Nocardia shimofusensis sp. nov., isolated from soil, and *Nocardia higoensis* sp. nov., isolated from a patient with lung nocardiosis in Japan

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Three actinomycete strains isolated from soils and one strain isolated from a patient with lung nocardiosis in 1999 and 2001 in Japan have been provisionally assigned to the genus *Nocardia* on the basis of morphological criteria. These isolates were further investigated to determine their specific taxonomic status. Detailed chemotaxonomic characterization and 16S rRNA gene sequence analysis of these isolates confirmed that they belong to the genus *Nocardia*. The 16S rRNA gene sequences of the four strains were most similar to that of *Nocardia farcinica*. However, the sequence similarity values between these four strains and *N. farcinica* were <98.9%. These four strains were susceptible to 5-fluorouracil, and they have the ability to decompose urea, which is a very characteristic trait. Furthermore, DNA–DNA relatedness data revealed that IFM 10311^T, IFM 10312 and IFM 10313 comprise a single novel species of *Nocardia*, that IFM 10084^T represents another novel species of *Nocardia* and that these two novel species could be distinguished from *N. farcinica*. The names *Nocardia shimofusensis* sp. nov. and *Nocardia higoensis* sp. nov. are proposed, with IFM 10311^T (=NBRC 100134^T=JCM 12122^T=DSM 44733^T) and IFM 10084^T (=NBRC 100133^T=JCM 12121^T=DSM 44732^T) as the respective type strains.

The genus *Nocardia* is a member of the family *Nocardiaceae* within the order *Actinomycetales* (Stackebrandt *et al.*, 1997). This genus belongs to the mycolic acid-containing group of actinomycetes represented by the suborder *Corynebacterineae* (Chun *et al.*, 1996; Kämpfer *et al.*, 1999; Linos *et al.*, 2002). Members of these genera can be distinguished from one another using a combination of biochemical, morphological and chemical characteristics (Goodfellow *et al.*, 1999; Linos *et al.*, 2002). The genus *Nocardia* currently encompasses 31 species with validly published names; 20 of these species have been described in the last 5 years. These species have been isolated from clinical samples and soils.

Nocardia asteroides is the major cause of nocardiosis; however, authors of many taxonomic studies (Schaal & Reuterberg, 1978; Orchard & Goodfellow, 1980) have considered *N. asteroides* as a heterogeneous species. Four strains that were similar to *N. asteroides* in their morphological and biochemical characteristics have been isolated in the present study. Further taxonomic studies revealed that the organisms consistently formed a distinct clade most closely associated with *Nocardia farcinica*. Comparative DNA–DNA relatedness data show that the four strains are distinguishable from the *N. farcinica* type strain and hence represent two novel species within the genus *Nocardia*. We propose the novel species designations *Nocardia shimofusensis* sp. nov. and *Nocardia higoensis* sp. nov.

Strains IFM 10311^T, IFM 10312 and IFM 10313 were isolated by the method of Khan *et al.* (1997) from soils in the city of Choshi and in the village of Chyosei, respectively, in Japan in 1999. Strain IFM 10084^T was isolated using Ogawa agar (Eiken) in 2001 from a 70-year-old Japanese female patient who had a history of autohepatitis and had received steroid therapy. She complained of chest pain, was

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains IFM 10311^T, IFM 10312 and IFM 10313 and IFM 10084^T are AB108775–AB108778, respectively.

An extended 16S rRNA gene-based phylogenetic tree and a comparison of properties of all *Nocardia* species are available as supplementary material in IJSEM Online.

hospitalized and her condition was diagnosed as pleurisy involving *Nocardia* species.

The four strains IFM 10311^T, IFM 10312, IFM 10313, IFM 10084^T and *N. farcinica* IFM 0284^T were cultured on Mueller–Hinton medium II (MHII; Difco) slants with 1 % glucose and 1 % glycerol for 1 week at 30 °C. They were also cultured on MHII agar plates with 1 % glucose and 1 % glycerol for 1 week at 30 °C for colonization. The isolated strains were cultured in brain heart infusion broth (Difco) with 0.1 % glucose and 0.1 % glycerol for 5 days at 30 °C prior to DNA extraction, amplification and sequencing. Bacterial strains were cultured in brain heart infusion broth with 2 % glucose and 2 % glycine for 3 days at 30 °C for use in DNA–DNA hybridization experiments. Morphological observations under a scanning electron microscope (model S-5200; Hitachi) were made on cultures grown on MHII agar with 0.2 % glucose or humic acid-MOPS gellan gum medium (Suzuki *et al.*, 2000) at 30 °C for 7–10 days.

Decomposition of adenine, casein, hypoxanthine, tyrosine, urea and xanthine was examined by 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 and growth at 37 and 45 °C were determined by using the modified method of Poonwan *et al.* (1995). The strains isolated were tested for their ability to grow on MHII 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 isomers using TLC (Staneck & Roberts, 1974). Whole-cell sugars were prepared as reported previously (Lechevalier & Lechevalier, 1980) and analysed by TLC (Miyadoh, 2001). Mycolic acids were prepared and analysed as described by Minnikin *et al.* (1980). Menaquinones were extracted from freeze-dried biomass (500 mg) and analysed as described by Chun & Goodfellow (1995). The strain was 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 the analyses. 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 package (Sasser, 1990). The following conditions were applied: carrier gas, ultra-high-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 (Kageyama *et al.*, 2002).

16S rRNA genes were amplified and sequenced using a PCR employing six prokaryotic 16S rRNA universal primers. The PCR was performed in 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. PCR products were purified using a Centri-Sep column (Princeton Separations). DNA sequences were determined using an automatic sequence analyser (ABI PRISM 3100; PE Applied Biosystems) with 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 the BLAST programs (nucleotide–nucleotide BLAST: <http://www.ncbi.nlm.nih.gov/BLAST>). Sequences of related species were also retrieved from GenBank. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura & Ohta, 1972) and phylogenetic trees were constructed using 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.

DNA was also isolated as described by Saito & Miura (1983) with modification for successive DNA base composition analysis as estimated by HPLC (Tamaoka & Komagata, 1984). Levels of DNA–DNA relatedness were determined by the method of Ezaki *et al.* (1989) using photobiotin and microplates.

The chemotaxonomic and morphological characteristics of these four isolates were consistent with their assignment to the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999). All strains contained galactose and arabinose as characteristic whole-cell sugars in addition to *meso*-diaminopimelic acid as the dominant cell-wall diamino acid. In addition, the strains contained mycolic acid that co-migrated (R_f value approx. 0.47) with that extracted from *Nocardia* type strains. The major menaquinone was MK-8($H_{4\alpha\text{-cyc}}$). Analysis of the fatty acids by GLC revealed the expected pattern that is diagnostic for members of the genus *Nocardia* and related taxa, i.e. straight-chain saturated and unsaturated fatty acids together with a diagnostic amount of tuberculostearic acid (10-methyl-branched octadecanoic acid) (Table 1).

Nearly complete 16S rRNA gene sequences were determined for the four isolated strains. A database search demonstrated that these strains belonged to the suborder *Corynebacterineae* of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997). It was clear from the phylogenetic tree (Fig. 1) that the four isolates formed a monophyletic clade loosely associated with *N. farcinica*. The sequence similarity values among IFM 10311^T, IFM 10312 and IFM 10313 were >99.9 %, and these three isolates and IFM 10084^T had

Table 1. Comparison of fatty acid composition of *N. higoensis* sp. nov. IFM 10084^T and *N. shimofusensis* sp. nov. IFM 10311^T with the type strains of the related species *N. farcinica* and *N. beijingensis*

Values are percentages of total fatty acids. ECL, Unknown fatty acid with equivalent chain-length. Abbreviations are exemplified by: *cis*-9 18:1, *cis*-9 octadecenoic acid (oleic acid); 10-methyl-18:0, 10-methyl-octadecanoic acid (tuberculostearic acid).

Fatty acid	IFM 10084 ^T	IFM 10311 ^T	<i>N. beijingensis</i>	<i>N. farcinica</i>
14:0	1.37	1.97	1.06	0.88
ECL 14:959	0.81	2.09	1.50	1.22
15:1	—	—	—	—
15:0	0.88	1.36	—	—
<i>cis</i> -7 16:1	—	—	0.27	—
<i>cis</i> -8 16:1	—	—	0.87	—
<i>cis</i> -9 16:1	17.12	16.93	8.69	15.35
16:0	23.78	26.15	30.01	22.88
<i>cis</i> -9 17:1	1.70	1.49	1.36	—
17:0	2.88	3.75	3.41	3.40
10-Methyl-17:0	0.86	—	—	—
<i>cis</i> -9 18:1	10.67	11.10	20.65	22.01
<i>cis</i> -11 18:1	—	—	0.79	—
18:0	4.31	3.52	13.59	9.25
10-Methyl-18:0	29.11	25.13	15.39	14.96
<i>cis</i> -9 19:1	1.22	1.46	0.69	—
19:0	—	—	0.49	—
ECL 19:399	1.85	1.81	—	—
<i>cis</i> -11 20:1	3.44	3.25	0.59	5.08
20:0	—	—	—	0.94

99.1–99.3 % identity; the sequence similarity values between these four strains and *N. farcinica* were <98.9 %. The distinction between these four strains and *N. farcinica* was supported by DNA–DNA relatedness data. The DNA relatedness levels among IFM 10311^T, IFM 10312 and IFM 10313 were between 88 and 96 %. IFM 10311^T, IFM 10312 and IFM 10313 showed DNA relatedness values of 17–32 % with IFM 10084^T. The four isolates revealed DNA–DNA relatedness values of <12 % with the type

strain of *N. farcinica* (Table 2). These values were well below the 70 % cut-off point for species classification as recommended by Wayne *et al.* (1987).

The four strains were also examined for a set of phenotypic characteristics in comparison with *Nocardia asiatica*, *Nocardia beijingensis*, *N. farcinica* and *Nocardia puris* (Table 3). From the investigations described, it was clear that the strains could be distinguished from other *Nocardia* species with validly published names by a combination of phenotypic characteristics (see supplementary table in IJSEM Online). It was possible to differentiate *Nocardia* species from the strains isolated using a combination of the results of the utilization of sorbitol and citrate, susceptibility to fluorouracil, imipenem, tobramycin and kanamycin and growth at 45 °C.

Table 2. Levels of DNA–DNA relatedness (%) among the isolated *Nocardia* strains and *N. farcinica*

Strain	1	2	3	4	5
1. IFM 10084 ^T	100	22	17	32	6
2. IFM 10311 ^T	28	100	96	93	7
3. IFM 10312	19	93	100	93	7
4. IFM 10313	21	88	96	100	5
5. <i>N. farcinica</i> IFM 0284 ^T	9	5	6	12	100

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 values for branch points. Only values above 50 % significance are indicated. An extended tree is available as supplementary material in IJSEM Online.

Table 3. Phenotypic properties of the isolated strains and phylogenetically related *Nocardia* species

Strains: 1, IFM 10084^T; 2, IFM 10311^T; 3, *N. asiatica* IFM 0245^T; 4, *N. beijingensis* JCM 10666^T; 5, *N. farcinica* ATCC 3318^T; 6, *N. puris* DSM 44599^T. Data were taken from Kageyama *et al.* (2002), Kinoshita *et al.* (2001), Wang *et al.* (2001), Yassin *et al.* (2000, 2001a, b, 2003) and this study. +, Positive; –, negative; ND, no data. All of the organisms were negative for decomposition of adenine, casein, hypoxanthine and tyrosine.

Characteristic	1	2	3	4	5	6
Decomposition of:						
Urea	+	+	–	–	–	+
Xanthine	–	–	–	+	–	–
Utilization of:						
Arabinose	–	–	–	+	–	–
Erythritol	–	–	–	+	–	–
Galactose	–	–	–	+	–	–
Glucose	–	–	+	+	+	+
Inositol	–	–	–	–	–	+
Maltose	–	–	+	+	+	+
Mannose	–	–	–	+	ND	ND
Rhamnose	–	–	+	+	+	–
Sorbitol	–	–	–	–	–	+
Citrate	–	–	+	+	–	+
Growth at:						
37 °C	+	+	+	–	+	+
45 °C	+	–	–	–	+	+
Susceptibility to:*						
Imipenem ^a	+++	+++	+++	+++	+++	ND
Tobramycin ^a	+++	+++	+++	+++	–	ND
5-Fluorouracil ^b	+	+	–	–	–	ND
Kanamycin ^c	+++	+++	++	+++	–	ND

*Scored as: a, + + +, highly susceptible (growth inhibition at 2.5 µg per disc), –, resistant (no growth inhibition at 10 µg per disc); b, +, susceptible (growth inhibition at 30 µg per disc), –, resistant (no growth inhibition at 30 µg per disc); c, + + +, highly susceptible (growth inhibition at 5 µg per disc), + +, moderately susceptible (growth inhibition at 10 µg per disc), –, resistant (no growth inhibition at 30 µg per disc).

On the basis of both phenotypic and genotypic data, it was concluded that the four strains formed two novel species within the genus *Nocardia*. We therefore propose that the names *Nocardia shimofusensis* sp. nov. and *Nocardia higoensis* sp. nov. should be used to describe these four strains of *Nocardia*.

Description of *Nocardia shimofusensis* sp. nov.

Nocardia shimofusensis (shi.mo.fus.en'sis. N.L. fem. adj. *shimofusensis* pertaining to Shimofusa, a traditional geographical name for a northern part of Chiba Prefecture in Japan, the source of the isolates).

Aerobic, Gram-positive, partially acid-fast, non-motile

actinomycetes that form an extensively branched substrate mycelium that fragments into rod-shaped elements (0.5–0.7 × 0.9–1.7 µm). Orange to reddish orange substrate mycelium carries white to reddish white aerial hyphae. No soluble pigment is produced. Colonies are 0.2–1.0 mm in diameter after 7 days at 30 °C on MHII medium with 0.2 % glucose. Arabinose, erythritol, galactose, glucose, inositol, maltose, mannose, rhamnose, sorbitol and citrate are not utilized. Adenine, casein, hypoxanthine, tyrosine and xanthine are not decomposed. Urea is decomposed. Does not grow at 45 °C. The G + C content of the DNA is 68–69 mol%.

The type strain, IFM 10311^T (=NBRC 100134^T=JCM 12122^T=DSM 44733^T), was isolated from soil at Choshi in Japan.

Description of *Nocardia higoensis* sp. nov.

Nocardia higoensis (hi.go.en'sis. N.L. fem. adj. *higoensis* pertaining to Higo, a traditional geographical name for Kumamoto Prefecture in Japan, the source of the type strain).

Aerobic, Gram-positive, partially acid-fast, non-motile actinomycetes that form an extensively branched substrate mycelium that fragments into rod-shaped elements (0.6–0.8 × 0.9–2.1 µm). Orange to reddish orange substrate mycelium carries white to reddish white aerial hyphae. Colonies without aerial hyphae may be produced, but they have a 16S rRNA gene sequence identical to that of the parent colonies (accession no. AB108778). No soluble pigment is produced. Colonies are 0.3–0.6 mm in diameter after 7 days at 30 °C on MHII medium with 0.2 % glucose. Arabinose, erythritol, galactose, glucose, inositol, maltose, mannose, rhamnose, sorbitol and citrate are not utilized. Adenine, casein, hypoxanthine, tyrosine and xanthine are not decomposed. Urea is decomposed. Grows at 45 °C. The G + C content of the DNA is 69 mol%.

The type strain, IFM 10084^T (=NBRC 100133^T=JCM 12121^T=DSM 44732^T), was isolated from a patient with lung nocardiosis.

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