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TAXONOMIC DESCRIPTION

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Nesterenkonia muleiensis sp. nov., a novel actinobacterium isolated from sap of *Populus euphratica*

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Abstract

A novel, Gram-stain-positive, aerobic, non-endospore-forming, non-motile and rod-shaped bacterium designated RB2^T was isolated from sap of *Populus euphratica* collected in Mulei county, Xinjiang province, PR China. RB2^T was able to grow at 10–45 °C (optimum 35 °C), pH 6.0–12.0 (optimum 8.0) and with 0–12% (w/v) NaCl (optimum 1 %). The genomic DNA G+C content was 63.5% (from the genome sequence). The results of the chemotaxonomic analysis indicated that the predominant isoprenoid quinones were MK-8 and MK-9. The major fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The major polar lipids of RB2^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and two glycolipids. The peptidoglycan type of RB2^T was A4α, L-Lys–Gly–L-Glu. The results of the phylogenetic analysis, along with the phenotypic and chemotaxonomic characteristics, indicate that strain RB2^T represents a novel species of the genus *Nesterenkonia*, for which the name *Nesterenkonia muleiensis* sp. nov. is proposed. The type strain is RB2^T (=MCCC 1K03528^T=KCTC 49017^T).

The genus *Nesterenkonia*, belonging to the family *Micrococcaceae*, was proposed by Stackebrandt *et al.*1995 [1] and the description of the genus was emended by Collins *et al.* [2] and Li *et al.* [3]. At the time of writing, the genus consists of 19 species with validly published names, and these species have been mainly isolated from alkaline lakes or soils, but have also been isolated from fermented seafoods, paper mill effluents, cotton pulp mill and plant tissues. Members of genus *Nesterenkonia* are non-spore-forming, rod-shaped bacteria. The DNA G+C contents of these strains ranged from 60.2 to 71.5 mol%. These members have A4α-type peptidoglycan with L-Lys–Gly–L-Glu, L-Lys–L-Glu or Lys–Gly–D-Asp in their peptidoglycans. The strains of members of the genus *Nesterenkonia* contained MK-8 and MK-9 as the predominant menaquinones. Most of the strains contained anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ as the major fatty acids. In this study, a novel species, isolated from sap of *Populus euphratica* collected in Mulei county, Xinjiang province, PR China, is described based on the results of polyphasic taxonomc analysis.

In June 2016, a study was conducted to investigate the diversity of endophytic culturable bacteria isolated from *Populus euphratica* samples collected from Mulei county, Xinjiang province, PR China. A sample was diluted using tenfold dilution series methods and spread on marine 2216 agar (MA; BD Difco) medium and incubated at 35–37 °C for 10 days, some colonies were picked and one yellow colony named strain RB2^T was subsequently purified on MA. The purified strain was preserved at −80 °C in marine broth 2216 (MB) medium supplemented with 30% (v/v) glycerol. Strain RB2^T has been deposited at the Korean Collection for Type Cultures (KCTC) and the Marine Culture Collection of China (MCCC).

Cell morphology and motility were observed by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL) after cells were incubated on MA at 35 °C for 3 days. Gram reaction was tested by using the Gram staining method as described previously [4]. The temperature range for growth was tested at 4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 45 and 50 °C. The pH range for growth was determined at different pH values (pH 4.5–12, at increments

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Keywords: *Nesterenkonia muleiensis* sp. nov; phylogenetic analysis.
Abbreviations: HPLC, high-performance liquid chromatography; MK-8, menaquinone 8; MK-9, menaquinone 9.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RB2^T is MF526963. The GenBank accession numbers for the whole genome sequences of strain RB2^T was QWLD00000000.
Three supplementary figures are available with the online version of this article.

Table 1. Comparison of characteristics of RB2^T and related members of the genus *Nesterenkonia*

Strains: 1, RB2^T; 2, *N. alkaliphila* F10^T; 3, *N. alba* CAAS 252^T; 4, *N. populi* GP10-3^T. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4
Morphology	Short rods	Short rods	Short rods	Short rods
Colony pigmentation	Yellow	White	White	Yellow
Motility	–	+	–	–
Optimal temperature (°C)	35	40	42	37
pH tolerance	6.0–12.0	7.0–12.0	8.0–12.0	8.0–12.0
NaCl tolerance (%)	0–12	0–12	0–6	3–15
Urease	–	–	–	+
ONPG test	+	–	+	–
Hydrolysis of:				
Gelatin	+	–	–	–
Tween 80	–	–	+	–
Carbon source utilization				
D-Glucose	w	–	+	w
D-Xylose	–	+	–	–
D-Fructose	–	–	–	+
Sucrose	+	–	+	+
Maltose	–	–	+	+
D-Galactose	+	–	–	–
Cellobiose	–	–	–	+
L-Arabinose	w	+	+	–
myo-Inositol	–	–	–	+
Psicose	+	–	–	–
α-Ketobutyric acid	+	–	–	–
Acid production from:				
l-Arabinose	+	+	+	–
D-Xylose	+	+	–	–
D-Galactose	+	+	–	–
Glucose	+	–	–	–
Mannitol	+	–	–	+
Sucrose	+	–	+	+
Menaquinones	MK-8, MK-9	MK-8, MK-9	MK-7, MK-8, MK-9	MK-7, MK-8, MK-9
Polar lipids	PG, DPG, PI, GL	PG, DPG, PI, GL	PG, DPG, PI, GL	PG, DPG, PI, GL
DNA G+C	63.5	66.2*	60.2†	67.4‡

*Data from Zhang *et al.* [36].
†Data from Luo *et al.* [37].
‡Data from Liu *et al.* [38].

Table 2. Cellular fatty acid contents of strain RB2^T and the reference strains

Strains: 1, RB2^T; 2, *N. alkaliphila* F10^T; 3, *N. alba* CAAS 252^T; 4, *N. populi* GP10-3^T. All data was obtained from this study. Fatty acids comprising less than 1% in all four strains are not shown.

Fatty acids	1	2	3	4
iso-$C_{15:0}$	5.7	2.3	1.5	1.4
anteiso-$C_{15:0}$	22.2	25.5	18.4	20.6
$C_{16:0}$	0.9	2.4	5.8	4.0
iso-$C_{16:0}$	9.0	9.3	2.3	4.0
iso-$C_{16:0}$G	1.5	0.1	–	–
iso-$C_{17:0}$	3.5	6.1	10.6	9.4
anteiso-$C_{17:0}$	46.2	50.7	54.6	56.3
anteiso-$C_{17:1}\omega 9c$	7.1	–	–	–

of 0.5 pH units) and using MB media supplemented with the buffering agents 40 mM MES (pH 4.5–6.0), PIPES (pH 6.5–7.5), Tricine (pH 8.0–8.5) and CAPSO (pH 9.0–10.0), respectively. Modified MB was used for NaCl tolerance tests, in which NaCl was omitted (0 %) or added at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 12 and 15.0% (w/v) final concentration. Growth under anaerobic and microaerobic conditions was determined with a microaerobic system (AnaeroPack; Mitsubishi Gas Chemical) using modified modified MB medium supplemented with 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite and 20 mM sodium nitrate [5]. Oxidase activity was determined using oxidase reagent (bioMérieux) and catalase activity was determined by observing bubble production in 3% (v/v) H_2O_2. Hydrolysis of casein and gelatin were tested on MA supplemented with 1% skimmed milk (Difco) and 1% gelatin, respectively. Degradation of starch was tested on MA supplemented with 0.2% soluble starch [6]. Hydrolysis of Tweens 20, 40, 60 and 80 was determined as described by Zhu *et al.* [7]. MA containing 0.5% L-tyrosine was used to test the degradation of L-tyrosine. H_2S and indole production tests were assayed according to the methods of Zhang *et al.* [8]. The methyl red and Voges–Proskauer tests were performed according to the methods of Lányi [9]. Other physiological and biochemical tests were performed using API ZYM and API 20E according to the manufacturer's instructions. Acid production was tested using API 50 CH (bioMérieux) systems, and modified MOF medium was used [10], which contained (per litre distilled water): Casitone (BD) 1 g, yeast extract 0.1 g, $(NH_4)_2SO_4$ 0.5 g, Tris buffer 0.5 g, phenol red 0.01 g, NaCl 13.75 g, $MgCl_2{\cdot}6H_2O$ 7.75 g, $MgSO_4{\cdot}7H_2O$ 2.0 g, $CaCl_2$ 0.5 g, KCl 1.0 g, $FeSO_4$ 0.001 g, adjusted to pH 7.5 with HCl. Carbon or nitrogen source utilization were tested using Biolog GN2 (Biolog).

For cellular fatty acid analysis, cells of RB2^T, *Nesterenkonia alkaliphila*, *Nesterenkonia alba* and *Nesterenkonia populi* were obtained and freeze-dried after incubation in MB at 35 °C for 48 h [11]. Fatty acids were then analysed according to the standard protocol of the Microbial Identification System. Isoprenoid quinones were analyzed by LC-MS (Agilent1200 and LCQ DECA XP MAX mass spectrometer, Thermo Finnigan) [12]. The amino-acid composition of the cell-wall peptidoglycan was determined and analyzed according to the method of Schleifer and Kandler [13]. The polar lipids were extracted and separated by two dimensional TLC on silica gel plates (10×10 cm; Merck 5554, Merck) [14]. The solvent systems of the two dimensions were prepared as described by Jia *et al.* [15]. The plates were then heated at 120 °C for 10–15 min after spraying with 50% (v/v) sulfuric acid/ethanol solution. Other reagents such as ninhydrin and molybdenum blue (Sigma) were used to detect aminolipids and phospholipids, respectively. In addition, the silica gel plates were sprayed with 5% phosphomolybdic acid and heated at 160 °C for 10–15 min to identify the total polar lipids.

The Quick Bacteria Genomic DNA Extraction Kit (DongSheng Biotech) was used to extract genomic DNA. The 16S rRNA gene was amplified by PCR using two universal primers, 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-AGAAAGGAGGTGATCCAGCC-3′) [16], and the PCR products were then purified and cloned into pMD 19 T vector (TaKaRa) for sequencing. The 16S rRNA gene sequence was identified on the ExTaxon-e service [17]. Multiple sequence alignment was accomplished via the CLUSTAL W program of the MEGA 5 package [18]. The neighbour-joining [19] maximum-likelihood [20] and maximum-parsimony [21] methods were used to reconstruct phylogenetic trees using the MEGA 5.0 software [18]. Bootstrap values of 1the three phylogenetic trees were based on 1000 replicates. The algorithm of Kimura's two-parameter model [22] was chosen for the neighbour-joining method. Phylogenomic analysis were performed as described by Xu *et al.* [23]. The ORFs were predicted by using Glimmer v.3.0 [24] and then annotated by the online Rapid Annotation using Subsystems Technology (RAST) server [25]. The predicted proteins of all strains were compared through OrthoMCL version 2.0 [26] to detect orthologous clusters (OCs). Single-copy shared OCs of all strains were filtered using in-house perl scripts. The filtered shared OCs were aligned by using MAFFT version 7 [27]. Aligned sequences were refined by using trimAL version 1.4.1 [28] and concatenated manually. The maximum-likelihood phylogenetic tree (Fig. S3, available in the online version of this article) was reconstructed using IQ-TREE 1.6.1 software [29] based on concatenated aligned single-copy OCs with bootstraps analysis set to 100 replicates, with the best amino acid substitutional model set as LG+F+R4 as proposed by IQ-TREE 1.6.1 software [29].

The genomes of strain RB2^T and *N. alkaliphile* F10^T were sequenced by HiSeq X-Ten PE150 platform at the Beijing Genomics Institute (BGI; Beijing, PR China). The sequencing generated 699 and 984 Mb clean data (approximate 250-fold genome coverage). The genomes of *N. alba* DSM 19423^T (ATXP00000000), *N. populi* GP10-3^T (VOIL00000000), *Nesterenkonia massiliensis* NP1^T (CBLL000000000), *Nesterenkonia aurantiaca* DSM 27373^T (SOAN00000000), *Nesterenkonia jeotgali* CD08_7 (LQBM00000000) and *Nesterenkonia*

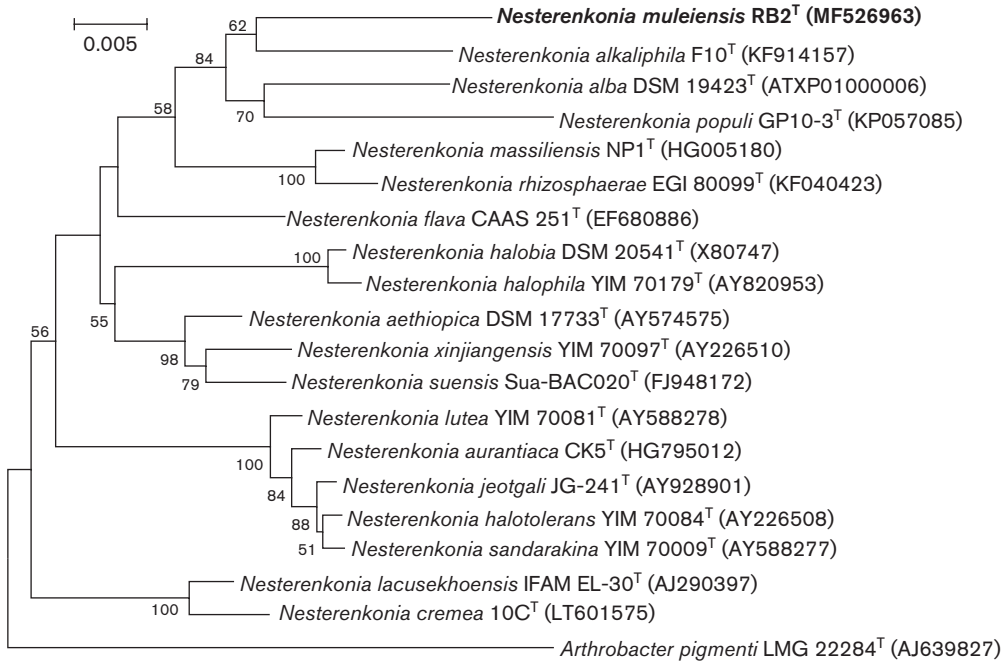

Fig. 1. Neighbour-joining tree reconstructed using the MEGA 5.0 program package based on 16S rRNA gene sequences, showing the phylogenetic relationship of RB2^T with the related taxa. Bootstrap values were based on 1000 replicates; only values ≥50% were shown. Bar, 0.005 substitutions per nucleotide position. *Arthrobacter pigmenti* LMG 22284^T was used as an outgroup.

sandarakina CG 35 (PVTY00000000) were retrieved from the NCBI database. The *de novo* assembly of the reads was performed using ABySS v2.0.2 [30]. The quality of microbial genomes was assessed using the bioinformatics tool CheckM v1.0.7 [31]. *In silico* DNA–DNA hybridization (DDH) values were calculated by using the Genome-to-Genome Distance Calculator [32]. The average nucleotide identity (ANI) values were calculated using the OrthoANIu algorithm by Chun Lab's online ANI calculator [33]. The DNA base composition (G+C content) was determined by counting G+C in full genome sequences.

Cells of RB2^T are Gram-stain-positive, aerobic, non-motile, non-endospore-forming and rod-shaped (0.7–1.2×0.3–0.6 µm) without flagella (Fig. S1). Colonies of RB2^T are 2 mm in diameter, circular, convex and pale yellow after growth on MA 35 °C for 3 days. RB2^T grew at 10–45 °C (optimum 35 °C), pH 6.0–12.0 (optimum 8.0) and with 0–12% (w/v) NaCl (optimum 1%). The physiological and biochemical characteristics of RB2^T in comparison to the reference strains are shown in Table 1. All strains were negative for indole and H_2S production and the results of methyl red and Voges–Proskauer tests were negative. Starch, Tween 20 and Tween 60 could not be hydrolyzed in all strains. However, some characteristics were found to discriminate RB2^T from the reference strains. RB2^T could grow at pH 6.5, but the reference strains could not. Unlike the reference strains, RB2^T could hydrolyze gelatin. Tween 80 could not be hydrolyzed by RB2^T, which was different from *N. alba* CAAS 252^T. The result of the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) test was positive for RB2^T, but negative for *N. alkaliphila* F10^T and *N. populi* GP10-3^T. As for the API 50 CH test, acid is produced from L-arabinose in RB2^T, unlike *N. populi* GP10-3^T. RB2^T could produce acid from D-xylose and D-galactose, but *N. alba* CAAS 252^T and *N. populi* GP10-3^T could not. In contrast to the closest phylogenetic neighbour *N. alkaliphila* F10^T, RB2^T could produce acid from glucose, mannitol and sucrose. In the carbon utilization test, RB2^T could utilize D-galactose, psicose and α-ketobutyric acid, which were different from the reference strains. In contrast to *N. populi* GP10-3^T, RB2^T could not utilize D-fructose, cellobiose and *myo*-inositol. Unlike *N. alba* CAAS 252^T, RB2^T could not utilize maltose. RB2^T could utilize sucrose but could not utilize D-xylose, which distinguished it from its from closest phylogenetic neighbour *N. alkaliphila* F10^T.

The fatty acid profiles of RB2^T and the reference strains are listed in Table 2.

The major fatty acids (≥5% of the total fatty acids) found in RB2^T were anteiso-$C_{17:0}$ (46.2 %), anteiso-$C_{15:0}$ (22.2 %), iso-$C_{16:0}$ (9.0 %), anteiso-$C_{17:1}\omega 9c$ (7.1 %) and iso-$C_{15:0}$ (5.7 %). The four strains had similarities in major components (≥5.0 %), such as anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$. However, there were some characteristics that distinguished RB2^T from the reference strains. Anteiso-$C_{17:1}\omega 9c$ was a major fatty acid in RB2^T but not in the other reference strains. Compared with *N. alba* CAAS 252^T, $C_{16:0}$ was not a major acid in RB2^T. The main respiratory quinone detected in RB2^T was MK-8, MK-9 was present as a minor component, as in *N. alkaliphila* F10^T. The

Table 3. Comparison of the genomic characteristics of RB2^T and the reference strains

Strains: 1, RB2^T; 2, *N. alkaliphila* F10^T; 3, *N. alba* CAAS 252 ^{T}; 4, *N. populi* GP10-3^T. 5, *N. massiliensis* NP1^T; 6, *N. aurantiaca* DSM 27373^T; 7, *N. jeotgali* CD08_7; 8, *N. sandarakina* GC35.

Genomic characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Size (bp)	3676111	3318774	2591866	2551278	2641000	2948026	2925195	3224976
DNA G+C content (%)	63.6	64.8	63.7	66.8	62.9	67.6	67.6	67.5
Number of Contigs (with PEGs)	57	103	36	2	175	25	2	56
N50	118097	60124	202348	2537625	27943	237171	731296	180192
Number of Coding Sequences	3552	3156	2367	2381	2596	2709	2692	3023
Number of RNAs	45	41	49	51	48	50	51	53
Subsystem feature counts								
Cofactors, Vitamins, Prosthetic Groups, Pigments	128	112	88	98	93	111	86	113
Virulence, Disease and Defense	24	39	22	21	36	26	27	34
Phages, Prophages, Transposable Elements, Plasmids	0	3	2	2	0	0	1	3
Membrane Transport	58	60	49	54	45	53	46	49
Iron Acquisition and Metabolism	8	4	5	4	7	4	4	8
Protein Metabolism	130	134	150	149	156	150	148	147
Secondary Metabolism	10	8	4	4	10	9	8	4
DNA Metabolism	49	64	39	50	68	47	48	62
Fatty Acids, Lipids, and Isoprenoids	85	52	24	31	40	60	59	56
Nitrogen Metabolism	11	8	6	7	11	13	8	8
Respiration	32	37	34	28	33	31	24	36
Stress Response	19	13	17	21	13	27	26	24
Metabolism of Aromatic Compounds	30	9	5	9	8	31	20	25
Amino Acids and Derivatives	275	273	197	221	226	251	241	241
Sulfur Metabolism	15	5	4	20	5	5	5	5
Carbohydrates	198	138	104	113	120	184	162	163

major polar lipids of strain RB2^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, two glycolipids and two unidentified lipids. (Fig. S2), a profile that was similar to those of reference strains of members of the genus *Nesterenkonia*. However, one glycolipid (PGL) was not detected in RB2^T but found in *N. alba* CAAS 252^T and *N. populi* GP10-3^T. Also, the absence of one amino lipid (AL) distinguished RB2^T from *N. alba* CAAS 252^T. In contrast to the closest phylogenetic neighbour *N. alkaliphila* F10^T, one glycolipid (GL2) and one lipid (L2) were present in strain RB2^T. The peptidoglycan structure of strain RB2^T was type A4*α*, L-Lys–Gly–L-Glu. This result was similar to those for the reference strains.

The genome completeness of RB2^T was 97.8%, with a contamination percentage of 0.86%. Genome sequences estimated to have ≥95% completeness, are considered as excellent reference genomes for future analyses [31]. The DNA G+C content of RB2^T was 63.5 mol%, which was similar to those of *N. alkaliphila* F10^T (66.2 mol%), *N. alba* CAAS 252^T (60.2 mol%) and *N. populi* GP10-3^T (67.4 mol%). On the basis of 16S rRNA gene sequence similarity, RB2^T represents a close relative of species of the genus *Nesterenkonia*, sharing 97.17, 97.03 and 96.00% similarity with the type strains of *N. alba* CAAS 252^T, *N. alkaliphila* F10^T and *N. populi* GP10-3^T, respectively. RB2^T formed a cluster with *N. alkaliphila* F10^T in the neighbour-joining tree (Fig. 1). The phylogenetic tree (Fig. S3) based on the phylogenomic analysis was reconstructed and RB2^T also formed a cluster with *N. alkaliphila* F10^T. Based on 16S rRNA gene sequences, the phylogenetic analysis revealed that RB2^T represented a member of the genus *Nesterenkonia*. The in *silico* DDH values indicated that RB2^T shared 21.7, 20.4 and 20.7 % DNA relatedness with *N. alkaliphila* F10^T, *N. alba* CAAS 252^T and *N. populi* GP10-3^T, respectively, which were lower than the threshold values of the Genome to Genome Distance calculator (GGDC; 70%) [34]. The ANI values between RB2^T and *N. alkaliphila* F10^T, *N. alba* CAAS 252^T and *N. populi* GP10-3^T were 78.2, 74.5 and 74.9%, respectively, which were lower than the threshold values of the species boundary (ANI 94–96%) [35]. The different genomic characteristics (Table 3) could be used to discriminate RB2^T from the reference strains.

On the basis of the phenotypic, phylogenetic and chemotaxonomic properties presented in this study, strain RB2^T represents a novel species in the genus *Nesterenkonia*, for which the name *Nesterenkonia muleiensis* sp. nov. is proposed.

DESCRIPTION OF *NESTERENKONIA MULEIENSIS* SP. NOV.

Nesterenkonia muleiensis (mu.lei.en'sis. N.L. fem. n. *muleiensis* pertaining to Mulei county, PR China, where the type strain was isolated).

Cells are Gram-stain-positive, aerobic, NON-motile, non-endospore-forming and rod-shaped (0.7–1.2×0.3–0.6 µm) without flagella. Colonies are 2 mm in diameter, circular, convex and pale yellow after growth on MA 35 °C for 3 days. Growth occurs at 10–45 °C (optimum 35 °C), pH 6.0–12.0 (optimum 8.0) and with 0–12% (w/v) NaCl (optimum 1%). Oxidase and catalase are negative and positive, respectively. Positive for ONPG test and gelatin hydrolysis, butnegative for urease, nitrate reduction, indole production, H_2S production, methyl red and Voges–Proskauer test and hydrolysis of starch, Tween 20, Tween 40, Tween 60, Tween 80, skimmed milk and L-tyrosine. Production of esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase and α-glucosidase are positive; alkaline phosphatase, lipase (C14), cystine arylamidase, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. Acid is produced from glycerol, D-arabinose, L-arabinose, D-xylose, D-galactose, glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose and tagatose. In utilization of carbon sources test, sucrose, D-galactose, D-sorbitol, L-arabinose, psicose and α-ketobutyric acid can be utilized. The major respiratory quinones are MK-8 and MK-9. The major fatty acids (≥5% of the total fatty acids) are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$, iso-$C_{17:0}$ and anteiso-$C_{17:1}\omega 9c$. The major polar lipids of strain RB2^T are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, two glycolipids and two unidentified lipids. The peptidoglycan structure of strain RB2^T is type A4α, L-Lys–Gly–L-Glu.

The type strain, RB2^T (=MCCC 1K03528^T=KCTC 49017^T) was isolated from sap of *Populus euphratica* collected in Mulei county, Xinjiang province, PR China. The DNA G+C content of the genomic DNA of the type strain was 63.5 mol% (by genome).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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