Nocardia jiangxiensis sp. nov. and *Nocardia miyunensis* sp. nov., isolated from acidic soils

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The taxonomic positions of two soil actinomycetes, strains 117^T and 43401^T, provisionally assigned to the genus *Nocardia* were determined in a polyphasic study. The organisms were found to have phenotypic properties typical of members of the genus *Nocardia*, and formed a distinct branch in the *Nocardia* 16S rRNA gene tree. It was evident from the phylogenetic data that the isolates were most closely, albeit loosely, associated with the type strains of *Nocardia nova* and *Nocardia pseudobrasiliensis*. However, all four of these organisms were readily distinguishable from one another using DNA–DNA relatedness and phenotypic data. It is evident from the genotypic and phenotypic data that the two isolates should be recognized as novel species of the genus *Nocardia*. It is proposed, therefore, that strains 117^T (= CGMCC 4.1904^T = JCM 12860^T) and 43401^T (= CGMCC 4.1905^T = JCM 12861^T) be classified in the genus *Nocardia* as the type strains of *Nocardia miyunensis* sp. nov. and *Nocardia jiangxiensis* sp. nov., respectively.

The genus *Nocardia* belongs to the family *Nocardiaceae*, a member of the suborder *Corynebacterineae*, proposed by Stackebrandt *et al.* (1997). At the time of writing, the genus encompasses 46 species with validly published names; the majority of these species have been described in the last 5 years by means of polyphasic taxonomic approaches (Maldonado *et al.*, 2000; Yassin *et al.*, 2001a, b; Kim *et al.*, 2002; Zhang *et al.*, 2003, 2004). Most of the novel species are associated with human and animal infections (Yassin *et al.*, 2000a, b, 2003; Hamid *et al.*, 2001; Kageyama *et al.*, 2004a, b, c, d); nearly all of the remaining novel species are from soil collected from geographically diverse locations (Maldonado *et al.*, 2000; Albuquerque de Barros *et al.*, 2003; Li *et al.*, 2004; Saintpierre-Bonaccio *et al.*, 2004; Zhang *et al.*, 2003, 2004).

Little is known about the functional roles of nocardiae in natural habitats, though it is clear that they form an integral part of the autochthonous soil microflora (Orchard, 1979, 1981). Nevertheless, there is evidence that nocardial species diversity in the soil ecosystem is greatly underestimated (Orchard & Goodfellow, 1980; Wang *et al.*, 1999; Maldonado

et al., 2000). It is important to establish the species richness of nocardiae in natural habitats for ecological reasons and for industrial purposes, as novel nocardiae are a source of commercially significant bioactive compounds (Kinoshita *et al.*, 2001; Bringmann *et al.*, 2005). The aim of the present study was to determine the taxonomic status of two *Nocardia*-like strains isolated from soil.

Strain 117^T was isolated from a pine-forest soil sample (about pH 5.5) collected in Miyun County, Beijing (China), and strain 43401^T was isolated from rhizosphere soil (about pH 3.5) of goose-grass (*Eleusine indica*) growing next to a copper mine in Wushan, Jiangxi Province, southern China. In each case, soil suspensions prepared using a dispersion and differentiation procedure (Wang *et al.*, 2003) were inoculated onto plates of an acidified selective isolation medium (Huang *et al.*, 2004), which were incubated for 3 weeks at 28 °C. The strains were maintained on oatmeal agar (ISP medium 3; Küster, 1959), adjusted to an initial pH of 5.5 using a citric acid (0.1 M)/Na₂HPO₄ (0.2 M) buffer.

The colonial properties of the isolates were observed on modified Sauton's agar (Mordarska *et al.*, 1972) and acidified oatmeal agar (as above) plates that had been incubated for 7 days at 28 °C. Micromorphological properties were recorded using samples taken from the Sauton's agar plates and examined by using light microscopy (Axioskop 20; Zeiss) and scanning electron microscopy (FEI QUANTA instrument). Well-established procedures were used to

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 117^T and 43401^T are AY639901 and AY639902, respectively.

Scanning electron micrographs of strains 117^T and 43401^T are available as a supplementary figure in IJSEM Online.

determine a range of phenotypic properties (Goodfellow, 1971). In addition, acid production from carbohydrates was determined using media and methods described by Gordon *et al.* (1974), use of sole carbon sources for energy and growth was assessed according to the methods of Yassin *et al.* (1995) and biochemical and biodegradation tests were performed using procedures described by Gordon & Mihm (1957). Oatmeal agar adjusted to initial pH values of 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5 using citric acid (0.1 M)/Na₂HPO₄ (0.2 M) and glycine (0.05 M)/NaOH (0.05 M) buffer systems was used to test the ability of strains to grow at different pHs. The chemotaxonomic properties of the isolates were determined by following standard chromatographic procedures as outlined by Zhang *et al.* (2003). Extraction of genomic DNA, PCR amplification of 16S rRNA and purification of the PCR products from strains 117^T and 43401^T were carried out by following the procedures described by Rainey *et al.* (1996); the purified PCR products were sequenced directly, according to the method of Lu *et al.* (2001). The 16S rRNA gene sequences of the strains were aligned manually with corresponding nucleotide sequences of the type strains of *Nocardia* species, retrieved from the GenBank/EMBL/DDBJ databases, using CLUSTAL X 1.8 software (Thompson *et al.*, 1997). Phylogenetic 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) tree-making algorithms from the PHYLIP suite of programs (Felsenstein, 1993). Evolutionary distance matrices were generated as described by Kimura (1980). The resultant unrooted tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings, using the SEQBOOT and CONSENSE options from the PHYLIP package.

Chromosomal DNA was extracted and purified from the two isolates and from the type strains of *Nocardia nova* and *Nocardia pseudobrasiliensis*. DNA–DNA relatedness values between the tested strains were estimated using the fluorometric microplate method of Ezaki *et al.* (1989). The hybridization experiments were carried out under stringent conditions in 50 % formamide at 50 °C. Fluorescence intensities were measured using a Fluostar Optima microplate reader (BMG LABTECH) at a wavelength of 360 nm for excitation and at 460 nm for emission. DNA–DNA relatedness values were calculated from quadruplicated hybridization experiments and expressed as means of the corresponding reciprocal values.

When the almost-complete 16S rRNA gene sequences of strains 117^T (1405 nt) and 43401^T (1409 nt) were examined, they were found to contain the signature nucleotides characteristic of the family *Nocardiaceae* (Stackebrandt *et al.*, 1997) and of the genus *Nocardia* (Chun & Goodfellow, 1995). It was apparent from the phylogenetic analyses that the two strains fell within the 16S rRNA *Nocardia* gene tree (data not shown). The organisms were also shown to have

phenotypic properties consistent with their classification in the genus *Nocardia* (Goodfellow *et al.*, 1999), i.e. the strains are aerobic, Gram-positive, partially acid–alcohol-fast actinomycetes that form extensively branched substrate mycelia that fragment into irregular, rod-shaped, non-motile, rod-shaped elements (see Supplementary Fig. S1 in IJSEM Online). In addition, each strain produced whole-organism hydrolysates rich in *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970) and contained major amounts of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (phospholipid type II *sensu* Lechevalier *et al.*, 1977), a predominance of hexahydrogenated menaquinones with eight isoprene units where the end two were cyclized, and fatty acid profiles consisting mainly of saturated, unsaturated and 10-methyl-branched components. They were also characterized by the presence of mycolic acids that co-migrated (*R_f* value around 0.47) with those from marker strains of *Nocardia*.

Fig. 1. An unrooted neighbour-joining tree (Saitou & Nei, 1987), based on nearly-complete 16S rRNA gene sequences, showing the positions of strains 117^T and 43401^T amongst representatives of closely related *Nocardia* species. The symbols f, m and p denote branches that were also recovered using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) tree-making algorithms, respectively. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50 % are given. Bar, 0.01 substitutions per nucleotide position.

It is apparent from the abridged phylogenetic tree (Fig. 1) that the two strains form a phylogenetic branch that is supported by three out of the four tree-making algorithms and by a bootstrap value of 84 % in the neighbour-joining analysis. The two organisms share a 16S rRNA gene sequence similarity of 98.5 %, a value that corresponds to 15 nt differences at 1405 locations. Each organism is most closely, albeit loosely, related to the type strains of *N. nova* and *N. pseudobrasiliensis*. Strain 117^T shares 16S rRNA gene sequence similarity of 98.1 % with *N. nova* JCM 6044^T and 98.2 % with *N. pseudobrasiliensis* ATCC 51512^T, values that are equivalent to 26 and 25 nt differences at 1405 positions; the corresponding values for strain 43401^T are 98.7 and 97.9 %, corresponding to 18 and 29 nt differences at 1395 positions. Much higher 16S rRNA gene sequence similarities than these have been found between the type strains of *Nocardia* species shown to have DNA–DNA relatedness values well below the 70 % threshold recommended for the delineation of genomic species (Wayne *et al.*, 1987). The type strains of *Nocardia brevicatena* and *Nocardia paucivorans*, for instance, were found to share a 16S rRNA gene sequence similarity of 99.6 % and had a DNA relatedness value of 61.9 % (Yassin *et al.*, 2000a).

It is evident from the DNA–DNA relatedness data that strains 117^T and 43401^T represent two distinct genomic species. These strains were found to have a mean DNA–DNA hybridization value of 58 % and corresponding relatedness values below 55 % with *N. nova* JCM 6044^T (54 % with strains 117^T and 46 % with strain 43401^T) and *N. pseudobrasiliensis* ATCC 51512^T (42 % with strain 117^T and 39 % with strain 43401^T). The two strains can also be distinguished from one another and from phylogenetically close relatives, including the type strains of *N. nova* and *N. pseudobrasiliensis*, by using a combination of phenotypic properties (Table 1).

It is clear from the genotypic and phenotypic data that strains 117^T and 43401^T merit recognition as novel *Nocardia* species: the names proposed for these novel taxa are *Nocardia miyunensis* sp. nov. and *Nocardia jiangxiensis* sp. nov., respectively.

Description of *Nocardia jiangxiensis* sp. nov.

Nocardia jiangxiensis (jiang.xi.en'sis. N.L. fem. adj. *jiangxiensis* referring to Jiangxi Province, southern China, the source of the type strain).

Table 1. Phenotypic characteristics that distinguish strains 117^T and 43401^T from one another and from the type strains of phylogenetically close *Nocardia* species

Strains: 1, strain 117^T; 2, strain 43401^T; 3, *Nocardia abscessus* DSM 44432^T; 4, *Nocardia africana* DSM 44491^T; 5, *Nocardia asteroides* ATCC 19247^T; 6, *Nocardia crassostreae* ATCC 700418^T; 7, *N. nova* JCM 6044^T; 8, *Nocardia otitidiscaviarum* NCTC 1934^T; 9, *N. pseudobrasiliensis* ATCC 51512^T; 10, *Nocardia transvalensis* DSM 43405^T; 11, *Nocardia vaccinii* DSM 43285^T; 12, *Nocardia vinacea* JCM 10988^T; 13, *Nocardia xishanensis* CGMCC 4.1860^T. Data were taken from this study and previous studies (Yassin *et al.*, 2000b, 2001b; Albuquerque de Barros *et al.*, 2003; Zhang *et al.*, 2003, 2004). +, Positive; –, negative; d, doubtful; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Hydrolysis of:													
Aesculin	+	+	–	–	+	+	+	+	+	+	+	D	+
Arbutin	–	+	+	–	+	ND	+	+	–	+	–	ND	+
Urea	–	–	+	–	+	–	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	–	+	+	–	+	+	+	+
Decomposition of (% w/v):													
Adenine (0.4)	–	+	–	–	–	–	–	–	+	–	–	–	–
Casein (1.0)	+	–	–	–	–	–	–	–	+	–	–	–	–
Elastin (0.3)	–	–	–	–	–	–	–	–	+	+	–	–	–
Hypoxanthine (0.4)	–	–	–	–	–	–	–	+	+	+	–	+	–
Tyrosine (0.5)	–	–	–	–	–	–	–	–	+	–	–	–	–
Xanthine (0.4)	–	–	–	–	–	–	–	+	–	–	–	–	–
Growth on sole carbon sources (% w/v):													
D-Mannitol (1.0)	–	+	–	–	+	–	+	+	+	+	+	+	–
D-Mannose (1.0)	–	+	ND	ND	–	ND	–	ND	+	+	ND	ND	+
α-L-Rhamnose (1.0)	+	+	+	–	–	+	–	–	–	+	+	–	+
D-Sorbitol (1.0)	+	+	–	–	–	–	+	–	+	+	–	+	+
Sodium acetate (0.1)	–	–	+	–	+	–	+	+	+	+	–	ND	+
Sodium citrate (0.1)	+	+	+	–	+	–	–	–	+	–	–	d	–
Growth at 45 °C	–	–	–	+	–	–	–	+	–	–	–	–	–

Aerobic, Gram-positive, catalase-positive, partially acid-alcohol-fast, non-motile actinomycete that forms an extensively branched substrate mycelium that fragments into rod-shaped elements. A pinkish white to orange substrate mycelium bears sparse to abundant, white to pink aerial hyphae on modified Sauton's agar. A yellowish brown substrate mycelium carrying white aerial hyphae is formed on oatmeal agar at an initial pH of 5.5. Diffusible pigments are not formed. Does not degrade starch, Tween 20 or 80 or xanthine. Acid is formed from D-arabinose, D-galactose, D-glucose, glycerol, *myo*-inositol, D-mannose and D-xylose, but not from D-lactose, D-maltose, D-mannitol, α -L-rhamnose or sodium citrate. D-Arabinose, D-cellobiose, *meso*-erythritol (weak), D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol (weak), D-lactose, D-maltose, D-melezitose, D-raffinose, D-ribose, D-sorbose, D-sucrose (weak), D-trehalose and D-xylose are used as sole carbon sources for energy and growth, but adonitol and sodium propionate are not. Growth occurs at an initial pH between 3.5 and 9.5, and at 17–37 °C, but not at 10 or 45 °C. Additional phenotypic properties are shown in Table 1. Chemotaxonomic properties are typical of the genus *Nocardia*. The major cellular fatty acids are C_{16:1} ω 7c (12.4%), C_{16:0} (24.8%), C_{18:1} ω 9c (12.3%), C_{18:0} (11.0%) and 10-methyl C_{18:0} (27.3%). The species description is based on a single strain, which therefore serves as the type strain.

The type strain, 43401^T (= CGMCC 4.1905^T = JCM 12861^T) was isolated from the rhizosphere of goose-grass (*Eleusine indica*) that was growing next to a copper mine in Wushan, Jiangxi Province, China.

Description of *Nocardia miyunensis* sp. nov.

Nocardia miyunensis (mi.yun.en'sis. N.L. fem. adj. *miyunensis* referring to Miyun County in Beijing, the source of the type strain).

Aerobic, Gram-positive, catalase-positive, partially acid-alcohol-fast, non-motile actinomycete that forms an extensively branched substrate mycelium that fragments into rod-shaped elements. A white to yellowish white substrate mycelium bears sparse to abundant, white to pink aerial hyphae on modified Sauton's agar. A yellowish brown substrate mycelium bearing white aerial hyphae is formed on oatmeal agar at an initial pH of 5.5. Diffusible pigments are not formed. Does not degrade starch, Tween 20 or 80 or xanthine. Acid is formed from D-galactose, D-glucose, glycerol, D-mannose and D-xylose, but not from D-arabinose, *myo*-inositol, D-lactose, D-maltose, D-mannitol, α -L-rhamnose or sodium citrate. D-Arabinose (weak), D-cellobiose, *meso*-erythritol (weak), D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, D-lactose, D-maltose, D-melezitose, α -L-rhamnose, D-raffinose, D-ribose, D-sorbose, D-sucrose (weak), D-trehalose and D-xylose are used as sole carbon sources for energy and growth, but adonitol, D-mannitol, D-mannose and sodium propionate are not. Growth occurs at an initial pH between 4.5 and 9.5, and

between 17 and 37 °C, but not at 10 or 45 °C. Additional phenotypic properties are shown in Table 1. Chemotaxonomic properties are typical of the genus *Nocardia*. The major cellular fatty acids are C_{16:1} ω 7t (16.4%), C_{16:0} (27.2%), C_{18:1} ω 9c (7.7%), C_{18:0} (6.4%) and 10-methyl C_{18:0} (33.0%). The species description is based on a single strain, which therefore serves as the type strain.

The type strain, 117^T (= CGMCC 4.1904^T = JCM 12860^T), was isolated from a pine-forest soil sample collected in Miyun County, Beijing, People's Republic of China.

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