Note

Nocardia cerradoensis sp. nov., a novel isolate from Cerrado soil in Brazil

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An isolate from Cerrado soil, provisionally assigned to the genus *Nocardia*, was shown to have chemical and morphological properties typical of nocardiae. The strain formed a distinct monophyletic clade in the 16S rDNA tree together with *Nocardia africana*, *Nocardia vaccinii* and *Nocardia veterana*, and showed a unique combination of phenotypic properties that distinguished it from representatives of all recognized species of *Nocardia*. DNA–DNA relatedness studies indicated that the isolate belongs to a genomic species that is readily distinguished from its nearest neighbours, the type strains of *N. africana* and *N. veterana*. The organism is considered to merit species status, and it is proposed that it be designated *Nocardia cerradoensis* sp. nov., with strain Y9^T (=CCT 5601^T =DSM 44546^T) as the type strain.

Complementary genotypic and phenotypic data have been used to clarify the systematics of the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999). The genus currently accommodates 26 recognized species, which form a clade within the evolutionary radiation occupied by mycolic-acid-containing actinomycetes, that is, by actinomycetes that comprise the suborder *Corynebacterineae* Stackebrandt *et al.* 1997. The taxonomic status of most of the species is supported by a rich combination of molecular systematic and numerical phenetic data (Goodfellow *et al.*, 1999; Hamid *et al.*, 2001; Maldonado *et al.*, 2001; Yassin *et al.*, 2001a, b). The improved classification of the genus provides a framework for the recognition of additional nocardial species.

It seems likely that soil is the primary reservoir of nocardiae (Cross *et al.*, 1976; Orchard *et al.*, 1977), though members of the genus are better known as causal agents of actinomycete mycetoma and nocardioses (McNeil & Brown, 1994; Goodfellow, 1998; Hamid *et al.*, 2001).

Little is known about the biology of nocardiae in natural habitats, though they have been considered to form part of the autochthonous soil microflora (Orchard, 1979, 1981). There is also evidence that nocardial species diversity in natural and artificial habitats is grossly underestimated (Orchard & Goodfellow, 1980; Wang *et al.*, 1999; Maldonado *et al.*, 2001). It is important to establish the species richness of nocardiae in natural ecosystems in order to determine the roles that specific nocardiae play in organic-matter turnover; representatives of such novel taxa also form high-quality biological material for exploitable biology (Bull *et al.*, 2000).

The Brazilian Cerrado is an extensive species-rich ecosystem that contains commercially significant actinomycetes (Huddleston *et al.*, 1997; Esposito *et al.*, 1998). In a search for novel actinomycetes indigenous to this ecosystem, an unusual strain was isolated and provisionally assigned to the genus *Nocardia* by using morphological criteria. The aim of the present study was to determine the taxonomic status of this organism by using a polyphasic taxonomic approach.

Strain Y9^T was isolated on an R5 agar plate (Hopwood *et al.*, 1985) that had been incubated for 4 days at 30 °C, following incubation with a soil sample collected from a Cerrado cultivated field of corn and *Brachiaria* grass; the sample had been kept for 5 months at 4 °C and treated with CaCO₃ prior

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The GenBank accession number for the 16S rDNA sequence of strain Y9^T (=CCT 5601^T =DSM 44546^T) is AF060790.

to the preparation of a dilution series. The isolate was maintained on modified Bennett's agar (Jones, 1949) at room temperature and as suspensions of mycelial fragments in glycerol (20 %, v/v) at -80°C . Biomass for chemotaxonomic studies was grown in shake flasks of modified Bennett's broth for 5 days at 28°C and harvested by centrifugation; the resultant pellets were washed twice with distilled water.

The colonial and micromorphological properties of the tested strain were observed using standard procedures following inoculation on modified Bennett's agar plates for 14 days at 30°C . The remaining phenotypic properties were scored following established procedures (Isik *et al.*, 1999). The isomeric form of diaminopimelic acid (A_2pm) of strain Y9^{T} was determined by TLC of whole-organism hydrolysates (Staneck & Roberts, 1974). Standard procedures were also used for the extraction and analysis of mycolic acids (Minnikin *et al.*, 1975), whole-organism sugars (Schaal, 1985) and isoprenoid quinones and polar lipids (Minnikin *et al.*, 1984), using appropriate controls. The base composition of DNA of the strain was determined using the reverse-phase HPLC method described by Tamaoka (1994) and the HPLC conditions outlined by Gerke *et al.* (1984). DNA–DNA hybridizations were made, in duplicate, between *Nocardia africana* SD769^T (=DSM 44491^T) and *Nocardia veterana* N1071^T (=DSM 44445^T) and between *Nocardia* strain Y9^{T} and the former two strains, under the auspices of the identification service at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), as described by Kim *et al.* (1999). Genomic DNA was extracted and amplified from single colonies grown on modified Bennett's agar using a procedure (Chun & Goodfellow, 1995) slightly modified from that of Pitcher *et al.* (1989). PCR amplification of the 16S rRNA gene was performed as described by Wang *et al.* (2001), and the resultant PCR product was purified by using the Wizard PCR purification system (Promega) according to the manufacturer's instructions. The purified PCR products were sequenced using a Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems) and two universal primers, as described previously (Lu *et al.*, 2001). Sequence gel electrophoresis was carried out and the nucleotide sequence obtained automatically by using an Applied Biosystems DNA sequencer (model 373A) and software provided by the manufacturer.

The 16S rDNA sequence of the test organism was aligned manually against nucleotide sequences of representative *Nocardia* strains retrieved from the Ribosomal Database Project (Maidak *et al.*, 1997) and the GenBank database, using the PHYDIT program (J. Chun, unpublished data). Evolutionary trees were inferred using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) routines from the PHYLIP suite of programs (Felsenstein, 1993). The algorithm

of Jukes & Cantor (1969) was used to generate evolutionary distance matrices for the least-squares and neighbour-joining methods. The robustness of the unrooted trees was evaluated by bootstrap analysis of 1000 resamplings, using the SEQBOOT and CONSENSE programs from the PHYLIP package (Felsenstein, 1993).

The almost complete 16S rDNA sequence (1460 nt) obtained for strain Y9^{T} was compared with those of related actinomycetes and found to contain all of the signature nucleotides expected for members of the suborder *Corynebacterineae* and the family *Nocardiaceae* (Stackebrandt *et al.*, 1997). The high 16S rDNA sequence similarities found between the tested strain and representatives of the genus *Nocardia* (94.0–98.8 %) support its inclusion in this taxon. The 16S rDNA nucleotide sequence of strain Y9^{T} also contains the signature nucleotides characteristic of members of the genus *Nocardia* (Chun & Goodfellow, 1995).

It is evident from the 16S rDNA sequence data that strain Y9^{T} forms a distinct subclade in the nocardial tree together with *N. africana*, *Nocardia vaccinii* and *N. veterana* (Fig. 1). The taxonomic integrity of this subclade was supported by

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rDNA sequences (1377 nt) showing relationships between strain Y9^{T} and representatives of the genus *Nocardia*. Asterisks indicate branches of the tree that were also found using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods. F and P respectively indicate branches that were also recovered using the least-squares and maximum-parsimony methods. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values $>55\%$ are shown. Bar, 0.1 substitutions per site.

all four treeing algorithms and by the 86 % bootstrap value obtained in the neighbour-joining analysis. Strain Y9^T shares 16S rDNA nucleotide similarity of 98.8 % with *N. africana*, 98.1 % with *N. vaccinii* and 99.0 % with *N. veterana*, values that respectively correspond to 17, 27 and 14 differences at 1420 nt sites. Higher 16S rDNA similarity values have been recorded between representatives of several validly described *Nocardia* species found to have DNA–DNA relatedness values well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the delineation of genomic species of bacteria. The type strains of *Nocardia brevicatena* and *Nocardia paucivorans*, for instance, were found to share 16S rRNA gene sequence similarity of 99.6 % and a DNA relatedness value of 61.9 % (Yassin *et al.*, 2000a). Similarly, the type strains of *Nocardia carnea* and *Nocardia flavorosea* were shown to share a high 16S rRNA nucleotide similarity value (99.2 % or 12 nucleotide differences) and a mean DNA relatedness value of 5 % when the *N. carnea* strain was used as the probe (Chun *et al.*, 1998). In the present study, *Nocardia* strain Y9^T showed a mean DNA–DNA relatedness value of 60.5 % with *N. africana* SD769^T and 58 % with *N. veterana* N1071^T;

the corresponding value between the *N. africana* and *N. veterana* strains was 47.8 %.

Strain Y9^T was found to have phenotypic properties typical of members of the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999). The organism is an aerobic, Gram-positive, acid–alcohol-fast actinomycete that forms an extensive branched substrate mycelium that fragments into irregular, rod-shaped, non-motile elements and supports pinkish-white aerial hyphae on modified Bennett's agar. The strain was also shown to produce whole-organism hydrolysates rich in *meso*-A₂pm, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), to have major amounts of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (phospholipid type II *sensu* Lechevalier *et al.*, 1977), to have mycolic acids that co-migrated (*R_f* value of about 0.47) with those extracted from marker *Nocardia* strains and to have DNA with a G+C content of 68.8 mol%. The isolate also contained predominant amounts of hexahydrogenated menaquinones with eight isoprene units where the end two were cyclized; this menaquinone is

Table 1. Phenotypic properties that distinguish strain Y9^T from the type strains of validly described *Nocardia* species

Strains are indicated as: 1, strain Y9^T; 2, *N. abscessus* DSM 44432^T; 3, *N. africana* DSM 44491^T; 4, *N. asteroides* ATCC 19247^T; 5, *N. beijingensis* IFO 16342^T; 6, *N. brasiliensis* ATCC 19246^T; 7, *N. brevicatena* DSM 43024^T; 8, *N. carnea* DSM 43397^T; 9, *N. crassostreae* ATCC 70418^T; 10, *N. cummideiens* DSM 44490^T; 11, *N. cyriageorgica* DSM 44484^T; 12, *N. farcinica* ATCC 3318^T; 13, *N. flavorosea* JCM 3332^T; 14, *N. fluminea* DSM 44488^T; 15, *N. ignorata* DSM 44496^T; 16, *N. nova* JCM 6044^T; 17, *N. otitidiscaviarum* NCTC 1934^T; 18, *N. paucivorans* DSM 44386^T; 19, *N. pseudobrasiliensis* ATCC 51512^T; 20, *N. salmonicida* JCM 4826^T; 21, *N. seriolae* JCM 3360^T; 22, *N. soli* DSM 44488^T; 23, *N. transvalensis* DSM 43405^T; 24, *N. uniformis* JCM 3224^T; 25, *N. vaccinii* DSM 43285^T; 26, *N. veterana* DSM 44445^T; 27, *N. vinacea* JCM 10988^T. ND, Not determined; d, doubtful. Data for reference strains were taken from Hamid *et al.* (2001), Kinoshita *et al.* (2001), Maldonado *et al.* (2001), Wang *et al.* (2001) and Yassin *et al.* (2000a, b) unless indicated otherwise.

Property	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Biochemical tests:																											
Aesculin hydrolysis	+	–	–	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	+	+	+	+	+	+	+	d	d
Nitrate reduction	+	+	+	+	+	+	+	–	+	+	ND	+	–	+	ND	+	+	–	–	+	+	+	+	+	+	–	+
Urea hydrolysis	+	+	–	+	+	+	–	–	–	+	+	+	–	–	ND	+	+	+	+	+	–	+	+	+	+	+	+
Decomposition of (% w/v):																											
Adenine (0.4)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	+	–	–	–	–	–	–	–	–
Casein (1.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Elastin (0.3)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hypoxanthine (0.4)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tyrosine (0.5)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Uric acid (0.5)	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	ND	–	–	–	–	–	–	–	–	–	–	–	–
Xanthine (0.4)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth on carbon sources (% w/v):																											
D(+)-Mannitol (1.0)	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
α-L-Rhamnose (1.0)	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D(+)-Sorbitol (1.0)	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D(+)-Xylose (1.0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sodium acetate (0.1)	+	+	–	+	+	+	+	+	–	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	–	–
Sodium citrate (0.1)	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth at 45 °C	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

*Data from this study.

characteristic of representatives of the genera *Nocardia* and *Skermania* (Chun *et al.*, 1997; Goodfellow *et al.*, 1999). The biochemical, degradative and growth profiles of strain Y9^T also serve to distinguish it from the type strains of the validly described species of *Nocardia* (Table 1).

It is clear from the genotypic and phenotypic data that strain Y9^T merits recognition as a novel species of the genus *Nocardia*. It is therefore proposed that this organism be classified in the genus *Nocardia* as *Nocardia cerradoensis* sp. nov.

Description of *Nocardia cerradoensis* sp. nov.

Nocardia cerradoensis (cer.ra.do.en'sis. N.L. fem. adj. *cerradoensis* pertaining to the Cerrado, soil of which was the source of the organism).

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. Pinkish-white aerial hyphae are formed. Orange to tan colonies are produced on modified Bennett's agar. Colony elevation is convex to irregular, and colony margins are filamentous. Diffusible pigments are not produced. Aesculin and urea are hydrolysed and nitrate is reduced. Does not degrade adenine, casein, elastin, hypoxanthine, tyrosine, uric acid or xanthine. Grows well at 30 °C but does not grow at 45 °C. D(–)-Arabinose, dulcitol, D(–)-fructose, D(+)-glucose, glycerol, inulin, D(+)-maltose, D(+)-mannose, D(+)-melibiose, methyl α -D-glucoside, D(–)-ribose, salicin, D(+)-sucrose and D(+)-trehalose (all at 1%, w/v), acetate, benzoate, fumarate, *m*-hydroxybenzoic acid, DL-malate, pyruvate and succinate (at 0.1%, w/v) are used as sole carbon sources for energy and growth, but adonitol, arbutin, D(+)-cellobiose, *meso*-erythritol, ethanol, D(–)-fucose, D(+)-galactose, glycogen, *meso*-inositol, D(+)-lactose, D(+)-melezitose, D(+)-raffinose, xylitol, D(+)-xylose, isoamyl alcohol, isobutanol (at 1%, w/v), benzoate, butyrate, gluconate and *p*-hydroxybenzoic acid (at 0.1%, w/v) are not. L-Glutamate, L-leucine, L-proline and L-valine are used as sole carbon and nitrogen sources, but acetamide, L-alanine, L-aspartic acid, gelatin, monoethanolamine, L-phenylalanine, uric acid and urea (all at 0.1%, w/v) are not. The G+C content of the DNA is 68.8 mol%. The organism was isolated from Cerrado soil in Brazil. The type strain is Y9^T (=CCT 5601^T =DSM 44546^T). The species description is based on a single strain and hence it serves as the type strain.

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