Nonomuraea antimicrobica sp. nov., an endophytic actinomycete isolated from a leaf of *Maytenus austroyunnanensis*

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A novel actinomycete strain, designated YIM 61105^T, was isolated from a leaf of *Maytenus austroyunnanensis* from the tropical rainforest in Xishuangbanna, Yunnan Province, south-west China. A 16S rRNA gene sequence analysis revealed that the organism belonged to the phylogenetic cluster of the genus *Nonomuraea* and was most closely related to *Nonomuraea candida* HMC10^T (98.2%), '*Nonomuraea aegyptia*' S136 (97.9%), *Nonomuraea kuesteri* GW 14-1925^T (97.5%) and *Nonomuraea turkmeniaca* DSM 43926^T (97.4%). The 16S rRNA gene sequence similarities to other *Nonomuraea* species were less than 97.4%. The main chemotaxonomic properties of strain YIM 61105^T, such as the principal amino acid of the peptidoglycan, the predominant menaquinone and the polar lipid profile, supported its classification within the genus *Nonomuraea*. Strain YIM 61105^T was also readily differentiated from closely related species on the basis of a broad range of phenotypic properties and DNA–DNA hybridization values. Thus, this isolate is considered to represent a novel species of the genus *Nonomuraea*, for which the name *Nonomuraea antimicrobica* sp. nov. is proposed. The type strain is YIM 61105^T (=DSM 45220^T=CCTCC AA 208016^T).

The genus *Nonomuraea* was proposed by Zhang *et al.* (1998) as a member of the family *Streptosporangiaceae*. Members of the genus *Nonomuraea* are aerobic, Gram-positive, non-acid-fast, non-motile actinomycetes that form an extensively branched substrate and aerial mycelium. The aerial hyphae differentiate into hooked, spiral or straight chains of spores, which show a folded, irregular, smooth or warty ornamentation. The genus is characterized chemotaxonomically by the presence of *meso*-diaminopimelic acid in the cell wall, madurose as a characteristic sugar in the whole-cell hydrolysates, considerable amounts of C_{17:0} 10-methyl and iso-C_{16:0} branched-chain fatty acids, major proportions of di-, tetra- and hexahydrogenated menaquinones with nine isoprene units and major amounts of diphosphatidylglycerol, hydroxylated phosphatidylethanolamine, uncharacterized glycolipids and a glucosamine-containing phospholipid (Nonomura & Ohara, 1971; Zhang *et al.*, 1998; Quintana *et al.*, 2003).

At the time of writing, the genus *Nonomuraea* comprised 21 species with validly published names and 2 subspecies

on the basis of a polyphasic approach. There is no record of the isolation of *Nonomuraea* species from the endophytic environment. Xishuangbanna is famous in China for its diverse flora, especially the rainforest plants, many of which have an indigenous pharmaceutical history. The rapidly diminishing tropical rainforests may hold the greatest possible resource for acquiring novel microorganisms and their products.

In the course of our study on endophytic actinomycetal diversity of the tropical-rainforest medicinal plants of Xishuangbanna, strain YIM 61105^T was isolated from a healthy leaf of *Maytenus austroyunnanensis*. After being thoroughly washed in tap water, healthy leaf samples were air-dried at room temperature and the surfaces were sterilized according to the five-step sterilization procedure described by Qin *et al.* (2008). The surface-sterilized samples were pulverized in a ceramic mortar, distributed onto HV agar (Hayakawa & Nonomura, 1987) and incubated at 28 °C for 3 weeks. The isolate was maintained as mycelial fragments in a 20% (v/v) glycerol suspension at –80 °C.

Extraction of genomic DNA and amplification of the 16S rRNA gene sequence from strain YIM 61105^T were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 61105^T is FJ157184.

performed as described by Li *et al.* (2007). An almost-full-length 16S rRNA gene sequence (1454 bp) was aligned with selected sequences obtained from the GenBank/EMBL/DBJ databases using CLUSTAL X version 1.8 (Thompson *et al.*, 1997). Neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) trees were constructed using MEGA version 3.1 (Kumar *et al.*, 2004). The reliability of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985). The phylogenetic tree (Fig. 1) based on the 16S rRNA gene sequences revealed that the closest relatives of strain YIM 61105^T were *Nonomuraea candida* HMC10^T (98.2%), '*Nonomuraea aegyptia*' S136 (97.9%), *Nonomuraea kuesteri* GW 14-1925^T (97.5%) and *Nonomuraea turkmeniaca* DSM 43926^T (97.4%). The 16S rRNA gene sequence similarity values between the isolate and other members of the genus *Nonomuraea* were lower than 97.4%. Strain YIM 61105^T formed a coherent and monophyletic clade with '*N. aegyptia*' S136 that was supported by a high bootstrap value of 84% in the neighbour-joining tree and also recovered with the maximum-parsimony algorithm.

Cultural and morphological characteristics were observed with strain YIM 61105^T grown on yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4) and glycerol-asparagine agar (ISP 5; Shirling & Gottlieb, 1966), as well as potato-glucose agar (PDA; Difco), Czapek's agar and nutrient agar (Waksman, 1967), for 3 weeks at 28 °C. The colour of both substrate and aerial mycelia and any soluble pigments produced were determined by comparison with chips from the colour charts of the Inter-Society Color Council (Kelly, 1964). The morphological features of the spore chains and mycelia were observed by using light microscopy (BH-2; Olympus)

and scanning electron microscopy (JSM-5600LV; JEOL). Ranges of conditions required for growth were tested using ISP 2 for pH 4.0–11.0 (at intervals of 0.5 pH units), temperature (0, 4, 10, 15, 20, 28, 37, 40, 45 and 55 °C) and 0–20% (w/v) NaCl (at intervals of 0.5%). The ability to use a range of carbon and nitrogen sources for energy and growth and other physiological and biochemical features was tested according to Gordon *et al.* (1974). Strain YIM 61105^T showed good growth on ISP 2–4 and PDA media, moderate growth on ISP 5 and Czapek's media, and poor growth on nutrient agar. It formed extensively branched substrate mycelia and white-to-pink aerial mycelia on the media tested, and spore chains borne on mycelia were spiral (Fig. 2). Pink diffusible pigments were produced on ISP 5 and PDA media. The physiological and biochemical properties of strain YIM 61105^T are listed in Table 1 and the species description.

Biomass for quantitative fatty acid analysis was prepared by scraping colonies from tryptic soy agar (TSA; Difco) after incubation at 28 °C for 10 days. Biomass for other chemotaxonomic studies was obtained after cultivation at 28 °C for 7–10 days in shaken cultures with ISP 2. The antimicrobial activity of strain YIM 61105^T was detected using the agar-well diffusion method (Hugo & Russell, 1983). *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium smegmatis* and *Candida albicans* were used as indicator organisms. Standard analytical procedures were used to extract and analyse the isomeric forms of diaminopimelic acid, whole-organism sugars (Hasegawa *et al.*, 1983), isoprenoid quinones (Groth *et al.*, 1997), polar lipids (Minnikin *et al.*, 1979; Collins & Jones, 1980) and fatty acids (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Strain YIM 61105^T contained *meso*-diaminopimelic

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationship between strain YIM 61105^T and its phylogenetic neighbours. Percentages at nodes (>40%) are levels of bootstrap support based on 1000 resamplings. Asterisks denote nodes that were also recovered using the maximum-parsimony method. *Thermopolyspora flexuosa* DSM 43186^T was used as the outgroup. Bar, 0.005 substitutions per nucleotide position.

Fig. 2. Scanning electron micrograph of spiral spore chains of strain YIM 61105^T after growth on ISP 2 medium at 28 °C for 3 weeks. Bar, 10 µm.

acid and madurose in whole-organism hydrolysates (cell-wall chemotype IIIB; Lechevalier & Lechevalier, 1970). The predominant menaquinone was MK-9(H₄) (78 %), and MK-9(H₂) (10 %), MK-9(H₆) (6 %) and MK-9 (6 %) were also present. The diagnostic phospholipids were phosphatidylethanolamine and a glucosamine-containing phospholipid (phospholipid type IV; Lechevalier *et al.*, 1977). The detailed fatty acid profile was iso-C_{14:0} (3.9 %), C_{14:0}

(4.2 %), iso-C_{15:0} (6.5 %), iso-C_{16:1} G (12.7 %), iso-C_{16:0} (32.2 %), C_{16:0} (11.3 %), C_{17:0} 10-methyl (17.1 %), ante-iso-C_{17:0} (2.7 %), C_{17:0} (2.4 %), C_{18:1} ω9c (3.0 %) and C_{18:0} 10-methyl (5.8 %). Strain YIM 61105^T showed antimicrobial activity against *Candida albicans*.

The genomic DNA G+C content of strain YIM 61105^T was 69.2 mol%, determined by using HPLC (Mesbah *et al.*, 1989). DNA–DNA relatedness values were measured fluorometrically using the microplate hybridization method (Ezaki *et al.*, 1989; He *et al.*, 2005). Strain YIM 61105^T showed relatively low DNA–DNA relatedness with *N. candida* DSM 45086^T (37 %) and '*N. aegyptia*' DSM 45082 (34 %). These results are below the 70 % cut-off point recommended for the delineation of genomic species (Stackebrandt & Goebel, 1994). It has been shown that *Nonomuraea* species exhibit high 16S rRNA gene sequence similarities (97.7–99.4 %), but low DNA–DNA relatedness values (<70 %) (Fischer *et al.*, 1983; Poscher *et al.*, 1985; Tamura *et al.*, 2000; Kämpfer *et al.*, 2005). Stackebrandt *et al.* (2001) reported DNA–DNA relatedness values between the type strains of *Nonomuraea africana*, *N. dietziae* and *N. recticatena* of 45–48 %, although these species shared 16S rRNA gene sequence similarities of 98.9–99.8 %. For this reason, DNA–DNA relatedness experiments between strain YIM 61105^T and type strains with 16S rRNA gene sequence similarities that were less

Table 1. Comparison of the phenotypic properties of strain YIM 61105^T and the nearest phylogenetically related species

Strains: 1, YIM 61105^T (*N. antimicrobica* sp. nov.); 2, '*Nonomuraea aegyptia*' S136; 3, *Nonomuraea candida* HMC10^T; 4, *Nonomuraea turkmeniaca* DSM 43926^T; 5, *Nonomuraea kuesteri* GW 14-1925^T. Data were taken from this study and Kämpfer *et al.* (2005), Hozzein & Goodfellow (2007) and Roes & Meyers (2008). +, Positive; –, negative; w, weak; ND, not determined.

Characteristic	1	2	3	4	5
Spore chains	Spirals	Straight to hooked/curled	Hooked/curled	Spirals	Spirals
Growth on ISP 3					
Aerial mycelium	White/pink	Trace	White (sparse)	Trace	Trace
Substrate mycelium	Moderate brown	Yellowish brown	Cream	Violet/red	Yellow
Soluble pigment	Pink	None	None	Pink/violet	None
Temperature range for growth (°C)	15–37	10–37	30–45	ND	ND
Aesculin hydrolysis	–	–	+	+	+
Nitrate reduction	–	ND	–	+	ND
H ₂ S production	–	ND	+	ND	ND
Degradation of:					
Casein	+	+	+	+	–
Hypoxanthine	–	+	–	+	–
Starch	–	ND	–	+	–
Gelatin	–	ND	w	+	–
Tyrosine	–	ND	–	+	–
Utilization of:					
L-Arabinose	–	–	w	+	+
Cellobiose	+	–	w	+	+
meso-Erythritol	+	–	ND	ND	+
myo-Inositol	–	ND	+	+	+
Sucrose	+	–	+	+	+
D-Xylose	–	+	+	+	+

than 97.4 % were not carried out. Recently, Stackebrandt & Ebers (2006) recommended an increase of about 2 % for the 16S rRNA gene sequence similarity threshold value used to determine the taxonomic status of a strain, from 97 to 98.7–99 %, provided that these data are supported by clear phenotypic differences. Thus, strain YIM 61105^T clearly represents a novel species in the genus *Nonomuraea*.

The morphological and chemotaxonomic characteristics of strain YIM 61105^T are consistent with its classification in the genus *Nonomuraea*. However, strain YIM 61105^T is phenotypically different from its closest phylogenetic neighbours, with differences, for example, in growth characteristics on ISP 3 medium, spore chain arrangement and biochemical and carbon-source utilization test results (Table 1). On the basis of the phenotypic and genotypic data, strain YIM 61105^T represents a novel species within the genus *Nonomuraea*. In view of its antimicrobial activity towards *Candida albicans*, the name *Nonomuraea antimicrobica* sp. nov. is proposed.

Description of *Nonomuraea antimicrobica* sp. nov.

Nonomuraea antimicrobica (an.ti.mi.cro'bi.ca. Gr. prep. *anti* against; N.L. n. *microbium* microbe; L. adj. suff. *-icus* -a -um suffix used with various meanings; N.L. fem. adj. *antimicrobica* antimicrobial).

Gram-positive, aerobic, non-acid-fast and non-acid-alcohol-fast actinomycete that forms extensively branched, brown substrate mycelia and white-to-pink aerial mycelia on ISP 2–5 and PDA media. Pink diffusible pigments are produced on ISP 5 and PDA media. After 14 days of incubation at 28 °C, spiral spore chains composed of smooth spores are observed on the aerial mycelium. Temperature range for growth is 15–37 °C, with optimal growth at 28 °C. pH range for growth is pH 6.0–9.0, with optimal growth at pH 7.0. No growth is observed with 5 % NaCl. Positive for catalase. Negative for oxidase, milk coagulation, milk peptonization, gelatin liquefaction, cellulose and starch hydrolysis, H₂S production and nitrate reduction. The diagnostic amino acid of the peptidoglycan is *meso*-diaminopimelic acid. Cell hydrolysates contain ribose, glucose, madurose and galactose. Polar lipids include diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides and the diagnostic phospholipids of phosphatidylethanolamine and an unknown glucosamine-containing phospholipid. The predominant menaquinone of the type strain is MK-9(H₄); MK-9(H₂), MK-9(H₆) and MK-9 are also present. Major fatty acids (>10 %) are iso-C_{16:0}, C_{17:0} 10-methyl, iso-C_{16:1} G and C_{16:0}. Additional phenotypic properties are shown in Table 1. The DNA G+C content of the type strain is 69.2 mol%.

The type strain, YIM 61105^T (=DSM 45220^T=CCTCC AA 208016^T), was isolated from a surface-sterilized leaf of *Maytenus austroyunnanensis* from the tropical rainforest of Xishuangbanna, Yunnan Province, south-west China.

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