Nocardia polyresistens sp. nov.

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A novel actinomycete strain YIM 33361^T was isolated from a soil sample collected from Yunnan, China. Comparative 16S rRNA gene sequencing showed that the strain constituted a distinct subclade within the genus *Nocardia*, displaying more than 3 % sequence divergence from established species. Based on its morphological, chemotaxonomic, phenotypic and genotypic characteristics, strain YIM 33361^T (=CCTCC AA 204004^T=KCTC 19027^T) is proposed as the type strain of a novel species, *Nocardia polyresistens* sp. nov.

The application of chemotaxonomic, numerical phenetic and molecular systematic methods has promoted a radical reappraisal of the genus *Nocardia* (Goodfellow, 1998; Goodfellow *et al.*, 1999; Lechevalier, 1976). This improved classification provides a sound framework for the recognition of additional species (Yassin *et al.*, 2001). Members of the genus form extensively branched mycelia and the substrate hyphae fragment into rod-shaped, non-motile elements; aerial hyphae are usually formed but are sometimes only visible microscopically (Goodfellow & Lechevalier, 1989; Gordon & Mihm, 1957, 1962). Nocardiae are also characterized by a number of chemical markers, including the presence of *meso*-diaminopimelic acid (DAP), arabinose and galactose, mycolic acids and a DNA G+C content of 64–72 % (Goodfellow & Lechevalier, 1989; Goodfellow, 1992).

Currently, the genus *Nocardia* encompasses 54 accepted species with validly published names. Much of the emphasis in nocardial systematics has focused on the causal agents of actinomycetoma and nocardiosis (Goodfellow, 1992, 1998; McNeil & Brown, 1994). Little is known about nocardial species diversity, functional activities and commercial value in natural habitats (Kämpfer *et al.*, 2004; Maldonado *et al.*, 2000; Orchard *et al.*, 1977; Orchard & Goodfellow, 1980; Saintpierre-Bonaccio *et al.*, 2004; Wang *et al.*, 2001; Zhang *et al.*, 2003). In the course of our screening programme for new antibiotics, several actinomycete strains, which contained both type I and type II polyketide biosynthetic pathway genes, were isolated from soil samples collected from Yunnan Province, China (Xu *et al.*, 2003a).

Strain YIM 33361^T was isolated from a soil sample after 2 weeks incubation at 28 °C on water/proline (1 % proline in tap water) agar. Biomass for molecular systematic and most of the chemotaxonomic studies was obtained after incubation at 28 °C for 7 days by growing in shake flasks of yeast extract-malt extract broth (ISP 2 broth; Shirling & Gottlieb, 1966) supplemented with the vitamin mixture of HV medium (Hayakawa & Nonomura, 1987). Cultural characteristics were determined after 2 weeks at 28 °C by methods used in the International *Streptomyces* Project (ISP; Shirling & Gottlieb, 1966) except for modified Sauton's agar (Mordarska *et al.*, 1972). Morphological observations of spores and mycelia were made by light microscopy (Olympus microscope BH-2) and scanning electron microscopy (model JEOL JSM 5600LV). Gram (Hucker's modification; Society for American Bacteriologists, 1957) and Ziehl–Neelsen (Gordon, 1967) preparations were also observed by light microscopy.

The test strain was examined for a range of phenotypic properties using standard procedures (Goodfellow, 1971; Williams *et al.*, 1983). In addition, acid production from carbohydrates was carried out using media and methods described by Gordon *et al.* (1974). The utilization of sole carbon and sole nitrogen sources was investigated after Gordon & Mihm (1957) and Tsukamura (1966). Resistance to lysozyme was determined by the method of Gordon *et al.* (1974). Tolerance to temperature (10, 27, 30, 37 and 45 °C), sodium chloride (4, 7, 10 and 13 %) and phenol (0·1, 0·2, 0·5 and 1·0 %) was tested using modified Bennett's agar (Williams *et al.*, 1983). Resistance to antibiotics was examined using amikacin (30 μg), aureomycin (30 μg), ciprofloxacin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin sulfate (10 μg), kanamycin (15 μg), netilmicin (10 μg), novobiocin (30 μg), oleandomycin

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Abbreviation: DAP, diaminopimelic acid.

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(10 μg), penicillin G (10 U), polymyxin B (300 U), streptomycin sulfate (10 μg), terramycin (30 μg), tetracycline (30 μg), tobramycin sulfate (10 μg) and vancomycin (10 μg) discs (Goodfellow & Orchard, 1974) with glucose-yeast extract agar (Gordon & Mihm, 1962) as the basal medium; the results were recorded following incubation at 28 °C for up to 14 days. Colours and hues were determined according to Kelly (1964).

Cell wells were purified and amino acids of peptidoglycan were analysed by TLC (Lechevalier & Lechevalier, 1980; Jiang *et al.*, 2001). Analysis of whole-cell sugar composition followed procedures described by Becker *et al.* (1965) and Lechevalier & Lechevalier (1980). Phospholipid analysis was carried out as described by Lechevalier *et al.* (1981). The acid methanolysis procedure was used to detect mycolic acids (Minnikin *et al.*, 1975). Menaquinones were determined using the procedures of Collins *et al.* (1977). Biomass for the quantitative fatty acid analysis was prepared by scraping growth from TSA plates [trypticase soy broth (BBL), 3 % (w/v); Bacto agar (Difco), 1·5 % (w/v)] that had been incubated for 3 days at 28 °C. The fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

Genomic DNA extraction and PCR amplification of the 16S rRNA gene from strain YIM 33361^T were carried out using procedures described by Xu *et al.* (2003b). The nearly complete resultant 16S rRNA gene sequence (1511 nucleotides) was aligned manually with corresponding almost-complete sequences of representative *Nocardia* species retrieved from the DDBJ, EMBL and GenBank databases by using BLAST (Altschul *et al.*, 1997) and BLAST 2 sequences (Tatusova & Madden, 1999). Phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignment of data by CLUSTAL X (Thompson *et al.*, 1997); distances (distance options) according to the Kimura two-parameter model (Kimura, 1980, 1983) and clustering with the neighbour-joining method (Saitou & Nei, 1987). The topology of the tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) using 1000 resamplings. *Arthrobacter globiformis* was used as the outgroup.

The chromosomal DNA for genomic DNA G+C content analysis was extracted as described by Marmur (1961). The DNA G+C base content of strain YIM 33361^T was determined by the thermal denaturation method (Mandel & Marmur, 1968).

Strain YIM 33361^T has phenotypic properties consistent with its classification in the genus *Nocardia* (Goodfellow *et al.*, 1999). The organism is aerobic, Gram-positive, slightly acid–alcohol-fast. It developed well on several media including ISP 2 agar, glycerol-asparagine agar (ISP 5 medium; Shirling & Gottlieb, 1966), potato agar (DSMZ medium 129) and modified Bennett's agar (Jones, 1949), showed moderate growth on oatmeal agar (ISP 3 medium; Shirling & Gottlieb, 1966), inorganic salts-starch agar (ISP 4 medium; Shirling & Gottlieb, 1966), modified Sauton's agar (Mordarska *et al.*, 1972) and nutrient agar (Waksman, 1961) and grew poorly on Czapek's agar (Shirling & Gottlieb, 1966) (Table 1). Diffusible pigments were not produced on any tested medium. Morphological features were observed on modified Bennett's agar. The substrate mycelium branched extensively and fragmented into non-motile, rod-shaped elements. The white aerial mycelium was also well developed. Sporotrichetes of up to seven spores were borne on both substrate and aerial hyphae.

Whole-organism hydrolysates of strain YIM 33361^T were rich in *meso*-DAP, arabinose and galactose (cell wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970) and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (phospholipid type II *sensu* Lechevalier *et al.*, 1977). The menaquinones were MK-8($H_4\omega$-cycl.) (85 %) and MK-8(H_2) (15 %). It was also characterized by the presence of mycolic acids that co-migrated (R_f value around 0·47) with those from marker strains of *Nocardia*. The fatty acid profile contained mainly straight-chain saturated, unsaturated and 10-methyl-branched fatty acids. The predominant

Table 1. Cultural characteristics of strain YIM 33361^T on various media

Colours were determined according to Kelly (1964).

Medium	Growth	Aerial mycelium	Substrate mycelium
Yeast extract-malt extract (ISP 2)	Good	White	Moderate orange–yellow
Oatmeal agar (ISP 3)	Moderate	−	Yellow–white
Inorganic salts-starch agar (ISP 4)	Moderate	Yellow–white	Yellow–white
Glycerol-asparagine agar (ISP 5)	Good	Pale yellow	Pale yellow
Czapek's agar	Poor	White	Yellow–white
Modified Sauton's agar	Moderate	White	Pale yellow
Modified Bennett's agar	Good	White	Pale yellow
Potato agar	Good	White	Pale yellow
Nutrient agar	Moderate	White	Light yellow

Table 2. Phenotypic properties that separate strain YIM 33361^T from related *Nocardia* species

Data were taken from this and previous studies (Maldonado *et al.*, 2000; Wang *et al.*, 2001; Zhang *et al.*, 2003; Yassin *et al.*, 2001; Albuquerque de Barros *et al.*, 2003; Kämpfer *et al.*, 2004; Saintpierre-Bonaccio *et al.*, 2004; Kageyama *et al.*, 2004a, b). Symbols: +, utilization; −, no utilization; D, doubtful; W, weak; ND, not determined.

Strain	Hydrolysis of:			Nitrate reduction	Decomposition of (%, w/v):						Growth on sole carbon sources (%, w/v)					Growth at 45 °C
	Aesculin	Arbutin	Urea		Adenine (0·4)	Casein (1·0)	Elastin (0·3)	Hypoxanthine (0·4)	Tyrosine (0·5)	Xanthine (0·4)	D(+)-Mannitol (1·0)	L-Rhamnose (1·0)	D(+)-Sorbitol (1·0)	Sodium acetate (0·1)	Sodium citrate (0·1)	
N. caishijiensis F829^T	−	−	+	+	−	−	−	−	−	−	−	+	−	+	−	−
N. abscessus DSM 44432^T	−	+	+	+	−	−	−	−	−	−	−	+	−	+	+	−
N. africana DSM 44491^T	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+
N. asteroides ATCC 19247^T	+	+	+	+	−	−	−	−	−	−	+	−	−	+	+	−
N. beijingensis JCM 10666^T	+	−	+	+	−	−	−	−	−	+	+	+	+	+	+	−
N. brasiliensis ATCC 19296^T	+	+	+	+	−	+	+	+	+	−	+	−	−	+	+	+
N. brevicatena DSM 43024^T	+	+	−	−	−	−	−	−	−	−	−	+	−	+	−	−
N. carnea DSM 43397^T	+	−	−	+	−	−	−	−	−	−	+	−	+	+	−	−
N. cerradoensis Y9^T	+	ND	+	+	−	−	−	−	−	−	−	+	+	+	−	−
N. crassostreae ATCC 700418^T	+	ND	−	−	−	−	−	−	−	−	−	+	−	−	−	−
N. cummidelens DSM 44490^T	+	+	+	+	−	−	−	−	−	−	−	−	−	+	−	−
N. cyriacigeorgica DSM 44484^T	+	ND	+	ND	−	−	−	−	−	−	−	−	−	+	−	−
N. farcinica ATCC 3318^T	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	+
N. flavorosea JCM 3332^T	−	+	−	−	−	−	−	−	−	−	+	−	+	+	+	−
N. fluminea DSM 44489^T	+	+	−	+	−	−	−	−	+	−	−	+	−	−	+	−
N. ignorata DSM 44496^T	+	ND	ND	ND	ND	−	−	−	−	−	+	−	ND	+	−	+
N. nova JCM 6044^T	+	+	+	+	−	−	−	−	−	−	+	−	+	+	−	−
N. otitidiscaviarum NCTC 1934^T	+	+	+	+	−	−	−	+	−	+	+	−	−	+	−	+
N. paucivorans DSM 44386^T	−	+	+	+	−	−	−	−	−	−	−	−	−	+	−	−
N. pseudobrasiliensis ATCC 51512^T	+	−	+	−	+	+	+	+	+	−	+	−	+	+	+	−
N. salmonicida JCM 4826^T	+	+	+	+	−	−	−	−	+	+	+	−	+	+	+	−
N. seriolae JCM 3360^T	+	+	−	+	−	−	−	−	−	−	−	−	−	+	+	−
N. soli DSM 44488^T	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−
N. transvalensis DSM 43405^T	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	−
N. uniformis JCM 3224^T	+	+	+	+	−	−	+	+	+	+	−	−	−	+	−	−
N. vaccinii DSM 43285^T	+	−	+	+	−	−	−	−	−	−	+	+	−	−	−	−
N. veterana DSM 44445^T	D	ND	+	−	−	−	−	−	−	−	−	+	−	−	−	+
N. vinacea JCM 10988^T	D	ND	+	+	−	−	−	+	−	−	+	−	+	ND	D	−
N. neocaledoniensis SBH_R OA6^T	+	ND	+	+	−	−	−	−	+	−	+	−	−	−	−	+
N. tenerifensis DSM 44704^T	+	ND	+	ND	−	+	ND	+	+	−	+	−	−	ND	+	−
N. asiatica IFM 0245^T	+	+	D	+	−	−	−	−	−	−	D	+	−	+	+	−
N. inohanensis IFM 0092^T	ND	ND	+	ND	−	−	ND	+	−	−	ND	−	−	ND	+	−
N. yamanashiensis IFM 0265^T	ND	ND	+	ND	−	−	ND	+	−	−	ND	−	−	ND	+	−
N. niigatensis IFM 0330^T	ND	ND	D	ND	−	−	ND	W	−	−	ND	−	−	ND	−	−
YIM 33361^T	−	ND	+	−	−	D	+	+	D	+	+	−	−	−	−	−

components, as a proportion of the total fatty acid composition, were i-$C_{15:0}$ (1·33 %), ai-$C_{15:0}$ (5·73 %), i-$C_{16:0}$ (1·76 %), *cis*7-$C_{16:1}$ (1·33 %), i-2-OH-$C_{15:0}$ (5·69 %), $C_{16:0}$ (22·14 %), i-$C_{17:0}$ (2·16 %), ai-$C_{17:0}$ (2·72 %), $C_{17:0}$ (1·53 %), *cis*6,9-$C_{18:2}$ (10·86 %), *cis*9-$C_{18:1}$ (10·05 %), $C_{18:0}$ (21·16 %) and 10-methyl-$C_{18:0}$ (10·5 %). The G+C content of genomic DNA was 65·6 mol%. Detailed results of the physiological features are indicated in Table 2 and in the species description.

A database search demonstrated that the strain YIM 33361^T belongs to the family *Nocardiaceae* (Stackebrandt *et al.*, 1997) and that the determined sequence contains all the signature nucleotides characteristic of the genus *Nocardia* (Chun & Goodfellow, 1995). The rooted phylogenetic tree (Fig. 1) indicated that strain YIM 33361^T formed a distinct subclade within the genus *Nocardia*. Low 16S rRNA gene sequence similarity values (<97 %) were found with all species with validly published names of the genus *Nocardia*. The closest relatives of strain YIM 33361^T were *Nocardia pseudobrasiliensis* DSM 44290^T (AF430042) and *Nocardia paucivorans* DSM 44386^T (AF430041) which both showed 96·92 % similarity (46 nucleotide differences in 1492 sites).

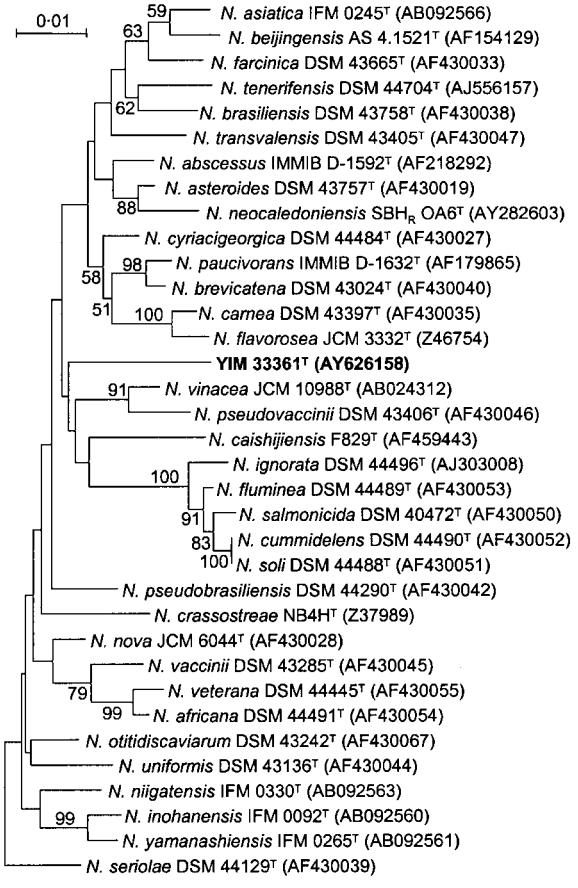

Fig. 1. Phylogenetic dendrogram obtained by neighbour-joining analysis based on 1396 bp of 16S rRNA gene sequences, showing the position of strain YIM 33361^T among its phylogenetic neighbours. Numbers on branch nodes are bootstrap percentages (1000 resamplings). Sequence accession numbers are given in parentheses. The sequence of *Arthrobacter globiformis* DSM 20124^T (X80736) was used as the root (not shown). Bar, 0·01 substitutions per nucleotide position.

This is also supported by phenotypic data, as at least three differences in phenotypic properties were observed between strain YIM 33361^T and the species with validly published names of the genus *Nocardia* (Table 2). Strain YIM 33361^T, *N. paucivorans* IMMIB D-1632^T and *N. pseudobrasiliensis* ATCC 51512^T can also be distinguished easily by their physiological properties. Each of the two strains shows seven differences in comparison with the characteristics of strain YIM 33361^T(Table 2).

In conclusion, the genotypic and phenotypic data show that strain YIM 33361^T forms a novel species of the genus *Nocardia*, for which we propose the name *Nocardia polyresistens* sp. nov.

Description of *Nocardia polyresistens* sp. nov.

Nocardia polyresistens [poly.re.sis′tens. Gr. adj. *polus* many; L. part. adj. *resistens* resisting; N.L. part. adj. *polyresistens* resisting many (antibiotics)].

Aerobic, Gram-positive, catalase-positive and slightly acid–alcohol-fast. Aerial mycelium and substrate mycelium are extensively branched and fragment irregularly into rod-shaped, non-motile elements. A pale-yellow to moderate orange–yellow substrate mycelium carries sparse to abundant, white aerial hyphae on yeast extract-malt extract agar, Czapek's agar, modified Sauton's agar, modified Bennett's agar, potato agar and nutrient agar. A pale-yellow to yellow–white substrate mycelium bears pale-yellow to yellow–white aerial hyphae on glycerol-asparagine or inorganic salts-starch agar. Diffusible pigments are not formed. Ribose, glucose, mannose, fructose, sorbose, melibiose, xylose, sucrose, arabinose, galactose, maltose, lactose, cellobiose, raffinose, melezitose, mannitol, inositol, xylitol, dulcitol, adonitol, salicin, glycerol and dextrin are utilized as sole carbon and energy sources, but not rhamnose, trehalose, sorbitol, arabitol, acetate, malonate, citrate, oxalate or tartrate. Acid is not formed from these tested carbon sources. L-Valine, L-proline (weak), L-asparagine, L-tyrosine (weak), L-alanine and L-histidine are used as sole nitrogen sources, but not acetamide, L-hydroxyproline, L-lysine, L-methionine, L-tryptophan, L-threonine, L-glutamic acid, glycine, L-arginine, L-cysteine or phenylalanine. Urea, xanthine, hypoxanthine, amygdalin, keratin and chitin are hydrolysed. Tweens 20 and 80 are degraded, but not cellulose, starch, allantoin, glucosamine, aesculin, DNA or adenine. Tests for gelatin hydrolysis, nitrate reduction, melanin production, milk coagulation and peptonization, H_2S production and resistance to KCN are negative. Grows between 28 and 37 °C, from pH 7 to 9, and in the presence of phenol at 0·1 %, but not in the presence of sodium chloride. Resistant to lysozyme, penicillin G, ciprofloxacin,

vancomycin, polymyxin B, erythromycin, terramycin, aureomycin (weak), tobramycin, gentamicin sulfate, amikacin, netilmicin, novobiocin, kanamycin and oleandomycin but sensitive to tetracycline, streptomycin and chloramphenicol. The cell wall of strain YIM 33361^T contains *meso*-DAP. Whole-cell sugars are galactose and arabinose. MK-8($H_4\omega$-cycl.) is the major menaquinone and a minor amount of MK-8(H_2) is also present. The phospholipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. The major cellular fatty acids are $C_{16:0}$ (22·14 %), *cis*6,9-$C_{18:2}$ (10·86 %), *cis*9-$C_{18:1}$ (10·05 %), $C_{18:0}$ (21·16 %) and 10-methyl-$C_{18:0}$ (10·5 %). The G+C content of genomic DNA of the type strain is 65·6 mol%.

The type strain, YIM 33361^T (=CCTCC AA 204004^T=KCTC 19027^T), was isolated from soil in Yunnan, China.

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