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Nonomuraea rhizophila sp. nov., an actinomycete isolated from rhizosphere soil

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A novel actinomycete, designated strain YIM 67092^T, was isolated from rhizosphere soil of the perennial vine *Tripterygium wilfordii* Hook. f. collected from Yunnan province, South-west China. The strain formed well differentiated aerial and substrate mycelia and grew in the presence of up to 7 % (w/v) NaCl. Phylogenetic analysis based on the 16S rRNA gene showed that strain YIM 67092^T belonged to the genus *Nonomuraea*, with highest sequence similarity to *Nonomuraea rosea* GW 12687^T (99.0 %). Sequence similarities between strain YIM 67092^T and other species of the genus *Nonomuraea* ranged from 97.8 % (*Nonomuraea dietziae* DSM 44320^T) to 93.8 % (*Nonomuraea kuesteri* GW 14-1925^T). Key morphological, physiological and chemotaxonomic characteristics of strain YIM 67092^T were congruent with the description of the genus *Nonomuraea*. The G+C content of the genomic DNA was 69.3 mol%. Based on comparative analysis of physiological, biochemical and chemotaxonomic data, including low DNA–DNA hybridization results, strain YIM 67092^T represents a novel species of the genus *Nonomuraea rhizophila* sp. nov. is proposed. The type strain is YIM 67092^T (=CCTCC AA 209044^T =DSM 45382^T).

The genus *Nonomuraea* was proposed by Zhang *et al.* (1998) and is part of the family *Streptosporangiaceae*. Members of the genus *Nonomuraea* are aerobic, Grampositive, nonacid-fast, non-motile actinomycetes which form extensively branched substrate and aerial mycelia. The aerial hyphae differentiate into hooked, spiral or straight chains of spores, which show a folded, irregular, smooth or warty surface ornamentation. The genus is characterized chemotaxonomically by the presence of *meso*-diaminopimelic acid in the cell wall, madurose as a characteristic sugar in whole-cell hydrolysates and hexahy-drogenated menaquinones with nine isoprene units as the predominant isoprenologues (Nonomura & Ohara, 1971;

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One supplementary figure and two supplementary tables are available with the online version of this paper.

Zhang *et al.*, 1998; Quintana *et al.*, 2003). The type species of the genus is *Nonomuraea pusilla* and, at the time of writing, the genus comprised 26 species with validly published names as well as two subspecies.

Plant roots release organic compounds into the rhizosphere, which can affect the microbial population (Lynch & Whipps, 1990). Bodelier *et al.* (1997) claimed that rhizosphere soil contains an increased microbial biomass and activity compared with non-rhizosphere soil. During the course of our research on new actinobacterial sources, we isolated bacterial strains from rhizosphere soil of the perennial vine *Tripterygium wilfordii* Hook. f. and obtained a novel isolate, designated YIM 67092^T, which, in initial studies, was found to have properties consistent with members of the genus *Nonomuraea*. The organism was the subject of a polyphasic taxonomic study, which showed that it represented a new species of the genus *Nonomuraea*.

Strain YIM 67092^{T} was isolated from rhizosphere soil of *T. wilfordii* Hook. f., collected from Yunnan province, South-west China, by using a standard serial dilution technique using HV agar plates (Hayakawa & Nonomura, 1987) and incubating at 28 °C for 2–3 weeks. A pure culture

Abbreviations: DPG, diphosphatidylglycerol; PME, phosphatidylmethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; GluNu, unknown glucosamine-containing phospholipid; PL, unknown phospholipid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 67092^{T} is HM755723.

of strain YIM 67092^T was obtained and the isolate was maintained on trypticase soy agar (TSA; 1.5% trypticase peptone, 0.5% soya peptone, 0.5% NaCl, 1.5% agar) slants at 4 °C and in 20% (v/v) glycerol suspensions at -80 °C. Biomass for chemical and molecular studies was obtained by cultivating in trypticase soy broth (TSB) medium in shake flasks (~200 r.p.m.) at 28 °C for 1 week.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene from strain YIM 67092^{T} were performed as described by Li *et al.* (2007). The resulting 16S rRNA gene sequence was compared with those available from GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/) to determine an approximate phylogenetic affiliation. Multiple alignments with sequences of the most closely related actinobacteria and calculations of levels of sequence similarity were carried out using CLUSTAL_X (Thompson et al., 1997). Phylogenetic trees were reconstructed by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) tree-making algorithms in the software packages MEGA version 4.0 (Tamura et al., 2007), PHYLIP version 3.6 (Felsenstein, 2002) and PHYML (Guindon & Gascuel, 2003). The topology of the phylogenetic trees was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The nearly complete 16S rRNA gene sequence of strain YIM 67092^T (1521 bp) was determined and compared with the corresponding sequences of other bacterial strains in the GenBank database. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YIM 67092^T was a member of the genus *Nonomuraea*. A phylogenetic tree, based on 16S rRNA gene sequence data

from strain YIM 67092^T and type strains of members of the genus Nonomuraea was constructed according to the neighbour-joining algorithm (Fig. 1) using Thermopolvspora flexuosa DSM 43186^T as an outgroup. Comparative analysis of 16S rRNA gene sequences and phylogenetic relationships showed that strain YIM 67092^T grouped in a subclade with Nonomuraea rosea GW 12687^T, supported by a bootstrap value of 100% (Fig. 1), with which it shared a 16S rRNA gene sequence similarity of 99%. 16S rRNA gene sequence similarities between strain YIM 67092^T and other species of the genus Nonomuraea were <98%, ranging from 93.8% (Nonomuraea kuesteri GW 14-1925^T) to 97.8% (Nonomuraea dietziae DSM 44320^T). The affiliation of strain YIM 67092^{T} with its closest neighbour, N. rosea GW 12687^T, was also corroborated by the trees reconstructed using the maximum-parsimony and maximum-likelihood algorithms and was supported by bootstrap values of 99.8 % (PHYLIP version 3.6) and 99.5 % (PHYML), respectively.

It has been shown that some species of the genus *Nonomuraea* share high 16S rRNA gene sequence similarities (ranging from 97.6 to 99.4%) but have low DNA– DNA relatedness values (Fischer *et al.*, 1983; Poscher *et al.*, 1985; Kämpfer *et al.*, 2005). For example, Stackebrandt *et al.* (2001) reported DNA–DNA relatedness values of 45–48% between the type strains of *Nonomuraea africana*, *N. dietziae* and *Nonomuraea recticatena*, despite the fact that these strains shared 16S rRNA gene sequence similarities of between 98.9 and 99.8%. Similarly, *Nonomuraea maheshkhaliensis* 16-5-14^T showed a 16S rRNA gene sequence similarity of 99.4% to the type strain of *N. kuesteri*, but displayed relatively low DNA–DNA relatedness values of 39.9–45.7% (Ara *et al.*, 2007). Because of the relatively lower

	Nonomuraea salmonea IFO 14687 ^T (U48982) Nonomuraea angiospora IFO 13155 ^T (U48943) Nonomuraea spiralis IFO 14097 ^T (U48983) Nonomuraea pusilla IFO 14684 ^T (U48978) Nonomuraea ferruginea IFO 14094 ^T (U48978) Nonomuraea ferruginea IFO 14094 ^T (U48978) Nonomuraea polychroma IFO 14345 ^T (U48977) 91 [*] Nonomuraea maheshkhaliensis 16-5-14 ^T (AB290014) Nonomuraea kuesteri GW 14-1925 ^T (AJ746362) Nonomuraea kuesteri GW 14-1925 ^T (AJ746362) Nonomuraea bangladeshensis 5-10-10 ^T (AB274966) 97 Nonomuraea tastidiosa IFO 14680 ^T (U48944) 85 [*] 97 Nonomuraea coxensis 5-38-42 ^T (AB274967) 81 [*] Nonomuraea venchangensis DSM 45477 ^T (FJ261959) 83 [*] Nonomuraea turinata' DSM 44505 ^T (AF277200) 'Nonomuraea turinata' DSM 44505 ^T (AF277201) 98 [*] Nonomuraea antimicrobica YIM 61105 ^T (FJ157184) 'Nonomuraea antimicrobica YIM 61105 ^T (FJ157184) Nonomuraea andopytica YIM 65601 ^T (GU367158) Nonomuraea and longicatena NRRL 15532 ^T (AB018787) 100 [*] Nonomuraea arcicana IFO 14745 ^T (U48842) Nonomuraea arcicana IFO 14745 ^T (U48979) 98 [*] Nonomuraea roseo/a IFO 14685 ^T (U48979) 98 [*] Nonomuraea roseo/a IFO 14685 ^T (U48979) 98 [*] Nonomuraea roseo/a IFO 14685 ^T (U48979) 100 [*] Thermopolyspora flexuosa DSM 43186 ^T (AY039253)	Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the relationship between strain YIM 67092 ^T and species of the genus <i>Nonomuraea. Thermopolyspora flexuosa</i> DSM 43186 ^T was used as an outgroup. Asterisks denote branches that were also recovered using the maximum-parsimony and maximum-likelihood methods. Bootstrap values >50% (based on 1000 replications) are shown at branch points. Bar, 0.005 substitutions per nucleotide position.
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sequence similarities (<98.0%) seen between strain YIM 67092^{T} and other strains of this genus, DNA–DNA relatedness experiments between strain YIM 67092^{T} and other species of the genus *Nonomuraea* were not carried out. DNA–DNA hybridization between strain YIM 67092^{T} and *N. rosea* GW 12687^T was carried out by applying the fluorometric micro-well method (Ezaki *et al.*, 1989; He *et al.*, 2005) at the optimal hybridization temperature (48 °C). Strain YIM 67092^{T} showed a DNA–DNA relatedness value of $38.5 \pm 2.0\%$ with *N. rosea* GW 12687^T, which is well below the 70% cut-off point recommended for the delineation of bacterial species (Stackebrandt & Goebel, 1994). This suggested that strain YIM 67092^{T} represented a different genomic species of the genus *Nonomuraea*.

Gram staining was carried out by using the standard Gram reaction method and cell motility was confirmed by the development of turbidity throughout a tube containing semisolid medium (Leifson, 1960). The morphological characteristics of strain YIM 67092^T, including spore-chain morphology, spore size and surface ornamentation, were determined by light and scanning electron microscopy (Philips XL30 ESEM-TMP) of 14-day-old cultures on International Streptomyces Project (ISP) 2 medium (Shirling & Gottlieb, 1966). Aerial spore-mass colour, substrate mycelium pigmentation and coloration of the diffusible pigments of strain YIM 67092^T were determined on ISP media (Shirling & Gottlieb, 1966), Czapek's agar, potato-glucose agar and nutrient agar prepared as described by Dong & Cai (2001). Colours were designated by comparing with colour chips from the ISCC-NBS colour charts (standard samples, no. 2106) (Kelly, 1964). Growth at 4, 10, 20, 28, 37, 45, 50 and 55 °C was tested on TSA by incubating the cultures for 21 days. Growth at pH 4, 5, 6, 7, 8, 9 and 10 (using the buffer system described by Xu et al. 2005) and in 0, 1, 3, 5, 7, 10, 15 and 20 % (w/v) NaCl was tested by culturing the strains in TSB at 28 °C for 14-21 days. Catalase, oxidase and gelatinase activities, starch hydrolysis, nitrate reduction and urease activity were assessed as described by Smibert & Krieg (1994). Other physiological and biochemical tests were performed as described by Gordon et al. (1974).

Cells of strain YIM 67092^T were Gram-reaction-positive, aerobic and non-motile. Strain YIM 67092^T grew well on ISP 2, ISP 3, ISP 4, Czapek's agar and potato-glucose agar media; moderate growth was recorded on ISP 5 and nutrient agar. A white aerial mycelium was produced on ISP 3, Czapek's agar and potato-glucose agar media but no aerial mycelium was formed on ISP 2, ISP 4, ISP 5 and nutrient agar. The substrate mycelium colour varied from orange through brownish yellow to brown on the media tested. No diffusible pigment was observed on any media tested (Supplementary Table S1, available in IJSEM Online). After approximately 14 days, strain YIM 67092^T showed morphological characteristics typical of members of the genus Nonomuraea; cells produced well-developed branched substrate and aerial mycelia which did not fragment into bacillary or coccoid elements. Spiral spore



Fig. 2. Scanning electron micrograph of spiral spore chains on aerial mycelium of strain YIM 67092^{T} grown on ISP 2 at 28 °C for 14 days. Bar, 2 μ m.

chains of strain YIM 67092^{T} were composed of ~7–10 nonmotile spores (0.9–1.2 µm in diameter) with a rough surface and were borne directly on the aerial mycelium (Fig. 2). The isolate grew at 10–37 °C, at pH 6–8 and in 0– 7 % (w/v) NaCl. Optimal growth was observed between 20 and 28 °C and at pH 7. The isolate was catalase-positive and oxidase-negative. Detailed physiological and biochemical properties are given in Table 1 and in the species description. It is evident from the results in Table 1 that there were significant phenotypic differences between strain YIM 67092^T and *N. rosea* GW 12687^T.

Table 1. Phenotypic properties of strain YIM 67092^{T} and closely related species of the genus *Nonomuraea*

Strains: 1, YIM 67092^{T} ; 2, *N. rosea* GW 12687^{T} . Both strains were able to utilize cellobiose, D-fructose, glucose, *myo*-inositol, lactose, maltose, D-mannose, raffinose and L-rhamnose but not ribose or sucrose as sole carbon sources. Both strains grew at pH 6–8, produced white aerial mycelia on on ISP 3 medium and formed spiral spore chains. +, Positive; -, negative; ND, not determined. Both stains were grown under the same conditions in this study.

Characteristic	1	2
Spore ornamentation	Rough	ND
Number of spores	7-10	4-10
Substrate mycelium	Brown-yellow	Pink-red
(on ISP 3 medium)		
Degradation of:		
Gelatin	_	+
Tween 20	+	_
Starch	_	+
Urea	+	_
Utilization of:		
L-Arabinose	_	+
Xylose	_	+
Temperature range for growth ($^{\circ}$ C)	10-37	10-45
Tolerance of NaCl (%, w/v)	7	5

The isomer of diaminopimelic acid and the whole-cell sugars were determined according to the procedures described by Hasegawa et al. (1983), Lechevalier & Lechevalier (1970) and Tang et al. (2009). Phospholipids were extracted, examined by two-dimensional TLC and identified using previously described procedures (Minnikin et al., 1979; Collins & Jones, 1980). Menaquinones were isolated according to Collins et al. (1977) and separated by HPLC (Tamaoka et al., 1983). For fatty acid analysis, cell biomass of strain YIM 67092^{T} and *N. rosea* GW 12687^{T} was obtained after cultivation in TSB medium at 28 °C for 7 days. Cellular fatty acids were extracted, methylated and analysed by using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions. The fatty acid methyl esters were then analysed by GC (Agilent Technologies 7890A GC System) by using the Microbial Identification software package (Sherlock Version 6.1; MIDI database: TSBA6).

Chemotaxonomic analyses revealed that strain YIM 67092^T exhibited characteristics which were typical of members of the genus Nonomuraea, such as the presence of meso-diaminopimelic acid and the presence of madurose, glucose, mannose, ribose and galactose as whole-cell sugars. MK-9(H_4) (82.4%) was the predominant menaquinone; MK-9(H₆) (12.6%) and MK-9(H₂) (5.0%) were minor components. The phospholipids consisted of diphosphatidylglycerol (DPG), phosphatidylmethylethanolamine (PME), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), an unknown glucosamine-containing phospholipid (GluNu) and some unknown phospholipids (PLs) (Supplementary Fig. S1). The major fatty acids were $C_{17:0}$ 10-methyl (26.66%), iso- $C_{16:0}$ (24.00%), iso- $C_{16:1}$ G (14.11%), $C_{17:1}\omega 6c$ (5.63%) and iso- $C_{15:0}$ (4.57%), similar to profiles described for recognized species of the genus Nonomuraea but different from that of N. rosea GW 12687^T (Supplementary Table S2).

The G+C content of the genomic DNA was determined by using the HPLC method (Mesbah *et al.*, 1989) with *Escherichia coli* JM-109 as the reference strain. The DNA G+C content of strain YIM 67092^{T} was 69.3 mol%, which is consistent with values seen for members of the genus *Nonomuraea*.

The phenotypic and chemotaxonomic properties, together with the results of 16S rRNA gene sequence analysis, support the proposal that strain YIM 67092^T represents a novel species of the genus *Nonomuraea*, for which the name *Nonomuraea rhizophila* sp. nov. is proposed.

Description of Nonomuraea rhizophila sp. nov.

Nonomuraea rhizophila (rhi.zo.phi'la. Gr. n. *rhiza* a root; Gr. adj. *philos* on loving; N.L. fem. adj. *rhizophila* root-loving).

Gram-reaction-positive, aerobic, non-motile actinomycete that forms extensively branched substrate and aerial mycelia. No diffusible pigments are observed on any of the tested media. Spiral spore chains composed of \sim 7–10

non-motile spores (0.9–1.2 μ m in diameter) with a rough surface are borne directly on the aerial mycelium. Grows at 10-37 °C (optimum 20-28 °C) and pH 6-8 (optimum pH 7). The NaCl tolerance range for growth is up to 7% (w/v). Positive for catalase, milk coagulation, milk peptonization and urea hydrolysis. Negative for oxidase, gelatin liquefaction, hydrogen sulfide production, cellulose and starch hydrolysis and nitrate reduction. Tween 20 is hydrolysed but Tweens 40 and 80 are not hydrolysed. Utilizes cellobiose, D-fructose, glucose, mvo-inositol, lactose, maltose, D-mannose, D-mannitol, raffinose and Lrhamnose as sole carbon sources; L-arabinose, D-galactose, glycerol, ribose, sodium acetate, D-sorbitol, sucrose and D-xylose are not utilized. Does not produce acids from any carbon sources tested. L-Alanine, L-arginine, L-asparagine, glycine, L-hydroxyproline, hypoxanthine, L-lysine, Lphenylalanine, L-serine, L-tyrosine, L-valine and xanthine can be used as sole nitrogen sources. The diagnostic amino acid of the peptidoglycan is meso-diaminopimelic acid. Cell hydrolysates contain madurose, glucose, mannose, ribose and galactose. Polar lipids include diphosphatidylglycerol (DPG), phosphatidylmethylethanolamine (PME), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), an unknown glucosaminecontaining phospholipid (GluNu) and some unknown phospholipids (PLs). The predominant menaquinone is MK-9(H₄); MK-9(H₆) and MK-9(H₂) are also present. Major fatty acids are C_{17:0} 10-methyl, iso-C_{16:0}, iso-C_{16:1} G, $C_{17:1}\omega 6c$ and iso- $C_{15:0}$.

The type strain, YIM 67092^{T} (=CCTCC AA 209044^{T} =DSM 45382^{T}), was isolated from rhizosphere soil of the perennial vine *Tripterygium wilfordii* Hook. f., collected from Yunnan Province, South-west China. The DNA G+C content of the type strain is 69.3 mol%.

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