Nocardia beijingensis sp. nov., a novel isolate from soil

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The taxonomic status of a soil isolate, provisionally assigned to the genus *Nocardia*, was established following a polyphasic study. An almost complete sequence of the 16S rDNA of the strain was determined and aligned with available sequences for nocardiae and phylogenetic trees were inferred using four tree-making algorithms. The organism was associated consistently with the type strains of *Nocardia brasiliensis* and *Nocardia farcinica* in the neighbour-joining analysis, albeit with low bootstrap values. It was also found to have chemotaxonomic and morphological properties consistent with its assignment to the genus *Nocardia*. The organism was readily separated from representatives of other *Nocardia* species by a broad range of phenotypic properties. The name proposed for the novel species is *Nocardia beijingensis* sp. nov. The type strain is AS4.1521^T (= IFO 16342^T = JCM 10666^T).

Keywords: *Nocardia beijingensis* sp. nov., polyphasic taxonomy, soil isolate

INTRODUCTION

The systematics of the genus *Nocardia* have been revolutionized by the application of modern taxonomic methods (Goodfellow, 1998; Goodfellow *et al.*, 1999). The revised genus encompasses 21 species that form a clade within the evolutionary radiation occupied by the mycolic-acid-containing actinomycetes, that is, by organisms that constitute the suborder *Corynebacterineae* (Stackebrandt *et al.*, 1997). The taxonomic integrity of most of these species is underpinned by a plethora of molecular systematic and numerical phenetic data (Goodfellow *et al.*, 1999; Hamid *et al.*, 2001; Maldonado *et al.*, 2000). The improved classification of the genus provides an invaluable framework for the recognition of additional species of *Nocardia*.

The primary reservoir of nocardiae is the soil (Cross *et al.*, 1976; Orchard *et al.*, 1977), though members of the genus are better known as causal agents of actinomycete mycetoma and nocardiosis (McNeil & Brown,

1994; Goodfellow, 1998). Little is known about nocardial species diversity in natural habitats, though there is evidence that it is grossly underestimated (Orchard & Goodfellow, 1980; Wang *et al.*, 1999; Maldonado *et al.*, 2000). Similarly, there is a lamentable dearth of knowledge about the functional activities of nocardiae, though their widespread distribution suggests important roles in the soil ecosystem (Orchard *et al.*, 1977); there is evidence that nocardiae form part of the autochthonous soil microflora (Orchard, 1979, 1981). It is important to unravel the species richness of nocardiae in soil in order to establish the activities of individual taxa in soil processes, such as organic matter turnover. The discovery of additional species will also help to provide high-quality biological material for exploitable biology (Bull *et al.*, 2000).

In a search for novel actinomycetes of commercial value, an unusual strain isolated from a soil sample was provisionally assigned to the genus *Nocardia* on the basis of colonial and staining properties. The aim of the present study was to determine the taxonomic status of this isolate using a polyphasic approach. The resultant data show that the isolate forms a distinct centre of taxonomic variation in the genus *Nocardia*. It is, therefore, proposed that the organism merits species status as *Nocardia beijingensis* sp. nov.

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The GenBank accession number for the 16S rDNA sequence of strain 02^T (= AS4.1521^T) is AF154129.

METHODS

Source, isolation, maintenance and cultivation of the isolate.

Strain 02^T was isolated on glucose/asparagine agar plates (5 g L-asparagine, 10 g glucose, 0.5 g K₂HPO₄, 20 g agar, 1 l distilled water, pH 7.2), which were incubated for 6 d at 28 °C, following inoculation with a suspension of a mud sample taken from a sewage ditch at Xishan mountain in Beijing. The isolate was maintained on slopes of modified Sauton's agar (Mordarska *et al.*, 1972) at room temperature and as suspensions of mycelial fragments in glycerol (20%, v/v) at -20 °C. Biomass for chemotaxonomic studies was grown in shake flasks of modified Sauton's broth for 7 d at 30 °C, harvested by centrifugation and washed twice with distilled water.

Cultural and morphological properties. The colonial properties of the isolate were observed on modified Sauton's agar plates (Mordarska *et al.*, 1972) after 7 d at 30 °C; the micromorphology of the substrate and aerial mycelium was examined using the coverslip technique of Kawato & Shinobu (1959). Growth on coverslips was fixed and examined following the procedure described by Zhou *et al.* (1998). Spore chain morphology was recorded following the examination of gold-coated, dehydrated preparations using a Hitachi S-570 scanning electron microscope.

Phenotypic properties. The test strain was examined for a range of phenotypic properties following procedures described by Isik *et al.* (1999).

Chemotaxonomy. The isomeric form of diaminopimelic acid and predominant whole-organism sugars were detected following standard procedures described by Lechevalier & Lechevalier (1980). Standard TLC and GC protocols were also used for the extraction and analysis of mycolic acids (Hecht & Causey, 1976) and polar lipids (Minnikin *et al.*, 1984). Non-hydroxylated fatty acids were extracted, purified by preparative TLC, separated, identified and quantified by GC as described by Yassin (1988). The small-scale procedure of Minnikin *et al.* (1984) was also used to extract isoprenoid quinones from freeze-dried biomass (about 30 mg); the purified menaquinones were analysed by HPLC following Collins *et al.* (1987).

DNA base composition. The G+C content of the DNA of the test strain was determined using the thermal denaturation method of Marmur & Doty (1962) using *Escherichia coli* AS1.365 as a control.

16S rDNA sequencing. Genomic DNA was isolated from single colonies grown on modified Sauton's agar using a procedure (Chun & Goodfellow, 1995) slightly modified from that of Pitcher *et al.* (1989). PCR amplification of the 16S rDNA was carried out as described by Kim *et al.* (1996) and the resultant PCR products were purified by using the Wizard PCR purification system (Promega), following the manufacturer's instructions. The purified products were sequenced directly using a Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems) and two universal primers described previously (Lu *et al.*, 2001). Sequence gel electrophoresis was carried out and the nucleotide sequence was obtained automatically by using an Applied Biosystems DNA sequencer (model 373A) and software provided by the manufacturer.

Phylogenetic analysis. The 16S rDNA sequence was aligned manually with corresponding sequences of representative *Nocardia* strains retrieved from the DDBJ/EMBL/GenBank databases by using the programs AL16S (Chun,

1995) and PHYDIT (J. Chun, unpublished). 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 treeing topologies of the neighbour-joining data were evaluated through bootstrap analyses of 1000 resamplings using the SEQBOOT and CONSENSE programs from the PHYLIP package (Felsenstein, 1993).

RESULTS AND DISCUSSION

An almost complete 16S rDNA sequence (1422 nt) was obtained for strain 02^T. Comparison of this sequence with those of related organisms showed clearly that it contains all of the signature nucleotides designated for the suborder *Corynebacterineae* and, more specifically, those characteristic of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997). The values for 16S rRNA gene sequence similarity to members of the genus *Nocardia* (94.5–97.6%) support the addition of the strain to this genus. This assignment is also supported by the fact that the 16S rDNA sequence contains the signature nucleotide characteristic for the genus *Nocardia* (Chun & Goodfellow, 1995). It is evident from the 16S rDNA trees (Fig. 1) that the isolate is loosely associated with *Nocardia brasiliensis* and *Nocardia farcinica*. It shares 16S rDNA similarity of 97.6% with the former and 97.5% with the latter, values that correspond to 35 and 36 nt differences out of 1421 positions. Higher similarity values have been recorded between several validly described *Nocardia* species, for instance, between *Nocardia brevicatena* and *Nocardia paucivorans* (Yassin *et al.*, 2000a) and *Nocardia carnea* and *Nocardia flavorosea* (Chun *et al.*, 1998); the DNA–DNA relatedness values shown between these respective sets of type strains were found to be well below the 70% cut-off point recommended for the circumscription of bacterial genomic species (Wayne *et al.*, 1987).

The chemotaxonomic, morphological and physiological properties of strain 02^T are consistent with its assignment to the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999). The organism is aerobic, Gram-positive and weakly acid-alcohol-fast and produces an orange substrate mycelium on modified Sauton's agar. The substrate mycelium, which fragments *in situ* into irregular rod-shaped elements, carries sparse to abundant, white aerial hyphae. Short sporotrichetes of up to four spores were borne on both substrate and aerial hyphae (Fig. 2). The formation of conidia on substrate and aerial mycelia, while unusual, has been reported for *N. brevicatena* and some *Nocardia asteroides* strains (Goodfellow & Lechevalier, 1989). The organism was found to have whole-organism hydrolysates rich in *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rDNA sequences (1420 nt) showing relationships between isolate 02^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. The symbols F and P respectively indicate branches that were also recovered using the least-square and maximum-parsimony methods. Accession numbers are given in parentheses. Numbers at the nodes indicate the level of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are shown. Bar, 0.02 substitutions per nucleotide position.

IV *sensu* Lechevalier & Lechevalier, 1970), contained major amounts of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (phospholipid type 2 *sensu* Lechevalier *et al.*, 1977) and DNA with a G+C content of 69 mol%. One-dimensional TLC of whole-organism acid methanolysates revealed the presence of two lipid spots on the chromatogram. The lower one corresponded to mycolic acids, as identified by its R_f value (about 0.47), and the higher spot to non-hydroxylated fatty acids. The GC profile of the trimethylsilyl ether derivatives of the purified mycolic acids revealed that the major components ranged from C₅₈ to C₆₂. The organism contained major proportions of straight-chain saturated, unsaturated and 10-methyl (tuberculostearic) branched-chain fatty acids and predominant amounts of hexahydrogenated menaquinones with eight isoprene units where the two end

units were cyclized; this menaquinone has been detected only in *Nocardia* and *Skermania* strains (Chun & Goodfellow, 1995; Chun *et al.*, 1997). The strain also showed a profile of phenotypic properties that distinguishes it from representatives of all validly described species of *Nocardia*, including *N. brasiliensis* and *N. farcinica* (Table 1).

The genotypic and phenotypic data indicate that strain 02^T merits recognition as a distinct species of *Nocardia*. It is, therefore, proposed that the organism be classified in the genus *Nocardia* as *Nocardia beijingensis* sp. nov.

Description of *Nocardia beijingensis* sp. nov.

Nocardia beijingensis (bei.jing.en'sis. N.L. masc. adj. *beijingensis* pertaining to Beijing, the source of the isolate).

Fig. 2. Scanning electron micrograph of strain 02^T showing short spirotrichetes on both substrate and aerial mycelium after 10 d of growth at 28 °C on modified Sauton's agar. Magnification ×7000.

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. Short chains of up to four conidia are formed on substrate and aerial hyphae. Colony elevation is convex to irregular and colony margins are filamentous. Diffusible pigments are not formed. Nitrate is reduced and aesculin and urea are hydrolysed. Uric acid is degraded but not adenine, casein, elastin, hypoxanthine, tyrosine or xanthine. The organism grows at between 18 and 35 °C and on D(–)-arabinose, D(+)-cellobiose, dulcitol (weak), *meso*-erythritol, ethanol, D(–)-fucose (weak), D(–)-fructose, D(+)-galactose, glycerol, glycogen, D(+)-glucose, inulin, D(+)-lactose, D(+)-maltose, D(+)-mannose, D(+)-raffinose, α-L-rhamnose, D(–)-ribose, salicin, D(+)-sucrose, D(+)-trehalose, D(+)-xylose (all at 1 %, w/v), acetate, benzoate, fumarate, gluconate, *p*-hydroxybenzoic acid, H-malate, pyruvate and succinate (at 0.1 %, w/v) as sole carbon sources for energy and growth but not on adonitol, arbutin, *meso*-inositol, D(+)-melezitose, D(+)-melibiose, α-methyl D-glucoside, xylitol, isoamyl alcohol, isobutanol, *n*-propanol (1 %, w/v or v/v), butyrate or *m*-hydroxybenzoic acid (0.1 %, w/v). L-Alanine (weak), 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,

Table 1. Phenotypic properties that distinguish strain 02^T from the type strains of *Nocardia* species

Taxa are identified as: 1, strain 02^T; 2, *N. abscessus* DSM 44432^T; 3, '*Nocardia africana*' DSM 44491; 4, *N. asteroides* ATCC 19247^T; 5, *N. brasiliensis* ATCC 19296^T; 6, *N. brevicatena* DSM 43024^T; 7, *N. carnea* DSM 43397^T; 8, *Nocardia crassostreae* ATCC 70418^T; 9, '*Nocardia cummideiens*' DSM 44490; 10, *N. farcinica* ATCC 3318^T; 11, *N. flavorosea* JCM 3332^T; 12, '*Nocardia fluminea*' DSM 44488; 13, *Nocardia nova* JCM 6044^T; 14, *Nocardia otitidiscaviarum* NCTC 1934^T; 15, *N. paucivorans* DSM 44386^T; 16, *Nocardia pseudobrasiliensis* ATCC 51512^T; 17, *Nocardia salmonicida* JCM 4826^T; 18, *Nocardia seriolae* JCM 3360^T; 19, '*Nocardia soli*' DSM 44488; 20, *Nocardia transvalensis* DSM 43405^T; 21, *Nocardia uniformis* JCM 3224^T; 22, *Nocardia vaccinii* DSM 43285^T. Data were taken from Hamid *et al.* (2001), Maldonado *et al.* (2000), Yassin *et al.* (2000b) and this study.

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

phenylalanine or urea (all at 0.1 %, w/v). The G + C content of the DNA is 69 mol %.

The organism was isolated from a mud sample taken from a sewage ditch at Xishan mountain in Beijing. The type strain is strain 02^T [= AS4.1521^T (China Culture Collection of Microorganisms, Beijing) = IFO 16342^T = JCM 10666^T]. The species description is based on a single strain and hence serves as the type strain description.

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