Nocardia vermiculata sp. nov. and *Nocardia thailandica* sp. nov. Isolated from Clinical Specimens

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The taxonomic positions of actinomycetes isolated from clinical specimens in Japan and Thailand were examined using a polyphasic approach. The strains IFM 0391^T and IFM 10145^T 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 two new species of the genus *Nocardia*: *Nocardia vermiculata* for IFM 0391^T (=NBRC 1000427^T = JCM 12345^T = DSM 44807^T), and *Nocardia thailandica* for IFM 10145^T (=NBRC 100428^T = JCM 12356^T = DSM 44808^T).

INTRODUCTION

Nocardia is a filamentous, Gram-positive, partially acid-fast, branched bacterium, with the typical habitat of a soil saprophyte, and classified as an aerobic actinobacteria^{1, 2)}. Most *Nocardia* species can cause human infections that are difficult to diagnose due to non-specific clinical and histological manifestations^{1–3)}. Therefore, for definitive diagnosis and effective therapy, identification of *Nocardia* species is required^{4–8)}. *Nocardia asteroides* is considered the major cause of nocardiosis; however, many taxonomic studies have repeatedly reported that *N. asteroides* is a heterogeneous species^{9, 10)}. Two strains with similar morphological and biochemical characteristics to *N. asteroides* were isolated from clinical specimens and used in this study. Present taxonomic studies revealed that the strains, IFM 10145^T and IFM 0391^T, were loosely associated with *N. asteroides*, *N. caishijiensis* and *N. neocaledoniensis*, and *N. africana*, *N. cerradoensis*, *N. nova*, *N. vaccinii*, *N. veterana*, and *N. vinacea*, respectively. However, these strains were found to be delineated from *N. asteroides* and *N. africana* and related species based on comparative DNA-DNA relatedness data. Therefore, these two strains were found to represent new species within the genus *Nocardia*. We propose the new species designations *Nocardia vermiculata* sp. nov. and *Nocardia thailandica* sp. nov.

MATERIALS AND METHODS

Organism and cultivation: IFM 0391^T was isolated from sputum of a 63-year-old Japanese male patient in 1985.

IFM 10145^T was isolated from pus of a Thai patient. Strain IFM 0391^T, IFM 10145^T, *N. asteroides*, *N. africana*, *N. cerradoensis*, *N. nova*, *N. vaccinii*, *N. veterana* and *N. vinacea* were cultured on Muller Hinton II (MH II) agar slant 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.

Biochemical and chemotaxonomical characterization: Decomposition of adenine, casein, hypoxanthine, tyrosine, urea, and xanthine were examined using the methods of Gordon *et al.*¹¹⁾ Acid production from carbohydrates, utilization of organic acids, and growth temperature were determined by the modified method of Poonwan *et al.*⁷⁾. Isolated strains were tested for their ability to grow in MH II agar with 0.2% glucose with each antibiotic TRIDISK (Eiken, Japan, Tokyo) at 32°C for 2 or 3 days¹²⁾.

Whole cell hydrolysates were analyzed for diaminopimelic acid isomers using thin layer chromatography (TLC)¹³⁾. Whole cell sugars were prepared as previously reported¹⁴⁾ and analyzed by TLC¹⁵⁾. Mycolic acids were prepared as previously reported¹⁶⁾. Menaquinones were extracted from freeze-dried biomass (500 mg) and analyzed as described by Chun & Goodfellow¹⁷⁾.

Fatty acid methyl-esters were prepared and analyzed as previously described¹⁸⁾ using the standard Microbial Identification System (Midi, Inc., Del., U.S.A.) for automated gas chromatographic analyses^{19,20)}.

DNA analysis: Genomic DNA samples for sequencing

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were prepared using the guanidine thiocyanate method^{21,22}. A nearly complete 16S rRNA gene sequence of the isolated strain was determined. The 16S rDNA was amplified and sequenced using PCR and prokaryotic 16S rDNA universal primers 8F (5'-AGAGTTTGATCCTG-GCTCAG-3') and 691R (5'-ACCGCTACACCAGGA-3'), 520F (5'-CAGCAGCCGCGGTAATAC-3') and 1100R (5'-GGGTTGCGCTGTTG-3'), and 926F (5'-AAACT-CAAAGGAATTGACGG-3') and 1542R (5'-ACAAAG-GAGGTGATC-3'). PCR was performed with a DNA thermal cycler (TaKaRa, Tokyo, Japan) using 35 cycles and 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. The PCR products were purified with a Centri-Sep column (Princeton Separations). The DNA sequences were determined with an automatic sequence analyzer (ABI PRISM™ 3100; PE Applied Biosystems, CA, U.S.A.) using a dye terminator cycle sequencing kit (PE Applied Biosystems).

BLAST analysis against various sequence databases was used to identify strains related to the IFM 0391^T and IFM 10145^T strains. Sequence data of related species were retrieved from GenBank. Nucleotide substitution rates (K_{NUC} values) were calculated²³ and phylogenetic trees were constructed by the neighbor-joining method²⁴. The topology of the trees was evaluated by bootstrap analysis of the sequence data using CLUSTAL W software²⁵. The DNAML program in the PHYLIP 3.5c package²⁶ was used for Maximum Likelihood analysis, with the default transition/transversion ratio of 2.000000. The sequence similarity values were calculated manually.

DNA was isolated as described by Saito & Miura²⁷ with modification. DNA base composition was estimated by high performance liquid chromatography (HPLC)²⁸. Levels of DNA-DNA relatedness were determined by the method of Ezaki *et al.*²⁹ using photobiotin and microplates.

RESULTS AND DISCUSSION

The chemotaxonomic and morphological characteristics of the two isolated strains are consistent with their assignment to the genus *Nocardia*^{30,31}. All of them contain galactose and arabinose as characteristic whole-cell sugars in addition to *meso*-diaminopimelic acid as the wall diamino acid, and a mycolic acid that co-migrated (*R_f* value of about 0.47) with those extracted from marker *Nocardia* strains. Major menaquinones were MK-8($H_{40\text{-cyc}}$). Analysis of the fatty acids of the two isolated strains IFM 0391^T and IFM 10145^T by GLC revealed the expected pattern that is diagnostic of members of the genus *Nocardia* and related taxa; that is, straight-chain saturated and unsaturated fatty acids together with a diagnostic amount of tuberculostearic acid (10-methyl-branched octadecanoid acid) (Table 1).

The physiological properties of strains IFM 0391^T and IFM 10145^T are shown in Table 2. Susceptibility testing

Table 1 Fatty acid composition of strains IFM 0391^T and IFM 10145^T

Fatty acid (%)	IFM 0391 ^T	IFM 10145 ^T
12:0	0.3	-
13:1	0.8	-
13:0	0.4	-
14:0	5.4	0.3
unknown ECL 14.959	3.1	-
cis-9-15:1	-	0.9
15:0	1.6	3.4
cis-8-16:1	1.6	-
cis-9-16:1	7.9	15.9
16:0	33.9	27.9
10-Methyl-16:0	-	0.2
cis-10-17:1	0.6	-
cis-9-17:1	1.7	1.9
17:0	6.0	9.7
10-Methyl-17:0	0.5	2.2
cis-9-18:1	12.7	3.1
cis-11-18:1	0.5	-
18:0	12.3	10.2
11-Methyl-18:0	1.1	-
10-Methyl-18:0	9.0	21.5
cis-9 19:1	0.4	2.1
19:0	-	0.6
20:0	-	0.2

Abbreviations: ECL, equivalent chain length

cis-9 18:1, cis-9 octadecenoic acid (oleic acid); 10-methyl-18:0, 10-methyl-octadecanoic acid (tuberculostearic acid)

of clinical isolates of *Nocardia* to antimicrobials is useful in grouping organisms. IFM 0391^T and IFM 10145^T were susceptible to 5-fluorouracil, and this characteristic was useful for discrimination of both strains from other reference *Nocardia* strains¹².

Almost complete 16S rDNA sequences were determined for IFM 0391^T (1367 bp) and IFM 10145^T (1440 bp), and their 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*³². It is clear from the phylogenetic trees (Figs. 1, 2) that IFM 0391^T is loosely associated with *N. africana*, *N. cerradoensis*, *N. nova*, *N. vaccinii*, *N. veterana*, and *N. vinacea*, and IFM 10145^T is loosely associated with *N. asteroides*, *N. caishijiensis* and *N. neocaledoniensis*. The sequence similarity value between IFM 0391^T and the 6 related species was 97.5–98.5%, between IFM 10145^T and *N. asteroides* was 98.7%, IFM 10145^T and *N. neocaledoniensis* was 97.8%, and IFM 10145^T and *N. caishijiensis* was 97.8%. These sequence similarity values are not so high for the same species. Therefore, we checked the similarity of these strains based on DNA-DNA hybridization with related valid species. The DNA-DNA relatedness between IFM 0391^T and related *Nocardia* species was < 46 % (Table 3) and between IFM 10145^T

Table 2. Phenotypic properties that distinguish isolate strains from the type strains of *Nocardia* species.

	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	28	29	30	31	32	33	34	35	36	37		
Decomposition of																																							
Adenine	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	ND	-	ND	-	ND	-	-	ND	+	ND	-	-	ND	-	-	-	-	-	-	-		
Casein	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	ND	ND	-	-	-	-	-	-		
Hypoxanthine	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-	ND	+	+	-	-	+	+	+		
Tyrosine	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	-	-	+	-	-	-	-	ND	-	+	+	-	-	-	-		
Urea	-	+	+	-	ND	+	+	-	+	+	-	+	+	+	ND	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Xanthine	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	ND	-	+	-	-	-	-	-		
Utilization of																																							
Arabinose	-	-	-	ND	-	-	-	ND	ND	ND	ND	-	ND	ND	-	-	ND	ND	ND	-	-	-	-	-	-	ND	-	-	ND	ND	ND	-	ND	+	ND	ND	-	-	
Erythritol	-	-	-	ND	-	-	-	ND	ND	ND	ND	-	ND	ND	-	-	ND	ND	ND	-	-	-	-	-	-	ND	ND	-	ND	ND	ND	+	ND	-	ND	ND	-	-	
Galactose	-	-	-	ND	-	-	+	ND	ND	ND	ND	-	ND	ND	-	-	ND	ND	ND	-	-	-	-	-	-	ND	-	+	ND	ND	+	ND	+	ND	+	ND	ND	-	-
Glucose	+	+	+	ND	+	+	+	ND	ND	ND	ND	+	ND	ND	-	+	ND	ND	ND	+	+	+	+	+	-	ND	+	+	ND	ND	ND	+	ND	+	ND	ND	+	+	
Inositol	-	-	-	ND	-	-	+	ND	ND	ND	ND	-	ND	ND	-	-	ND	ND	ND	+	ND	-	-	-	+	-	ND	+	-	ND	ND	+	ND	-	ND	ND	+	+	
Maltose	-	-	+	ND	ND	-	+	ND	ND	ND	ND	ND	ND	ND	-	+	ND	ND	ND	-	ND	+	-	-	-	ND	-	-	ND	ND	ND	+	ND	-	ND	ND	-	-	
Mannose	-	+	ND	ND	-	-	-	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	
Rhamnose	-	-	+	-	+	-	-	+	+	+	+	+	ND	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	-	-	-	
Sorbitol	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Citrate	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	ND	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	
Gluconate	-	-	ND	ND	ND	ND	ND	ND	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	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Growth at 45°C	-	-	-	+	-	-	+	-	-	-	-	-	-	-	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Susceptibility test																																							
IPM ^{a)}	3+	3+	1+	3+	3+	3+	-	ND	3+	ND	3+	ND	ND	3+	3+	3+	ND	ND	ND	ND	3+	-	ND	3+	-	3+	3+	ND	3+	ND	3+	ND	3+	ND	3+	3+	3+	3+	
TOB ^{a)}	3+	3+	3+	1+	ND	3+	3+	ND	3+	ND	3+	ND	ND	3+	3+	-	ND	ND	ND	ND	3+	3+	ND	-	1+	3+	3+	ND	3+	ND	-	ND	ND	-	ND	ND	-	1+	
5-FU ^{b)}	+	+	+	+	-	-	-	ND	-	ND	-	ND	ND	-	-	-	ND	ND	ND	ND	ND	ND	+	-	-	-	-	ND	-	ND	-	ND	+	+	+	+	+	+	
KM ^{c)}	3+	3+	2+	3+	ND	1+	-	ND	3+	ND	3+	ND	ND	-	-	-	ND	ND	ND	ND	3+	3+	ND	-	3+	3+	-	ND	1+	ND	-	ND	ND	2+	-	3+	+		

+, positive; -, negative; IPM, imipenem; TOB, tobramycin; 5-FU, 5-fluorouracil; KM, kanamycin

^aa, 3+ (highly susceptible, growth inhibition at 2.5 µg per disc), 1+ (slightly susceptible, growth inhibition at 10 µg per disc), - (not susceptible, no growth inhibition at 10 µg per disc); b, + (susceptible, growth inhibition at 30 µg per disc), - (not susceptible, no growth inhibition at 30 µg per disc); c, 3+ (highly susceptible, growth inhibition at 5.0 µg per disc), 2+ (moderately susceptible, growth inhibition at 10 µg or less per disc), 1+ (slightly susceptible, growth inhibition at 30 µg per disc), - (not susceptible, no growth inhibition at 30 µg per disc).

1, IFM 0391^T; 2, IFM 10145^T; 3, *Nocardia abscessus* DSM 44432^T; 4, *Nocardia africana* DSM 44491^T; 5, *Nocardia asiatica* IFM 0245^T; 6, *Nocardia asteroides* ATCC 19247^T; 7, *Nocardia brasiliensis* ATCC 19296^T; 8, *Nocardia brevicatena* DSM 43024^T; 9, *Nocardia beijingensis* JCM 10666^T; 10, *Nocardia caishijiensis* JCM 11508^T; 11, *Nocardia carneae* DSM 43397^T; 12, *Nocardia cerraensis* DSM 44546^T; 13, *Nocardia crassostreae* ATCC 70418^T; 14, *Nocardia cummideiensis* DSM 44490^T; 15, *Nocardia cyriacigeorgica* DSM 44484^T; 16, *Nocardia farcinica* ATCC 3318^T; 17, *Nocardia flavorosea* JCM 3332^T; 18, *Nocardia fluminea* DSM 44488^T; 19, *Nocardia neocaledoniensis* DSM 44717^T; 20, *Nocardia inohanensis* IFM 0092^T; 21, *Nocardia nilgatisensis* IFM 0330^T; 22, *Nocardia ignorata* DSM 44496^T; 23, *Nocardia nova* JCM 6044^T; 24, *Nocardia otitidiscavarium* NCTC 1934^T; 25, *Nocardia parvivorans* DSM 44386^T; 26, *Nocardia pseudobrasiliensis* ATCC 51512^T; 27, *Nocardia puris* DSM 44599^T; 28, *Nocardia salmonicida* JCM 4826^T; 29, *Nocardia verticillata* JCM 3360^T; 30, *Nocardia soli* DSM 44488^T; 31, *Nocardia tenerifensis* DSM 44704^T; 32, *Nocardia transvalensis* DSM 43405^T; 33, *Nocardia uniformis* JCM 3224^T; 34, *Nocardia vaccini* DSM 43285^T; 35, *Nocardia veterana* DSM 44445^T; 36, *Nocardia vinacea* JCM 10302^T; 37, *Nocardia yamanashiensis* IFM 0265^T. Data were taken from Albuquerque *et al.*⁽³³⁾, Gurtler *et al.*⁽³⁴⁾, Hamid *et al.*⁽³⁵⁾, Kageyama *et al.*^(21, 22), Kämpfer *et al.*⁽³⁶⁾, Kinoshita *et al.*⁽³⁷⁾, Maldonado *et al.*⁽³⁸⁾, Saintpierre-Bonaccio *et al.*⁽³⁹⁾, Wang *et al.*⁽⁴⁰⁾, Yassin *et al.*⁽⁴¹⁻⁴⁴⁾, Zhang *et al.*⁽⁴⁵⁾ and this study.

and *N. asteroides* was < 12%, showing that these two isolated strains could each represent a new species.

Based on the phylogenetic and phenotypic data described above, we concluded that IFM 0391^T and IFM 10145^T are two new species in the genus *Nocardia*, and we propose the new species names of *Nocardia vermiculata* sp. nov. for IFM 0391^T and *Nocardia thailandica* sp. nov. for IFM 10145^T.

Description of *Nocardia vermiculata* sp. nov.

Nocardia vermiculata (L. fem. part. adj. *vermiculata*, in the form of worms, referring to the morphology of the aerial mycelium).

Aerobic, Gram-positive, partially acid-fast, non-motile actinomycetes forming beige colored substrate mycelium. The cells have typical *Nocardia* micromorphology of

branched vegetative hyphae, fragmented into oval to rod-shaped elements (0.4–0.7 by 0.7–1.0 µm). The aerial mycelium is moderate and orange to pale orange brown. Colonies are 0.3–1.0 mm in diameter after 7 days at 30°C on MH11 agar with 0.2% glucose. Short chains of conidia are found on the aerial mycelium. The aerial mycelium shows primitive spiral-like structures (worm-like structures). They produce faint brown soluble pigment. Glucose is utilized, but arabinose, erythritol, galactose, inositol, maltose, mannose, rhamnose, sorbitol, gluconate, and citrate are not. Adenine, casein, hypoxanthine, tyrosine, urea, and xanthine are not decomposed. The strain grows at 37°C but not 45°C. The G+C content of the DNA is 67-mol%. The strain is a clinical isolate from sputum of a Japanese male patient. The type strain of *Nocardia vermiculata* is IFM 0391^T (=NBRC 100427^T = JCM 12345^T = DSM 44807^T).

Fig. 1. Phylogenetic tree derived from 16S rDNA sequences. The tree was created using the neighbor-joining method and KNUC values. The numbers on the tree indicate bootstrap values for the branch points. Only values above 50% significance are indicated.

Fig. 2. Phylogenetic tree derived from 16S rDNA sequences. The tree was created using Maximum Likelihood analysis.

Table 3. Levels of DNA-DNA relatedness among IFM 0391^T strain and related *Nocardia* species.

	1	2	3	4	5	6	7
1. IFM 0391 ^T	100	8	9	10	6	7	2
2. <i>N. africana</i> IFM 10147 ^T	10	100	11	3	10	3	3
3. <i>N. cerradoensis</i> IFM 10366 ^T	9	12	100	11	43	38	13
4. <i>N. nova</i> IFM 290 ^T	11	2	10	100	10	7	2
5. <i>N. vaccinii</i> IFM 10284 ^T	10	10	41	13	100	46	13
6. <i>N. veterana</i> IFM 10086 ^T	1	4	37	12	42	100	5
7. <i>N. vinacea</i> IFM 10175 ^T	2	2	13	3	5	3	100

Description of *Nocardia thailandica* sp. nov.

Nocardia thailandica (N.L. fem. adj. *thailandica*, referring to Thailand, where the type strain was isolated).

Colonies are grayish orange to tan. Cells show typical *Nocardia* micromorphology (branched vegetative mycelium that fragment into bacteroid, rod-shaped elements; 0.4–0.6 by 0.8–1.6 μm). The aerial mycelium is visible and long in most media tested such as BHI agar plate. No soluble pigment is produced. Colonies are 0.2–1.2 mm in diameter after 7 days at 30°C on MH11 agar with 0.2% glucose. Glucose and mannose are utilized, but arabinose, erythritol, galactose, inositol, maltose, mannose, rhamnose, sorbitol, xylose, citrate and gluconate are not. Adenine, casein, hypoxanthine, tyrosine, xanthine, and elastine are not decomposed. Urea is not decomposed. Strain grows at 37°C but not at 45°C. The G+C content of the DNA is 72mol%. The strain is a clinical isolate. The type strain of *Nocardia thailandica* is IFM 10145^T (=NBRC 100428^T=JCM 12356^T = DSM 44808^T).

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