Nocardia pigrifrangens sp. nov., a novel actinomycete isolated from a contaminated agar plate

Liming Wang,¹ Yamei Zhang,¹ Ying Huang,¹ Luis A. Maldonado,² Zhiheng Liu¹ and Michael Goodfellow²

Correspondence

Zhiheng Liu

zhliu@sun.im.ac.cn

¹State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, People's Republic of China

²School of Biology, University of Newcastle, Newcastle upon Tyne NE1 7RU, UK

A polyphasic study was undertaken to establish the taxonomic position of an actinomycete strain isolated from a contaminated agar plate. The strain, designated 7031^T, had morphological and chemotaxonomic properties typical of the genus *Nocardia*. An almost-complete 16S rRNA gene sequence determined for the strain was aligned with available sequences for nocardiae, and phylogenetic trees were inferred using three tree-generating algorithms. Strain 7031^T clustered with the type strains of *Nocardia carnea* and *Nocardia flavorosea*, showing low 16S rRNA gene sequence similarities to these species (97.2 and 97.5 %, respectively). The strain was also distinguished from the closest species by a range of phenotypic properties. It is proposed that the strain be recognized as a novel species of *Nocardia*, *Nocardia pigrifrangens* sp. nov., the type strain of which is 7031^T (=AS 4.1808^T=JCM 11884^T).

The genus *Nocardia* is well defined as a result of the application of complementary genotypic and phenotypic methods (Goodfellow *et al.*, 1999; Yassin *et al.*, 2003). The improved classification provides an invaluable framework for the delineation of additional species. The genus encompasses 36 recognized species at the time of writing, 20 of which have been described in the last 5 years. Members of most of the latter have been isolated either from clinical material (Yassin *et al.*, 2000a, b, 2001a, b, 2003; Gürtler *et al.*, 2001; Hamid *et al.*, 2001; Kageyama *et al.*, 2004a, b) or from environmental samples, notably soil (Maldonado *et al.*, 2000; Kinoshita *et al.*, 2001; Wang *et al.*, 2001; Kämpfer *et al.*, 2004; Saintpierre-Bonaccio *et al.*, 2004; Albuquerque de Barros *et al.*, 2003). Despite this rapid expansion in the number of described species, it is evident that additional nocardial diversity has still to be formally described (Orchard & Goodfellow, 1980; Wang *et al.*, 1999; Maldonado *et al.*, 2000; Roth *et al.*, 2003).

In the course of ongoing studies on unusual actinomycetes, a strain isolated from a contaminated Bennett's agar plate (Zhang *et al.*, 2003) was considered to belong to the genus

Nocardia based on morphological criteria. The aim of the present study was to determine the taxonomic position of this isolate using a polyphasic taxonomic approach.

The colonial properties of strain 7031^T were recorded from a modified Bennett's agar plate (Zhang *et al.*, 2003) that had been incubated for 7 days at 28 °C. It was also examined for a broad range of phenotypic properties using standard procedures (Goodfellow, 1971). The biochemical and degradative tests were carried out after Gordon & Mihm (1957) and the carbon utilization tests according to Yassin *et al.* (1995). Biomass for most of the chemotaxonomic tests was prepared in Sauton's broth as described by Zhang *et al.* (2003). Established TLC procedures were used to determine the diagnostic isomers of diaminopimelic acid (Lechevalier & Lechevalier, 1980) and menaquinone (Collins *et al.*, 1987), polar lipid (Yassin *et al.*, 1993) and whole-organism sugar patterns (Lechevalier & Lechevalier, 1980). Mycolic acids were detected using the acid methanolysis procedure (Minnikin *et al.*, 1975). Biomass for the quantitative fatty acid analysis was prepared from trypticase soy broth agar plates after Zhang *et al.* (2003). The non-hydroxylated fatty acids were extracted, purified, methylated, identified and quantified by GC using the standard MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

Chromosomal DNA from strain 7031^T was isolated and purified following the procedure described by Yassin *et al.* (2000a). The G+C content of the DNA was determined

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16S rRNA signatures of representative *Nocardia* species are available as supplementary data in IJSEM Online.

using the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* AS 1.365 as control. Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR products were carried out following the procedures of Rainey *et al.* (1996). Purified PCR products were sequenced directly using a *Taq* Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems) and universal primers as described by Lu *et al.* (2001). Sequence gel electrophoresis was carried out and nucleotide sequences were obtained automatically using an Applied Biosystems DNA sequencer (model 377) and software provided by the manufacturer.

An almost-complete 16S rRNA gene sequence of strain 7031^T (1419 nt) was aligned manually with corresponding nucleotide sequences of representatives of recognized *Nocardia* species, available from public databases, using the PHYDIT program (Chun, 1995). Evolutionary trees were inferred using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. The robustness of the resultant trees was evaluated by a bootstrap analysis (Felsenstein, 1985) of the neighbour-joining dataset using the SEQBOOT and CONSENSE options from the PHYLIP package.

Strain 7031^T exhibited a range of phenotypic properties typical of members of the genus *Nocardia* (Goodfellow *et al.*, 1999). The organism is an aerobic, Gram-positive, slightly acid–alcohol-fast actinomycete, which forms an extensively branched orange substrate mycelium that fragments into rod-shaped, non-motile elements and supports abundant white aerial hyphae on modified Bennett's agar. Whole-organism hydrolysates of the organism are rich in *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970) and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositolmannosides (phospholipid lipid type II *sensu* Lechevalier *et al.*, 1977). The isolate contains hexahydrogenated menaquinones with eight isoprene units, in which the end two are cyclized; this menaquinone is restricted to members of the genera *Nocardia* and *Skermania* (Chun *et al.*, 1997; Goodfellow *et al.*, 1999). The fatty acid profile mainly consists of straight-chain saturated, unsaturated and 10-methyl branched components. The G+C content of the DNA is 68.7 mol%.

The unrooted phylogenetic tree (Fig. 1) shows that strain 7031^T and the type strains of *Nocardia carnea* and *Nocardia flavorosea* form a clade that is loosely associated with a taxon that encompasses the type strains of *Nocardia brevicatena* and *Nocardia paucivorans*. The taxonomic integrity of the two clades is supported by analyses based on all three tree-making algorithms and by a high bootstrap value with the neighbour-joining method. It is known that the type strains of *N. carnea* and *N. flavorosea* share a high 16S rRNA gene sequence similarity (99.2% or 12 nt differences out of 1472 positions) but belong to genomic species with a mean

Fig. 1. Unrooted neighbour-joining tree (Saitou & Nei, 1987), based on almost-complete 16S rRNA gene sequences, showing the position of strain 7031^T within the genus *Nocardia*. Asterisks indicate the branches of the tree that were also recovered using both the least-squares (Fitch & Margoliash, 1967) and maximum-parsimony (Kluge & Farris, 1969) tree-making algorithms. Numbers at the nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are given.

DNA–DNA relatedness value of 5% (Chun *et al.*, 1998). It is, therefore, evident that the low 16S rRNA gene sequence values found between strain 7031^T and the type strains of *N. carnea* and *N. flavorosea*, namely 97.2 and 97.5%, respectively, indicate that the test strain forms a distinct centre of taxonomic variation in the *N. carnea* clade. It is clear from Table 1 that strain 7031^T can be distinguished readily from the type strains of *N. carnea* and *N. flavorosea* using a combination of phenotypic properties. These organisms can also be distinguished by a number of 16S rRNA signatures (results are available as supplementary data in IJSEM Online).

Table 1. Phenotypic characteristics that distinguish strain 7031^T from representatives of phylogenetically close *Nocardia* species

Strains: 1, 7031^T; 2, *N. carnea* DSM 43397^T; 3, *N. flavorosea* JCM 3332^T. Data were taken from this and previous studies (Orchard & Goodfellow, 1980; Chun *et al.*, 1998; Zhang *et al.*, 2003). +, Positive; –, negative.

Character	1	2	3
Aesculin hydrolysis	–	+	–
Arbutin hydrolysis	+	–	+
Nitrate reduction	+	+	–
Urea hydrolysis	+	–	–
Decomposition of:			
Uric acid	+	–	–
Growth on sole carbon sources (% w/v):			
(+)–D-Glucose (1·0)	+	+	–
(+)–D-Mannitol (1·0)	–	+	+
(+)–D-Sorbitol (1·0)	–	+	+
Sodium acetate (0·1)	+	–	+
Sodium benzoate (0·1)	+	–	–
Sodium citrate (0·1)	–	–	+
Growth at 45 °C	–	–	+

The combined genotypic and phenotypic data indicate that strain 7031^T merits recognition as a novel species in the genus *Nocardia*; the name proposed for this new taxon is *Nocardia pigrifrangens* sp. nov.

Description of *Nocardia pigrifrangens* sp. nov.

Nocardia pigrifrangens (L. adj. *piger* slow; L. part. adj. *frangens* from L. v. *frango* to break up small; N.L. part. adj. *pigrifrangens* slow to break up; *frango* referring to the fact that the substrate mycelium remains stable for up to 14 days before undergoing fragmentation).

Aerobic, Gram-positive, catalase-positive, slightly acid–alcohol-fast, non-motile actinomycete that produces an orange substrate mycelium that fragments *in situ* into irregular rod-shaped elements and which bears sparse white to pinkish aerial hyphae on modified Bennett's, modified Sauton's and oatmeal agars. Colonies are convex with filamentous margins. Does not degrade adenine, casein, elastin, hypoxanthine, tyrosine or xanthine. Growth occurs between 18 and 35 °C. (–)-D-Arabinose, (+)-D-cellobiose, dulcitol, meso-erythritol, ethanol, (–)-D-fructose, glycerol, glycogen, inulin, (+)-D-mannose, (–)-D-ribose, (+)-D-salicin, (+)-D-sucrose, (+)-D-trehalose, (+)-D-xylose (all at 1 %, w/v), gluconate, *p*-hydroxybenzoic acid, sodium H-malate, pyruvate and succinate (all at 0·1 %, w/v) are used as sole carbon sources for energy and growth, but not adonitol, (+)-D-galactose, (+)-D-lactose, (+)-D-maltose, (+)-D-melezitose, (+)-D-melibiose, methyl α -D-glucopyranoside, (+)-D-raffinose, α -L-rhamnose, xylitol (all at 1 %, w/v), iso-amyl alcohol, iso-butanol, n-propanol (all at 1 %, v/v), butyrate or *m*-hydroxybenzoic acid.

L-Alanine, L-aspartate, L-glutamate, monoethanolamine, L-proline, uric acid and L-valine are used as sole carbon and nitrogen sources, but not acetamide, gelatin, L-leucine, L-phenylalanine or urea (all at 0·1 %, w/v). Additional phenotypic properties are shown in Table 1. The major cellular fatty acids are C_{16:1} ω 7c (8·9 %), C_{16:0} (18·0 %), C_{18:1} ω 9c (17·6 %), C_{18:0} (30·5 %) 10-methyl-C_{18:0} (21 %) and C_{21:1} ω 9c (4·1 %). The G + C content of the DNA is 68·7 mol%.

The type strain, 7031^T (=AS 4.1808^T=JCM 11884^T), was isolated from a contaminated agar plate of a clinically significant strain in Shanghai, People's Republic of China.

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