Nonomuraea fusirolea sp. nov., an actinomycete isolated from the rhizosphere soil of rehmannia (Rehmannia glutinosa Libosch)

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A novel actinomycete, designated strain NEAU-dht8T, was isolated from the rhizosphere soil of rehmannia (Rehmannia glutinosa Libosch) and characterized using a polyphasic approach. The organism was found to have morphological and chemotaxonomic characteristics typical of the genus Nonomuraea. The G+C content of the DNA was 68.47 mol%. On the basis of 16S rRNA gene sequence similarity studies, strain NEAU-dht8T was most closely related to Nonomuraea maheshkhaliensis 16-5-14T (99.31 %), Nonomuraea kuesteri GW 14-1925T (98.77 %), Nonomuraea coxensis JCM 13931T (98.71 %), Nonomuraea wenchangensis 210417T (98.44 %), Nonomuraea bangladeshensis 5-10-10T (98.36 %) and Nonomuraea salmonea DSM 43678T (98.0 %); similarities to other species of the genus Nonomuraea were lower than 98 %.

Two tree-making algorithms based on 16S rRNA gene sequences showed that the isolate formed a phyletic line with its closest neighbour N. maheshkhaliensis 16-5-14T. However, the low level of DNA–DNA relatedness allowed the novel isolate to be differentiated from N. maheshkhaliensis 16-5-14T. Strain NEAU-dht8T could also be differentiated from other species of the genus Nonomuraea showing high 16S rRNA gene sequence similarity (98–98.77 %) by morphological and physiological characteristics. Thus, strain NEAU-dht8T is considered to represent a novel species of the genus Nonomuraea, for which the name Nonomuraea fusirolea sp. nov. is proposed. The type strain is NEAU-dht8T (=CGMCC 4.7104T =DSM 45880T).

The genus Nonomuraea, the name of which was corrected by Chiba et al. (1999) from the original spelling, Nonomuria, was originally proposed by Zhang et al. (1998) as a member of the family Streptosporangiaceae (Goodfellow et al., 1990; Stackebrandt et al., 1997). Members of the genus Nonomuraea are aerobic, Gram-staining-positive, non-acid-fast, non-motile actinomycetes that can 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 ornamentation (Quintana et al., 2003; Kämpfer et al., 2005). 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 (wall chemytype IIIB sensu Lechevalier & Lechevalier, 1970), di-, tetra- and hexa-hydrogenated menaquiones with nine isoprene units as predominant isoprenologues (Nonomura & Ohara, 1971; Zhang et al., 1998; Quintana et al., 2003), and major amounts of diphostatidylglycerol, hydroxylated phosphatidylethanolamine, uncharacterized glycolipids and a glucosamine-containing phospholipid (phospholipid type IV sensu Lechevalier & Lechevalier, 1970). The type species of the genus is Nonomuraea pusilla (Nonomura & Ohara, 1971). At the time of writing, the genus comprised 36 species with validly published names. As part of a programme to discover actinomycetes with novel antibiotic production properties, an aerobic actinomycete, strain NEAU-dht8T, was isolated. In this study, we performed a polyphasic taxonomic study on strain NEAU-dht8T, and propose that the isolate represents a novel species of the genus Nonomuraea.

Strain NEAU-dht8T was isolated from the rhizosphere soil of rehmannia (Rehmannia glutinosa Libosch) collected
from Shijiazhuang, Hebei Province, north China (38° 3′ N 114° 26′ E). The strain was isolated using the standard dilution plate method and grown on humic acid-vitamin agar (HV) (Hayakawa & Nonomura, 1987) supplemented with cycloheximide (50 mg l⁻¹) and nalidixic acid (20 mg l⁻¹). After 21 days of aerobic incubation at 28 °C, colonies were transferred and purified on oatmeal agar [International Streptomyces Project (ISP) 3 medium; Shirling & Gottlieb, 1966] and maintained as glycerol suspensions (20 %, v/v) at −80 °C.

The morphological properties of the isolate were observed by light microscopy (Nikon ECLIPSE E200) and scanning electron microscopy (Hitachi S-3400N) using cultures grown on ISP3 agar at 28 °C for 16 days. For cultural characterization, the isolate was grown for 14 days at 28 °C on various agar media (Table S1, available in the online Supplementary Material) as described by Waksman (1950, 1961), Shirling & Gottlieb (1966) and Asano & Kawamoto (1986). ISCC-NBS colour charts (Kelly, 1964) were used to determine the colours of substrate and aerial mycelia. Growth at different temperatures (4, 16, 18, 22, 28, 37 and 40 °C) was determined on ISP3 medium after incubation for 14 days. pH range (pH 4, 5, 6, 7, 8, 9, 10, 11 and 12) and NaCl tolerance (0, 1, 2, 3, 4, 5 and 6 %, w/v) for growth were determined in modified YEME medium (yeast extract, 3 g; sucrose, 103 g; tryptone, 5 g; malt extract, 3 g; glucose, 10 g; distilled water, 1 l; pH 7.2) at 28 °C for 7–14 days on a rotary shaker. Production of catalase, esterase and urease were tested as described by Smibert & Krieg (1994). Decomposition of cellulose, hydrolysis of starch and aesculin, reduction of nitrate, coagulation and peptonization of milk, production of H₂S and liquefaction of gelatin were assessed following the procedures described by Gordon et al. (1974) and Yokota et al. (1993). Utilization of carbohydrates as sole carbon sources was tested by using ISP medium 9 as a basal medium according to the method of Shirling & Gottlieb (1966). Nitrogen utilization experiment was performed as described by Williams et al. (1983). Production of melanin was examined using tyrosine agar (ISP medium 7; Shirling & Gottlieb, 1966).

Morphological observation of a 16-day-old culture of strain NEAU-dht8ᵀ grown on ISP3 agar revealed that it was consistent with those members of the genus Nonomuraea. Aerial and substrate mycelium were well-developed without fragmentation. Spiral spore chains (Fig. S1) of strain NEAU-dht8ᵀ were composed of about 5 to 11 non-motile spores (0.92 × 0.78 μm) with a smooth surface that were borne directly on aerial mycelia (Fig. 1). Sporangia were not detected. Good growth occurred on ISP2, ISP3, ISP6, ISP7, nutrient and Seino agar media; moderate growth on ISP4, glucose-yeast extract, water and Bennett agar; poor growth on glucose-asparagine agar; no growth on ISP5 agar. The substrate mycelium colour varied from pale yellow to strong brown on the media tested. White aerial mycelia and sporulation occurred on ISP3, ISP4 and water agar after 14 days of incubation at 28 °C. No diffusible pigment was observed on any of the media tested (Table S1). Strain NEAU-dht8ᵀ grew well between pH 6.0 and 9.0, with optimal growth at pH 7.0. The temperature range for growth was 16–37 °C, with the optimum temperature being 28 °C. Growth was observed in the presence of 0–2.0 % (w/v) NaCl. Detailed physiological and biochemical properties are presented in the species description.

Biomass for chemotaxonomic studies was prepared by growing strain NEAU-dht8ᵀ in modified YEME medium on a rotary shaker at 250 r.p.m. for 7 days at 28 °C; cells were harvested by centrifugation, washed twice with distilled water, recentrifuged and freeze-dried. The isomer of diaminopimelic acid in the cell wall peptidoglycan was analysed by an HPLC method using an Agilent TC-C₁₈ column (250 × 4.6 mm, internal diameter 5 μm) with a mobile phase consisting of acetonitrile/0.05 M phosphate buffer (pH 7.2) (15 : 85) at a flow rate of 0.5 ml min⁻¹. The peak detection used an Agilent G1321A fluorescence detector with a 365 nm excitation and 455 nm longpass emission filters (McKerrow et al., 2000). The whole-cell sugars were analysed according to the procedures developed by Lechevalier & Lechevalier (1980). Phospholipids in cells were examined by two-dimensional TLC and identified using the method of Minnikin et al. (1984). Menaquinones were extracted from freeze-dried biomass and purified according to the protocol of Collins (1985). Extracts were analysed by an HPLC-UV method using an Agilent Extend-C₁₈ column (150 × 4.6 mm, internal diameter 5 μm), typically at 270 nm. The mobile phase was acetonitrile/propyl alcohol (60:40, v/v), the flow rate was set to 1.0 ml min⁻¹ and the run time was 60 min. The injection volume was 20 μl, and the chromatographic column was controlled at 40 °C (Wu et al., 1989). Mycolic acids were checked by the acid methanolysis method as described by Minnikin et al. (1980). Cellular fatty acids were analysed by GC-MS using the method of Xiang et al. (2011).

Strain NEAU-dht8ᵀ contained meso-diaminopimelic acid as diamino acid. Whole-cell hydrolysates were found to contain glucose and madurose. The predominant
menaquinones detected were MK-9 (H₄) (53.93 %), MK-9 (H₂) (27.95 %), MK-8 (H₆) (9.43 %) and MK-9 (H₆) (8.69 %). The phospholipid profile was found to consist of diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, hydroxy-phosphatidylinositol, phosphatidylethanolamine and two unknown phospholipids (phospholipid type IV; Lechevalier & Lechevalier, 1970) (Fig. S2). Mycolic acids were not detected. The cellular fatty acid profile was determined to be composed of C₁₆ : 0 (39.08 %), C₁₈ : 0 (20.78 %), 10-methyl C₁₇ : 0 (17.17 %), 10-methyl C₁₆ : 0 (17.02 %), C₁₅ : 0 (4.64 %) and C₁₄ : 0 (1.31 %) (Fig. S3). Strain NEAU-dht8ᵀ shared many chemotaxonomic characteristics with other species of the genus *Nonomuraea*. However, the fatty acid profile of strain NEAU-dht8ᵀ was evidently different from those of other species of the genus *Nonomuraea* by the absence of iso-C₁₆ : 0. This result might be because of the GY medium used to culture cells for cellular fatty acid analysis. Another study in our lab also produced a similar result (Zhang et al., 2014).

Genomic DNA of strain NEAU-dht8ᵀ was extracted as described by Lee et al. (2003) and PCR amplification of the 16S rRNA gene was carried out using the method of Lqman et al. (2009). The PCR product was purified and cloned into the vector pMD19-T (Takara), sequenced by using an Applied Biosystems DNA sequencer (model 3730XL) and software provided by the manufacturer. The almost full-length 16S rRNA gene sequence (1510 nt) was obtained and aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using CLUSTAL x 1.83 software. The alignment was manually verified and adjusted prior to reconstruction of phylogenetic trees. Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Tamura et al., 2007) methods using MEGA software version 5.05 (Tamura et al., 2011). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses based on 1000 resamplings (Felsenstein, 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). 16S rRNA gene sequence similarities between strains were calculated on the basis of pairwise alignment using the EzTaxon-e server (Kim et al., 2012). DNA–DNA relatedness tests between strain NEAU-dht8ᵀ and *Nonomuraea maheshkhalienis* 16-5-14ᵀ and *Nonomuraea kuesteri* GW 14-1925ᵀ were carried out as described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in-situ temperature probe (Varian).

EzTaxon-e analysis of the 16S rRNA gene sequence revealed that strain NEAU-dht8ᵀ belonged to the genus *Nonomuraea*. The most closely related strains were *N. maheshkhalienis* 16-5-14ᵀ (99.31 % 16S rRNA gene sequence similarity), *N. kuesteri* GW 14-1925ᵀ (98.77 %), *Nonomuraea coxensis* JCM 13931ᵀ (98.71 %), *Nonomuraea wenchangensis* 210417ᵀ (98.44 %), *Nonomuraea bangladesensis* 5-10-10ᵀ (98.36 %) and *Nonomuraea salmonae* DSM 43678ᵀ (98.0 %); lower sequence similarities (<97.87 %) were found with the type strains of all other members of the genus *Nonomuraea* with validly published names. The phylogenetic tree (Fig. 2) based on 16S rRNA gene sequences showed that strain NEAU-dht8ᵀ formed a phyletic line with its closest neighbour *N. maheshkhalienis* 16-5-14ᵀ, an association that was supported by a bootstrap value of 64 % in the neighbour-joining tree and also recovered with the maximum-likelihood algorithm. However, strain NEAU-dht8ᵀ showed DNA relatedness values of 50.37 ± 2.21 % to *N. maheshkhalienis* 16-5-14ᵀ and 48.29 ± 0.82 % to *N. kuesteri* GW 14-1925ᵀ (based on a mean of three determinations), clearly well below the threshold value of 70 % recommended by Wayne et al. (1987) for assignment of strains to the same species. Besides the genotypic evidence, strain NEAU-dht8ᵀ could also be distinguished from its close relatives (similarity >98 %) by phenotypic characteristics (Table 1). For example, the spore chain arrangement, spore ornamentation, nitrate reduction, degradation of gelatin, hydrolysis of starch and utilization of sole carbon sources were clearly different between strain NEAU-dht8ᵀ and the most closely related type strains of the genus *Nonomuraea*.

The DNA G+C content of the genomic DNA was determined by the thermal denaturation (Tₘ) method as described by Mandel & Marmur (1968), and *Escherichia coli* JM109 was used as the reference strain. The genomic DNA G+C content of strain NEAU-dht8ᵀ was 68.47 ± 0.25 mol %, which is consistent with values seen for members of the genus *Nonomuraea*.

In conclusion, it is evident from the genotypic, chemotaxonomic and phenotypic data that strain NEAU-dht8ᵀ represents a novel species of the genus *Nonomuraea*, for which the name *Nonomuraea fuscirosea* sp. nov. is proposed.

**Description of *Nonomuraea fuscirosea* sp. nov.**

*Nonomuraea fuscirosea* (fus.ci.ro’se.a. L. adj. fuscus dark-coloured, brown; L. adj. roseus pink; N.L. fem. adj. fuscirosea brownish-pink).

Aerobic, Gram-staining-positive actinomycete that forms branched, non-fragmenting substrate mycelium. Abundant aerial mycelia are present on ISP3 agar. Spore chains are spiral with one or two turns (5–11 spores) and the spore (0.92 × 0.78 μm) surface is smooth. Sporangia are not found. No diffusible pigment or melanin is observed on any of the tested media. Temperature range for growth is 16–37 °C, with optimal growth at 28 °C. pH range for growth is 6.0–9.0, with optimal growth at pH 7.0. The NaCl tolerance range for growth is up to 2.0 % (w/v). Positive for production of catalase, hydrolysis of aesculin, decomposition of cellulose, nitrate reduction, milk coagulation and milk peptonization. Negative for production of...
urease, esterase and H₂S, liquefaction of gelatin and hydrolysis of starch. L-Arabinose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-mannitol, D-rafifinose, D-rhamnose and sucrose are utilized as sole carbon sources but inositol, D-fructose, D-ribose, D-sorbitol and D-xylose are not utilized. L-Alanine, L-asparagine, L-aspartic acid, L-creatine and L-serine are utilized as sole nitrogen sources but L-glutamine, L-threonine, L-tyrosine, L-arginine, glycine and L-glutamic acid are not. Cell walls contain meso-diaminopimelic acid as diagnostic diamino acid and the whole cell sugars are glucose and madurose. The predominant menaquinones are MK-9 (H₂) and MK-9 (H₄). The polar lipid profile contains diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, hydroxy-phosphatidylmonomethylethanolamine, hydroxy-phosphatidylethanolamine, phosphatidylinositol, diphosphatidylglycerol, phosphatidylethanolamine, hydroxy-phosphatidylmonomethylethanolamine, and phosphatidylinositol.

**Fig. 2.** Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the relationship between strain NEAU-dht8T and all the species of the genus Nonomuraea with validly published names. Thermopolyspora flexuosa DSM 43186T was used as an out-group. Asterisks indicate branches that were also recovered using the maximum-likelihood algorithm. Numbers at nodes indicate bootstrap percentages (based on 1000 replicates); only values above 50% are shown. Bar, 0.005 substitutions per nucleotide position.
The type strain, NEAU-dht8T (= CGMCC 4.7104T = DSM 45880T), was isolated from the rhizosphere soil of *Rehmannia glutinosa* (Libosch) collected from Shijiazhuang, Hebei Province, China. The DNA G+C content of the type strain is 68.47 ± 0.25 mol%.

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**References**


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