**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 43397\(^T\) 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 3332\(^T\).

**Keywords:** *Nocardia flavorosea*, classification, soil bacteria, actinomycetes

### INTRODUCTION

The taxonomy of the genus *Nocardia* Trevisan 1889 has undergone marked revision with the application of chemo taxonomic, 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 chemo-type 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 3332\(^T\),...
which had been characterized and provisionally assigned to the genus *Nocardia* as 'Nocardia flavorosea' 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 flavorosea* sp. nov. is proposed.

**METHODS**

**Bacterial strain and culture conditions.** Strain JCM 3332(T) (=NRRRL B-16176(T)) 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 stocks in flasks containing modified Sauton’s broth (Modarska *et al.*, 1972).

**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.*, 1979; 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 μg), 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 sloppy 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 Stanek & Roberts (1974). The small-scale method of Minnikin *et al.* (1984) was used to extract menaquinones from freeze-dried biomass (about 100 μg).

**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 additional sequence data were obtained from the GenBank/EMBL databases. Phylogenetic 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(T) and *Nocardia carnea* DSM 43397(T) 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

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**Fig. 1.** Unrooted neighbour-joining tree based on 1408 unambiguously aligned nucleotide positions. Asterisks indicate the branches that were also recovered in the remaining three methods employed in this study (see text). The numbers at the nodes are the levels of bootstrap support based on neighbour-joining analyses of 1000 resampled data sets. The scale bar represents 0.01 nucleotide substitutions per position.
hybridized membranes were washed according to the manufac-
turer'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 mor-
phological 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, in-
cluding 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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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 Nocardi a. It is, therefore, proposed that the organism be classified in the genus Nocardi a as Nocardi a flavorosea sp. nov.

Description of Nocardi a flavorosea sp. nov.

Nocardi a 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 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 used. D-melezitose, D-melibiose, raffinose, L-rhamnose, salicin, cin, D-sorbitol, sucrose, D-trehalose, xylitol or D-dextrin, D-fructose, D-galactose, D-glucose, meso-inositol, D-lactose, D-maltose, D-mannitol, D-mannose, D-melezitose, D-melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose, D-trehalose, xylitol or D-levans. Propionate, pyruvate and succinate are used as growth factors. Growth occurs at pH 7.0–9.0. The type strain is Nocardi a flavorosea ATCC 23865 = DSM 44925.

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