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Nesterenkonia ebinurensis sp. nov., a Novel Actinobacterium Isolated From *Populus euphratica*

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Abstract

During our studies focused on the microorganism diversity and community structure of *Populus euphratica* at Ebinur lake wetland nature reserve in Xinjiang Uyghur Autonomous Region, PR China, a Gram-positive, aerobic, short rod-shaped bacterium without flagellum, designated as MD2^T, was isolated from a piece of resina on *Populus euphratica.* The isolate grew at temperature of 10–45 °C (optimum 37 °C), pH of 7.0–12.0 (optimum pH 8.0) and NaCl concentration of 1–18% (optimum 3%, w/v). Based on the 16S rRNA gene sequences and the phylogenetic analysis, the strain shared the highest sequence similarities to *Nesterenkonia alkaliphila* JCM 19766^T (96.3%), *Nesterenkonia populi* KCTC 29119^T (95.9%), *Nesterenkonia alba* CCTCC AB 207011^T (95.5%), and was placed within the radiation of Nesterenkonia species in the phylogenetic trees. The draft genome of the isolate was sequenced, which comprised 3,739,891 bp with G+C content of 63 mol%, and was annotated to contain 3614 protein-coding genes, 44 tRNA genes and 5 rRNA genes. Chemotaxonomic analysis indicated that the main respiratory quinones were MK-8 and MK-9, the predominant cellular fatty acids were anteiso-$C_{17:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$, the major polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol andphosphatidylinositol. According to the phenotypic, chemotaxonomic and phylogenetic features, strain MD2^T is considered to represent a novel species, for which the name *Nesterenkonia ebinurensis* sp. nov. is proposed. The type strain is MD2^T (= KCTC 52999^T = MCCC 1K03343^T).

Introduction

The genus *Nesterenkonia*, a species-rich genus in the class *Micrococcaceae*, with *Nesterenkonia halobia* as the type strain, was originally proposed in 1995 by Stackebrandt et al. [1]. The description of this genus was later emended by Collins et al. in 2002 [2] and Li et al. in 2005 [3], respectively. At the time of writing, the genus *Nesterenkonia* contained 24 validly published species according to List of Prokaryotic names with Standing in Nomenclature (https://lpsn.dsmz.de/genus/nesterenkonia). Members within the genus are Gram-positive, non-spore-forming, short rod-shaped, aerobic, and distributed in various saline environments including saline soda lakes [4, 5], saline soils [6–8], pulp mills [9, 10], seafood [11] and ocean [12].

In this study, we described a novel species MD2^T, which has been isolated from a piece of resina on *Populus euphratica* at Ebinur lake in Xinjiang Uyghur Autonomous Region, in China. The strain is considered to represent a novel species of the genus *Nesterenkonia*, for which the name *Nesterenkonia ebinurensis* sp. nov. is proposed on the basis of the polyphasic taxonomic approach.

Materials and Methods

Isolation and Preservation

An alkaliphilic, moderate halophilic actinobacterium was isolated from a piece of resina on *P. euphratica* stands at the Ebinur lake (83°31′10″E 44°34′58″N) in June, 2016.The resina was covered by a thin layer of white salt and located near a wound, where briny water leaked out. The brackish resina was ground into powder, suspended and diluted by sterile water. According to the conventional dilution-plating method [14], serially diluted (tenfold dilutions each) samples were made and spread on the marine 2216 agar (MA; Difco) medium and incubated at 35–37 °C for up to 10 days, a light-yellow colony was picked and named MD2^T.After using repeated plate streaking on the same medium, the pure cultures were obtained. The strain was maintained at 4 °C on

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Luria–Bertani agar slants supplemented with 5% (w/v) NaCl at pH 8.0 (LB-NaCl) and preserved as aqueous glycerol suspensions (20%, v/v) at − 20 °C [15].The closely related type strains *N. alkaliphila* JCM 19766^T, *N. populi* KCTC 29119^T and *N. alba* CCTCC AB 207011^T were obtained from the Japan Collection of Microorganisms (JCM), the Korean Collection for Type Cultrues (KCTC) and the China Center for Type Culture Collection (CCTCC), respectively for the physiological, biochemical and chemotaxonomic characteristics comparison. Routine cultivation of strain MD2^T and reference type strains were performed on modified LB-NaCl agar.

Phenotypic Characterization

Morphological characteristics were observed after incubation at 37 °C for 5 days on LB-NaCl agar by transmission electron microscopy (JEM-1230; Jeol) after uranyl acetate (0.5%, w/v) staining under 80 kV and optical microscopy (BX40; Olympus) after Gram staining (Fig. S1). Anaerobic growth was determined with anaerobic system (AnaeroPack-MicroAero, 2.5l, MGC, Japan) using LB-NaCl agar, which 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite and 20 mM sodium nitrate were added as electron acceptors [16]. The motility of cells was tested by the semi-solid agar puncture method [14].

Temperature range for growth was tested at 4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 42, 45, 50 °C in LB medium (original pH 7.0). The pH range for growth (5–10, with interval of 0.5) was determined in LB medium using the appropriate biological buffers: citrate/phosphate (pH 5.0 and 5.5), MOPS (Sigma) (pH 6.0–8.0), boricacid/borax (pH 8.5 and 9.0) and borax/NaOH (pH 9.5 and 10.0), and each at a final concentration of 50 mM [17]. The salinity range supporting growth was determined in NaCl-free LB medium (according to LB formula, but without NaCl) at various NaCI concentrations (0–28%, w/v, with interval of 1%). The optimal growth conditions were measured after 5 days of incubation and the growth limits were tested after 14 days of incubation [18].

Catalase activity was determined by bubble production after the addition of a drop of 3% H_2O_2 to bacteria. Oxidase activity was detected using the API oxidase reagent (bioMe'rieux) according to the manufacturer's instructions. Degradation of starch, hydrolysis of Tween 20, 40, 60, 80, hypoxanthine, xanthine, nitrate reduction and urease activities, the ability to hydrolyze casein, chitin, carboxymethyl cellulose (CMC), filter paper and gelatin, H_2S production, methyl red and Voges-Proskauer reactions were tested as described previously [19]. Other enzyme activities were determined by using the API ZYM, API 20E and 20NE kits (bioMe'rieux), which acid production was tested using marine oxidation-fermentation (MOF) medium supplemented with 1% sugars [20]. Carbon or nitrogen source utilization were studied by using the GENIII MicroPlates (Biolog). Resistance to (μg/ml) streptomycin (10), kanamycin (30), chloramphenicol (50), lincomycin (2), ampicillin (10), cefalotin (30), penicillin (10), tetracycline (30), gentamicin (10), polymyxin B (300), novobiocin (5), neomycin (30) and rifampicin (25) in LB-NaCl agar medium was also evaluated [21].

Chemotaxonomic Characterization

Cells of strain MD2^T and reference strains were harvested during the third quadrants on LB agar medium (38 °C, 3d) for cellular fatty acids analysis. Cellular fatty acids were extracted and analyzed according to the standard protocol of the Sherlock Microbial Identification systemversion 6.0 [22] and the peaks were identified using the peak naming table TSBA6 6.00. A purified cell-wall preparation was obtained and was hydrolysed as described by Schleifer and Kandler [23], and the amino acid composition and whole-cell sugars were analysed according to the procedures developed by Hasegawa et al. [24]. For the analysis of respiratory quinones and the polar lipids, biomass was obtained when cells were lyophilized duringthe late-exponential phase. Polar lipids were extracted from 100 mg freeze-dried cell material by using two-stage method and the results were further analyzed according to Liu et al. [13]. Respiratory quinones were extracted as described by Collins et al. [25] and identified by HPLC analysis according to Minnikin et al. [26].

Molecular Characterization and Phylogenetic Analysis

Genomic DNA was extracted and purified by using a Wizard Genomic DNA purification kit (Promega). PCR was performed using universal primer pair 27F (5′-GAGAGTTTG ATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTT GTTACGAC-3′) to amplify the 16S rRNA gene. The purified PCR products were cloned into the vector pMD19-T (TaKaRa) and then sequenced.

The 16S rRNA gene sequence was used for pairwise sequence alignment performed by the BLASTN program (http://www.ncbi.nlm.nih.gov) and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net) [27]. Multiple sequences alignment was performed with CLUSTAL_W 1.8 program of the MEGA 6 package [28]. The evolutionary distances were calculated using the Kimura two-parameter model [29]. Phylogenetic trees were reconstructed from 1000 replicates each for bootstrap analysis with the neighbor-joining (Fig. 1), maximum-likelihood (Fig.S2) and minimum evolution (Fig.S3) methods by using MEGA6 version [28]. The

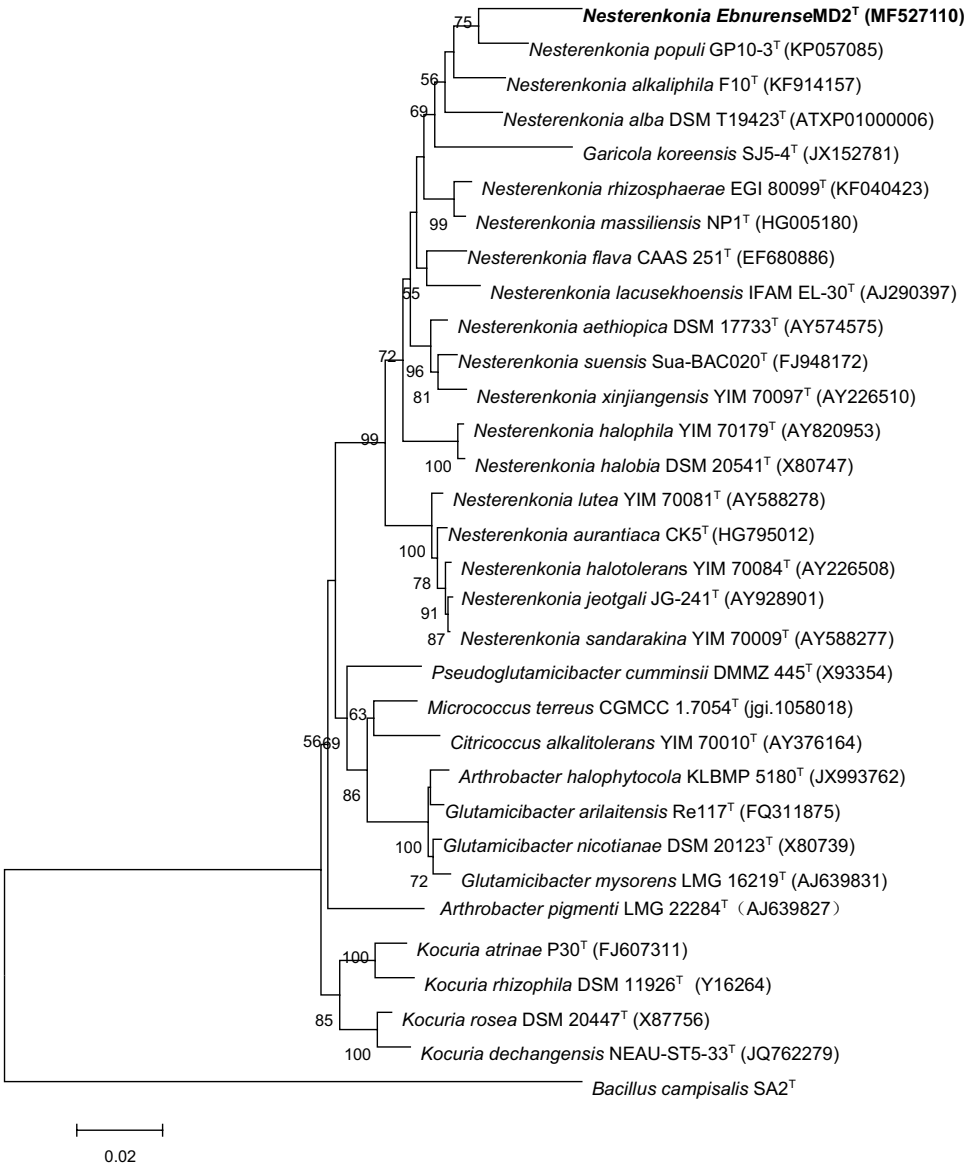

Fig. 1 Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences of strain MD2^T and representatives of related taxa. Bootstrap values were expressed as a percentage of 1000 replicates and only those higher than 50% were given at the branch points. Bar, 0.02 substitutions per nucleotide position

topology of the phylogenetic trees was assessed by the bootstrap analysis based on 1000 replications [30].

Genome Sequencing and Analysis

The genome of strain MD2^T was sequenced by Solexa PE150 sequencing technology with the HiSeq platform (Beijing Genomics Institute). The de novo assembly of the reads was performed using ABySS 1.5.2 [31]. The assembly k-value was tested from 32 to 64 to find the optimal one using abyss-prescript. The quality of microbial genomes was assessed using the bioinformatics tool CheckM 1.0.8 [32]. The gene prediction was performed by the Glimmer 3 (http://www.cbcb.umd.edu/software/glimmer/) with Hidden Markov models. The rRNA and tRNA genes were predicted using the RNAmmer [33] and tRNAscan-SE [34], respectively. For the annotation of the open reading frames (ORFs), the Rapid Annotation using Subsystem Technology (RAST) server online was used [35]. The function annotation was proceeded by using the BLAST searches of Non-Redundant (NR) protein sequences from the NCBI [36], Protein Families (Pfam) [37], a manually annotated and reviewed protein sequence (SwissProt) [38], Gene Ontology (GO) [39], Kyoto Encyclopedia of Genes and Genomes (KEGG) [40] and Clusters of Orthologous Groups (COG) [41] databases. The carbohydrate-active enzymes were detected by comparing against the Carbohydrate Active Enzymes (CAZy) database [42]. The DNA G + C content of strain MD2^T was calculated from the draft genome sequence.

Results and Discussion

Molecular Characterization and Phylogenetic Analysis

Phylogenetic analysis of strain MD2^T based on 16S rRNA gene sequences indicated that the novel strain belonged to the genus *Nesterenkonia* and exhibited 16S rRNA gene sequence similarities to the type strains *N. alkaliphila*JCM 19766^T of 96.3%, *N. populi* KCTC 29119^T of 95.9% and *N. alba* CCTCC AB 207011^T of 95.5%, respectively. In the neighbor-joining tree analysis, strain MD2^{T}fell within the clade of the genus *Nesterenkonia* and occupied a branch together with *N. populi* KCTC 29119^T (Fig. 1). In addition, with the almost same topological structure, the maximum-likelihood and minimum evolution trees also showed the closely phylogenetic relationships of strain MD2^T and the reference strains (Figs. S2, S3).

Morphological, Physiological, and Biochemical Analysis

Strain MD2^T, which has been isolated from a piece of resina on *P. euphratica*, a plant with saline-alkali tolerance and drought resistance. What is remarkable was that the references strain *N. populi* KCTC 29119^T has also been isolated from a piece of bark on *P. euphratica.* Detailed morphological, physiological, and biochemical characteristics of strain MD2^{T}were given in the species description of Table 1. Strain MD2^T shared most of features with other members of the genus *Nesterenkonia*. But there are some differences between the novel isolate and its closely related species, for instance, strain MD2^{T}were positive for gelatin, sorbitol, D-galactose, D-sorbitol, D-mannose, while other strains were not (Table 1).

Chemotaxonomic Characterization

The detailed fatty acid profiles of strain MD2^T and the reference strains were shown in Table S1. The fatty acid profiles obtained in this study for the reference strains were similar to those given in the original descriptions. As showed in Fig. S4, strain MD2^{T}polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, two unknown glycolipids and two unknown lipids. MK-8 and MK-9 were the predominant respiratory quinones in strain MD2^T. The amino acids lysine, alanine, glutamic acid, glycine, and aspartate were detected in the total hydrolysates of the cell-wall peptidoglycan, and galactose as major whole-cell sugars (minor amounts of glucose and ribose were also detected, and trace amounts or no mannose), (Supplementary Fig. S5, S6). This result was similar to those for the reference strains.

Genome Sequencing and Analysis

The draft genome of strain MD2^T was deposited at DDBJ/EMBL/GenBank under the accession number VWNF00000000. The genome completeness of strain MD2^T was 99.52% with 0.48% contamination, which considered as good reference genome for further analysis (≥ 95% completeness, ≤ 5% contamination) [43]. Draft genome sequence of strainMD2^T comprised 3,739,891 base pairs (bp) and produced 50 contigs with the largest contig of 652,652bpafter assembly. N50 value of the strain was 339,101 bp. The genomic DNA G + C was 63 mol%. A total of 3614 proteincoding genes were predicted. Among these, 3367 genes (94.9%) were annotated by querying the NR database. In addition, 2621 (73.9%), 2374 (66.9%), 2336 (65.8%), 1509 (42.5%) and 125 (3.4%) genes were annotated by querying the Pfam, SwissProt, GO, KEGG and CAZy databases, respectively. Among the 3614 coding sequences (CDSs), 2485 (70.0%) CDSs were classified into COG categories suggesting that approximate30% CDSs were cryptic. The major categories were transcription (11.4%), amino acid transport and metabolism (8.9%), inorganic ion transport and metabolism (7.2%), carbohydrate transport and metabolism (7.1%), energy production and conversion (6.2%), translation, ribosomal structure, and biogenesis (6.2%). The genomewas also annotated to contain 5 rRNA genes and 44 tRNA genes. The genomic features of strain MD2^T were shown in Table 2.

In conclusion, the results of the phylogenetic analysis, morphological and chemotaxonomic investigations strongly supported the affiliation of strain MD2^T to the genus *Nesterenkonia*. Many different characteristic features, such as source of isolation, biochemical characterization, fatty acid profiles and 16S rRNA gene sequence similarity could be used to distinguish the isolate from phylogenetically related taxa. Therefore, based on the results obtained in this study, strain MD2^T represents a novel species within the genus *Nesterenkonia*. Hence, *Nesterenkonia ebinurensis* sp. nov., with strain MD2^T as the type strain, is proposed.

Description of *Nesterenkonia ebinurensis*sp. nov.

Nesterenkonia ebinurensis (E.bi.nur.en'sis. N.L. fem. adj. e*binurensis* pertaining to Ebinur lake in Xinjiang Uyghur Autonomous Region, PR China, where the type strain was isolated).

Colonies on LB-NaCl agar medium arecircular, convex, smooth, entire and cream-coloured. Cells are Gram-stain-positive, aerobic, non-motile,

Table 1 Differential characteristics of strain MD2^T and type strains of its closest relatives in the genus *Nesterenkonia*

Characteristic	1	2	3	4
Morphology	Short rods	Short rods	Short rods	Short rods
Pigmentation	Light-yellow	White	Sulfur-yellow	White
Motility	−	+	−	−
Temperature	37	40	37	42
PH	7–12	7–12	8–12	8–12
NaCl	3–18	0–12	3–15	0–6
Oxidase activity	−	−	−	−
Gelatin	+	−	−	−
Sorbitol	+	−	−	−
Starch	−	−	−	+
Gelatin	+	+	+	+
Tween80	+	+	−	+
L-Arabinose	+	+	−	−
D-Glucose	−	−	W	+
Cellobiose	+	−	+	−
myo-Inositol	−	−	+	+
D-Fructose	+	−	+	−
D-Galactose	+	−	−	−
Raffinose	+	+	−	+
D-Sorbitol	+	−	w	−
D-Mannose	+	−	−	−
Lactose	w	−	−	−
Maltose	+	−	+	+
Esterase(C4)	W	+	+	+
D-Mannitol	−	+	−	+
Menaquinones	MK-8 MK-9	MK-8 MK-9	MK-7 MK-8 MK-9	MK-7 MK-8 MK-9
Fatty-acids	Anteiso-C17: 0 Anteiso-C15: 0	Anteiso-C17: 0 Anteiso-C15: 0	Anteiso-C1: 0 Anteiso-C15: 0	Anteiso-C17: 0 Anteiso-C15: 0
Polar lipid	PG, DPG, PI, GL	PG, DPG, PI, GL	DPG, PG, PI, GL	PG, DPG, PI, GL
Peptidoglycan type	L-Lys-Gly-L-Glu	L-Lys-Gly-L-Glu	L-Lys-L-Glu	L-Lys-Gly-D-Asp
DNA G + C content (mol %)	64.6	66.2	67.4	60.2
Habitat	Resina on *P. euphratica*	Deep-sea sediment	Bark on *P. euphratica*	Black liquortreatment system

Strain: 1 Strain MD2^T; 2 *N. alkaliphila*JCM 19766T; 3 *N. populi* KCTC 29119T; 4 *N. alba* CCTCC AB 207011; + Positive; − negative; *W* weak. Data for both MD2^T and *N. alkaliphila* JCM 19766T were obtained in this study. Data for other two reference strains were taken from [19, 13]

nonspore-forming,short-rod-shaped, alkaliphilic, moderately halophilic, approximately 0.2–0.5 µm width and 0.4–0.7 µm length. Temperature, pH and NaCl tolerance ranges for growth are 12–42 °C, pH 7.0–12.0 and 1–18% (w/v), respectively. Optimum growth occurs on LB medium supplemented with 3% (w/v) NaCl at 37 °C and pH 8.0. Positive for catalase, β-galactosidase, lipid esterase (C8), valine aromatase, naphthol-AS-BI-phosphate hydrolase, esculin hydrolase, Tween 80, gelatin and nitrate reduction. Negative for oxidase and urease, production of H_2S and indole, cellulose degradation,hydrolysis of starch. L-arabinose, cellobiose, D-fructose, D-galactose, raffinose, D-maltose, L-alanine, D-cellobiose and D-fucose can be utilized as carbon sources, but D-mannose, D-arabitol, L-arginine and D-salicin cannot be utilized. Acid is produced from D-xylose, rhamnose, mannitol and raffinose. The peptidoglycan structure is type A4a, L-Lys-Gly-L-Glu. The predominant menaquinones are MK-8 and MK-9. The main cellular fatty acids are anteiso-$C_{17:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylglycolipid, phosphatidylcholine, phosphatidylinositol, glycolipid, and an unidentified phospholipid. The genomic DNA G + C was 63 mol%.

The type strain is MD2^T (= KCTC 52999^T = MCCC 1K03343^T), which was isolated from a piece of resina on *Populus euphratica*, a plant with saline-alkaline tolerance

and drought resistance in Ebinur lake (44°25′N, 83°41′E), Xinjiang Province, China.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MD2^T is MF527110. The GenBank accession number for the whole genome sequence of strain MD2^{T}isVWNF01000000.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-021-02597-4.

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