Nonomuraea wenchangensis sp. nov., isolated from mangrove rhizosphere soil

Fan Wang,^{1,2} Xiao-Xiong Xu,¹ Zhi Qu,¹ Cheng Wang,¹ Hai-Peng Lin,¹ Qing-Yi Xie,¹ Ji-Sheng Ruan,¹ Ming Sun² and Kui Hong¹

Correspondence

Kui Hong
k1022@163.net

¹Laboratory of Tropical Microbial Resource, Hainan Province, Key Laboratory of Tropical Crop Biotechnology, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agriculture Sciences, Haikou 571101, PR China

²College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430000, PR China

Strain 210417^T, which forms highly branched substrate and aerial mycelia, is a Gram-positive, aerobic and non-motile actinomycete isolated from mangrove rhizosphere soil. 16S rRNA gene sequence analysis showed that the strain should be classified in the genus *Nonomuraea*, being most closely related to the type strains of *Nonomuraea coxensis* (99.6%) and *Nonomuraea bangladeshensis* (99.3%). Chemotaxonomic properties [madurose as the major sugar in the cell wall; meso-diaminopimelic acid and N-acetylmuramic acid in the peptidoglycan; MK-9(H₄) as the major menaquinone; iso-C_{16:0} (24.1%) as major fatty acid; and phospholipid pattern type IV] are consistent with the assignment of strain 210417^T to the genus *Nonomuraea*. Strain 210417^T could be differentiated from the closely related species *N. coxensis* and *N. bangladeshensis* by morphological, physiological, biochemical and chemotaxonomic properties, 16S rRNA gene sequence analysis and DNA–DNA hybridization results. It is therefore proposed that strain 210417^T represents a novel species of the genus *Nonomuraea*, for which the name *Nonomuraea wenchangensis* sp. nov. is given; the type strain is 210417^T (=CGMCC 4.5598^T =DSM 45477^T).

The genus *Nonomuria*, which was proposed by Zhang *et al.* (1998), belongs to the family *Streptosporangiaceae*, a member of the suborder *Streptosporangineae* within the order *Actinomycetales*. Chiba *et al.* (1999) subsequently corrected the spelling of the genus name to *Nonomuraea*. At the time of writing, this genus comprised 24 species with validly published names and two subspecies (Euzéby, 2010).

During the process of searching for actinomycetes that can produce commercially important secondary metabolites, a novel actinomycete, strain 210417^T was isolated from a rhizosphere soil sample of the mangrove *Bruguiera sexangula* in Wenchang, Hainan, China. The strain produced metabolites that showed activity against the SMMC-7721 cancer cell line. The soil sample was heat-treated at 100 °C for 1 h after being air-dried at room temperature for 7 days and treated with a solution of chloramine-T (1%, w/v) (Hayakawa *et al.*, 1997). The

pretreated soil sample was diluted 1:10 (v/v) with sterile 1/4 Ringer's solution (K₂HPO₄, 0.38%; KH₂PO₄, 0.12%; MgSO₄·7H₂O, 0.51%; NaCl, 0.25%; Fe₂(SO₄)₃·7H₂O, 0.005%; MnSO₄, 0.005%) and serial dilutions were prepared down to 10^{−3}. One hundred microlitres of the 10^{−1} to 10^{−3} suspensions were spread, in triplicate, on glucose-aspartic acid-vitamin medium (Hayakawa *et al.*, 1991) supplemented with cycloheximide (50 mg l^{−1}), nystatin (50 mg l^{−1}) and nalidixic acid (50 mg l^{−1}). After 21 days of aerobic incubation at 28 °C, the strain was purified and maintained as a working culture on yeast extract-malt extract agar [International *Streptomyces* Project (ISP) medium 2; Shirling & Gottlieb, 1966]. The aim of this study was to determine the taxonomic position of isolate 210417^T using a polyphasic approach. For comparative purposes, *Nonomuraea coxensis* JCM 13931^T and *Nonomuraea bangladeshensis* JCM 13930^T were used as reference strains in this study.

Genomic DNA extraction, PCR amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Hong *et al.* (2009). The almost-complete 16S rRNA gene sequence of strain 210417^T (1484 nt) was aligned with nucleotide sequences obtained from GenBank/EMBL/DDBJ using CLUSTAL X software

Abbreviations: DAP, diaminopimelic acid; GlcNU, unknown glucosamine-containing phospholipid; ISP, International *Streptomyces* Project; PE, phosphatidylethanolamine; PME, phosphatidylmethylethanolamine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Nonomuraea wenchangensis* 210417^T is FJ261959.

A supplementary table is available with the online version of this paper.

(Thompson *et al.*, 1997). A phylogenetic tree was reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods from the software package MEGA version 3.1 (Kumar *et al.*, 2004). Confidence in tree topology was evaluated by using bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. Sequence similarity values between strain 210417^T and related species were calculated using EzTaxon (<http://www.eztaxon.org/>; Chun *et al.*, 2007).

Cell morphology was observed by light microscopy (80i, Nikon) and scanning electron microscopy (FEI QUANTA) of cultures grown for 21 days at 28 °C on oatmeal agar (ISP 3). Cultural characteristics were determined by the methods proposed by Shirling & Gottlieb (1966) after 3 weeks at 28 °C. ISCC–NBS colour charts (Kelly, 1964) were used to determine the colours of substrate and aerial mycelia. Growth at various temperatures (4–45 °C) was determined on ISP 2 for 14–21 days. NaCl tolerance and the pH range for growth were determined in cells grown in 0–8 % NaCl and at pH 4–12, respectively, on ISP 2 at 28 °C for 14–21 days. The ability to use carbohydrates as sole carbon sources was tested according to the methods of Gordon *et al.* (1974). Decomposition of casein (skimmed milk), gelatin, hypoxanthine, tyrosine and xanthine was assessed following the procedures described by Williams *et al.* (1983).

For chemotaxonomic analysis, biomass was obtained from ISP 2 broth after incubation at 28 °C for 5–7 days in shake flasks (200 r.p.m.). The isomeric form of diaminopimelic acid (DAP) and sugars in whole-cell hydrolysates were determined based on standard procedures, as described previously (Hasegawa *et al.* 1983; Lechevalier & Lechevalier, 1980). The *N*-acyl type of muramic acid in the peptidoglycan was analysed according to the method of Uchida & Aida (1984). Menaquinones were extracted from freeze-dried cells and purified according to Minnikin *et al.* (1984); composition was determined by HPLC using an ODS-BP C18 column (4.6 × 250 mm). Fatty acids were extracted by the method of Sasser (1990) and analysed by GC (Oliver & Colwell, 1973). Phospholipid analysis was carried out by TLC according to Minnikin *et al.* (1984).

Genomic DNA for DNA–DNA hybridization was extracted using a CTAB method, as described by Wang & Hong (2010). The DNA G + C content was determined by HPLC (Mesbah *et al.*, 1989). Levels of DNA–DNA relatedness were measured on nylon membranes using the method described by Wang *et al.* (2011).

Strain 210417^T exhibited white aerial mycelium on ISP 2, ISP 3, ISP 5, modified Bennett's agar (Jones, 1949) and glucose-yeast extract media. Good growth occurred on ISP 2, ISP 6, ISP 7 and glucose-yeast extract agar media; moderate growth was observed on ISP 1, ISP 3, nutrient agar and modified Bennett's agar media; and poor growth was seen on ISP 5 medium. No diffusible pigment was detected on any media tested. After approximately 14 days,

strain 210417^T showed morphological characteristics that are typical of the genus *Nonomuraea*, i.e. the formation of extensively branched aerial and substrate mycelia and spiral spore chains borne on the aerial mycelium. The spore chains of strain 210417^T were covered with rough sheaths (Fig. 1), which are similar to those of *N. bangladeshensis* JCM 13930^T but different from those of *N. coxensis* JCM 13931^T. In addition, the sheaths of *N. bangladeshensis* JCM 13930^T were smooth, whereas those of strain 210417^T were rough (Fig. 1). The physiological and biochemical characteristics of strain 210417^T are given in Table 1 and the species description.

Chemotaxonomic characteristics support the classification of strain 210417^T as a member of the genus *Nonomuraea*. Whole-cell hydrolysates yielded madurose as the diagnostic sugar. The cell-wall peptidoglycan contained *meso*-DAP and *N*-acetylmuramic acid (cell-wall chemotype III B; Lechevalier & Lechevalier, 1970). The predominant menaquinone was MK-9(H₄) (83 %). Small amounts of MK-9(H₆) (10 %), MK-9(H₂) (5 %) and MK-9(H₀) (2 %) were also detected. Large amounts of iso-C_{16:0} (24.1 %) in combination with C_{17:0} (12.5 %), 10-methyl C_{18:0} (TBSA; 12.3 %) and 10-methyl C_{17:0} (7.1 %) were present as major fatty acids; minor fatty acids were iso-C_{18:0} (6.8 %), iso-C_{15:0} (5.7 %), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c) (5.0 %) and C_{15:0} (3.6 %) (Supplementary Table S1 available in IJSEM Online). The diagnostic phospholipid phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME) and an unknown glucosamine-containing phospholipid (GlcNU) were detected (phospholipid type IV; Lechevalier *et al.*, 1977). The DNA G + C content was 72 mol%.

The almost-complete 16S rRNA gene sequence (1484 nt) of isolate 210417^T was compared with sequences of representatives of the genus *Nonomuraea*. Highest sequence similarities

Fig. 1. Scanning electron micrograph of spiral spore chains on aerial mycelia of *Nonomuraea wenchangensis* strain 210417^T grown on oatmeal agar (ISP 3) at 28 °C for 21 days. Bar, 1 µm.

Table 1. Comparison of the phenotypic properties of strain 210417^T and phylogenetically most closely related species

Strains: 1, 210417^T; 2, *N. coxensis* JCM 13931^T; 3 *N. bangladeshensis* JCM 13930^T. +, Positive; −, negative; ±, weakly positive. Data from this study except where marked.

Characteristic	1	2	3
Spore-chain morphology			
Spore chain*	S	Str, H†	S†
Spore ornamentation	Rough	Smooth†	Smooth†
Number of spores	6–10	12–17†	8–12†
Growth on ISP 3 medium			
Aerial mycelium	White	Pink to white†	Pale brown†
Substrate mycelium	Pale pink	Orange†	Pale brown†
Growth at 45 °C	−	+†	+†
pH range for growth	6–10	5–9†	5–9†
Major menaquinone(s)	MK-9(H ₄), MK-9(H ₆)	MK-9(H ₆), MK-9(H ₄)†	MK-9(H ₄)†
Nitrate reductase	+	+	−
Degradation of:			
Starch	−	+	+
Tyrosine	+	−	+
Production of:			
Urease	+	+	−
β-Galactosidase	+	−	−
Utilization of:			
L-Arabinose	+	−	−
Sucrose	+	+	±
D-Xylose	+	−	−
myo-Inositol	+	−	−
D-Mannose	−	+	+
D-Fructose	+	−	−
L-Rhamnose	+	+	+
Raffinose	+	−	−
Cellobiose	−	±	−

*H, Hooks, curled; S, spirals of one or two turns; Str, straight.

†Data from Ara *et al.* (2007).

were shown to the type strains of *N. coxensis* (99.6 %) and *N. bangladeshensis* (99.3 %). A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences indicated that strain 210417^T formed a monophyletic clade with *N. coxensis* JCM 13931^T and *N. bangladeshensis* JCM 13930^T (Fig. 2).

It has been reported that *Nonomuraea* species have high 16S rRNA gene sequence similarities (97.6–99.4 %), but low DNA–DNA relatedness values (Fischer *et al.*, 1983; Poschner *et al.*, 1985; Tamura *et al.*, 2000; Kämpfer *et al.*, 2005; Qin *et al.*, 2009). The type strains of *Nonomuraea africana*, *Nonomuraea dietziae* and *Nonomuraea recticatena* shared high 16S rRNA gene sequence similarities (within the range 98.9–99.8 %), whereas the highest DNA–DNA relatedness values between them have been reported as 45–48 % (Stackebrandt *et al.*, 2001). For these reasons, DNA–DNA hybridizations were only carried out between strain 210417^T, *N. coxensis* JCM 13931^T and *N. bangladeshensis* JCM 13930^T. Mean DNA–DNA hybridization values of 56.5 ± 2.3 % and 36.9 ± 1.9 % were observed for strain 210417^T with *N. coxensis* JCM 13931^T and *N. bangladeshensis* JCM 13930^T, respectively. These values are signifi-

cantly lower than the 70 % threshold for species delineation as recommended by Wayne *et al.* (1987).

Strain 210417^T can be distinguished from *N. coxensis* JCM 13931^T and *N. bangladeshensis* JCM 13930^T based on spore surface ornamentation, growth characteristics on ISP 3 medium (the colour of aerial and substrate mycelium), physiological and biochemical reactions (nitrate reduction, some degradation tests and carbon utilization), major menaquinones (Table 1) and fatty acid composition (Supplementary Table S1). Both phenotypic and genotypic data show that strain 210417^T differs from previously described species of the genus *Nonomuraea*. It is therefore proposed that strain 210417^T represents a novel species within the genus *Nonomuraea*, *Nonomuraea wenchangensis* sp. nov.

Description of *Nonomuraea wenchangensis* sp. nov.

Nonomuraea wenchangensis (wen.chan.gen'sis. N.L. fem. adj. *wenchangensis* of or pertaining to Wenchang, from where the type strain was isolated).

Fig. 2. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (1484 nt) showing relationships between strain 210417^T and all species of the genus *Nonomurea* with validly published names. *Thermopolyspora flexuosa* DSM 43186^T was used as an outgroup. Asterisks indicate branches that were also recovered using the maximum-parsimony (Fitch, 1971) tree-making algorithm. Bootstrap values are expressed as percentages of 1000 replications. Only bootstrap values of more than 50 % are shown. Bar, 0.005 substitutions per site.

Aerobic, Gram-positive, non-acid-fast actinomycete that forms highly branched substrate and aerial mycelia. After approximately 14 days, the aerial mycelium produces spiral spore chains, each containing 6–10 spherical spores that are covered with a rough sheath. Sporangia are not detected. The colour of the substrate mycelium on various agar plates is pale pink to dark brown. White aerial mycelia are observed on ISP 2, ISP 3, ISP 5, modified Bennett's agar and glucose-yeast extract media. Diffusible pigments are not produced. Grows at 20–37 °C and pH 6.0–10.0, with optimal growth occurring at 28 °C and pH 7.2. Growth occurs in the presence of 0–4 % (w/v) NaCl, but no growth is observed at 5 % (w/v) NaCl. Degrades casein and tyrosine, but not hypoxanthine, starch, xanthine or gelatin. Positive for aesculin hydrolysis, nitrate reduction, urease, gelatinase, arginine dihydrolase and β -galactosidase production, but negative for citrate lyase, lysine decarboxylase, ornithine decarboxylase and acetoin production. L-Arabinose, sucrose, D-xylose, *myo*-inositol, D-fructose, L-rhamnose and raffinose are utilized as sole carbon sources, but not D-mannose or cellobiose. *meso*-DAP is the diagnostic diamino acid in the peptidoglycan. Whole cell-hydrolysates contain madurose, galactose, glucose, mannose and ribose. The predominant menaquinone of the type strain is MK-9(H₄); small amounts of MK-9(H₆), MK-9(H₂) and MK-9(H₀) are also detected. The major

fatty acids are iso-C_{16:0}, C_{17:0}, 10-methyl C_{18:0} (TBSA), 10-methyl C_{17:0}, iso-C_{18:0}, iso-C_{15:0}, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1} ω 7c) and C_{15:0} (Supplementary Table S1). The polar lipids comprise PE, PME and GlcNU.

The type strain is 210417^T (=CGMCC 4.5598^T =DSM 45477^T), isolated from mangrove rhizosphere soil collected from Wenchang, Hainan, China. The DNA G+C content of the type strain is 72 mol%.

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