Nocardia amamiensis sp. nov., isolated from a sugar-cane field in Japan

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An actinomycete, strain TT 00-78^T, was isolated from soil from a sugar-cane field on Amami Island in Japan, using an SDS/yeast extract pre-treatment method, and the taxonomy was studied using a polyphasic approach. The chemotaxonomic and morphological characterizations clearly demonstrated that the strain belongs to the genus *Nocardia*. 16S rRNA gene sequencing studies showed that the strain was closely related to the type strains of *Nocardia pneumoniae* (98.6%), *Nocardia araoensis* (98.1%), *Nocardia arthritidis* (97.9%) and *Nocardia beijingensis* (97.7%). However, the results of DNA–DNA hybridization and physiological and biochemical tests showed that strain TT 00-78^T could be differentiated from its closest phylogenetic relatives both genotypically and phenotypically. Therefore this strain represents a novel species of the genus *Nocardia*, for which the name *Nocardia amamiensis* sp. nov. is proposed. The type strain is TT 00-78^T (=NBRC 102102^T = DSM 45066^T = KCTC 19208^T).

The genus *Nocardia* is a member of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997) and currently contains 63 species with validly published names, including the recently described species *Nocardia jiangxiensis*, *Nocardia jejuensis* and *Nocardia harenae* (Cui *et al.*, 2005; Lee, 2006; Seo & Lee, 2006). In recent years, many species have been isolated from clinical specimens and are considered to be opportunistic pathogens in animal infections (Watanabe *et al.*, 2006; Brown-Elliott *et al.*, 2006). In contrast, the natural habitats of nocardiae are widely distributed throughout terrestrial and aquatic ecosystems, including, for instance, scumming activated sludge and sediments from moats, lakes and marine environments (Attwell & Colwell, 1981; Yamamura *et al.*, 2005). Additionally, they are considered to play a significant role as saprophytic organisms in the turnover of naturally occurring organic substances (Goodfellow *et al.*, 1999). *Nocardia* species have industrial uses as well, as they are known to produce bioactive agents such as nocardicin, tubelactomicin A and brasilicardin A (Aoki *et al.*, 1976; Komaki *et al.*, 1999; Igarashi *et al.*, 2000).

Strain TT 00-78^T was isolated from soil in a sugar-cane field on Amami Island in Japan, using an SDS/yeast extract pre-treatment method (Hayakawa & Nonomura, 1989) and HV agar (Hayakawa & Nonomura, 1987) containing nalidixic acid (20 mg l⁻¹). The strain formed thin, flat colonies with sparse, white aerial hyphae on HV agar containing nalidixic acid. The aim of the present study was to determine the

taxonomic position of strain TT 00-78^T by using a polyphasic approach.

The colonial properties of strain TT 00-78^T were recorded from colonies grown on a plate containing modified Bennett's agar (Jones, 1949) and incubated for 14 days at 28 °C. Spore motility was examined in hanging drops by means of light microscopy. Gram staining was examined by using Hucker's method (Gerhardt, 1981). Acid–alcohol-fastness was examined by using a modified version of the Ziehl–Neelsen method (Gordon, 1967) in which 0.5% (v/v) sulfuric acid was used for decolorization. The hydrolysis of complex substrates and the utilization of carbon sources were examined by using well-established procedures (Gordon *et al.*, 1974; Isik *et al.*, 1999). The tests for aesculin and arbutin hydrolysis (Williams *et al.*, 1983), nitrate reduction (Gordon & Mihm, 1962) and urea hydrolysis (Gordon *et al.*, 1974) were performed using established procedures. Growth at 45 °C was recorded on GYEA medium (Gordon & Mihm, 1962).

Diaminopimelic acid isomers and sugars in whole-cell hydrolysates were analysed on the basis of the methods established by Hasegawa *et al.* (1983) and Schaal (1985), respectively. Standard procedures were also used for the extraction and analysis of mycolic acids (Schaal, 1985), fatty acids (Tamura *et al.*, 1994), isoprenoid quinones and polar lipids (Minnikin *et al.*, 1984); comparisons were made with the appropriate controls. Chromosomal DNA from strain TT 00-78^T was isolated and purified by the method of Saito & Miura (1963) with a minor modification (Hatano *et al.*, 2003). The DNA G + C content of the strain was determined

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TT 00-78^T is AB275164.

by HPLC, as described by Tamura *et al.* (1994). DNA–DNA hybridization was carried out as described by Kusunoki *et al.* (1991), using biotinylated DNA.

PCR amplification of the 16S rRNA gene from strain TT 00-78^T was carried out according to the procedures described by Tamura & Hatano (2001) and directly sequenced using an ABI Prism BigDye Terminator cycle sequencing kit (PE Applied Biosystems) and an automatic DNA sequencer (model 3100 Genetic Analyzer; PE Applied Biosystems). The 16S rRNA gene sequence obtained in the present study was aligned with reference sequences for the genus *Nocardia* (available from EMBL/GenBank/DDBJ) by using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic trees were constructed with MEGA, version 3.1 (Kumar *et al.*, 2001) and CLUSTAL_X (Thompson *et al.*, 1997), using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution and maximum-parsimony methods (Takahashi & Nei, 2000). The topography of the resulting tree was evaluated by means of bootstrap analysis based on 1000 replicates (Felsenstein, 1985).

The 16S rRNA gene sequence derived from strain TT 00-78^T contained the signature nucleotides characteristic of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997). On the basis of the phylogenetic analysis, the strain falls within the radiation of the genus *Nocardia* (data not shown). The chemotaxonomic and morphological characteristics of strain TT 00-78^T were consistent with its assignment to the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999). The whole-cell hydrolysate of the test strain contained *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970). The major menaquinones were MK-8(H₄ω-cycl.) (30.3 %) and MK-8(H₂) (16.8 %). The major polar lipids found were phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol (phospholipid type PII *sensu* Lechevalier *et al.*, 1977). In addition, the TLC analysis revealed that the strain contained mycolic acid with an *R*_f value (0.46) identical to that of the reference strain used as a control. The major cellular fatty acids were hexadecanoate (43 %), hexadecanoate (20 %), tuberculostearic acid (10-methyl octadecanoate; 17 %) and *cis*-9 octadecanoate (10 %). The formation of branched substrate hyphae, fragmenting into rod-shaped elements (Goodfellow & Lechevalier, 1989), and relatively short aerial hyphae with chains of arthrospores were observed by microscopy (Fig. 1).

The almost-complete 16S rRNA gene sequence (1476 nt) of strain TT 00-78^T was compared with sequences from recognized species of *Nocardia*. The phylogenetic tree obtained using the neighbour-joining method showed that the strain forms a monophyletic clade with *Nocardia pneumoniae*, *Nocardia beijingensis*, *Nocardia arthritidis* and *Nocardia araoensis* (Fig. 2). The 16S rRNA gene sequence similarities with phylogenetic neighbours were in the range 97.7–98.6 %. The closest phylogenetic species was *N. pneumoniae*, with 98.6 % sequence similarity to the type

Fig. 1. Scanning electron micrograph of hyphae of strain TT 00-78^T grown on modified Bennett's agar at 28 °C for 14 days. Bar, 1 μm.

strain. The taxonomic integrity of strain TT 00-78^T was supported by the DNA relatedness data. DNA relatedness values of 6.9–20.3 % were obtained with respect to the type strains of related *Nocardia* species (*N. pneumoniae*, 20.3 %; *N. beijingensis*, 6.9 %; *N. arthritidis*, 20.3 %; *N. araoensis*, 18.3 %), the values being well below the 70 % cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne *et al.*, 1987). Strain TT 00-78^T was also distinguishable from its phylogenetic neighbours in comparisons of biochemical and phenotypic characteristics (Table 1).

On the basis of phenotypic and genotypic data, therefore, strain TT 00-78^T represents a novel species within the genus *Nocardia*, for which the name *Nocardia amamiensis* sp. nov. is proposed.

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequences, showing the relationships of strain TT 00-78^T with the closest *Nocardia* species. The tree was constructed using the neighbour-joining method and *K*_{nuc} values (Saitou & Nei, 1987). Asterisks indicate branches of the tree that were also recovered using the minimum-evolution and maximum-parsimony methods (Takahashi & Nei, 2000). Bar, 0.01 *K*_{nuc}.

Table 1. Phenotypic properties that serve to distinguish strain TT 00-78^T from the type strains of related *Nocardia* species

Strains: 1, TT 00-78^T; 2, *N. pneumoniae* IFM 0784^T; 3, *N. beijingensis* AS4.1521^T; 4, *N. arthritidis* IFM 10035^T; 5, *N. araoensis* IFM 0575^T. Data were from this study (columns 1 and 2) or from Wang *et al.* (2001) and Kageyama *et al.* (2004a, b) (columns 3–5, unless indicated). All strains are positive for urea hydrolysis and negative for degradation of casein.

Characteristic	1	2	3	4	5
Arbutin hydrolysis	–	–	–	+	–
Nitrate reduction	+	+	+	+	–
Decomposition of:					
Testosterone	+	+	–*	+	+
Uric acid	–	–	–	+	–*
Xanthine	–	–	+	–	–
Growth with sole carbon sources					
L-Arabinose	–	–	+	–	–
meso-Erythritol	–	–	+	–	–
D-Galactose	–	–	+	–	–
D-Lactose	–	–	+	–*	–*
Maltose	+	–	+	–	–
D-Mannitol	–	–	+	+	+
D-Mannose	+	–	+	–	–
L-Rhamnose	–	–	+	–	–
D-Xylose	–	–	+	+	–*
Growth at 45 °C	–	+	–	+	+

*Data from this study.

Description of *Nocardia amamiensis* sp. nov.

Nocardia amamiensis (a.ma.mi.en'sis. N.L. fem. adj. *amamiensis* pertaining to Amami Island, from where the organism was first isolated).

Aerobic, Gram-positive, partially acid–alcohol-fast, non-motile actinomycete that forms moderately white aerial mycelium that fragments into rod-shaped elements. Diffusile pigments are not produced. Aesculin is hydrolysed and nitrate is reduced. Arbutin and urea are not hydrolysed. Degrades uric acid, but does not degrade adenine, casein, elastin, hypoxanthine, tyrosine or xanthine. Grows at 15, 28 and 37 °C but does not grow at 5, 10 or 45 °C. Growth occurs in the presence of 0–3 % NaCl, but not at 5 % NaCl (w/v). Assimilates fumarate, L-glutamate, sebacic acid, sodium citrate, succinate (at 0.1 %, w/v), 1,2-propanediol, 2,3-butanediol, isoamyl alcohol, D-glucose, maltose, D-mannose and D-trehalose (at 1 %, w/v) as sole carbon sources, but not adipic acid, pimelic acid, sodium acetate, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid (at 0.1 % w/v), L-arabinose, D-cellobiose, dulcitol, meso-erythritol, D-galactose, guanine, gluconate, myo-inositol, inulin, D-lactose, D-mannitol, D-melezitose, D-melibiose, methyl D-glucoside, D-raffinose, L-rhamnose, salicin, D-sorbitol, D-xylitol and D-xylose (at 1 %, w/v). The major fatty acids are hexadecanoate,

hexadecenoate, tuberculostearic acid (10-methyl octadecanoate) and *cis*-9 octadecanoate. The G+C content of the DNA is 67.4 mol%.

The type strain, TT 00-78^T (=NBRC 102102^T=DSM 45066^T=KCTC 19208^T), was isolated from a soil sample collected from a sugar-cane field on Amami Island in Japan.

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