Nocardia jinanensis sp. nov., an amicoumacin B-producing actinomycete

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A novel actinomycete, strain 04-5195^T, that produces amicoumacin B, which targets bone morphogenetic protein-2, was isolated from a soil sample collected in Jinan, Shandong Province, China. Strain 04-5195^T had morphological, biochemical, physiological and chemotaxonomic properties that were consistent with its classification in the genus *Nocardia* and it formed a phyletic line in the *Nocardia* 16S rRNA gene tree. It was evident from the phylogenetic data that strain 04-5195^T was most closely associated with *Nocardia speluncae* N2-11^T. However, the two organisms were distinguishable from one another using DNA–DNA relatedness and phenotypic data. The isolate was readily differentiated from other related *Nocardia* strains by a set of phenotypic properties and by its phylogenetic position. Therefore, it is proposed that the isolate represents a novel species in the genus *Nocardia*, *Nocardia jinanensis* sp. nov.; the type strain is 04-5195^T (=CGMCC 4.3508^T =DSM 45048^T).

The genus *Nocardia* was proposed by Trevisan (1889) with *Nocardia farcinica* as the original type species (the type species is now *Nocardia asteroides*). The genus belongs to the mycolic-acid-containing group of actinomycetes, members of which form extensively branched mycelia and substrate hyphae that fragment into rod-shaped, non-motile elements (Goodfellow & Lechevalier, 1989). The application of chemotaxonomic, numerical phenetic and molecular systematic methods has led to improved classification of members of the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999). At the time of writing, the genus contained 71 species with validly published names. Many *Nocardia* species have been shown to be agents of human disease, such as *N. asteroides*, *N. farcinica* and *Nocardia nova* (Schaal & Lee, 1992; Wallace *et al.*, 1991), although it has also been shown that some species produce secondary metabolites of potential industrial value (Isik *et al.*, 1999; Kinoshita *et al.*, 2001), e.g. *Nocardia uniformis*, which can produce nocardicin. In the course of screening micro-organisms for new anti-oste-

porosis agents targeting bone morphogenetic protein-2 (BMP-2), an active compound (5195A) with the potential to increase expression of the BMP-2 gene was found in fermentation broth of a nocardioform actinomycete, strain 04-5195^T, that had been isolated from soil. The compound 5195A was identified as amicoumacin B (Yang *et al.*, 2007), which has been reported previously to be produced by *Bacillus pumilus* (Itoh *et al.*, 1982). A polyphasic taxonomic investigation based on genotypic and phenotypic characteristics revealed that isolate 04-5195^T represents a novel species of the genus *Nocardia*.

Strain 04-5195^T was isolated on a modified Sauton's agar plate (Mordarska *et al.*, 1972) that had been incubated at 28 °C for 2 weeks following inoculation with a suspension of a soil sample collected from Jinan, Shandong Province, China. The isolate was maintained on yeast extract-malt extract agar (ISP 2; Shirling & Gottlieb, 1966) slopes at 4 °C and as glycerol suspensions (20%, v/v) at –20 °C. All cultures were incubated at 28 °C unless otherwise indicated. Biomass for chemotaxonomic and molecular genetic studies was prepared as described previously (Sun *et al.*, 2007).

The colonial properties of the isolate were observed on ISP 2 agar plates that had been incubated for 8 days. Morphological properties were detected following growth on ISP 2 plates and examined by using light microscopy (Axioskop 20; Zeiss) and scanning electron microscopy (Quanta; FEI). Well-established methods were used to determine a range of phenotypic properties (Goodfellow,

Abbreviation: BMP-2, bone morphogenetic protein-2.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 04-5195^T is DQ462650.

A scanning electron micrograph of cells of strain 04-5195^T, an extended 16S rRNA gene sequence-based neighbour-joining tree and a comparison between the cultural characteristics of strain 04-5195^T and *N. speluncae* DSM 45078^T are available as supplementary material with the online version of this paper.

1971; Williams *et al.*, 1983). Acid production from carbohydrates was determined using media and methods described by Gordon *et al.* (1974) and the utilization of sole carbon sources was investigated according to Gordon & Mihm (1957). pH, temperature and NaCl tolerances were determined on ISP 2 agar plates incubated for up to 14 days. Established TLC procedures were used to determine diagnostic diaminopimelic acid isomers (Hasegawa *et al.*, 1983), whole-cell sugar composition (Lechevalier & Lechevalier, 1980) and polar lipids (Minnikin *et al.*, 1984). Menaquinones were extracted and estimated using the methods of Collins (1985). The acid methanolysis procedure was used for extraction and analysis of mycolic acids (Minnikin *et al.*, 1975). Fatty acids were extracted, methylated and estimated by GC using the standard Sherlock MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

Chromosomal DNA was extracted from biomass of strain 04-5195^T grown in modified Sauton's broth for 5 days at 28 °C and purified following the method of Rainey *et al.* (1996). The purified PCR product was sequenced according to the method of Gu *et al.* (2006). The DNA G+C content was determined using the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* K-12 as a control. Levels of DNA–DNA relatedness between strain 04-5195^T and related type strains were determined using the fluorometric microplate method of Ezaki *et al.* (1989). 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 wavelengths of 360 nm for excitation and 460 nm for emission. Phylogenetic analysis was performed by first using the BLAST search program available at NCBI. The corresponding sequences of strains of representative species were then analysed using the software packages MEGA version 3.1 (Kumar *et al.*, 2004) and PHYLIP version 3.5c (Felsenstein, 1993). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971), least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1981) methods. Evolutionary distance matrices were generated according to the method of Kimura (1980). Bootstrap values were determined based on 1000 replications.

The phenotypic properties of strain 04-5195^T were consistent with its assignment to the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999). The organism was an aerobic, Gram-positive, slightly acid–alcohol-fast actinomycete. It developed well on a series of media including ISP 2, 4 and 5 agars (Shirling & Gottlieb, 1966) and yeast extract–starch agar (DSMZ medium 1027); it showed moderate growth on ISP 6 agar (Shirling & Gottlieb, 1966), Czapek–Dox agar (DSMZ medium 130) and modified Bennett's agar (Jones, 1949), but grew poorly on ISP 7 agar (Shirling & Gottlieb, 1966) (see Supplementary Table S1, available in IJSEM Online). The substrate mycelium branched extensively and fragmented into non-motile, rod-shaped elements (see Supplementary Fig. S1).

The isolate contained mycolic acids. Whole-cell hydrolysates contained *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970). The polar phospholipids were phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and minor amounts of phospholipids of unknown structure containing glucosamine (phospholipid type II *sensu* Lechevalier *et al.*, 1977). The predominant menaquinone was MK-8 (H₄, ω -cycl.). The major cellular fatty acids were C_{16:0} (28.5 %), C_{18:0} (8.3 %), C_{16:1} ω 7c (5.7 %), iso-C_{15:0} 2-OH (5.8 %), C_{18:1} ω 9c (29.7 %) and 10-methyl C_{18:0} (10.9 %).

An NCBI database search demonstrated that strain 04-5195^T belonged to the genus *Nocardia*. 16S rRNA gene sequence similarities between strain 04-5195^T and the closely related type strains *Nocardia speluncae* N2-11^T, *N. carnea* DSM 43397^T, *N. flavorosea* JCM 3332^T, *N. testacea* JCM 12235^T and *N. sienata* IFM 10088^T were 99.0 % (14 nt differences at 1397 sites), 98.2 % (25/1404), 97.8 % (31/1406), 97.7 % (33/1406) and 97.8 % (31/1402), respectively. It is clear from the phylogenetic analysis that strain 04-5195^T forms a distinct phyletic line with *N. speluncae* N2-11^T in the *Nocardia* 16S rRNA gene tree (Fig. 1; see also Supplementary Fig. S2). This line was supported by all four tree-making algorithms and by a 100 % bootstrap value. Moreover, the four tree-making algorithms and the high bootstrap value supported the position of the strain 04-5195^T/*N. speluncae* N2-11^T phyletic line in the same clade as the other four related type strains above. The shortest phylogenetic distance (0.008) was observed between strain 04-5195^T and *N. speluncae* N2-11^T. DNA–DNA hybridization was conducted between strain 04-5195^T and *N. speluncae* DSM 45078^T; DNA–DNA relatedness values between these two strains were 22.5 $\pm$ 2.1 %, which is well below the 70 % cut-off point generally recognized for genomic species (Wayne *et al.*, 1987). A number of phenotypic properties (Table 1) also separated strain 04-5195^T from the type strains of the most closely related species.

The genotypic and phenotypic data indicate that strain 04-5195^T merits recognition as a representative of a novel species of *Nocardia*. It is therefore proposed that the isolate be classified in the genus *Nocardia* as the type strain of *Nocardia jinanensis* sp. nov.

Description of *Nocardia jinanensis* sp. nov.

Nocardia jinanensis (ji.nan.en'sis. N.L. fem. adj. *jinanensis* pertaining to Jinan, the capital city of Shandong Province, China, soil of which was the source of the type strain).

Aerobic, Gram-positive, catalase-positive, partially acid–alcohol-fast, non-motile actinomycete that forms branched substrate mycelium that fragments *in situ* into irregular rod-shaped elements. White to yellow substrate hyphae that bear sparse to abundant, white to yellowish aerial hyphae are formed on a number of agar media. Diffusible pigments are not produced. Aesculin is hydrolysed. Nitrate is not reduced to nitrite. Amylase and gelatinase are not produced. No

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) derived from aligned 16S rRNA gene sequences showing the position of strain 04-5195^T among its nearest phylogenetic neighbours. Branches that were also recovered using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods are indicated by the letters f, p and m, respectively; asterisks indicate branches that were recovered using all three methods. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50 % are indicated. Bar, 0.01 substitutions per nucleotide position. An extended tree is available as Supplementary Fig. S2.

antibiosis is observed against strains of *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa* or *Staphylococcus epidermidis*. Grows in the presence of 3 % NaCl, but not in 5–10 % NaCl. Acid is formed from D-fructose, D-galactose, mannitol, melezitose, raffinose, D-ribose and salicin, but not from D-arabinose, inositol, inulin, D-mannose, L-rhamnose or D-xylose. Dextrin, D-galactose, D-glucose, D-mannose and trehalose are utilized (all at 1 %, w/v), but L-cysteine (0.1 %, w/v), D-ribose (1 %, w/v), sodium citrate (0.1 %, w/v) and D-xylose are not. Negative for decomposition of elastin (0.3 %, w/v) and Tween 60. Growth occurs at an initial pH of 5.5–10.5 and between 15 and 37 °C, but not at pH 3.5, pH 4.5 or 45 °C. Sensitive to filter-paper discs soaked in tobramycin (10 µg ml⁻¹) and kanamycin (30 µg ml⁻¹). Additional phenotypic properties are shown in Table 1. The predominant cellular fatty acids are C_{18:1}ω9c, C_{16:0} and 10-methyl C_{18:0}. The species description is based on a single strain, which therefore serves as the type strain.

The type strain is 04-5195^T (=CGMCC 4.3508^T =DSM 45048^T), isolated from a soil sample collected from Jinan city, Shandong Province, northern China. The DNA G+C content of the type strain is 65.0 mol%.

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Table 1. Phenotypic properties that distinguish strain 04-5195^T from type strains of related *Nocardia* species

Strains: 1, strain 04-5195^T; 2, *N. speluncae* DSM 45078^T; 3, *N. carnea* DSM 43397^T; 4, *N. flavorosea* JCM 3332^T; 5, *N. testacea* DSM 44765^T; 6, *N. sientata* DSM 44766^T. Data are from this study. +, Positive; –, negative; w, weakly positive. All strains are negative for decomposition of adenine (0.4 %, w/v), guanine (0.4 %, w/v), hypoxanthine (0.4 %, w/v), tyrosine (0.5 %, w/v), xanthine (0.4 % w/v) and Tweens 20, 40 and 80.

Property	1	2	3	4	5	6
Aesculin hydrolysis	+	w	+	–	+	+
Nitrate reduction	–	+	+	–	–	–
Decomposition of 0.5 % (w/v) uric acid	–	+	–	–	–	–
Growth on sole carbon sources:*						
Cellobiose	+	–	–	–	+	–
D-Galactose	+	–	+	+	w	+
Methyl α-D-glucopyranoside	+	–	–	–	–	–
Inulin	+	–	–	–	–	–
D-Mannitol	w	+	+	+	–	–
Melezitose	+	–	–	–	–	–
α-L-Rhamnose	w	–	–	–	+	–
D-Sorbitol	+	–	–	–	+	–
Sodium acetate	–	+	+	+	+	+
Growth on sole carbon and nitrogen sources (0.1 %, w/v):						
L-Phenylalanine	+	–	–	–	–	–
L-Valine	–	+	–	–	–	–

*All at 1.0 % (w/v) apart from sodium acetate (0.1 %, w/v).

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