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ORIGINAL PAPER

Actinoallomurus bryophytorum sp. nov., an endophytic actinomycete isolated from moss (*Bryophyta*)

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Abstract A novel endophytic actinomycete, strain NEAU-TX1-15^T, was isolated from moss, collected from Wuchang, Heilongjiang province, north China. A polyphasic taxonomic study was carried out to establish the status of strain NEAU-TX1-15^T. Morphological and chemotaxonomic properties of strain NEAU-TX1-15^T are consistent with the description of the genus *Actinoallomurus*. Strain NEAU-TX1-15^T was observed to form short spiral or looped spore chains on aerial hyphae. The cell wall peptidoglycan was found to contain lysine and *meso*-diaminopimelic acid. The major menaquinones were identified as MK-9(H_6) and MK-9(H_8). The only phospholipid identified was phosphatidylglycerol. The major fatty acid was identified as iso-$C_{16:0}$. Analysis of the 16S rRNA gene sequence supports the assignment of the novel strain to the genus *Actinoallomurus*, as it exhibits 99.2 % gene sequence similarity to that of *Actinoallomurus yoronensis* NBRC 103686^T. However, the low level of DNA–DNA relatedness allowed the strain to be differentiated from its close relative. Moreover, strain NEAU-TX1-15^T could also be differentiated from *A. yoronensis* NBRC 103686^T and other *Actinoallomurus* species showing high 16S rRNA gene sequence similarity (>98.0 %) by cultural and physiological characteristics. Therefore, the combination of phenotypic and chemotaxonomic data, and the DNA–DNA hybridization value, indicated that strain NEAU-TX1-15^T represents a novel species of the genus *Actinoallomurus* for which the name *Actinoallomurus bryophytorum* sp. nov. is proposed. The type strain is NEAU-TX1-15^T (=CGMCC 4.7200^T = JCM 30340^T).

Keywords *Actinoallomurus bryophytorum* sp. nov. · Polyphasic taxonomy · 16S rRNA gene

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Introduction

The genus *Actinoallomurus* was first proposed by Tamura et al. (2009), with *Actinoallomurus spadix* designated as the type species of the genus. Members of the genus *Actinoallomurus* produce chains of

hooked or looped spores on aerial hyphae. The genus is characterised chemotaxonomically by the presence of *meso*-diaminopimelic acid and lysine in the cell wall, madurose as diagnostic sugar in the whole cell hydrolysates, phosphatidylglycerol and diphosphatidylglycerol as diagnostic phospholipids, and MK-9(H_6) and MK-9(H_8) as major menaquinones (Tamura et al. 2009; Thamchaipenet et al. 2010; Indananda et al. 2011). At the time of writing, the genus *Actinoallomurus* comprises 15 species with validly published names (http://www.bacterio.net/actinoallomurus.html).

As part of a programme to research the diversity of endophytic actinomycetes in moss and to discover novel actinomycetes, an aerobic actinomycete, strain NEAU-TX1-15^T, was isolated. In this study, we performed polyphasic taxonomy on this strain, and propose that strain NEAU-TX1-15^T represents a new species of the genus *Actinoallomurus*, named *Actinoallomurus bryophytorum* sp. nov.

Materials and methods

Isolation and maintenance of the organism

Strain NEAU-TX1-15^T was isolated from moss collected from Wuchang, Heilongjiang province, north China (44°15′N, 127°54′E). The plant sample was processed as described by Wang et al. (2013) and placed on a plate of humic acid-vitamin agar (Hayakawa and Nonomura 1987) supplemented with cycloheximide (50 mg l^{-1}) and nalidixic acid (20 mg l^{-1}). After 14 days of aerobic incubation at 28 °C, colonies were transferred and purified on International *Streptomyces* Project (ISP) 3 medium (Shirling and Gottlieb 1966) and maintained as glycerol suspensions (20 %, v/v) at −80 °C. The type strain of *Actinoallomurus yoronensis* was purchased from NITE Biological Resource Center (NBRC) and cultured under the same conditions for comparative analysis.

Morphological, cultural and physiological characteristics

Morphology properties were observed by light microscopy (Nikon ECLIPSE E200) and electron microscopy (Hitachi S-3400N) using cultures grown on ISP 3 agar at 28 °C for 5 weeks. Cultural characteristics were determined after 3 weeks at 28 °C using International *Streptomyces* Project (ISP) media 2–7 (Shirling and Gottlieb 1966), oatmeal-nitrate agar (JCM medium 52) and 1/10 yeast extract-starch (YS) agar (NBRC medium No.1053). The colours of colonies and soluble pigment were determined using the ISCC-NBS colour charts standard samples No 2106 (Kelly 1964). Growth at different temperatures (4, 10, 15, 20, 28, 37, 40 and 45 °C) was determined on ISP 3 medium after incubation for 21 days. The pH range for growth (pH 4–12, at intervals of 1 pH units) was tested in ISP 2 broth using the buffer system described by Xu et al. (2005), and NaCl tolerance was determined in ISP 2 broth supplemented with 1–7 % NaCl (w/v) at 28 °C for 21 days on a rotary shaker. Production of catalase, esterase and urease were tested as described by Smibert and Krieg (1994). The utilisation of sole carbon and nitrogen sources, decomposition of cellulose, hydrolysis of starch and aesculin, reduction of nitrate, peptonization and coagulation of milk, liquefaction of gelatin and production of H_2S were examined as described previously (Gordon et al. 1974; Yokota et al. 1993).

Chemotaxonomic characterization

Biomass for chemical studies was prepared by growing the strain in ISP 2 broth in Erlenmeyer flasks at 28 °C for 21 days. Cells were harvested by centrifugation, washed with distilled water and freeze-dried. The isomers of diaminopimelic acid (DAP) in the whole cell hydrolysates were derivatised according to McKerrow et al. (2000) and analysed by a HPLC method using an Agilent TC-C_{18} Column (250 × 4.6 mm i.d. 5 μm) with a mobile phase consisting of acetonitrile: 0.05 mol l^{-1} phosphate buffer pH 7.2 (15:85, v/v) at a flow rate of 0.5 ml min^{-1}. An Agilent G1321A fluorescence detector was used for peak detection with a 365 nm excitation and 455 nm longpass emission filters. The whole cell sugars were analysed according to the procedures developed by Lechevalier and Lechevalier (1980). The acyl type of muramic acids in the cell wall was analysed by using the method of Uchida and Aida (1984). The phospholipids 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 Collins (1985).

Extracts were analysed by a HPLC–UV method using an Agilent Extend-C_{18} Column (150 × 4.6 mm, i.d. 5 μm), typically at 270 nm. The mobile phase was acetonitrile: iso-propyl alcohol (60:40, v/v) and 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 previously (Minnikin et al. 1980). To determine cellular fatty acid composition, strain NEAU-TX1-15^T was cultivated in ISP 2 medium in shake flasks at 28 °C for 21 days. Fatty acid methyl esters were extracted from the biomass as described by Gao et al. (2014) and analysed by GC–MS using the method of Xiang et al. (2011).

DNA preparation, amplification and determination of 16S rRNA gene sequence

Extraction of chromosomal DNA and PCR amplification of the 16S rRNA gene sequence were carried out using a standard procedure (Kim et al. 2000). The PCR product was purified and cloned into the vector pMD19-T (Takara) and sequenced by using an Applied Biosystems DNA sequencer (model 3730XL). An almost full-length 16S rRNA gene sequence (1508 nt) was obtained. The EzTaxon server (Kim et al. 2012) was employed to identify the phylogenetic neighbours and calculate the pairwise 16S rRNA gene sequence similarities. The 16S rRNA gene sequence was aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using Clustal X 1.83 software. Phylogenetic trees were generated with the neighbour-joining (Saitou and Nei 1987) and maximum-likelihood (Felsenstein 1981) algorithms using molecular evolutionary genetics analysis (MEGA) software version 5.05 (Tamura et al. 2011). The stability of the clades in the trees was appraised using a bootstrap value of 1000 (Felsenstein 1985). A distance matrix was generated using the Kimura's two-parameter model (Kimura 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

DNA base composition and DNA–DNA hybridization

The G+C content of the genomic DNA was determined by the thermal denaturation method as described by Mandel and Marmur (1968), and *Escherichia coli* JM109 was used as the reference strain. DNA–DNA relatedness test between strain NEAU-TX1-15^T and *A. yoronensis* NBRC 103686^T was 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). The concentration and purity of DNA samples were determined by measuring the optical density at 260, 280 and 230 nm. The DNA samples used for hybridization were diluted to OD_{260} around 1.0 using 0.1 × SSC (saline sodium citrate buffer), then sheared using a JY92-II ultrasonic cell disruptor (ultrasonic time 3 s, interval time 4 s, 90 times). The DNA renaturation rates were determined in 2 × SSC at 70 °C. The experiments were performed with three replications and the DNA–DNA relatedness values were expressed as mean of the three values.

Results and discussion

The morphological characteristics of strain NEAU-TX1-15^T are consistent with those of members of the genus *Actinoallomurus*. Strain NEAU-TX1-15^T was observed to produce branched and non-fragmented well-developed substrate mycelium. The tips of the aerial mycelium divide into short hooked or looped spore chains with smooth surfaces at maturity (Fig. 1). Sporangia were not observed. Cultural characteristics of strain NEAU-TX1-15^T are given in Supplementary Table S1. Good growth was observed on ISP 3 and oatmeal-nitrate agar; moderate growth on ISP 2, ISP 5 and ISP 7 agar; no growth on ISP 4, ISP 6 agar and 1/10 yeast extract-starch (YS) agar. Sparce whitish aerial mycelium was found to be present on ISP 3 and oatmeal-nitrate agar. The colour of the substrate mycelium on the media tested was observed to vary from yellowish white to olive black. Soluble or melanoid pigments were not observed to be produced on all tested media. The growth of strain NEAU-TX1-15^T was observed at 20–37 °C (optimum 28 °C), pH ranges 6–8 (optimum pH 7) and in the presence of 0–1 % NaCl (w/v). Detailed physiological characteristics are presented in the species description.

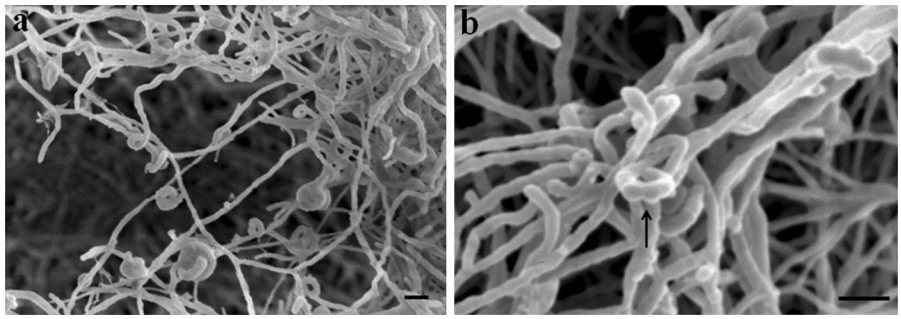

Fig. 1 Scanning electron micrograph of strain NEAU-TX1-15^T grown on ISP 3 agar for 5 weeks at 28 °C; *bar* 1 μm

The cell wall of strain NEAU-TX1-15^T was found to contain lysine and *meso*-diaminopimelic acid as the diamino acids of peptidoglycan. Whole cell hydrolysate was found to contain galactose and madurose. Phosphatidylglycerol was detected but diphosphatidylglycerol was not found (Supplementary Fig. S1). Strain NEAU-TX1-15^T was found to contain MK-9(H_6) (53.4 %) and MK-9(H_8) (40.2 %) as major menaquinones, along with a small amount of MK-9(H_2) (6.5 %). The major fatty acids were identified as iso-$C_{16:0}$ (32.8 %), $C_{17:1}\omega7c$ (14.2 %) and anteiso-$C_{17:0}$ (10.5 %) (Supplementary Table S2). The acyl type of the muramic acid was determined to be *N-acetyl*. Mycolic acids were not detected. It is evident that the chemotaxonomic characteristics of strain NEAU-TX1-15^T also support its classification as a member of the genus *Actinoallomurus*.

The almost-complete 16S rRNA gene sequence (1508 nt) of strain NEAU-TX1-15^T was determined and deposited as KJ425409 in the GenBank/EMBL/DDBJ databases. Based on analysis using EzTaxon-e, strain NEAU-TX1-15^T was affiliated to the genus *Actinoallomurus* and to be closely related to *A. yoronensis* NBRC 103686^T (99.2 %); lower sequence similarities (<99.0 %) were found with the type strains of all other members of the genus *Actinoallomurus* with validly published names. The phylogenetic tree based on 16S rRNA gene sequences showed that strain NEAU-TX1-15^T formed a distinct branch with its closest neighbour *A. yoronensis* NBRC 103686^T, supported by a bootstrap value of 96 % in the neighbour-joining tree (Fig. 2) and also recovered with the maximum-likelihood algorithm (Supplementary Fig. S2). However, DNA–DNA relatedness between strain NEAU-TX1-15^T and *A. yoronensis* NBRC 103686^T revealed a value of 47.2 ± 0.4 % (mean value of three hybridizations), which is well below the 70 % considered to be the threshold for assigning strains to the same prokaryotic species (Wayne et al. 1987). Comparison of cultural and physiological characteristics between strain NEAU-TX1-15^T and *A. yoronensis* NBRC 103686^T were performed to differentiate them. Differential characteristics include their clearly different colony colours after growth on ISP 3 and ISP 7 agar at 28 °C for 21 days (Supplementary Fig. S3), pH and temperature ranges of growth, liquefaction of gelatin, decomposition of urea and patterns of carbon and nitrogen utilisation (Table 1). In addition, a comparative study between strain NEAU-TX1-15^T and closely related type strains of the genus *Actinoallomurus* (>98.0 %) revealed that it differed from them in physiological and biochemical characteristics as summarised in Table 1.

In conclusion, it is evident from the genotypic and phenotypic data that strain NEAU-TX1-15^T represents a novel species of the genus *Actinoallomurus*, for which the name *Actinoallomurus bryophytorum* sp. nov. is proposed.

Description of *Actinoallomurus bryophytorum* sp. nov.

Actinoallomurus bryophytorum (bry.o.ph.y.to'rum. N.L. pl. n. *Bryophyta* name of a botanical phylum. N.L. gen. pl. n. *bryophytorum* of *Bryophyta*, referring to the isolation of the type strain from a member of the phylum *Bryophyta).*

Aerobic, Gram-stain positive actinomycete that produces a branched and non-fragmented well-developed substrate mycelium and forms short hooked or

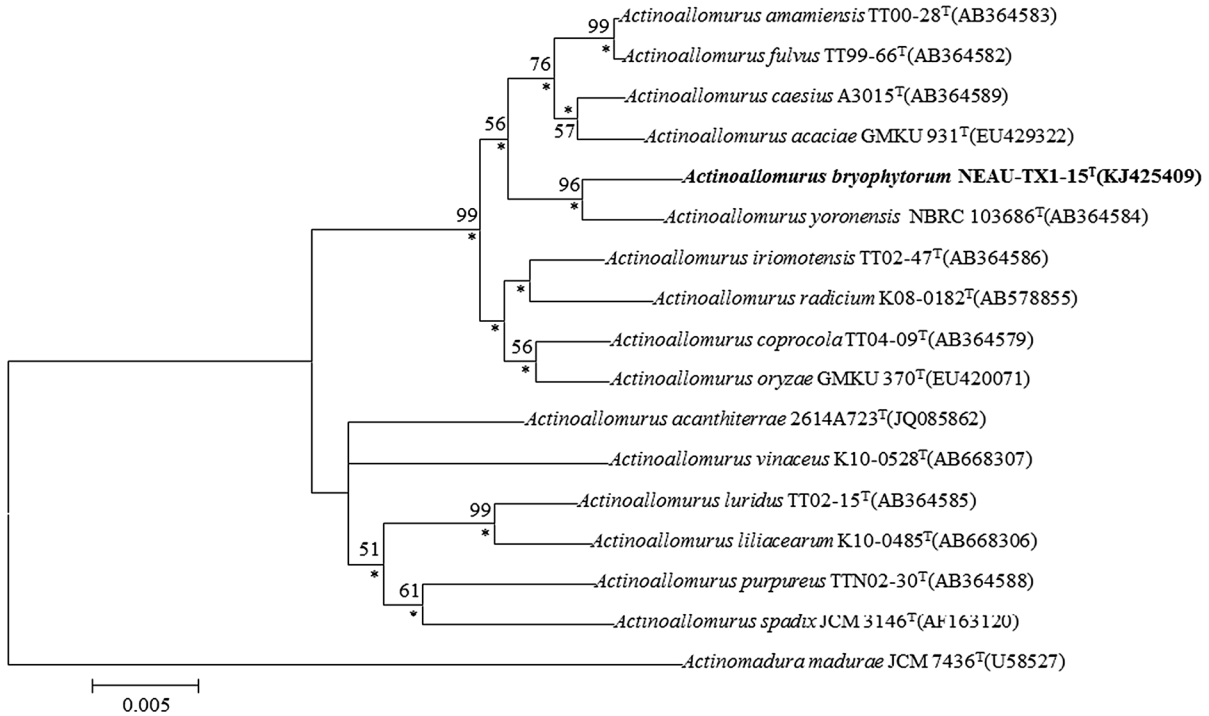

Fig. 2 Neighbour-joining tree based on 16S rRNA gene sequences (1306 nt) showing relationships between strain NEAU-TX1-15^T and members of the genus *Actinoallomurus*. The out-group used was *Actinomadura madurae* JCM 7436^T (U58527). Only bootstrap values above 50 % (percentages of 1000 replications) are indicated. *Asterisks* indicate branches also recovered in the maximum-likelihood tree. *Bar* 0.005 nucleotide substitutions per site

Table 1 Comparison of physiological characteristics of strain NEAU-TX1-15^T and closely related *Actinoallomurus* strains

Characteristics	1	2	3[a]	4[a]	5[a]	6[b]	7[a]	8[a]	9[c]	10[d]
Growth temperature range (°C)	20–37	15–37	20–37	20–37	20–37	12–41	20–37	20–37	21–45	12–36
pH range for growth	6–8	5–6	5–6	5–7	5–7	5–8	5–7	5–6	5–9	5–7
Tolerance of NaCl (%)	1	1	3	2	2	3	3	2	4	1
Hydrolysis of starch	+	+	+	+	−	W	+	+	−	−
Liquefaction of gelatin	−	+	+	+	+	−	+	+	W	+
Decomposition of urea	−	+	−	−	−	+	+	−	ND	−
Utilization of:										
L-arabinose	−	−	+	+	−	−	+	+	−	−
Inositol	+	−	−	+	−	+	+	+	+	−
Lactose	+	−	+	+	−	−	+	+	+	ND
D-mannitol	−	+	+	−	+	−	+	+	−	+

All of the other recorded properties were acquired in the present study

Strains 1 NEAU-TX1-15^T, *2 A. yoronensis* NBRC 103686^T, *3 A. amamiensis* TT00-28^T, *4 A. fulvus* TT99-66^T, *5 A. caesius* A3015^T, *6 A. acaciae* GMKU 931^T, *7 A. coprocola* TT04-09^T, *8 A. iriomotensis* TT02-47^T, *9 A. oryzae* GMKU 370^T, *10 A. radicium* K08-0182^T

+ positive; − negative, *W* weakly positive, *ND* no data available

[a] Data was taken from Tamura et al. (2009)

[b] Data was taken from Thamchaipenet et al. (2010)

[c] Data was taken from Indananda et al. (2011)

[d] Data was taken from Matsumoto et al. (2012)

looped spore chains with smooth surfaces on the tips of the aerial mycelium at maturity. Growth occurs in the pH range 6–8 with optimum growth at pH 7. Tolerates up to 1 % NaCl (w/v). The temperature range for growth is 20–37 °C, with the optimum temperature being 28 °C. L-Alanine, L-arginine, L-glutamine, proline, L-serine, L-threonine are utilised as sole nitrogen sources, but L-asparagine, L-aspartic acid, creatine, L-glutamic acid, glycine and L-tyrosine are not. D-Galactose, D-glucose, inositol, lactose, D-maltose, D-sucrose, D-raffinose are utilised as sole carbon sources, but L-arabinose, D-fructose, D-mannitol, D-mannose, D-ribose, L-rhamnose, D-sorbitol and D-xylose are not. Positive for hydrolysis of starch and aesculin, production of catalase and coagulation of milk but negative for decomposition of cellulose, hydrolysis of Tween 80 and urea, liquefaction of gelatin, production of H_2S and reduction of nitrate. The diagnostic amino acids of the cell wall are lysine and *meso*-diaminopimelic acid. The whole cell sugars are galactose and madurose. The major menaquinones are MK-9(H_6) and MK-9(H_8). The sole phospholipid is phosphatidylglycerol. The major fatty acids found are iso-$C_{16:0}$, $C_{17:1}\omega7c$ and anteiso-$C_{17:0}$ (>10 %). The acyl type of the muramic acid is *N-acetyl.* The DNA G + C content of the DNA of the type strain is 70.8 ± 0.3 mol%.

The type strain, NEAU-TX1-15^T (=CGMCC 4.7200^T = JCM 30340^T), was isolated from moss collected from Wuchang, Heilongjiang province, north China. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NEAU-TX1-15^T is KJ425409.

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