Nocardia flavorosea sp. nov.

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An actinomycete strain, ‘Nocardia flavorosea’ JCM 3332, was found to have properties consistent with its classification in the genus Nocardia. An almost complete gene sequence of the 16S rDNA of the strain was determined following cloning and sequencing of the amplified gene. The sequence was aligned with those available for nocardiae and phylogenetic trees were inferred using four tree-making algorithms. The organisms consistently formed a distinct clade with the type strain of Nocardia carnea. However, DNA relatedness experiments showed that the strain and N. carnea DSM 43397T belonged to two distinct genomic species. The organism was also distinguished from representatives of all of the validly described species of Nocardia using a combination of phenotypic properties. These genotypic and phenotypic data show that the strain merits recognition as a new species of the genus Nocardia. The name proposed for the new species is Nocardia flavorosea sp. nov. The type strain is JCM 3332T.

Keywords: Nocardia flavorosea, classification, soil bacteria, actinomycetes

INTRODUCTION

The taxonomy of the genus Nocardia Trevisan 1889 has undergone marked revision with the application of chemotaxonomic, molecular systematic and numerical phenetic methods (Goodfellow, 1992, 1998). Indeed, the proposal to recognize actinomycetes previously classified as Nocardia pinensis as Skermania piniformis (Chun et al., 1997) leaves the genus as a homogeneous taxon for the first time in its long and chequered taxonomic history (Goodfellow, 1998; Lechevalier, 1976). The genus Nocardia encompasses eleven validly described species, forms a monophyletic clade within the evolutionary radiation occupied by mycolic acid-containing actinomycetes (the mycolata), and has a phylogenetic depth comparable to that of other mycolata genera (Chun & Goodfellow, 1995; Chun et al., 1996; Goodfellow, 1998).

Members of the redefined genus Nocardia (Goodfellow, 1992, 1998) produce extensively branched substrate hyphae which fragment in situ or on mechanical disruption into rod-shaped to coccoid, non-motile elements. Aerial hyphae, at times only visible microscopically, are abundant and always found. Nocardiae have: meso-diaminopimelic acid, arabinose and galactose in the cell wall (wall chemotype IV; sensu Lechevalier & Lechevalier, 1970); muramic acid in an N-glycolyl form; diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides as predominant phospholipids; major amounts of straight-chain, unsaturated and tuberculostearic fatty acids; mycolic acids with 44-60 carbon atoms; and hexahydrogenated menaquinone with eight isoprene units where the end two are cyclized as the predominant menaquinone. The G+C content of the DNA lies within the range 64–72 mol%.

The present investigation was designed to clarify the taxonomic position of a soil isolate, strain JCM 3332T.
which had been characterized and provisionally assigned to the genus _Nocardia_ as ‘_Nocardia flavosea_’ by Liu et al. (1983). Genotypic and phenotypic data show that the strain should be formally recognized as a new species of the genus _Nocardia_ for which the name _Nocardia flavosea_ sp. nov. is proposed.

**METHODS**

**Bacterial strain and culture conditions.** Strain JCM 3332<sup>T</sup> (=NRRRL B-16176<sup>T</sup>) was grown at 30 °C for 7 d in shake flasks containing modified Sauton’s broth (Modarska et al., 1972). Biomass for the chemotaxonomic studies was collected by centrifugation, washed twice with distilled water and freeze-dried. The strain was maintained as glycerol suspensions (20%, v/v) at −20 °C.

**Phenotypic characterization.** The cultural and staining properties of the test strain were determined from glucose/yeast extract agar (GYEA; Jones, 1949) plates incubated at 30 °C for 3 d. After incubation, colonies were examined both by eye and microscopically. Unless otherwise stated, the remaining tests were read after incubation at 30 °C. The degradation tests were carried out using GYEA as the basal medium and the results were read after 4 weeks following the established procedure (Boiron et al., 1993; Gordon et al., 1974). The temperature tests were read from GYEA plates incubated for 4 weeks (10 °C) and 2 weeks (25, 35, 45 and 50 °C). The biochemical tests and acid production from sugars were carried out after Gordon et al. (1974).

Utilization of organic acids was determined according to Koser (1924). Resistance to antibiotics was examined using bacitracin (10 U), gentamicin (10 μg), penicillin (10 μg), streptomycin (10 μg) and tobramycin (10 μg) disks (Oxoid), and GYEA as basal medium. Readings were taken at 14, 21 and 28 d. The ability to inhibit the growth of _Escherichia coli_ HB101 was observed using an overlay technique. Multi-inoculated colonies on nutrient agar plates were inverted over 1.5 ml chloroform for 40 min. Killed colonies were then overlaid with 5 ml slopy agar (0–7%, w/v; Oxoid Nutrient Agar No. 2) inoculated with _E. coli_. Zones of inhibition were measured after 24 h at 30 °C.

**Chemotaxonomy.** Isomers of _Apm_ were analysed by the method of Staneck & Roberts (1974). The small-scale method of Minnikin et al. (1984) was used to extract menaquinones from freeze-dried biomass (about 100 mg). Purified menaquinones were analysed using an electron-impact mass spectrometer (JEOL SX-102), as described earlier (Chun et al., 1997). Freeze-dried biomass (about 50 mg) of the test strain was also degraded by acid-methanolysis (Minnikin et al., 1980) and the resultant fatty acid and mycolic acid methyl esters were purified by preparative TLC. The fatty acid methyl esters were examined using the MIDI system (Sasser, 1990), and the composition of the mycolic acid methyl esters was determined using electron-impact mass spectrometry according to Collins et al. (1982).

The analysis of sugars as their alditol acetates was carried out after Saddler et al. (1991).

**Determination of DNA base composition.** DNA was prepared according to Chun & Goodfellow (1995). The G+C content of the DNA was determined by the thermal denaturation method (Mandel & Marmur, 1968).

**16S rDNA sequencing.** Isolation of chromosomal DNA, and PCR, cloning and sequencing of the 16S rDNA were carried out using the _Taq_ DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an Applied Biosystems 373A DNA sequencer as described previously (Chun & Goodfellow, 1995). The resultant 16S rDNA sequence was aligned manually against sequences of representative mycolic acid-containing actinomycetes using the AL 16S program (Chun, 1995). The additional sequence data were obtained from the GenBank/EMBL databases. Phylegetic trees were inferred using four tree-making algorithms, namely the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant unrooted tree topology was evaluated in bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

**DNA–DNA hybridization.** Levels of genomic relatedness between strain JCM 3332<sup>T</sup> and _Nocardia carnea_ DSM 43397<sup>T</sup> were determined by the DNA–DNA slot-blot hybridization method (Kafatos et al., 1979). Chromosomal DNA was extracted from test strains according to Chun & Goodfellow (1995). Duplicated aliquots containing 1 μg each genomic DNA were denatured by boiling for 10 min in 0·4 M NaOH, transferred onto positively charged nylon membranes (Amersham) using a slot-blot apparatus (Bio-Rad), and immobilized using UV crosslinking. Prehybridization was carried out at 62 °C for 2 h in hybridization buffer containing 3 × SSC (1 × SSC is 0·15 M NaCl plus 0·015 M sodium citrate), 35% formamide, 1% blocking agent (Boehringer Mannheim) and 100 μg denatured and sheared salmon sperm DNA (Sigma) per ml. The probes were labelled with digoxigenin using a Random Prime Labelling kit (Boehringer Mannheim) and added (10 ng/ml) to fresh hybridization solution containing the loaded membranes. Hybridization was performed at 62 °C for 20 h and...
hybridized membranes were washed according to the manufacturer's instruction. Chemoluminescence was detected using a Boehringer Mannheim detection kit and exposed to Hyperfilm (Amersham). The chemoluminescent intensity of each blot was quantified using a densitometer (Molecular Dynamics). After detection, the membrane was washed for reprobing according to the manufacturer's instructions (Boehringer Mannheim). The signal produced by self-hybridization of the probe with homologous target DNA was taken as 100% and the percentage homology values were calculated for the duplicated slots.

RESULTS AND DISCUSSION

An almost complete 16S rDNA sequence (1472 nt) was obtained for the test strain. When this sequence was compared with those of representative actinomycetes, it was apparent that strain JCM 3332T belonged to the genus Nocardia (data not shown). This assignment is also supported by the chemotaxonomic and morphological data. Strain JCM 3332T forms a substrate mycelium which undergoes fragmentation. It contains: meso-diaminopimelic acid as the wall diamino acid; arabinose and galactose as major wall sugars; major amounts of saturated, monounsaturated and tuberculostearic fatty acids; mycolic acids with 5–56 carbon atoms; hexahydrogenated menaquinones with eight isoprene units where the end two are cyclized [i.e. major peak at m/z 720 (the molecular ion) and a significant peak at m/z 584 (molecular ion minus a terminal cyclized two isoprene moieties)] as the major isoprenologue; and DNA with a G+C content of 69 mol%. All of these properties are consistent with classification of the strain in the genus Nocardia (Chun & Goodfellow, 1995; Chun et al., 1997; Goodfellow, 1998).

The unrooted evolutionary tree (Fig. 1) shows that strain JCM 3332T forms a monophyletic clade with N. carnea. This relationship was highlighted in the analyses based on all four tree-making algorithms, by the high nucleotide similarity value (99.2% or 12 differences out of 1472 nt positions) and the 100% bootstrap value recorded with the neighbour-joining method. However, it is clear from the DNA–DNA relatedness study that strain JCM 3332T and N. carnea DSM 43397T belong to separate genomic species. The mean DNA relatedness value was 5% when N. carnea DSM 43397T was used as the probe and 2% in the corresponding experiment where strain JCM 3332T was labelled. It is also apparent that strain JCM 3332T can be distinguished from representatives of all of the validly described species of the genus Nocardia, including N. carnea, using a combination of phenotypic properties (Table 1). These observations are in good

### Table 1. Phenotypic characteristics that differentiate strain JCM 3332T from 11 other nocardiae

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Data obtained from Boiron et al. (1993), Goodfellow (1992, 1998), Goodfellow & Lechevalier (1989), Kudo et al. (1988) and Ruimy et al. (1996). Symbols: +, ≥ 90% strains are positive; -, ≤ 90% strains are negative; v, variable; ND, not determined.

agreement with the results of a numerical phenetic survey which showed that ‘Nocardi a flavorosea’ JCM 3332T formed a single-membered cluster (Yano et al., 1990).

It is clear from the genotypic and phenotypic data that strain JCM 3332T merits species status in the genus Nocardia. It is, therefore, proposed that the organism be classified in the genus Nocardia as Nocardia flavorosea sp. nov.

**Description of Nocardia flavorosea sp. nov.**

*Nocardia flavorosea* (fla.vo.ro.se.a. L. adj. flavus yellow; L. fem. adj. rosea rose-coloured; M.L. adj. flavorosea yellow rose).

Aerobic, Gram-positive, catalase-positive, partially acid-alcohol-fast, non-motile actinomycetes which form an extensively branched substrate mycelium which fragments in situ into rod-shaped to coccoid elements. The orange substrate mycelium carries white to pinkish aerial hyphae. Colony elevation is convex to irregular and colony margins are filamentous. Diffusible pigments are not formed. Growth occurs between 25 and 50 °C, but not at 10 °C. Starch is degraded but not adenine, casein, gelatin, tyrosine or xanthine. Allantoin, hippuric acid and urea are not utilised. Xylose, propionate, pyruvate and succinate are used as sole carbon sources for energy and growth, but benzoate, citrate, gluconate, lactate, mucate and oxalate are not. The organism is resistant to bacitracin and tobramycin. Antibiosis activity is evident against *E. coli*.

The organism contains major amounts of mycolic acids with 50–56 carbon atoms and 2–4 double bonds (C\(_{50:3}\), C\(_{52:2}\), C\(_{52:3}\), C\(_{52:4}\), C\(_{54:2}\) and C\(_{56:3}\)). The major cellular fatty acids are hexadecanoic (29% of total fatty acids), octadecenoic (23%), 10-methyloctadecanoic (tuberculostearic, 17%) and hexadecenoic (9%) acids. The remaining chemical properties of the organism were given earlier. The G+C content of the DNA of strain JCM 3332T is 69 mol%, as determined by the thermal denaturation method. The type strain is JCM 3332T (=NRRL B-16176\(^T\)). This strain was isolated from soil collected in Yunnan Province, China.

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**References**


