Microbispora bryophytorum sp. nov., an actinomycete isolated from moss (Bryophyta)

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A novel endophytic actinomycete, designated strain NEAU-TX2-2T, was isolated from moss and characterized using a polyphasic approach. The isolate was found to have morphological characteristics typical of the genus Microbispora. The isolate formed longitudinally paired spores on the tips of short sporophores that branched from aerial hyphae. Analysis of the 16S rRNA gene sequence supported the assignment of the novel strain to the genus Microbispora, and strain NEAU-TX2-2T exhibited 99.08 and 98.62% gene sequence similarities to Microbispora amethystogenes JCM 3021T and Microbispora rosea subsp. rosea JCM 3006T, respectively. However, two tree-making algorithms supported the position that strain NEAU-TX2-2T formed a distinct clade with M. rosea subsp. rosea JCM 3006T. A low level of DNA–DNA relatedness allowed the isolate to be differentiated from M. amethystogenes JCM 3021T and M. rosea subsp. rosea JCM 3006T. Moreover, strain NEAU-TX2-2T could also be distinguished from its closest phylogenetic relatives by morphological and physiological characteristics. Therefore, it is proposed that strain NEAU-TX2-2T represents a novel species of the genus Microbispora for which the name Microbispora bryophytorum sp. nov. is proposed. The type strain is NEAU-TX2-2T (=CGMCC 4.7138T=DSM 46710T).

The genus Microbispora was proposed by Nonomura & Ohara (1957) as a member of the family Streptosporangiaceae. Most members of the genus Microbispora produce longitudinally paired spores on aerial mycelia, but Microbispora mesophila JCM 3151T and Microbispora thailandensis NN276T are the only two members of the genus Microbispora that produce single spherical spores formed on short sporophores (Duanmal et al., 2012). At the time of writing, the genus Microbispora contains seven species with validly published names, namely Microbispora amethystogenes (Nonomura & Ohara, 1960; Miyadoh et al., 1990; Boondaeng et al., 2009), Microbispora corallina (Nakajima et al., 1999), Microbispora mesophila (Zhang et al., 1998), Microbispora siamensis (Boondaeng et al., 2009), Microbispora hainanensis (Xu et al., 2012) M. thailandensis (Duanmal et al., 2012) and Microbispora rosea with two subspecies Microbispora rosea subsp. rosea (an earlier heterotypic synonym of Microbispora chromogenes, Microbispora diastatica, Microbispora indica, Microbispora karna-
takensis and Microbispora parva) and Microbispora rosea subsp. aerata (an earlier heterotypic synonym of Microbispora thermodiastatica and Microbispora thermorosea) according to Miyadoh et al. (1990). Moss (Bryophyta) is a diverse group of land plants that usually colonize habitats with moist or extremely variable conditions, and inevitably biosynthesize secondary metabolites against biotic or abiotic stress (Xie & Lou, 2009; Singh et al., 2011). However, few studies have been carried out on the endophytes of mosses. As part of a programme to research the diversity of endophytic actinomycetes in moss and discover novel actinomycetes, an aerobic actinomycete, strain NEAU-TX2-2T, was isolated. In this study, we performed a polyphasic taxonomic analysis on this strain, and propose strain NEAU-TX2-2T as a representative of a novel species of the genus Microbispora.

Strain NEAU-TX2-2T was isolated from moss collected from Wuchang, Heilongjiang province, north China (44° 15' N 127° 54' E). The plant samples were processed as described by Wang et al. (2013) and incubated on humic acid-vitamin agar (HV) (Hayakawa & Nonomura, 1987) supplemented with cycloheximide (50 mg l⁻¹) and naldixic acid (20 mg l⁻¹). After 14 days of aerobic incubation at 28 °C, colonies were transferred and purified on
The morphological characteristics of strain NEAU-TX2-2^T were consistent with those of members of the genus Microbispora. Strain NEAU-TX2-2^T produced branched and non-fragmented, well-developed substrate mycelium. Longitudinal pairs of spores were borne on short sporophores branching from aerial hyphae. Non-motile spores (0.9–1.76 μm) were oval and the surface was smooth (Fig. 1). Sporangia were not observed. Cultural characteristics of strain NEAU-TX2-2^T are shown in Table S1 (available in the online Supplementary Material). Good growth was observed on ISP 2, ISP 3 and modified Bennett’s agar; moderate growth was observed on ISP 1, ISP 4, ISP 6 and nutrient agar; poor growth was observed on ISP 5 and ISP 7 agar. The colony colours varied from pinkish grey to dark reddish brown. Diffusible pigments or melanin were not formed on all media tested. Growth of strain NEAU-TX2-2^T was observed at 20–40 °C (optimum 28 °C), pH 6–10 (optimum pH 7) and in the presence of 0–2% (w/v) NaCl. Detailed physiological characteristics are presented in the species description.

Biomass for chemical studies was prepared by growing the strain in ISP 2 medium in Erlenmeyer flasks at 28 °C for 5 days. Cells were harvested by centrifugation, washed with distilled water and freeze-dried. The isomers of diamino-pimelic acid in the whole-cell hydrolysates were derivatized according to McKerrow et al. (2000) and analysed by an 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 365 nm excitation and 455 nm longpass emission filters. The whole-cell sugars were analysed according to the procedures developed by Lechevalier & Lechevalier (1980). 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 the protocol of Collins (1985). Extracts were analysed by an 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/propanol 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 methanalysis method as described by Minnikin et al. (1980). To determine cellular fatty acid compositions, strain NEAU-TX2-2^T was cultivated in ISP 2 medium in shake flasks at 28 °C for 5 days. Fatty acid methyl esters were extracted from the biomass as described by Gao et al. (2014) and were analysed by GC-MS using the method of Xiang et al. (2011).
The cell wall of strain NEAU-TX2-2T was observed to contain meso-diaminopimelic acid as the diagnostic diamino acid of the cell-wall peptidoglycan. The whole-cell hydrolysate was found to contain glucose and madurose. The phospholipids profile was found to consist of diphasphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylglycerol phosphate, phosphatidylserine, phosphatidylinositol, phosphatidylinositol mannose and phosphoglycolipid (phospholipid type IV sensu; Lechevalier et al., 1977) (Fig. S1). The predominant menaquinones were MK-9(H2) (40.3 %), MK-9(H4) (34.8 %) and MK-9(H6) (24.9 %). The fatty acids found were iso-C15:0 (29.3 %), 10-methyl C17:0 (22.7 %), C16:0 (13.5 %), C18:0 (13.3 %), 10-methyl C16:0 (8.8 %), C17:0 (5.6 %), 10-methyl C18:0 (3.7 %) and iso-C15:0 (3.3 %). Mycolic acids were not detected.

Extraction of chromosomal DNA and PCR amplification of the 16S rRNA gene 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 (1513 nt) was obtained. The EzTaxon-e server (Kim et al., 2012) was employed to identify the phylogenetic neighbours and calculate 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 & Nei, 1987) and maximum-likelihood (Felsenstein, 1985) algorithms using 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 Kimura’s two-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

The G + C content of the genomic DNA was determined by the thermal denaturation method as described by Mandel & Marmur (1968), and Escherichia coli JM109 was used as the reference strain. DNA–DNA relatedness tests between strain NEAU-TX2-2T and M. amethystogenes JCM 3021T and M. rosea subsp. rosea JCM 3006T 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 x 6 multicell changer and a temperature controller with in-situ temperature probe (Varian).

Based on analysis using EzTaxon-e, strain NEAU-TX2-2T was affiliated to the genus Microbispora, and was most closely related to M. amethystogenes JCM 3021T (99.08 %) and M. rosea subsp. rosea JCM 3006T (98.62 %). The phylogenetic tree based on 16S rRNA gene sequences showed that strain NEAU-TX2-2T formed a distinct branch with M. rosea subsp. rosea JCM 3006T that was supported by a bootstrap value of 69 % in the neighbour-joining tree (Fig. 2) and also recovered with the maximum-likelihood algorithm (Fig. S2). To determine whether strain NEAU-TX2-2T does indeed represent a novel genomic species, DNA–DNA hybridization was employed to further clarify the relatedness between strain NEAU-TX2-2T and M. amethystogenes JCM 3021T and M. rosea subsp. rosea JCM 3006T. The levels of DNA–DNA relatedness between strain NEAU-TX2-2T and these reference strains were 35.4 ± 0.4 and 24.7 ± 0.4 %, respectively. These values are lower than the 70 % threshold for species delineation recommended by Wayne et al. (1987).

In addition, strain NEAU-TX2-2T could be distinguished clearly from M. amethystogenes JCM 3021T and M. rosea subsp. rosea JCM 3006T by several cultural and physiological characteristics, such as the clearly different colony colours on ISP 1, ISP 2, ISP 3 and modified Bennett’s agar at 28 °C for 14 days (Fig. S3), temperature range for growth, starch hydrolysis, gelatin liquefaction, nitrate reduction, milk coagulation and utilization of L-arabinose, D-fructose and D-ribose (Table 1).

In conclusion, it is evident from the genotypic and phenotypic data that strain NEAU-TX2-2T represents a novel species of the genus Microbispora, for which the name Microbispora bryophytorum sp. nov. is proposed.

### Description of Microbispora bryophytorum sp. nov.

Microbispora bryophytorum (bry.o.phy.to’rum. N.L. gen. pl. n. bryophytorum pertaining to the botanical phylum Bryophyta).

Aerobic, Gram-staining-positive actinomycete that forms longitudinal pairs of spores branching from aerial hyphae. Non-motile spores (0.9–1.76 × 0.9–1.05 μm) are oval with a smooth surface. Grows well on ISP 2, ISP 3 and modified Bennett’s agar; moderately on ISP 1, ISP 4, ISP 6 and nutrient agar; poorly on ISP 5 and ISP 7 agar. Colony colour varies from pinkish grey to dark reddish brown. Diffusible pigments or melanin are not formed on all media tested. L-Arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-ribose and sucrose are utilized as sole carbon sources but inositol, raffinose, L-rhamnose, D-sorbitol and D-xylose are not utilized. L-Alanine, L-arginine, L-asparagine, L-aspartic acid, creatine, L-glutamic acid, L-glutamine, proline, serine, L-threonine and L-tyrosine can be used as sole nitrogen sources; glycine cannot. Growth occurs at pH 6–10 and with 0–2 % (w/v) NaCl, with optimum growth at pH 7 and with 1 % (w/v) NaCl. The temperature range for growth is 20–40 °C, with the optimum temperature being 28 °C. 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 H2S and reduction of nitrate. The diagnostic amino acid of the cell wall is meso-diaminopimelic acid and the whole-cell sugars are madurose and glucose. The major menaquinones are MK-9(H2), MK-9(H4), MK-9(H6), MK-9(H8) and MK-9(H10).
and MK-9(H₄). The phospholipid profile comprises diphasphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and phosphoglycolipid. Major fatty acids are iso-C₁₆:0, 10-methyl C₁₇:0, C₁₆:0 and C₁₈:0 (9.10 %).

The type strain is NEAU-TX2-2ᵀ (CGMCC 4.7138ᵀ | DSM 46710ᵀ), isolated from moss collected from Wuchang, Heilongjiang province, north China. The DNA G+C content of the DNA of the type strain is 70.4 ± 0.2 mol%.

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**Table 1.** Differential physiological characteristics of strain NEAU-TX2-2ᵀ and the most closely related species of the genus *Microbispora*

Strains: 1, NEAU-TX2-2ᵀ; 2, *M. amethystogenes* JCM 3021ᵀ; 3, *M. rosea* subsp. *rosea* JCM 3006ᵀ. All data are from this study. +, Positive; −, negative.

<table>
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<td>Use as sole carbon source</td>
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Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences (1382 nt) showing relationship between strain NEAU-TX2-2ᵀ, the type strains of species of the genus *Microbispora* and selected members of the family *Streptosporangiaceae* with which strain NEAU-TX2-2ᵀ shared gene sequence similarities > 97 % based on analysis using EzTaxon-e. The out-group used was *Actinomadura madurae* JCM 7436ᵀ (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.
References


