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ORIGINAL PAPER

Pseudonocardia nematodicida sp. nov., isolated from mangrove sediment in Hainan, China

Min Liu · Shan-shan Xing · Wei-dao Yuan · Hua Wei · Qian-guang Sun · Xiang-zhi Lin · Hui-qin Huang · Shi-xiang Bao

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Abstract Two aerobic, Gram-stain positive actinobacterial strains with nematicidal activity, designated HA11164^T and HA12591, were isolated from mangrove sediments in Hainan, China. Phylogenetic analysis based on the 16S rRNA gene sequences indicated that strains HA11164^T and HA12591 belong to the genus *Pseudonocardia* and are closely related to *Pseudonocardia carboxydivorans* (with the similarities of 98.30 and 98.24 %, respectively), *Pseudonocardia alni* (98.23 and 98.16 %, respectively) and *Pseudonocardia antimicrobica* (98.10 and 98.03 %, respectively). The major polar lipids of the strain HA11164^T, as a representative strain of the two strains, were found to consist of phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, five unidentified glycolipids and four unidentified polar lipids. The predominant menaquinone of strain HA11164^T was identified as MK-8 (H_4), and the major fatty acids were identified as iso-$C_{16:0}$, $C_{17:1}$ ω10, $C_{16:0}$ and $C_{16:1}$ ω9. The G+C content of strain HA11164^T was determined to be 74.9 mol%. The DNA–DNA relatedness values between strains HA11164^T and *P. alni*, *Pseudonocardia tropica*, *Pseudonocardia antarctica*, *P. carboxydivorans* and *Pseudonocardia parietis* were 58.3, 56.2, 50.0, 57.1 and 46.0 %, respectively. Based on the results of this polyphasic study, strains HA11164^T and HA12591 are considered to represent a novel species of the genus *Pseudonocardia*, for which the name *Pseudonocardia nematodicida* sp. nov. is proposed. The type strain is HA11164^T (=CGMCC 4.7118^T = DSM 45940^T).

Keywords *Pseudonocardia nematodicida* sp. nov. · 16S rRNA gene · Polyphasic taxonomy

Introduction

The genus *Pseudonocardia* first proposed by Henssen (1957) for nocardioform actinomycetes that lack mycolic acids and have a type IV cell wall, reflecting the presence of meso-diaminopimelic acid and the sugars arabinose and galactose (Evtushenko et al. 1989; Prabahar et al. 2004; Qin et al. 2008; Park et al.

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M. Liu · S. Xing · W. Yuan · H. Wei · Q. Sun · H. Huang (✉) · S. Bao (✉)
Key Laboratory of Biology and Genetic Resources of Tropical Crops of Ministry of Agriculture, Institute of Tropical Biosciences and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, Hainan, People's Republic of China
e-mail: huanghuiqin@itbb.org.cn

S. Bao
e-mail: baoshixiang@itbb.org.cn

X. Lin
Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, Fujian, People's Republic of China

2008; Schäfer et al. 2009; Qin et al. 2010), comprises 53 species at the time of writing (http://www.bacterio.net/pseudonocardia.html). Bacterial species of this genus have been isolated from various environments, such as soil (Henssen et al. 1983; Lee et al. 2002; Park et al. 2008; Qin et al. 2008; Ara et al. 2011), plant roots (Duangmal et al. 2009; Kaewkla and Franco 2011; Zhao et al. 2011), plant stem (Qin et al. 2010), coastal sediment (Liu et al. 2006), deep-sea sediment (Tian et al. 2013), industrial sludge (Mahendra and Alvarez-Cohen 2005) and a gold mine cave (Lee et al. 2001). Mangrove ecosystems, as highly productive wetlands distributed along estuaries, provide refuge, growing, breeding and feeding zones for many marine organisms (Holguin et al. 2001). During our study on isolation and diversity of bacteria with nematicidal activity from mangrove sediments, several novel strains were obtained. In this study, the taxonomic positions of novel *Pseudonocardia* strains isolated from mangrove sediments in Dongzhaigang Mangrove Forest were identified using a polyphasic approach.

Materials and methods

Isolation and maintenance of bacterial strains

Strains HA11164^T and HA12591 were isolated from sediments in *Bruguiera sexangula* and *Bruguiera gymnorrhiza* forest zones in Dongzhaigang Mangrove Forest (19°57′N, 110°35′E), respectively. 10 g air-dried sediment samples were heated at 55 °C for 6 min, then suspended in 100 mL sterile aged seawater and stirred for 30 min. Bacterial strains were isolated by the standard dilution-plating technique on modified Gause inorganic agar with 20 g soluble starch, 1 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 15 g agar, 1 L aged seawater (pH 7.2) (Gause et al. 1983). Plates were incubated at 28 °C for 7 days. Single colonies were picked from the plates and purified on Gause inorganic agar.

The bacterial cultures were kept temporarily on Gause inorganic slants and in glycerol suspensions (20 %, v/v) at –80 °C for permanent preservation. Strain HA11164^T has been deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany, as strain DSM 45940^T and in the China General Microbiological Culture Collection Center (CGMCC), China, as strain CGMCC 4.7118^T. *Pseudonocardia tropica* YIM 61452^T, *Pseudonocardia antarctica* DVS 5a1^T, *Pseudonocardia alni* DSM 44104^T and *Pseudonocardia parietis* 04-St-002^T were obtained from the DSMZ and *Pseudonocardia carboxydivorans* Y8^T from the Korean Culture Center of Micro-organisms (KCCM), and cultured under the same conditions for comparative analysis.

Morphological, physiological and biochemical characterization

Cultural characteristics, including the colour and growth of the aerial and substrate mycelia and the soluble pigments, were investigated after 7–15 days incubation at 28 °C on oatmeal agar (DSMZ medium 609), glycerol-asparagine agar (Shirling and Gottlieb 1966), Gause inorganic agar, inorganic salt starch (ISP 4) agar (Shirling and Gottlieb 1966), potato-dextrose agar (DSMZ medium 129) and on yeast extract-malt extract agar (ISP 2), respectively. The morphological characteristics of mycelia and spores were observed by light microscopy (DM6000B; Leica) and scanning electron microscopy (Phenom TM; Quantum Design). Gram staining was performed by using the Gram stain kit (Beijing Land Bridge). To test for aerotactic ability, cells were inoculated by mixing them with semisolid medium in tubes at 28 °C for 14 days. The temperature (4, 10, 15, 20, 25, 30, 35, 40, 42 °C) and pH (4–12, with 1 unit increments) range for growth, and the tolerance of NaCl [at 0–12 % with 1 % increments (w/v)], were examined on ISP 2 medium.

Antibiotic susceptibility tests were performed on ISP 2 medium by Sensi-Discs (6 mm; BBL) with the following antibiotics (μg per disc): ampicillin (10), chloromycetin (30), erythromycin (15), gentamycin (10), kanamycin (30), nalidixic acid (30), neomycin (30), novobiocin (30), rifampicin (5), tetracycline (30). The physiological and biochemical characteristics, such as utilisation of carbon source, gelatin liquefaction, H_2S and melanin production, catalase and oxidase activities, nitrate reduction, decomposition of starch, cellulose and milk were tested as previously described by Smibert and Krieg (1994).

For detection of nematicidal activities, each isolated bacterial strain was cultured in a liquid fermentation medium (yeast extract-malt extract broth) with shaking at 180 rpm at 28 °C. The fermentation broth was centrifuged and the supernatant was tested for

nematicidal activity using 24-well cell culture plates (Zeng et al. 2013). The target nematodes were *Meloidogyne Arenaria* and *Meloidogyne incognita*.

Chemotaxonomic characterization

For chemotaxonomic and molecular systematic studies, the strains were grown in yeast extract-malt extract broth at 28 °C with shaking cultivation for 5 days. Biomass was harvested by centrifugation, washed with sterile distilled water three times, re-centrifuged and freeze-dried. Polar lipids were analysed according to the previous methods (Minnikin et al. 1979; Collins and Jones 1980). Menaquinones were isolated according to Minnikin et al. (1984) and analysed by HPLC (Collins 1994).The fatty acid profile was determined by using GC/MS (model GC-2010; Shimadzu) (Kuykendall et al. 1988).

Phylogenetic analyses and DNA–DNA hybridization

Genomic DNA was extracted using a Bacterial Genomic DNA Isolation Kit (Foregene Biosciences, China) according to the manufacturer's protocol. The 16S rRNA genes of the strains were amplified using universal eubacterial primers 27 f (5′-AGAGTTTGATCATGGC TCAG-3′) and 1492 r (5′-GGTTACCTTGTTACGACT T-3′) (Weisburg et al. 1991). The 16S rRNA gene sequences were aligned with representative sequences of members of the genus *Pseudonocardia* obtained from the GenBank/EzBioCloud (http://www.ezbiocloud.net/; (Kim et al. 2012)) databases using the CLUSTAL X 1.8 program (Thompson et al. 1997). Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) using MEGA 5 program (Tamura et al. 2011) with bootstrap values based on 1000 replications (Felsenstein 1985).

The G+C content of DNA was determined by HPLC (Mesbah et al. 1989). DNA–DNA hybridization was carried out as described by Ezaki et al. (1989) and Christensen et al. (2000).

Results and discussion

Two bacterial strains were isolated from sediments in Dongzhaigang Mangrove Forest (strain HA11164^T from a *B. sexangula* forest zone and strain HA12591 from a *B. gymnorrhiza* forest zone). Both strains were found to have nematicidal activity. The strains were observed to be Gram-positive, aerobic, and to grow well on oatmeal agar, Gause inorganic agar, potato-dextrose agar and yeast extract-malt extract agar. On Gause inorganic agar, the aerial mycelia were observed to be white and the substrate mycelia to be pale yellow. The aerial mycelium was observed to fragment into rod-shaped spores with smooth surfaces (Supplementary Fig. S1). No soluble pigment was observed to be produced. The temperature and pH range for growth were determined to be 15–30 °C and 5–10, with the optimal growth at 25 °C and pH 7.0, respectively. Growth of the strains was observed at NaCl concentrations between 0 and 8 %, with an optimum of 3 %. Nitrate reduction was found to be negative. The strains are not able to degrade cellulose, gelatin and milk but starch was found to be hydrolysed. H_2S and melanin are not produced. Catalase and oxidase activities were found to be negative. The strains were found to utilise D-trehalose, inositol and starch but not L-arabitol, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, *a*-lactose, maltose, D-mannitol, D-mannose, D-raffinose, D-sorbitol or sucrose. The strains were found to be susceptible to ampicillin, chloromycetin, erythromycin, neomycin, novobiocin, kanamycin, rifampicin, and tetracycline, but resistant to gentamycin and nalidixic acid. Other detailed physiological and biochemical properties are given in Table 1 and the species description below.

The polar lipids of the strain HA11164^T, as a representative strain of the two strains, were found to consist of phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, five unidentified glycolipids and four unidentified polar lipids (Supplementary Figs. S2, S3). The predominant menaquinone of strain HA11164^T was identified as MK-8 (H_4). The major components of fatty acid (>10 %) were identified as iso-$C_{16:0}$ (44.8 %), $C_{17:1}$ ω10 (13.7 %), $C_{16:0}$ (10.5 %), and $C_{16:1}$ ω9 (10.2 %) (Supplementary Table S1). Some other characteristics and differential properties compared with phylogenetically closely related *Pseudonocardia* species are shown in Table 2.

Phylogenetic analysis indicated that strains HA11164^T and HA12591 share 99.7 % similarity of 16S rRNA gene sequence and are clustered into the same phylogenetic branch (Fig. 1). The two strains

Table 1 Physiological and biochemical properties that differentiate strains HA11164^T and HA12591 from their closely related species

Characteristics	1	2	3	4	5	6
NaCl range (%)	0–8	0–10	0–12	0–12	0–12	0–10
Temperature range (°C)	15–30	15–37	10–40	10–40	10–30	10–40
pH range	5–10	6–9	4–10	4–10	5–10	4–11
Utilization of						
L-arabitol	−	−	−	+	−	−
Cellobiose	−	+	−	+	−	−
D-fructose	−	+	−	+	+	−
D-galactose	−	+	+	+	+	−
D-glucose	−	+	+	+	+	+
Glycerol	−	+	+	+	+	+
Inositol	+	+	−	+	−	−
a-Lactose	−	+	+	+	w	+
Maltose	−	+	+	+	+	+
D-mannitol	−	+	w	+	+	+
D-mannose	−	+	+	+	+	+
D-raffinose	−	+	+	+	+	+
D-sorbitol	−	+	−	+	+	−
Starch	+	+	+	+	+	+
Sucrose	−	+	−	+	+	−
D-trehalose	+	+	−	+	+	+

All data from the present study

Strain 1 HA11164^T and HA12591, *2 Pseudonocardia tropica* YIM 61452^T, *3 Pseudonocardia antarctica* DVS 5a1^T, *4 Pseudonocardia alni* DSM 44104^T, *5 Pseudonocardia parietis* 04-St-002^T, *6 Pseudonocardia carboxydivorans* Y8^T

+ positive, − negative, *w* weak

Table 2 Chemotaxonomic properties that differentiate strain HA11164^T, as the representative strain, from closely related species

Characteristics	1	2	3	4	5	6
Menaquinone	MK-8 (H_4)	MK-8 (H_4)[a]	MK-8 (H_4)[a]	MK-8 (H_4)[a]	MK-8(H_4), MK-8(H_2)[a]	MK-9[a]
DNA G+C content (mol%)	74.9	72.4[a]	71.0[a]	72.0[a]	ND[a]	77.0[a]
Polar lipids	PME, PG, DPG, PC, PI, five unidentified glycolipids, four unidentified polar lipids	DPG,PC, PE, PG, PI, PIM[a]	PC, PI, PG, DPG, PE, PME[a]	PC, PME[a]	DPG, PME, PC, PG, PI, PIM, four unidentified glycolipids, four unidentified polar lipids and two unidentified phospholipids[a]	ND

Strain 1 HA11164^T, 2 *Pseudonocardia tropica* YIM 61452^T, 3 *Pseudonocardia antarctica* DVS 5a1^T, 4 *Pseudonocardia alni* DSM 44104^T, 5 *Pseudonocardia parietis* 04-St-002^T, 6 *Pseudonocardia carboxydivorans* Y8^T. *ND* not determined or no data available, *PME* phosphatidylmethylethanolamine, *PC* phosphatidylcholine, *PI* phosphatidylinositol, *DPG* diphosphatidylglycerol, *PE* phosphatidylethanolamine, PG phosphatidylglycerol, *PIM* phosphatidylinositol-mannosides

[a] Data are taken from Prabahar et al. (2004), Park et al. (2008), Evtushenko et al. (1989), Qin et al. (2010), Schäfer et al. (2009). All other data are taken from this study

were found to be closely related to members of the genus *Pseudonocardia*, and exhibited highest 16S rRNA gene sequence similarity to *P. carboxydivorans* (98.30 and 98.24 %, respectively), *P. alni* (98.23 and 98.16 %, respectively), *Pseudonocardia antimicrobica* (98.10 and 98.03 %, respectively), *P. parietis* (97.94 and 97.72 %, respectively) and *P. tropica* (97.31 and 97.24 %, respectively). The phylogenetic relationship between the strains HA11164^T and HA12591 and the other *Pseudonocardia* species was found in the neighbour-joining (Fig. 1), maximum parsimony (Supplementary Fig. S4) and maximum-

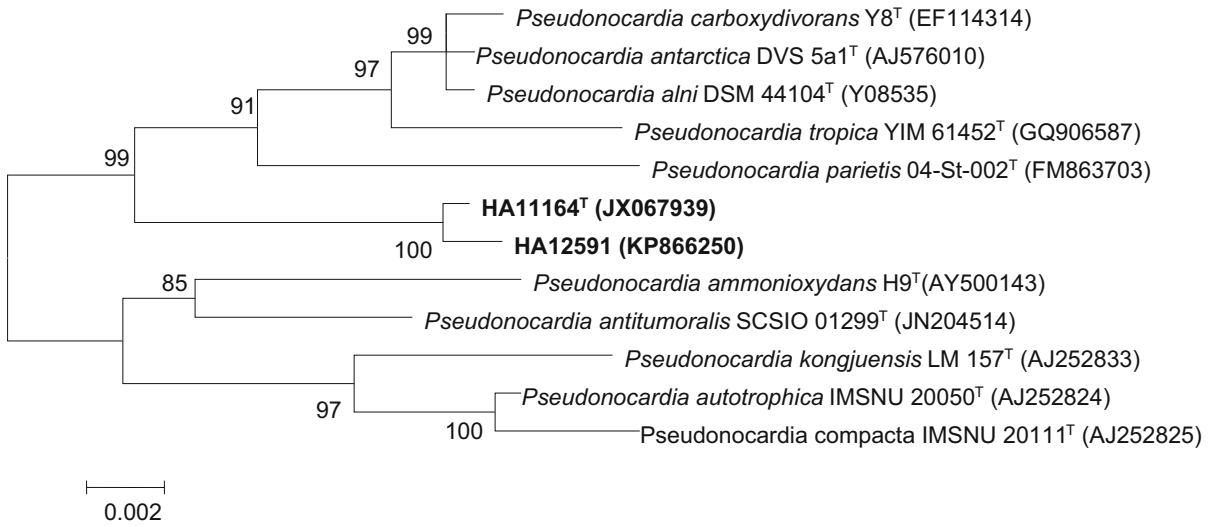

Fig. 1 Neighbour-joining tree based on 16S rRNA gene sequences, showing the relationships between strains HA11164^T, HA12591 and the type strains of the genus *Pseudonocardia*. Bootstrap values (1000 replicates) are shown as percentages at each node for values; values >50 % were shown. The *scale bar* represents 0.002 nucleotide substitutions per position

likelihood (Supplementary Fig. S5) trees. The three phylogenetic trees presented the same topology profile. Strains HA11164^T and HA12591 group with *P. carboxydivorans*, *P. alni*, *P. antarctica*, *P. tropica*, and *P. parietis* into one cluster. The phylogenetic analysis clearly shows that the isolated strains belong to the genus *Pseudonocardia*.

The G+C content of strain HA11164^T was determined to be 74.9 mol%. The DNA–DNA hybridization value between strains HA11164^T and HA12591 was 85.1 ± 3.3 %, which indicates that they are members of same species (Stackebrandt et al. 2002). The DNA–DNA relatedness values between strain HA11164^T and *P. alni, P. tropica, P. Antarctica, P. carboxydivorans* and *P. parietis* were 58.3 ± 2.3 % (mean ± SD of three hybridizations), 56.2 ± 3.1, 50.0 ± 3.6, 57.1 ± 2.8 and 46.0 ± 4.2 %, respectively. These hybridization values are clearly below the 70 % threshold that is considered the threshold for the delineation of separate prokaryotic species, as proposed by Wayne et al. (1987).

On the basis of physiological characteristics, polar lipid and fatty acid profiles, and DNA–DNA relatedness, the strain HA11164^T should be classified as representing a novel *Pseudonocardia* species, for which the name *Pseudonocardia nematodicida* sp. nov. is proposed. Strain HA12591 is considered to be a second strain of this species.

Description of *Pseudonocardia nematodicida* sp. nov

Pseudonocardia nematodicida (ne.ma.to.di.ci'da. N.L. pl. n. *Nematoda* nematodes; L. suff. -cida (from L. v. *caedere*, to kill), killer; N.L. n. *nematodicida* nematode-killing).

Aerobic, Gram-stain positive actinomycete. The aerial mycelia are white and the substrate mycelia are pale yellow on Gause inorganic agar. The aerial mycelia fragment into rod-shaped spores with smooth surfaces. Grows at 15–30 °C and pH 5–10, with 0–8 % (w/v) NaCl concentration. No soluble pigments are produced. Nitrate reduction, catalase and oxidase activities are negative. Cellulose, gelatin and milk are not degraded. H_2S and melanin are not produced. Starch is hydrolysed. D-Trehalose, inositol and starch cannot be utilised. The main polar lipids are phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, five unidentified glycolipids and four unidentified polar lipids. The major fatty acids (>10 %) are iso-$C_{16:0}$, $C_{17:1}$ ω10, $C_{16:0}$ and $C_{16:1}$ ω9, and the predominant menaquinone is MK-8 (H_4). The DNA G+C content of the type strain is 74.9 mol%.

The type strain HA11164^T (=CGMCC 4.7118^T = DSM 45940^T) was isolated from mangrove sediment in

Dongzhaigang Mangrove Forest, Hainan, China. The GenBank accession number for the 16S rRNA gene sequence of strain HA11164^T is JX067939.

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