·9 mol%.

The type strain is strain IFM 0245$^T$ (=NBRC 100129$^T$=JCM 11892$^T$=DSM 44668$^T$). The five strains described were isolated from Japan and Thailand.

**Table 1.** Phenotypic properties that distinguish the novel strains from type strains of *Nocardia* species

Strains: 1, strains IFM 0245$^T$, IFM 0263, IFM 0425, IFM 0731 and IFM 0860; 2, *N. abscessus* DSM 44432$^T$; 3, *N. africana* DSM 44491$^T$; 4, *N. asteroides* ATCC 19247$^T$; 5, *N. brasiliensis* ATCC 19296$^T$; 6, *N. brevicatena* DSM 43024$^T$; 7, *N. beijingensis* JCM 10666$^T$; 8, *N. carnea* DSM 43397$^T$; 9, *N. crassostreae* ATCC 70418$^T$; 10, *N. cummidelens* DSM 44490$^T$; 11, *N. cyriacigeorgica* DSM 44484$^T$; 12, *N. farcinica* ATCC 3318$^T$; 13, *N. flavorosea* JCM 3332$^T$; 14, *N. fluminea* DSM 44489$^T$; 15, *N. ignorata* DSM 44496$^T$; 16, *N. nova* JCM 6044$^T$; 17, *N. otitidiscaviarum* NCTC 1934$^T$; 18, *N. paucivorans* DSM 44386$^T$; 19, *N. pseudobrasiliensis* ATCC 51512$^T$; 20, *N. salmonicida* JCM 4826$^T$; 21, *N. seriolae* JCM 3360$^T$; 22, *N. soli* DSM 44488$^T$; 23, *N. transvalensis* DSM 43405$^T$; 24, *N. uniformis* JCM 3224$^T$; 25, *N. vaccinii* DSM 43285$^T$; 26, *N. veterana* DSM 44445$^T$; 27, *N. vinacea* JCM 10302$^T$. Data were taken from Gürtler *et al.* (2001), Hamid *et al.* (2001), Kinoshita *et al.* (2001), Maldonado *et al.* (2000), Wang *et al.* (2001), Yassin *et al.* (2001a, b) and this study. ND, No data available; V, strain-dependent results.

| Property | 1* | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nitrate reduction | + | − | + | + | + | − | + | + | ND | + | + | + | − | + | ND | + | + | ND | − | + | + | + | + | + | + | − | + |
| Decomposition of: | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Adenine | − | − | − | − | − | − | − | − | ND | − | − | − | − | − | ND | − | − | − | + | − | − | − | − | − | − | − | − |
| Aesculin | + | − | − | + | + | + | + | + | + | + | + | + | − | + | + | + | + | − | + | + | + | + | + | + | + | ND | ND |
| Arbutin | + | ND | − | + | + | + | ND | − | ND | ND | ND | + | + | + | ND | + | + | ND | − | + | + | + | + | + | − | ND | ND |
| Casein | − | − | + | − | + | − | − | − | − | − | − | − | − | − | + | − | − | − | + | − | − | − | − | − | − | − | − |
| Elastin | − | − | − | − | + | − | − | − | ND | − | − | − | − | − | − | − | − | − | + | − | − | − | + | + | − | ND | − |
| Hypoxanthine | − | − | − | − | + | − | − | − | − | − | − | − | − | − | − | − | + | − | + | − | − | − | + | + | − | − | + |
| Testosterone | + | + | ND | + | + | + | ND | + | ND | ND | + | + | + | ND | + | + | − | − | − | + | − | + | + | + | − | ND | + |
| Tyrosine | − | − | − | − | + | − | − | − | − | − | − | − | − | + | − | − | − | − | + | + | − | − | − | + | − | − | − |
| Urea | V[a] | ND | − | − | − | − | − | − | − | − | ND | − | + | − | ND | − | + | ND | + | − | − | − | + | + | − | ND | + |
| Xanthine | − | − | − | − | − | − | + | − | − | − | − | − | − | − | − | − | + | − | − | ND | − | − | − | + | − | − | − |
| Utilization of: | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Acetate | + | + | − | + | + | + | + | + | ND | + | + | + | + | − | + | + | + | + | + | + | + | + | + | + | − | − | ND |
| Adipic acid | − | − | ND | − | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Mannitol | V[b] | ND | − | + | + | − | + | + | − | − | − | − | − | − | + | + | + | − | + | + | − | − | + | − | + | − | + |
| Rhamnose | + | + | − | − | − | + | + | − | ND | − | − | + | − | + | − | − | − | − | − | − | − | + | + | − | + | + | − |
| Sorbitol | − | − | − | − | − | − | + | + | ND | − | ND | − | − | − | ND | + | − | − | + | + | − | − | + | − | − | ND | + |
| Citrate | + | + | − | + | + | − | + | − | − | − | − | − | − | + | − | − | − | − | + | + | + | − | − | − | − | − | ND |
| Arylsulfatase activity | − | − | ND | − | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Growth at 37 °C | + | ND | ND | ND | ND | ND | ND | ND | − | ND | ND | ND | ND | ND | ND | + | ND | ND | ND | − | − | − | ND | ND | ND | ND | ND |
| Growth at 45 °C | − | − | + | − | + | − | − | − | − | − | ND | + | + | − | ND | − | + | ND | − | − | − | − | − | − | − | + | − |
| Susceptibility tests:† | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Imipenem[a] | + + + | + | + + + | + + + | − | ND | + + + | + + + | ND | + + + | + + + | + + + | ND | ND | ND | + + + | ND | + + + | + + + | ND | ND | + + + | + + + | ND | ND | + + + | + + + |
| Tobramycin[a] | V[c] | + + + | + | + + + | + + + | ND | + + + | + + + | ND | + + + | + + + | − | ND | ND | ND | − | ND | + + + | + + + | ND | ND | + + + | − | ND | ND | − | + + + |
| 5-Fluorouracil[b] | − | − | + | − | − | ND | − | − | ND | − | − | − | ND | ND | ND | + | ND | − | − | ND | ND | − | − | ND | ND | + | − |

*Strain-dependent results indicated as: *a*, IFM 0245$^T$ and IFM 0731 negative, other strains positive; *b*, IFM 0245$^T$ positive, other strains negative; *c*, IFM 0731 scored as + +, other strains scored as + + +.

†Scored as: *a*, growth at 10 (+ + +), 5 (+ +) or 2·5 (+) μg per disk or no growth with 2·5 μg per disk (−); *b*, growth (+) or no growth (−) with 60 μg per disk.

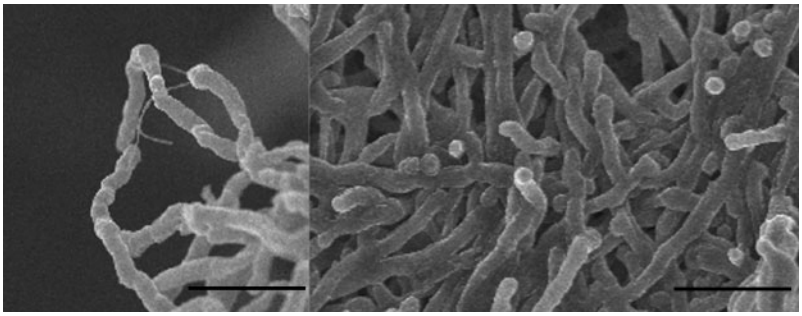

**Fig. 2.** Scanning electron micrographs of strain IFM 0245$^T$ grown on Mueller–Hinton II agar with 0·2% glucose at 30 °C for 7 days. Bars, 2·0 μm.

**Table 2.** Levels of DNA–DNA relatedness among the five novel strains and *N. beijingensis*

| Strain | DNA–DNA reassociation (%) with: | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| 1. IFM 0245$^T$ | 100 | 93 | 82 | 82 | 79 | 48 |
| 2. IFM 0263 | 93 | 100 | 81 | 94 | 83 | 53 |
| 3. IFM 0425 | 79 | 80 | 100 | 87 | 98 | 52 |
| 4. IFM 0731 | 80 | 80 | 84 | 100 | 82 | 48 |
| 5. IFM 0860 | 78 | 73 | 90 | 81 | 100 | 49 |
| 6. *N. beijingensis* | 33 | 36 | 49 | 36 | 43 | 100 |

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