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ORIGINAL ARTICLE

Micromonospora zeae sp. nov., a novel endophytic actinomycete isolated from corn root (*Zea mays* L.)

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A novel actinomycete, designated strain NEAU-gq9^T, was isolated from corn root (*Zea mays* L.) and characterized using a polyphasic approach. The organism was found to have morphological and chemotaxonomic characteristics typical of the genus *Micromonospora*. On the basis of 16S rRNA gene sequence similarity studies, strain NEAU-gq9^T was most closely related to *Micromonospora zamorensis* CR38^T (99.3%), *Micromonospora jinlongensis* NEAU-GRX11^T (99.2%), *Micromonospora saelicesensis* Lupac 09^T (99.2%), *Micromonospora chokoriensis* 2-19(6)^T (98.9%), *Micromonospora coxensis* 2-30-b(28)^T (98.6%) and *Micromonospora lupini* Lupac 14N^T (98.5%). Phylogenetic analysis based on the 16S rRNA gene and *gyrB* gene demonstrated that strain NEAU-gq9^T is a member of the genus *Micromonospora* and supported the closest phylogenetic relationship to *M. zamorensis* CR38^T, *M. jinlongensis* NEAU-GRX11^T, *M. saelicesensis* Lupac 09^T, *M. chokoriensis* 2-19(6)^T and *M. lupini* Lupac 14N^T. A combination of DNA–DNA hybridization, morphological and physiological characteristics indicated that the novel strain could be readily distinguished from the closest phylogenetic relatives. Therefore, it is proposed that strain NEAU-gq9^T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora zeae* sp. nov. is proposed. The type strain is NEAU-gq9^T (=CGMCC 4.7092^T=DSM 45882^T).
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INTRODUCTION

Micromonospora proposed by Ørskov[1] is the type genus of the family *Micromonosporaceae* and has gradually been recognized as an important source of secondary metabolites.[2] Many antibiotics, such as gentamicin, netamicin, lomaiviticins A and B, tetrocarcin A and diazepinomicin have been isolated from this genus.[2,3] Thus, their impact on medicine is considerable. Endophytic *Micromonospora* species have recently been reviewed with respect to their broad distribution and their potential for use as probiotics.[4] In the last few years, six novel endophytic species of genus *Micromonospora* were isolated from different plants, including *M. coriariae*,[5] *M. lupini*,[6] *M. saelicesensis*,[6] *M. pisi*,[7] *M. avicenniae*[8] and *M. sonneratiae*.[9] Two novel anthraquinones, lupinacidins A and B with significant antitumor activity were isolated from *M. lupini*.[10] As part of a program to discover actinomycetes with novel antibiotic production properties, strain NEAU-gq9^T was isolated from corn root (*Zea mays* L.). In this study, the taxonomic status of this strain is reported on the basis of phylogenetic and phenotypic evidences. It is proposed that strain NEAU-gq9^T is a new species of the genus *Micromonospora*, for which the name *Micromonospora zeae* sp. nov. is proposed.

MATERIALS AND METHODS

Isolation and cultivation

Strain NEAU-gq9^T was isolated from corn root (*Zea mays* L.) collected from Harbin, Heilongjiang province, North China (45°45′ N, 126°41′ E). The corn root was tagged outdoors and stored in a clean plastic bag until used (~24 h). The root sample was air dried for 24 h at room temperature and then washed in water with an ultrasonic step (160 W, 15 min) (KH-160TDV, Hechuang, China) to remove the surface soils and adherent epiphytes completely. After drying, the sample was cut into pieces of 5–10 mm in length and then subjected to a seven-step surface sterilization procedure: a 60-second wash in sterile tap water containing cycloheximide (100 mg l^{-1}) and nalidixic acid (20 mg l^{-1}), followed by a wash in sterile water, a 5-min wash in 5% (v/v) NaOCl, a 10-min wash in 2.5% (w/v) $Na_2S_2O_3$, a 5-min wash in 75% (v/v) ethanol, a wash in sterile water and a final rinse in 10% (w/v) $NaHCO_3$ for 10 min. After being thoroughly dried under sterile conditions, the surface-sterilized samples were subjected to continuous drying at 100 °C for 15 min. The sample was then cut up in a commercial blender and ground with a mortar and pestle, employing 1 ml of 0.5 M potassium phosphate buffer (pH 7.0) per 100 mg tissue. Tissue particles were allowed to settle down at 4 °C for 20–30 min, and the supernatant was spread on a plate of humic acid-vitamin agar[11] 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

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transferred and purified on International *Streptomyces* Project (ISP) medium 3[12] and incubated at 28 °C for 2–3 weeks.

Morphological and physiological characteristics

Morphological properties were observed by light microscopy (Nikon ECLIPSE E200, Japan) and scanning electron microscopy (Hitachi S-3400N, Japan) using cultures grown on ISP 3 agar for 2 weeks at 28 °C. Cultural characteristics were determined by growth on SA1 agar,[13] N-Z amine agar,[6] Bennett's agar[14] and ISP media 2–7[12] at 28 °C for 2 weeks. The ISCC-NBS color charts were used to determine the names and designations of colony colors.[15] Growth at different temperatures (4, 16, 18, 22, 28, 37, 45 °C) was determined on ISP 3 agar after incubation for 2 weeks. pH range (pH 4, 5, 6, 7, 8, 9, 10) and NaCl tolerance (0, 1, 2, 3, 4, 5, 6, 7 and 8%, w/v) for growth were determined on GY medium[16] in shake flasks (250 r.p.m.) at 28 °C for 7 days. Nitrate reduction, starch hydrolysis, milk coagulation, cellulose decomposition, gelatin liquefaction, production of H_2S and catalase, degradation of urea and aesculin were examined as described by Gordon *et al.*[17] Utilization of sole carbon source was tested on ISP 9 medium.[12] Utilization of amino acids as nitrogen sources was tested as described by Williams *et al.*[18]

Chemotaxonomic analysis

The freeze-dried cells used for chemotaxonomic analysis were obtained from cultures grown in GY medium on a rotary shaker for 7 days at 28 °C. Cells were harvested by centrifugation, washed with distilled water and freeze-dried. The isomers of diaminopimelic acid in peptidoglycan were analyzed by a HPLC method using Agilent TC-C_{18} Column (250 × 4.6 mm i.d. 5 μm) (USA) 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}. The peak detection used was an Agilent G1321A fluorescence detector with a 365 nm excitation and 455 nm longpass emission filters.[19] The whole-organism sugars were analyzed according to the procedures developed by Lechevalier and Lechevalier.[20] Phospholipids in cells were examined by two-dimensional TLC and identified using the method of Minnikin *et al.*[21] Menaquinones were extracted from freeze-dried biomass and purified according to Collins.[22] The extracts were analyzed 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-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.[23] Mycolic acids were checked by the acid methanolysis method as described previously.[24] Biomass for fatty acids analysis was obtained by growing strain NEAU-gq9^T in GY medium on a rotary shaker for 5 days at 28 °C and the fatty acids were analyzed by GC–MS using the method of Gao *et al.*[25]

Phylogenetic analysis

Extraction of chromosomal DNA and PCR-mediated amplification of the 16S rRNA gene were carried out using a standard procedure.[26] The PCR product was purified and cloned into the pMD19-T (Takara, Dalian, China) vector and sequenced by using an Applied Biosystems DNA sequencer (model 3730XL) and software provided by the manufacturer. 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. Phylogenetic trees were generated with the neighbor-joining[27] and maximum-likelihood algorithms[28] using Molecular Evolutionary Genetics Analysis software version 5.05.[29] The stability of the clades in the trees was appraised using a bootstrap value with 1000 repeats.[30] A distance matrix was generated using the Kimura's two-parameter model.[31] All positions containing gaps and missing data were eliminated from the data set (complete deletion option). 16S rRNA gene sequence similarities between the type strains were calculated on the basis of pairwise alignment using the EzTaxon-e server.[32] PCR amplification of the *gyrB* gene sequence (1162 nt) was carried out using primers GYF1 and GYR3B[33] by the PCR program for 16S rRNA gene. Sequencing and phylogenetic analysis of *gyrB* gene were performed as described above. *Actinoplanes regularis* IFO 12514^T was used as an outgroup. The G + C content of the genomic DNA was determined by the thermal denaturation method as described by Mandel and Marmur,[34] and *Escherichia coli* JM109 was used as the reference strain. DNA–DNA relatedness tests between strain NEAU-gq9^T and *Micromonospora zamorensis* CR38^T, *Micromonospora jinlongensis* NEAU-GRX11^T, *Micromonospora saelicesensis* Lupac 09^T, *Micromonospora chokoriensis* 2-19(6)^T and *Micromonospora lupini* Lupac 14N^T were carried out as described by De Ley *et al.*[35] under consideration of the modifications described by Huss *et al.*[36] using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an in-situ temperature probe (Varian).

RESULTS AND DISCUSSION

Morphological and physiological characteristics

The morphological and cultural properties of strain NEAU-gq9^T are consistent with its classification as a member of the genus *Micromonospora*. Strain NEAU-gq9^T produced well-developed and branched substrate hyphae on ISP 3 medium. Nonmotile and oval spores (0.56–0.66 × 0.61–0.71 μm) were borne singly on the substrate mycelium and the spore surface was smooth (Figure 1). The novel isolate showed good growth on SA1, Bennett's, ISP 2, ISP 3 and ISP 6 agar, moderate growth on N-Z amine, ISP 4 and ISP 7 agar, and no growth on ISP 5 agar. Aerial mycelia were not detected on any of the tested media. The substrate mycelium of strain NEAU-gq9^T varied from orange to blackish red on all media tested (Supplementary Table S1). No diffusible pigment was detected. Strain NEAU-gq9^T was observed to grow well between pH 6.0–9.0, with an optimum pH of 7.0. The range of temperature was determined to be 18–37 °C, with the optimum growth temperature at 28 °C. Strain NEAU-gq9^T was observed to grow in the presence of 0–3% NaCl (w/v). Detailed physiological characteristics are presented in the species description.

Chemotaxonomic characteristics

Chemotaxonomic characteristics of strain NEAU-gq9^T also supported its classification as a member of the genus *Micromonospora*. Cells of strain NEAU-gq9^T were observed to contain *meso*-diaminopimelic acid and glycine as diagnostic amino acids. Whole-cell hydrolysates were found to contain xylose and glucose. The major menaquinones detected were MK-10(H_2) (42.3%), MK-10(H_4) (37.2%), MK-8(H_8) (13.7%) and MK-9(H_4) (6.8%). The phospholipid profile was found to consist of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol (Supplementary Figure S1). The cellular fatty acid profile was determined to be composed of $C_{16:0}$ (35.1%), $C_{17:1}\omega7c$ (19.0%), $C_{15:0}$ (14.1%), $C_{18:0}$ (7.4%), $C_{17:0}$ 10-methyl (5.7%), anteiso-$C_{17:0}$ (4.5%), $C_{18:1}\omega7c$ (4.2%), $C_{16:1}\omega7c$ (2.7%),

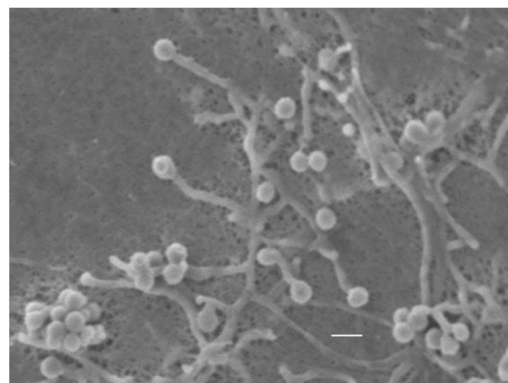

Figure 1 Scanning electron micrograph of spores on substrate mycelium of strain NEAU- gq9^T grown on ISP 3 agar for 2 weeks at 28 °C. Bar, 1 μm.

$C_{17:0}$ (2.4%), iso-$C_{17:0}$ (1.5%), $C_{18:1}\omega5c$ (1.2%), $C_{18:0}$ 10-methyl (1.0%), iso-$C_{14:0}$ (0.6%), $C_{14:0}$ (0.6%) and $C_{17:1}\omega8c$ (0.6%). The low proportions of iso-branched fatty acids were different from most *Micromonospora* species, but consistent with the description by Gao *et al.*[25] and Li *et al.*[37] Mycolic acids were not detected. The G + C content of the DNA was 68.8 ± 0.3 mol%.

Phylogenetic characteristics

The almost-complete 16S rRNA gene sequence (1510 nt) of strain NEAU-gq9^T was determined and deposited as KC287242 in the GenBank/EMBL/DDBJ databases. On the basis of EzTaxon-e analysis, the species most closely related to the novel isolate were *M. zamorensis* CR38^T (99.3%), *M. jinlongensis* NEAU-GRX11^T (99.2%), *M. saelicesensis* Lupac 09^T (99.2%), *M. chokoriensis* 2-19(6)^T (98.9%), *M. coxensis* 2-30-b(28)^T (98.6%) and *M. lupini* Lupac 14N^T (98.5%). The phylogenetic tree (Figure 2) based on 16S rRNA gene sequences showed that strain NEAU-gq9^T formed a distinct phyletic line with *M. zamorensis* CR38^T, *M. jinlongensis* NEAU-GRX11^T, *M. saelicesensis* Lupac 09^T, *M. chokoriensis* 2-19(6)^T and *M. lupini* Lupac 14N^T, an association that was supported by maximum-likelihood algorithm employed and by a 78% bootstrap value in the neighbor-joining analysis. The similarities of *gyrB* gene (1162 nt) between strain NEAU-gq9^T and *M. zamorensis* CR38^T, *M. jinlongensis* NEAU-GRX11^T, *M. saelicesensis* Lupac 09^T, *M. chokoriensis* 2-19(6)^T and *M. lupini* Lupac 14N^T were 98.6%, 98.4%, 96.7%, 95.5% and 97.6%, respectively. Phylogenetic analysis of *gyrB* gene sequence supported that strain NEAU-gq9^T was placed in the genus *Micromonospora*, also near to *M. zamorensis* CR38^T, *M. jinlongensis* NEAU-GRX11^T, *M. saelicesensis* Lupac 09^T, *M. chokoriensis* 2-19(6)^T and *M. lupini* Lupac 14N^T by a 78% bootstrap value (Supplementary Figure S2).

Further study showed that the DNA relatedness values between strain NEAU-gq9^T and *M. zamorensis* CR38^T, *M. jinlongensis* NEAU-GRX11^T, *M. saelicesensis* Lupac 09^T, *M. chokoriensis* 2-19(6)^T and *M. lupini* Lupac 14N^T were 51.8 ± 1.8, 41.3 ± 2.2, 48.5 ± 0.8, 53.0 ± 1.8% and 43.4 ± 2.9%, respectively. These values were below the threshold value of 70% recommended by Wayne *et al.*[38] for assigning strains to the same genomic species. In addition, a comparative study between strain NEAU-gq9^T and the five related *Micromonospora* type strains revealed that it differed from them in morphological, physiological and biochemical characteristics as summarized in Table 1 and Supplementary Figure S3. Therefore, strain NEAU-gq9^T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora zeae* sp. nov. is proposed.

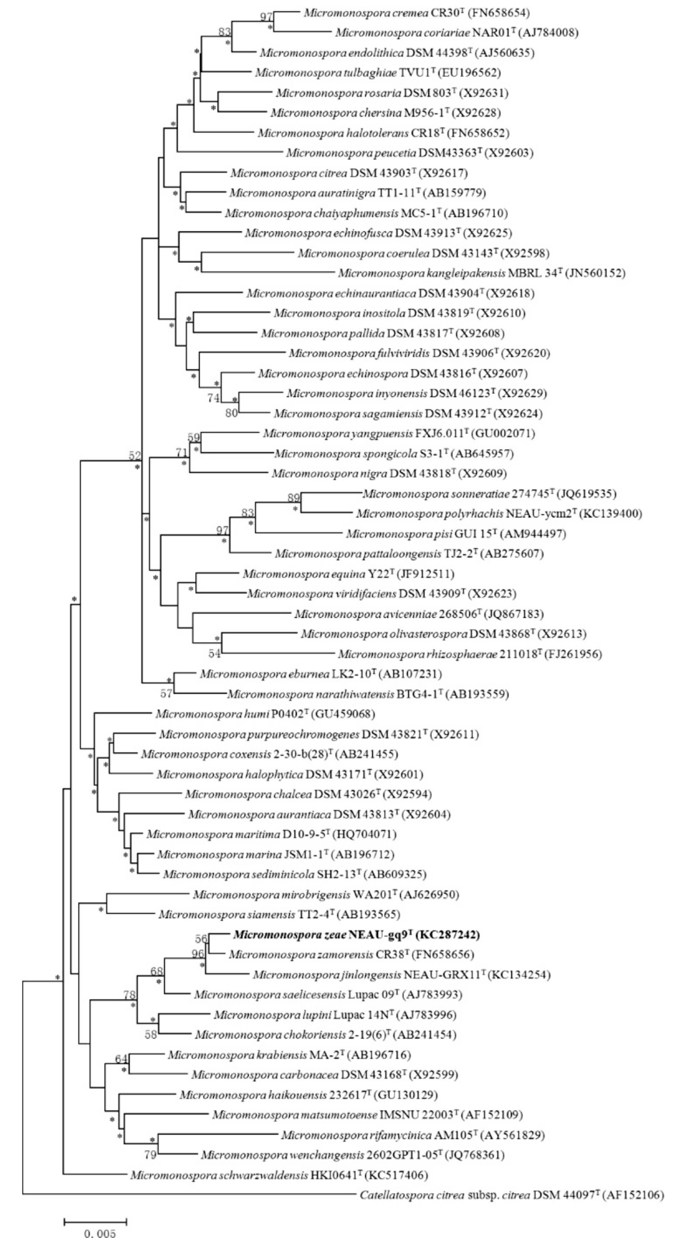

Figure 2 Neighbor-joining phylogenetic tree showing the position of strain NEAU-gq9^T (1510 nt) and the type strains of all other members of the genus *Micromonospora* based on 16S rRNA gene sequences. *Catellatospora citrea* subsp. *citrea* DSM 44097^T was used as an outgroup. Asterisks (*) indicate branches of the tree that were also recovered using the maximum-likelihood method. Bootstrap values > 50% (based on 1000 replications) are shown at branch points. Bar, 0.005 substitutions per nucleotide position.

Description of *Micromonospora zeae* sp. nov.

Micromonospora zeae (ze.ae. N.L. masc. n. *Zea*, botanical genus name; N.L. gen. n. *zeae*, of *Zea*, referring to the isolation of the organism from *Zea mays* L.).

Micromonospora zeae sp. nov. is aerobic, Gram-staining-positive actinomycete that forms well-developed and branched substrate hyphae. Colonies are orange color-series. No diffusible pigment is detected on any of the tested media. Single, nonmotile and oval spores (0.56–0.66 × 0.61–0.71 μm) with a smooth surface are produced. *M. zeae* is positive for gelatin liquefaction, aesculin hydrolysis, production of catalase; and negative for starch hydrolysis, milk coagulation, nitrate reduction, cellulose decomposition, urea degradation and production of H_2S. L-arabinose, D-fructose, D-galactose, D-glucose, lactose, D-maltose, D-mannose, D-mannitol, D-raffinose, L-rhamnose, D-sucrose and D-xylose are utilized as sole carbon sources but inositol, D-ribose and D-sorbitol are not utilized. L-alanine, L-arginine, L-aspartic acid, L-asparagine, creatine, L-glutamine, L-serine, L-threonine and L-tyrosine are utilized as sole nitrogen sources but glycine and L-glutamic acid are not utilized. Tolerates up to 3% NaCl and grows at temperatures between 18 and 37 °C, with an optimum temperature of 28 °C. Growth occurs at initial pH values between 6.0 and 9.0, the optimum being pH 7.0. The predominant menaquinones are MK-10(H_2) and MK-10(H_4). The major fatty acids (> 10%) are $C_{16:0}$, $C_{17:1}\omega7c$ and $C_{15:0}$. The G + C content of the DNA is 68.8 ± 0.3 mol%. The type strain is NEAU-gq9^T (= CGMCC 4.7092^T = DSM 45882^T), which was isolated from corn root (*Zea*

Table 1 Differential characteristics of strain NEAU-gq9^T and the closest related *Micromonospora* species

Characteristics	*1*	*2*	*3*	*4*	*5*	*6*
Color of streak plating/diffusible pigments on						
ISP 3	Blackish red/none	Deep brown/none	Very dark purple/ deep purple	Dark red/ moderate reddish orange	Moderate brown/ none	Brilliant orange yellow/none
ISP 7	Moderate brown/ none	Moderate orange/ brownish orange	Strong violet/none	Light orange yellow/ none	Light brown/none	Light orange yellow/none
SA1	Vivid orange/none	Strong brown/none	Moderate brown/ moderate violet	Moderate orange/ none	Dark brown/deep brown	Light orange/ light orange
Cellulose decomposition	−	−	−	+	+	+
Production of catalase	+	+	+	+	−	−
Use as sole nitrogen source:						
L-threonine	+	+	+	+	−	+
L-glutamic acid	−	−	+	+	−	−
Use as sole carbon source:						
D-raffinose	+	−	+	+	+	+
D-mannitol	+	−	+	−	+	−
D-fructose	+	−	−	+	+	+

Abbreviation: ISP, International *Streptomyces* Project.
Strains: 1, NEAU-gq9^T; 2, *M. zamorensis* CR38^T; 3, *M. jinlongensis* NEAU-GRX11^T, 4, *M. saelicesensis* Lupac 09^T; 5, *M. chokoriensis* 2-19(6)^T; 6, *M. lupini* Lupac 14N^T. +, positive; −, negative. All data are from this study.

mays L.). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *gyrB* gene of strain NEAU-gq9^T are KC287242 and KF053533, respectively.

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