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

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Two bacterial strains isolated from different hospitals in Japan were subjected to a polyphasic analysis. Strains IFM 0406 and IFM 0706T, 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(H4-cyc). The 16S rRNA gene sequence similarity of strains IFM 0406 and IFM 0706T 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 0706T (=JCM 13033T = DSM 44935T = NBRC 100888T).

The genus *Nocardia* belongs phylogenetically to the suborder *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 brasiliardin and brasiliolides (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(H4-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 0706T) 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 0706T was
isolated from a sputum sample of a patient in the intensive care unit at Chiba university hospital in Japan.

Strains IFM 0406, IFM 0706T, *N. nova* IFM 0290T and *N. vaccinii* IFM 10284T 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 RNA–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* nucleotide 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 0706T 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 0706T was 99.9%. The highest sequence similarity of IFM 0706T to a previously deposited sequence was shown with *N. nova* JCM 6044T [97.5% (1422/1461)]. A phylogenetic tree constructed using the neighbour-joining method depicting the phylogenetic placement of strains IFM 0406 and IFM 0706T 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 44952T. DNA–DNA relatedness between strain IFM 0406 and IFM 0706T was 91–93%. The relatedness values of these two strains with *N. nova* IFM 0290T 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 0706T 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 0706T ranged from C<sub>52</sub> to C<sub>60</sub> (Supplementary Table S1). These chemotaxonomic characteristics of strains IFM 0406 and IFM 0706T were consistent with their assignment to the genus *Nocardia* (Goodfellow, 1998; Goodfellow et al., 1999). The fatty acid pattern of strain IFM 0706T was composed of straight-chain saturated and unsaturated fatty acids plus tuberculostearic 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(9H,4-cyc) (49%), although substantial amounts of MK-8(9H) (32%) and MK-9(9H) (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(9H) and MK-9(9H) in substantial amounts, it was considered that these isolates should be assigned to the genus *Nocardia* within a novel
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. terpene\(\) termene; 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 \(\mu\)m \(\times\) 6–1.4 \(\mu\)m) (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-A\(_2\)-pm, arabinoic 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\(^T\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>IFM 0706(^T)</th>
<th>JCM 6044(^T)</th>
<th>DSM 44952(^T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decomposition</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Adenine</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Acid from (Gordon test):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adonitol</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Inositol</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of citrate</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>+</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Susceptibility to:*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipenem()</td>
<td>–</td>
<td>3 +</td>
<td>3 +</td>
</tr>
<tr>
<td>Tobramycin()</td>
<td>3 +</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5-Fluorouracil()</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Kanamycin()</td>
<td>–</td>
<td>1 +</td>
<td>1 +</td>
</tr>
</tbody>
</table>

*Scored as follows. \(a\): 3 +, highly susceptible, growth inhibition at 2.5 \(\mu\)g per disc; –, not susceptible, no growth inhibition at 10 \(\mu\)g per disc. \(b\): +, susceptible, growth inhibition at 30 \(\mu\)g per disc; –, not susceptible, no growth inhibition at 30 \(\mu\)g per disc. \(c\): 1 +, slightly susceptible, growth inhibition at 30 \(\mu\)g per disc; –, not susceptible, no growth inhibition at 30 \(\mu\)g per disc.
Predominant menaquinone is MK-8(H4
-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, brasilinolides, and macrolide-type antifungal antibiotics, brasilinolides A, B and C, with antifungal activity against Aspergillus niger.

The type strain is strain IFM 0706T (JCM 13033T = DSM 44935T = NBRC 100888T), isolated from a sputum sample of a patient with nocardiosis. Strain IFM 0406 is also assigned to this species. Strain IFM 10152.

References


