Nocardia terpenica sp. nov., isolated from Japanese patients with nocardiosis

Yasutaka Hoshino,^{1,2} Kayo Watanabe,¹ Soji Iida,¹ Shin-ichi Suzuki,³ Takuji Kudo,⁴ Takahisa Kogure,¹ Katsukiyo Yazawa,¹ Jun Ishikawa,² Reiner M. Kroppenstedt⁵ and Yuzuru Mikami¹

Correspondence

Yuzuru Mikami

mikami@faculty.chiba-u.jp

¹Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan

²National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640, Japan

³Discovery Research Laboratories, Tanabe Seiyaku Co., Ltd, 2-2-50 Kawagishi, Toda, Saitama 335-8505, Japan

⁴JCM, RIKEN BioResource Center, Wako, Saitama 351-0198, Japan

⁵DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany

Two bacterial strains isolated from different hospitals in Japan were subjected to a polyphasic analysis. Strains IFM 0406 and IFM 0706^T, producers of novel terpenoid antibiotics, were found to have morphological, biochemical, physiological and chemotaxonomic properties consistent with their classification in the genus *Nocardia*, except for the presence of MK-8(H₄) as one of the predominant menaquinones in addition to the major menaquinone MK-8(H_{4ω-cyc}). The 16S rRNA gene sequence similarity of strains IFM 0406 and IFM 0706^T was 99.9 %, and the closest members of *Nocardia* to these strains were the type strains of *Nocardia nova* and *Nocardia mexicana*, showing similarity of 97.5 and 97.1 %, respectively. Based on their characteristic phenotypic and phylogenetic properties, a novel species of the genus *Nocardia*, *Nocardia terpenica* sp. nov. is proposed for the two strains. The type strain is IFM 0706^T (= JCM 13033^T = DSM 44935^T = NBRC 100888^T).

The genus *Nocardia* belongs phylogenetically to the sub-order *Corynebacterineae* (Stackebrandt *et al.*, 1997) and, among species of the genus *Nocardia*, *Nocardia asteroides*, *N. brasiliensis*, *N. farcinica*, *N. nova* and *N. otitidiscaviarum* have been considered to be major pathogens that cause clinical diseases in humans (Goodfellow, 1998; Ishikawa *et al.*, 2004). However, in recent years, many species of the genus *Nocardia* have been proposed, and over 60 species of *Nocardia* have validly published names (Seo & Lee, 2006). Although most of them have been isolated from clinical specimens, some were isolated from soils and reported to be producers of a novel antibiotic (Kinoshita *et al.*, 2000). Our studies on secondary metabolites from pathogenic *Nocardia* suggested that two clinically isolated *Nocardia* strains

produce novel terpenoid-type immunosuppressive and macrolide-type antifungal antibiotics, respectively designated brasiliocardins and brasilinolides (Komatsu *et al.*, 2004). These antibiotic-producing *Nocardia* strains were originally identified as *N. brasiliensis* (Tanaka *et al.*, 1997), but our detailed chemotaxonomic studies suggested that these *Nocardia* strains have MK-8(H_{4ω-cyc}) (49 %) as the predominant menaquinone and also contain MK-8(H₄) (32 %) and MK-9(H₄) (19 %) as minor menaquinones. Further phylogenetic studies supported the conclusion that these strains should be classified into one phylogenetic group within the genus *Nocardia*.

In this paper, we report on the morphological, physiological and biochemical characteristics, analysis of cellular composition, DNA–DNA hybridization and 16S rRNA gene sequence of two *Nocardia* isolates (IFM 0406 and IFM 0706^T) in comparison with those of reference strains of *Nocardia*. The aim of the present study was to determine the taxonomic position of the two strains using a polyphasic taxonomic analysis.

Strain IFM 0406 was isolated in 1993 from a patient with lung nocardiosis at Okayama, Japan. Strain IFM 0706^T was

Abbreviation: A₂pm, diamminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains IFM 0706^T and IFM 0406 are respectively AB201298 and AB201299.

An extended phylogenetic tree, details of the mycolic acid and fatty acid profiles of strain IFM 0706^T and a comparison of physiological characters of IFM 0706^T and other *Nocardia* type strains are available as supplementary data with the online version of this paper.

isolated from a sputum sample of a patient in the intensive care unit at Chiba university hospital in Japan.

Strains IFM 0406, IFM 0706^T, *N. nova* IFM 0290^T and *N. vaccinii* IFM 10284^T were cultured on Mueller–Hinton II (MH II) agar slants with 1 % glucose and 1 % glycerol for 1 week at 27 °C. For extraction and sequencing of DNA and DNA–DNA hybridization, bacterial strains were cultured on brain heart infusion broth (Difco) with 2 % glucose and 2 % glycine for 3 days at 32 °C. Colony morphology and micromorphological properties of the two strains were observed by light and scanning electron microscopy according to previously described procedures (Kageyama *et al.*, 2004a, b). 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 methods of Poonwan *et al.* (2005). Decomposition of arbutin, elastin, aesculin and testosterone was examined by using the methods of Goodfellow & Pirouz (1982). Utilization of nitrogen sources was tested using an N-Buiyon set (Eiken). Drug susceptibility tests were performed with TRIDISK (Eiken Chemical Co.) using the method described by Kageyama *et al.* (2004a, b). Strains were stained by a modified method of Chapin & Murray (1999) using 0.5 % sulfuric acid for determination of acid–alcohol-fastness.

16S rRNA genes were amplified and sequenced by PCR employing six prokaryotic 16S rRNA gene universal primers and sequenced following procedures described previously (Kageyama *et al.*, 2004a, b). Species related to the novel isolates were identified by performing a nucleotide sequence database search using BLAST programs from GenBank. Sequences of related species were also retrieved from GenBank. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura & Ohta, 1972). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) and 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 through visual comparison and manual calculation. Preparation of genomic samples for DNA–DNA hybridization was performed using a modified method described by Kageyama *et al.* (2004a, b). DNA base composition was estimated by HPLC (Tamaoka & Komagata, 1984). Level of DNA–DNA relatedness were determined by the method of Ezaki *et al.* (1989) using photobiotin and microplates.

Whole-cell hydrolysates were analysed for diaminopimelic acid (A₂pm) isomers using TLC (Staneck & Roberts, 1974). Whole-cell sugars were prepared as reported in Lechevalier & Lechevalier (1980) and analysed by TLC (Miyadoh, 2001). Mycolic acids were prepared according to the methods of Minnikin *et al.* (1980) for TLC and Klatte *et al.* (1994) for GLC, respectively. Fatty acid methyl esters were prepared and analysed as described previously (Klatte *et al.*, 1994) using the standard Microbial Identification System (MIDI

Inc.) for automated GC analyses (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Menaquinones were extracted from freeze-dried biomass and analysed as described by Chun & Goodfellow (1995).

Comparison of the almost complete 16S rRNA gene sequences of strains IFM 0406 and IFM 0706^T with those of members of genera classified in the suborder *Corynebacterineae* showed they contained all of the signature nucleotides expected for members of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997) and the genus *Nocardia* (Chun & Goodfellow, 1995). 16S rRNA gene sequence similarity between strains IFM 0406 and IFM 0706^T was 99.9 %. The highest sequence similarity of IFM 0706^T to a previously deposited sequence was shown with *N. nova* JCM 6044^T [97.5 % (1422/1461)]. A phylogenetic tree constructed using the neighbour-joining method depicting the phylogenetic placement of strains IFM 0406 and IFM 0706^T within a subset of the genus *Nocardia* is shown in Fig. 1 (the full tree from which Fig. 1 was taken is available as Supplementary Fig. S1 in IJSEM Online). This phylogenetic tree shows that these two strains formed a monophyletic clade that is loosely associated with *N. mexicana* DSM 44952^T. DNA–DNA relatedness between strain IFM 0406 and IFM 0706^T was 91–93 %. The relatedness values of these two strains with *N. nova* IFM 0290^T was 48 %. The value is below the 70 % cut-off point for species classification recommended by Wayne *et al.* (1987).

Whole-cell hydrolysates of strains IFM 0406 and IFM 0706^T contained *meso*-A₂pm as the only diamino acid of the peptidoglycan and arabinose plus galactose as major whole-cell sugars (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1980). The mycolic acid chain lengths determined for strain IFM 0706^T ranged from C₅₂ to C₆₀ (Supplementary Table S1). These chemotaxonomic characteristics of strains IFM 0406 and IFM 0706^T were consistent with their assignment to the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999). The fatty acid pattern of strain IFM 0706^T was composed of straight-chain saturated and unsaturated fatty acids plus tuberculos-tearic acid (10-methyl octadecanoic acid) (Supplementary Table S2). Although this pattern is roughly the same in all members of *Nocardia*, there are qualitative and quantitative species-specific and often intraspecific differences (Klatte *et al.*, 1994).

The predominant menaquinone of these two strains was MK-8(H_{4co-cyc}) (49 %), although substantial amounts of MK-8(H₄) (32 %) and MK-9(H₄) (19 %) were present, suggesting differentiation of these isolates from *Nocardia* species reported to date. The amounts of menaquinones produced depended on the medium used, but similar ratios between the menaquinone types were always observed. Although there have been no reports up to now that *Nocardia* strains synthesize MK-8(H₄) and MK-9(H₄) in substantial amounts, it was considered that these isolates should be assigned to the genus *Nocardia* within a novel

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. Bar, 0.5 inferred substitutions per 100 nucleotides. An extended tree is available as Supplementary Fig. S1 in IJSEM Online.

species because of the agreement found in the other chemotaxonomic and phylogenetic markers.

Strains IFM 0406 and IFM 0706^T were also examined for a set of biochemical and physiological characteristics in comparison with *N. nova* IFM 0290^T and *N. vaccinii* IFM 10284^T. It was found that both strains could be readily distinguished from these strains by a combination of physiological and biochemical characteristics, including the decomposition of adenine, casein, hypoxanthine, tyrosine and urea (Table 1). They also differed phenotypically from other *Nocardia* species (Supplementary Table S3).

On the basis of the genotypic and phenotypic evidence described above, it is concluded that strains IFM 0406 and IFM 0706^T represent a novel species within the genus *Nocardia*. We therefore propose the name *Nocardia terpenica* sp. nov.

Description of *Nocardia terpenica* sp. nov.

Nocardia terpenica (ter.pe'ni.ca. N.L. n. *terpenum* terpene; L. suff. -icus -a -um suffix used with various meanings; N.L. fem. adj. *terpenica* referring to the ability to produce terpenoid antibiotics).

Aerobic, Gram-positive, acid-alcohol-fast, non-motile actinomycete which forms colourless to beige substrate mycelium that fragments into irregular, long rod-shaped elements during ageing (0.2–0.5 × 0.6–1.4 µm) (Fig. 2). Aerial mycelium is scanty or lacking on most media tested. Sclerotium-like structures are observed on MH II agar (Fig. 2). Grows at 37 °C, but not at 45 °C. Colonies are 1.0–3.5 mm in diameter after 7 days at 30 °C on MH II agar supplemented with 0.2 % glucose. Adonitol, galactose, glucose, inositol, sorbitol and citrate are utilized but arabinose, erythritol, maltose, mannose and rhamnose are not. Nitrate is not reduced. Adenine, casein, hypoxanthine and urea are decomposed but xanthine is not. Highly susceptible

to tobramycin, but not to imipenem, kanamycin or 5-fluorouracil. Contains *meso*-A₂pm, arabinose and galactose (cell-wall chemotype IV *sensu* Lechevalier & Lechevalier).

Table 1. Phenotypic properties of strain IFM 0706^T, *N. nova* JCM 6044^T and *N. mexicana* DSM 44952^T

Strain IFM 0406 gave identical results to strain IFM 0706^T. +, Positive; –, negative; ND, not determined. All strains were positive for acid production from glucose and sorbitol and negative for acid production from arabinose, maltose and mannose.

Characteristic	IFM 0706 ^T	<i>N. nova</i> JCM 6044 ^T	<i>N. mexicana</i> DSM 44952 ^T
Decomposition of:			
Adenine	+	–	+
Casein	+	–	–
Hypoxanthine	+	–	+
Tyrosine	+	–	+
Acid from (Gordon test):			
Adonitol	+	–	+
Galactose	+	–	+
Inositol	+	–	+
Rhamnose	–	–	+
Utilization of citrate	+	–	+
Nitrate reductase	–	+	ND
Susceptibility to:			
Imipenem ^a	–	3+	3+
Tobramycin ^a	3+	–	–
5-Fluorouracil ^b	–	+	–
Kanamycin ^c	–	1+	1+

*Scored as follows. *a*: 3+, highly susceptible, growth inhibition at 2.5 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*: 1+, slightly susceptible, growth inhibition at 30 µg per disc; –, not susceptible, no growth inhibition at 30 µg per disc.

Fig. 2. Scanty aerial mycelium on MH 11 agar medium with sclerotium-like structures (a) and long, fragmented substrate mycelium (b). Bars, 10 µm (a) and 1 µm (b).

Predominant menaquinone is MK-8(H_{4ω-cyc}) (49%), although substantial amounts of MK-8(H₄) (32%) and MK-9(H₄) (19%) are also present. Major fatty acids are straight-chain saturated and unsaturated fatty acids plus tuberculostearic acid. The G+C content of the DNA is 65.4 mol%. Mycolic acids with 52–60 carbons are present. Produces terpenoid immunosuppressive antibiotics, brasili-cardins, and macrolide-type antifungal antibiotics, brasili-nolides A, B and C, with antifungal activity against *Aspergillus niger*.

The type strain is strain IFM 0706^T (=JCM 13033^T=DSM 44935^T=NBRC 100888^T), isolated from a sputum sample of a patient with nocardiosis. Strain IFM 0406 is also assigned to this species.

References

- Chapin, K. C. & Murray, P. R. (1999). Stains. In *Manual of Clinical Microbiology*, pp. 1674–1686. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Tenover. Washington, DC: American Society for Microbiology.
- Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of genus *Nocardia* with 16S rRNA gene sequences. *Int J Syst Bacteriol* **45**, 240–245.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Goodfellow, M. (1998). *Nocardia* and related genera. In *Topley and Wilson's Microbiology and Microbial Infections*, 9th edn, vol. 2, *Systematic Bacteriology*, pp. 463–489. Edited by A. Balows & B. I. Duerden. London: Arnold.
- Goodfellow, M. & Pirouz, T. (1982). Numerical classification of sporoactinomycetes containing *meso*-diaminopimelic acid in the cell wall. *J Gen Microbiol* **128**, 503–527.
- Goodfellow, M., Isik, K. & Yates, E. (1999). Actinomycete systematics: an unfinished synthesis. *Nova Acta Leopold* **80**, 47–82.
- Gordon, R. E., Barnett, D. A., Handerhan, J. E. & Pang, C. H.-N. (1974). *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. *Int J Syst Bacteriol* **24**, 54–63.
- Ishikawa, J., Yamashita, A., Mikami, Y., Hoshino, Y., Kurita, H., Hotta, K., Shiba, T. & Hattori, M. (2004). The complete genomic sequence of *Nocardia farcinica* IFM 10152. *Proc Natl Acad Sci U S A* **101**, 14925–14930.
- Kageyama, A., Poonwan, N., Yazawa, K., Mikami, Y. & Nishimura, K. (2004a). *Nocardia asiatica* sp. nov., isolated from patients with nocardiosis in Japan and clinical specimens from Thailand. *Int J Syst Evol Microbiol* **54**, 125–130.
- Kageyama, A., Yazawa, K., Nishimura, N. & Mikami, Y. (2004b). *Nocardia inohanensis* sp. nov., *Nocardia yamanashiensis* sp. nov. and *Nocardia niigatensis* sp. nov., isolated from clinical specimens. *Int J Syst Evol Microbiol* **54**, 563–569.
- Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.
- Kimura, M. & Ohta, T. (1972). On the stochastic model for estimation of mutational distance between homologous proteins. *J Mol Evol* **2**, 87–90.
- Kinoshita, N., Homma, Y., Igarashi, M., Ikeno, S., Hori, M. & Hamada, M. (2000). *Nocardia vinacea* sp. nov. *Actinomycetologica* **15**, 1–5.
- Klatte, S., Rainey, F. A. & Kroppenstedt, R. M. (1994). Transfer of *Rhodococcus aichiensis* Tsukamura 1982 and *Nocardia amarae* Lechevalier and Lechevalier 1974 to the genus *Gordona* as *Gordona aichiensis* comb. nov. and *Gordona amarae* comb. nov. *Int J Syst Bacteriol* **44**, 769–773.
- Komatsu, K., Tsuda, M., Tanaka, Y., Mikami, Y. & Kobayashi, J. (2004). Absolute stereochemistry of immunosuppressive macrolide brasili-nolide A and its congener brasili-nolide C. *J Org Chem* **69**, 1535–1541.
- Lechevalier, M. P. & Lechevalier, H. A. (1980). The chemotaxonomy of actinomycetes. In *Actinomycete Taxonomy*, pp. 227–291. Edited by A. Dietz & D. W. Thayer. Fairfax, VA: Society for Industrial Microbiology.
- Minnikin, D. E., Hutchinson, I. G., Caldicott, A. B. & Goodfellow, M. (1980). Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. *J Chromatogr* **188**, 221–233.
- Miyadoh, M. (2001). Identification procedure at the genus level. In *Identification Manual of Actinomycetes*, pp. 9–19. Edited by S. Miyadoh, M. Hamada, K. Hotta, T. Kudo, A. Seino, K. Suzuki & A. Yokota. Tokyo: Business Center for Academic Societies Japan.

- Poonwan, N., Mekha, N., Yazawa, K., Thunyaharn, S., Yamanaka, A. & Mikami, Y. (2005). Characterization of clinical isolates of pathogenic *Nocardia* strains and related actinomycetes in Thailand from 1996 to 2003. *Mycopathologia* **159**, 361–368.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids. *USFCC Newsl* **20**, 1–6.
- Seo, J. P. & Lee, S. D. (2006). *Nocardia harenae* sp. nov., an actinomycete isolated from beach sand. *Int J Syst Evol Microbiol* **56**, 2203–2207.
- Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.
- Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Tanaka, Y., Komaki, H., Yazawa, K., Mikami, Y., Nemoto, A., Kadowaki, K., Shigemori, H. & Kobayashi, J. (1997). A new macrolide antibiotic produced by *Nocardia brasiliensis*: producing strain, isolation and biological activity. *J Antibiot* **50**, 1036–1041.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.