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ORIGINAL PAPER

Micromonospora jinlongensis sp. nov., isolated from muddy soil in China and emended description of the genus *Micromonospora*

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Abstract A novel actinomycete, designated strain NEAU-GRX11^T, was isolated from muddy soil collected from a stream of Jinlong Mountain in Harbin, north China. The organism was found to have morphological and chemotaxonomic characteristics typical of the genus *Micromonospora*. The 16S rRNA gene sequence of strain NEAU-GRX11^T showed highest similarity to *Micromonospora zamorensis* CR38^T (99.2 %), *Micromonospora saelicesensis* Lupac 09^T (99.0 %), *Micromonospora chokoriensis* 2-19/6^T (98.7 %), *Micromonospora coxensis* 2-30-b/28^T (98.5 %), *Micromonospora aurantiaca* ATCC 27029^T (98.4 %) and *Micromonospora lupini* lupac 14N^T (98.3 %). Phylogenetic analysis based on the 16S rRNA gene and *gyrB* gene demonstrated that strain NEAU-GRX11^T was a member of the genus *Micromonospora* and supported the closest phylogenetic relationship to *M. zamorensis* CR38^T, *M. saelicesensis* Lupac 09^T, *M. chokoriensis* 2-19/6^T and *M. lupini* lupac 14N^T. A combination of DNA–DNA hybridization and some phenotypic characteristics indicated that the novel strain could be readily distinguished from these closest phylogenetic relatives. Therefore, it is proposed that NEAU-GRX11^T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora jinlongensis* sp. nov. is proposed. The type strain is NEAU-GRX11^T (=CGMCC 4.7103^T=DSM 45876^T).

Keywords *Micromonospora jinlongensis* sp. nov. · Polyphasic taxonomy · 16S rRNA gene

Ruixia Gao and Chongxi Liu contributed equally to this work.

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Introduction

The genus *Micromonospora* was first proposed by Ørskov (1923) for actinomycetes that formed single, non-motile spores directly on the substrate mycelium, but no aerial mycelia were produced. Subsequently, this genus was well classified on the basis of chemotaxonomic characteristics, *gyrB* and 16S rRNA gene sequence analyses (Kroppenstedt 1985; Koch et al. 1996; Kasai et al. 2000). *Micromonospora* are widely distributed in nature, inhabiting such disparate environments as soil, water, marine environments and plant tissues (Lũdemann and Brodsky 1963; Kawamoto 1989;

Mincer et al. 2002; Maldonado et al. 2009; Trujillo et al. 2010; Li et al. 2013a). Up to now, the genus *Micromonospora* comprises 56 species including recently described *Micromonospora sediminicola* (Supong et al. 2013a), *Micromonospora spongicola* (Supong et al. 2013b), *Micromonospora equina* (Everest and Meyers 2013), *Micromonospora maritima* (Songsumanus et al. 2013), *Micromonospora schwarzwaldensis* (Gurovic et al. 2013), *Micromonospora avicenniae* (Li et al. 2013a), *Micromonospora sonneratiae* (Li et al. 2013b), *Micromonospora halotolerans* (Carro et al. 2013) and *Micromonospora kangleipakensis* (Nimaichand et al. 2013). During the investigation of exploring potential sources of novel species and novel natural products, strain NEAU-GRX11^T was isolated from muddy soil collected from a stream of Jinlong Mountain in Harbin, north China. In this study, the taxonomic status of this strain is reported based on phylogenetic, chemotaxonomic and physiological evidences. It is proposed that strain NEAU-GRX11^T is a new species of the genus *Micromonospora*.

Materials and methods

Isolation and maintenance of the organism

Strain NEAU-GRX11^T was isolated from muddy soil collected from a stream of Jinlong Mountain in Harbin, north China (45°30′N, 127°06′E), using the standard dilution plate method and grown on humic acid-vitamin agar (HV) (Hayakawa and Nonomura 1987) supplemented with nystatin (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 and Gottlieb 1966) and maintained as glycerol suspensions (20 %, v/v) at −80 °C.

Morphological, cultural and physiological characteristics

Morphological properties were observed by light microscopy (Nikon ECLIPSE E200) and scanning electron microscopy (Hitachi S-3400 N) using cultures grown on ISP 3 agar for 21 days at 28 °C. Cultural characteristics were determined by growth on Czapek's agar (Waksman 1967), nutrient agar (Waksman 1961) and ISP media 2–7 (Shirling and Gottlieb 1966) at 28 °C for 14 days. The ISCC–NBS colour charts were used to determine the names and designations of colony colours (Kelly 1964). Growth at different temperatures (4, 10, 15, 18, 22, 28, 32, 37, 40, 45 °C) was determined on ISP 3 agar after incubation for 14 days. Growth tests for pH range (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) were carried out by using the buffer system described by Xie et al. (2012), and NaCl tolerance was determined in GY medium (Jia et al. 2013) supplemented with 0–6 % NaCl (w/v) at 28 °C for 7 days on a rotary shaker. Production of catalase, esterase and urease were tested as described by Smibert and Krieg (1994). The utilization of sole carbon and nitrogen, decomposition of cellulose, hydrolysis of starch and aesculin, reduction of nitrate, peptonization 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

The freeze-dried cells used for chemotaxonomic analysis were obtained from cultures grown in GY medium on a rotary shaker for 4 days at 28 °C. Cells were harvested by centrifugation, washed with distilled water and freeze-dried. The isomers of diaminopimelic acid (DAP) in peptidoglycan were analysed by HPLC method using 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 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 N-acyl group of muramic acid in peptidoglycan was determined by the method of Uchida et al. (1999). The whole-organism sugars were analyzed according to the procedures developed by Lechevalier and Lechevalier (1980). Phospholipids in cells were extracted and identified by using the method of Minnikin et al. (1984). Menaquinones were extracted from freeze-dried biomass and purified according to Collins (1985). Extracts were analyzed by HPLC–UV method using 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 (Wu et al. 1989). The presence of mycolic acids was checked by the acid methanolysis method as described previously (Minnikin et al. 1980). Cells used for cellular fatty acids analysis were obtained with the same cultural condition above, then cells were harvested by centrifugation (6,000 rpm, 10 min), soaked for 24 h and centrifuged (3,000 rpm, 10 min). The supernatant was concentrated and placed in test tubes. To each tube, 2 ml of 1 M NaOH in methanol was added, and the tubes were sealed with nitrogen gas, vortexed, and placed in a 70 °C water bath for 30 min. The tubes were cooled to room temperature, 3 ml of 20 % boron trifluoride was added, then the tubes were sealed, vortexed, and placed in water bath with the same condition above. The tubes were cooled to room temperature, 5 ml of saturated NaCl and 2 ml *n*-heptane was added and centrifuged (4,000 rpm, 10 min). The heptane phase was used for cellular fatty acids analysis. The composition was analysed by GC–MS using the method of Xiang et al. (2011).

DNA preparation, amplification and determination of 16S rRNA and gyrB gene sequences

Extraction of chromosomal DNA and PCR-mediated 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) and software provided by the manufacturer. Almost full-length 16S rRNA gene sequence (1,507 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 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 with 1,000 repeats (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). PCR amplification of the *gyrB* gene was carried out using primers GYF1 and GYB3 (Garcia et al. 2010) by the PCR program for 16S rRNA gene. Sequencing and phylogenetic analysis was performed as described above. *Actinoplanes regularis* IFO 12514^T was used as an outgroup.

DNA base composition and DNA–DNA hybridization

The G+C content of the genomic DNA was determined by the thermal denaturation (*Tm*) method as described by Mandel and Marmur (1968), and *Escherichia coli* JM109 DNA was used as the reference strain. DNA–DNA relatedness tests between isolate NEAU-GRX11^T and *Micromonospora saelicesensis* Lupac 09^T, *Micromonospora zamorensis* CR38^T, *Micromonospora chokoriensis* 2-19/6^T and *Micromonospora lupini* lupac 14N^T 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).

Results and discussion

The almost-complete 16S rRNA gene sequence (1,507 nt) of strain NEAU-GRX11^T was determined and deposited in the GenBank/EMBL/DDBJ databases as KC134254. Based on EzTaxon-e analysis, the species showing most closely related to the novel isolate were *M. zamorensis* CR38^T (99.2 %), *M. saelicesensis* Lupac 09^T (99.0 %), *M. chokoriensis* 2-19/6^T (98.7 %), *Micromonospora coxensis* 2-30-b/28^T (98.5 %), *Micromonospora aurantiaca* ATCC 27029^T (98.4 %) and *M. lupini* lupac 14N^T (98.3 %). The phylogenetic tree (Fig. 1) based on 16S rRNA gene sequences showed that strain NEAU-GRX11^T formed a distinct phyletic line with *M. zamorensis* CR38^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 (Supplementary Fig. S1) and by a 79 % bootstrap value in the neighbour-joining analysis. The similarity of *gyrB* gene (1,162 nt) between strain

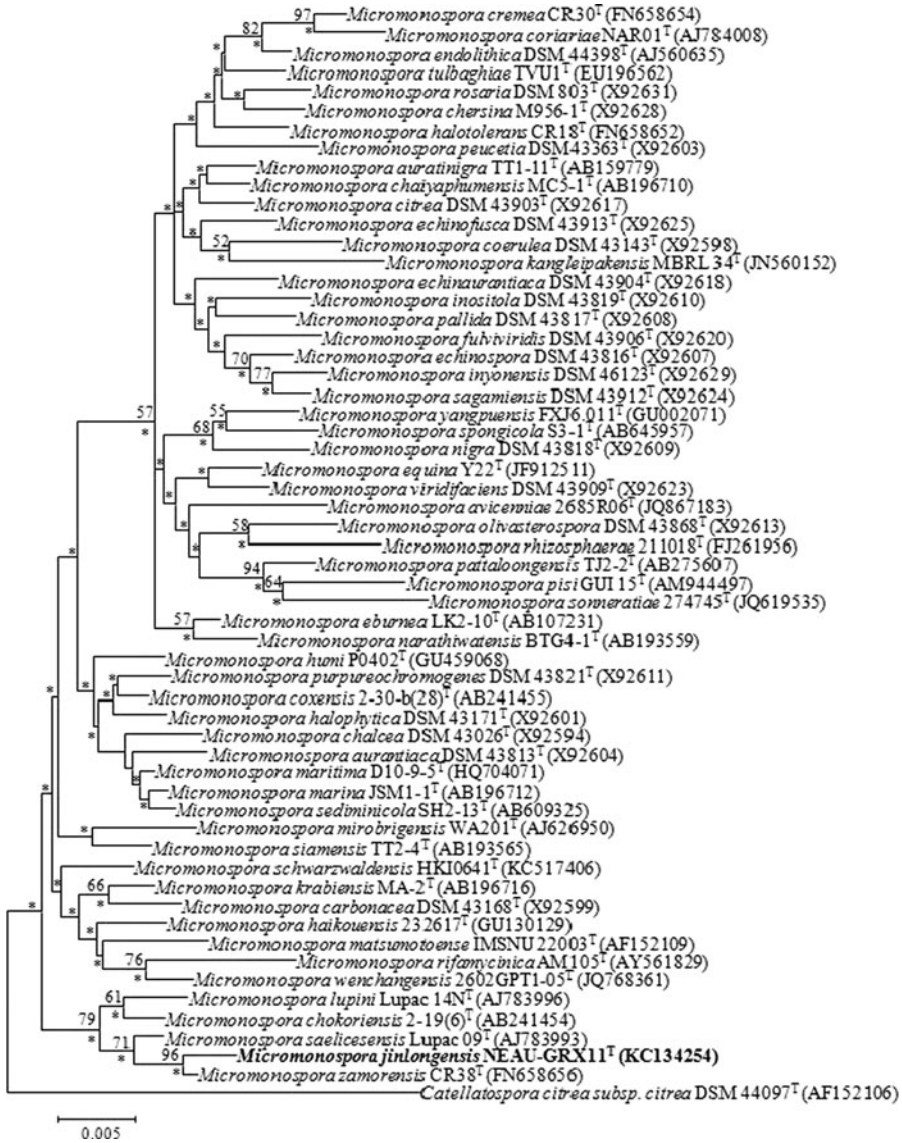

Fig. 1 Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequence (1,507 nt) showing the relationship between strain NEAU-GRX11^T and all species of the genus *Micromonospora*. *Catellatospora citrea* subsp. *citrea* DSM 44097^T was used as an outgroup. *Asterisks* indicate branches of the tree that were also recovered using the maximum-likelihood method. Bootstrap values >50 % (based on 1,000 replications) were shown at branch points. *Bar* 0.005 substitutions per nucleotide position

NEAU-GRX11^T and *M. zamorensis* CR38^T, *M. saelicesensis* Lupac 09^T, *M. chokoriensis* 2-19/6^T and *M. lupini* lupac 14N^T were 98.5, 96.7, 95.7 and 97.4 %, respectively. Phylogenetic analysis of *gyrB* gene sequence supported that strain NEAU-GRX11^T was placed in the genus *Micromonospora*, also near to *M. zamorensis* CR38^T, *M. saelicesensis* Lupac 09^T, *M. chokoriensis* 2-19/6^T and *M. lupini* lupac 14N^T by a 76 % bootstrap value (Supplementary Fig. S2). Further study showed that the DNA relatedness between strain NEAU-GRX11^T and *M. zamorensis* CR38^T, *M. saelicesensis* Lupac 09^T, *M. chokoriensis* 2-19/6^T and *M. lupini* lupac 14N^T were 55.5 ± 0.8, 34.6 ± 1.2, 57.3 ± 1.1 and 59.5 ± 2.0 %, respectively. These values were below the threshold value of 70 % recommended by Wayne et al. (1987) for assigning strains to the same species.

The morphological and cultural properties of strain NEAU-GRX11^T are consistent with its classification as a member of the genus *Micromonospora* (Kawamoto 1989). Strain NEAU-GRX11^T produced well-developed and branched substrate hyphae on ISP 3 medium. Non-motile and oval spores (0.6 × 0.8 μm) were borne singly on the substrate mycelium and the spore surface was smooth (Fig. 2). The novel isolate showed good growth on ISP 3 and ISP 6 agar,

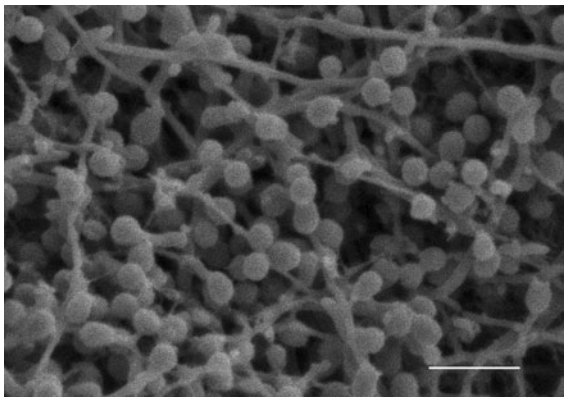

Fig. 2 Scanning electron micrograph of spores on substrate mycelium of strain NEAU-GRX11^T grown on ISP 3 agar for 21 days at 28 °C. *Bar* 2 μm

moderate growth on ISP 4 and ISP 7 agar, poor growth on Czapek's, nutrient and ISP 5 agar, and no growth on ISP 2 agar. The colour of colonies on different media was very dark purple (ISP 3), dark grayish purple (ISP 6), pale purple (ISP 5), strong orange yellow (ISP 4), pale yellow (ISP 7) and pale greenish yellow (nutrient and Czapek's agar). No aerial mycelium was seen on these media tested. Deep purple soluble pigment was produced on ISP 3 agar. Melanin was not observed on ISP 6 and ISP 7 agar. Strain NEAU-GRX11^T was observed to grow well between pH 6.0–9.0, with an optimum pH of 8.0. The temperature range for growth was determined to be 18–37 °C, with the optimum growth temperature at 28 °C. Strain NEAU-GRX11^T was observed to grow in presences of 0–3 % NaCl (w/v). Detailed physiological characteristics are presented in the species description.

Hydrolysates of strain NEAU-GRX11^T were found to contain glycine and *meso*-diaminopimelic acid (DAP). The whole-cell sugars were rhamnose, xylose and glucose (Supplementary Fig. S3). The acyl type of the cell wall polysaccharides was glycolyl. Mycolic acids were not detected. The predominant menaquinones were MK-9(H_4) (60.4 %), MK-9(H_6) (31.5 %) and MK-9(H_8) (8.1 %). The phospholipid profile consisted of diphosphatidylglycerol (DPG), phosphatidylmonomethylethanolamine (PME), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Supplementary Fig. S4), corresponding to phospholipid type PII of Lechevalier et al. (1977). The cellular fatty acid profile was determined to be composed of $C_{16:0}$ (39.3 %), $C_{15:0}$ (16.3 %), $C_{17:1}$ ω8c (11.8 %), $C_{18:0}$ (9.2 %), 10-methyl $C_{17:0}$ (8.6 %), $C_{17:0}$ (4.4 %), anteiso-$C_{17:0}$ (4.3 %), $C_{18:1}$ ω12c (2.9 %), $C_{16:1}$ ω9c (1.9 %) and $C_{13:0}$ (1.2 %) (Supplementary Fig. S5; Table S1). The DNA G+C content of the strain was 70.04 ± 0.21 mol%.

Strain NEAU-GRX11^T presented a phenotypic profile that clearly distinguished it from its closest phylogenetic neighbours, in particular soluble pigment on various media, hydrolysis of starch, urea hydrolysis and patterns of carbon and nitrogen utilization, and the phospholipids (presence of PME) and major cellular fatty acid compositions ($C_{16:0}$ and $C_{15:0}$) of strain NEAU-GRX11^T could differentiate it from its most related type strains of the genus *Micromonospora* (Table 1). Furthermore, a low level of DNA–DNA relatedness was observed between strain NEAU-GRX11^T and its closest phylogenetic relatives. It is evident from the genotypic and phenotypic data presented above that strain NEAU-GRX11^T is distinguishable from previously described *Micromonospora* species. Therefore, strain NEAU-GRX11^T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora jinlongensis* sp. nov. is proposed.

Emended description of the genus *Micromonospora*

All the descriptions are similar to that given by Ørskov (1923), Lechevalier et al. (1977), Kroppenstedt (1985) and Kawamoto (1989) except for the following changes. *Micromonospora* strains generally have large amounts of iso- and anteiso-fatty acids in their cell membranes, but $C_{16:0}$ and $C_{15:0}$ may be major fatty acids. The phospholipid profile may consist of PME.

Description of *Micromonospora jinlongensis* sp. nov

Micromonospora jinlongensis (jin.long.en′sis. N.L. masc. adj. *jinlongensis* of or pertaining to Jinlong, the name of a mountain in Harbin, Heilongjiang Province, north China, from where the type strain was isolated).

Aerobic, Gram-stain positive actinomycete that forms well-developed and branched substrate hyphae. Colonies are in the yellow and purple color-series. Grows well on ISP 3 and ISP 6 agar; moderately on ISP 4 and ISP 7 agar; poorly on Czapek's, nutrient and ISP 5 agar; no growth is observed on ISP 2 agar. Deep purple soluble pigment is produced on ISP 3 agar.

Table 1 Differential characteristics of strain NEAU-GRX11^T and the closest related *Micromonospora* species

Characteristics	1	2	3	4	5
Soluble pigment					
ISP 2	No growth	None	Light orange	Deep red	None
ISP 3	Deep purple	None	None	None	None
ISP 7	None	Light brown	None	None	None
Hydrolysis of starch	+	−	−	−	−
Urea hydrolysis	−	+	+	+	+
Liquefaction of gelatin	+	+	+	+	−
Production of catalase	+	+	+	−	−
Decomposition of cellulose	−	−	+	+	+
Growth at pH 5	−	−	+	−	−
Utilization of					
Use as sole carbon source					
D-Xylose	−	+	+	+	+
D-Maltose	+	−	−	+	+
D-Mannose	−	−	+	+	+
D-Raffinose	+	−	+	+	+
D-Sorbitol	+	−	−	−	−
D-Galactose	−	+	+	+	+
D-Fructose	−	−	+	+	+
Inositol	+	−	−	−	−
D-Mannitol	+	−	−	+	−
Use as sole nitrogen source					
Creatine	−	+	+	+	+
L-Alanine	−	+	+	+	+
L-Glutamic acid	+	−	+	−	−
Chemotaxonomic markers					
Major fatty acids (>5.0 %)	$C_{16:0}$, $C_{15:0}$, $C_{17:1}$ ω8c, $C_{18:0}$, 10-methyl $C_{17:0}$	$C_{17:1}$ ω8c, anteiso-$C_{15:0}$, 10-methyl $C^a_{17:0}$	iso-$C_{15:0}$, cis-8 $C_{17:1}$, iso-$C^b_{16:0}$	iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, $C^c_{17:0}$	iso-$C_{16:0}$, iso-$C^b_{15:0}$
Whole-cell sugars	Rha, Xyl, Glu	Glu, Man, Rib, Xyl[a]	Glc, Man, Ara, Xyl, Rib, Rha[b]	Rib, Man, Xyl, Gal, Glu, Ara[c]	Glc, Man, Ara, Xyl, Rib[b]
Phospholipids	DPG, PME, PE, PI	DPG, PE, PI[a]	DPG, PE, PI[b]	DPG, PE, PG, PI, PIMs[c]	DPG, PE, PI[b]
Menaquinones	MK-9($H_{4,6,8}$)	MK-10($H_{2,4,6,8}$), MK-9(H_4)[a]	MK-10($H_{4,6}$)[b]	MK-10($H_{4,6}$), MK-9($H_{4,6}$)[c]	MK-10($H_{4,6}$), MK-9(H_4)[b]

Data were obtained from this study, except as labeled

+ Positive; − negative; *Ara* arabinose; *Gal* galactose; *Glc* glucose; *Glu* glucose; *Man* mannose; *Rha* rhamnose; *Rib* ribose; *Xyl* xylose

Strains: 1, NEAU-GRX11^T; 2, *M. zamorensis* CR38^T; 3, *M. saelicesensis* Lupac 09^T; 4, *M. chokoriensis* 2-19/6^T; 5, *M. lupini* lupac 14N^T

[a] Data from Carro et al. (2012)

[b] Data from Trujillo et al. (2007)

[c] Data from Ara and Kudo (2007)

Single, non-motile and oval spores (0.6 × 0.8 μm) with a smooth surface are produced. Positive for production of catalase and esterase, hydrolysis of aesculin and starch and liquefaction of gelatin and negative for decomposition of cellulose and urea, reduction of nitrate, peptonization of milk and production of H_2S. D-Glucose, D-sorbitol, D-maltose, D-sucrose, D-mannitol, inositol, L-rhamnose, L-arabinose, D-raffinose and lactose are utilized as sole carbon sources but D-mannose, D-ribose, D-fructose, D-xylose and D-galactose are not utilized. L-Arginine, L-asparagine, L-glutamine, L-glutamic acid, L-aspartic acid, L-serine and L-threonine are utilized as sole nitrogen sources but creatine, L-alanine, glycine and L-tyrosine 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 8.0. Cell walls contain *meso*-diaminopimelic acid as diagnostic diamino acid and the whole cell sugars are rhamnose, xylose and glucose. The acyl type of the cell wall polysaccharides is glycolyl. Mycolic acids are not detected. The predominant menaquinones are MK-9(H_4) and MK-9(H_6). The phospholipid profile consists of DPG, PME, PE and PI. The predominant fatty acids are $C_{16:0}$, $C_{15:0}$, $C_{17:1}$ ω8c, $C_{18:0}$ and 10-methyl $C_{17:0}$ (>5.0 %). The DNA G+C content of the type strain is 70.04 ± 0.21 mol%.

The type strain is NEAU-GRX11^T (=CGMCC 4.7103^T=DSM 45876^T), which was isolated from muddy soil collected on Jinlong Mountain in Harbin, China. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *gyrB* gene of strain NEAU-GRX11^T are KC134254 and KF318646, respectively.

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