INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Chen *et al.*, *Int J Syst Evol Microbiol*
DOI 10.1099/ijsem.0.002585

MICROBIOLOGY SOCIETY

Halomonas endophytica sp. nov., isolated from liquid in the stems of *Populus euphratica*

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Abstract

A Gram-stain-negative, aerobic, motile and rod-shaped bacterium, designated MC28^T, was isolated from storage liquid collected from the stems of *Populus euphratica* in the Xinjiang province of China. The growth range of NaCl concentration was 0.5–6.0 % (w/v), with an optimum at 3.0 % (w/v), the temperature range for growth was 10–45 °C, with an optimum at 40 °C, and the pH range for growth was 6.0–9.0, with an optimum around pH 8.5. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain MC28^T formed a distinct lineage in the clade of genus *Halomonas* and is closely related to *Halomonas desiderata* DSM 9502^T (96.4 %), *Halomonas heilongjiangensis* DSM 26881^T (96.2 %) and *Halomonas urumqiensis* JCM 30202^T (95.2 %). The average nucleotide identity, average amino acid identity and *in silico* DNA–DNA hybridization values between strain MC28^T and the references strains were 77.2–80.3, 65.8–76.8 and 21.6–25.6 %, respectively. Chemotaxonomic analysis indicated that the main respiratory quinones were Q-9 and Q-8, the predominant cellular fatty acids were summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$), summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:1}\omega9c$, $C_{16:0}$ and $C_{17:1}\omega9c$, the major polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, phosphatidylinositol mannoside, an aminophospholipid, an unidentified phospholipid, an unidentified aminolipid and two unidentified lipids. Based on its phenotypic, chemotaxonomic and phylogenetic characteristics, strain MC28^T is considered to represent a novel species, for which the name *Halomonas endophytica* sp. nov. is proposed. The type strain is MC28^T (=KCTC 52999^T=MCCC 1K03343^T).

The genus *Halomonas*, is the largest genus in the family *Halomonadaceae* within the class *Gammaproteobacteria*; the type species is *Halomonas elongata*, which was originally proposed by Vreeland *et al.* [1]. At time of writing, it contained 95 species with validly published named (www.bacterio.net/-allnamesdl.html). A prominent feature of the genus *Halomonas* is salt tolerance, many *Halomonas* strains can survive in an environment from 0.1 to 32.5 % sodium chloride [2]. Most species of the genus *Halomonas* have been isolated from saline habitats, such as alkaline soil [3], saline–alkaline lake [4], industrial brine [5], solar saltern sediment [6], deep-sea environment [7], fermented seafood [8], salty leaves [9], dialysis machines of a renal care centre [10] and Antarctica [11]. Various characteristics of numerous strains of *Halomonas* are recognized for applications in biotechnology, such as degradation of toxic compounds [11, 12], metal tolerance [7], producing exoenzymes [13, 14], and denitrification [15]. The most members of the genus *Halomonas* are Gram-stain-negative, rod-shaped and non-sporulating [4]. They are chemoorganotrophs, with a predominantly respiratory metabolism, using oxygen, nitrate or nitrite as electron acceptors [16].

Liquid samples were collected from stems of *Populus euphratica*, a plant with saline–alkali tolerance and drought resistance in Ebinur Lake Wetland Nature Reserve (44° 25′ N, 83° 41′ E), Xinjiang Province, China, in Spring 2016, and stored at 4 °C until used. Samples were resuspended and diluted by sterile water, using a tenfold dilution series method, and then spread onto marine 2216 agar

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Keywords: *Halomonas endophytica* sp. nov.; polyphasic taxonomy; storage liquid from *Populus euphratica*.
Abbreviations: AAI, average amino acid identity; ANI, average nucleotide identity; DDH, DNA–DNA hybridization; MA, marine 2216 agar; MB, marine 2216 broth.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MC28^T is MF850257. The GenBank accession numbers for the genome sequence of strain MC28^T, *Halomonas heilongjiangensis* DSM 26881^T and *Halomonas urumqiensis* JCM 30202^T are PNRF00000000, PNRE00000000 and PNRG00000000, respectively.
One supplementary table and four supplementary figures are available with the online version of this article.

(MA; Difco) plates, which were incubated at 28 °C [17]. After 5 days of incubation, numerous small single colonies were purified by repeated streaking two times on new MA plant, a yellow-coloured colony was picked out and named MC28^T. To data, MC28^T may be the first *Halomonas* strain isolated from storage liquid of plant which is far from the ocean. Strain MC28^T was preserved at −80 °C in marine 2216 broth (MB; Difco) supplemented with 20 % (v/v) glycerol for further study.

Genomic DNA was extracted and purified using the Wizard Genomic DNA purification kit (Promega). The 16S rRNA gene of strain MC28^T was amplified with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [18]. PCR products were cloned into the pMD 19 T vector (TaKaRa) for sequencing. The 16S rRNA gene sequence (1452 bp) obtained in this study was used for pairwise sequence alignment performed by the BLASTN program (www.ncbi.nlm.nih.gov) and the EzTaxon-e server (https://www.ezbiocloud.net/) [19]. According to the EzTaxon server, the sequence of strain MC28^T was most closely related to *Halomonas desiderata* DSM 9502^T (96.4 %), and showed 96.3–94.8 % sequence similarity to the type strains of other species of the genus *Halomonas*. Similarity values between the 16S rRNA gene sequences of strain MC28^T and the type strains of other genera were lower than 90 %. Multiple sequence aligment based on 16S rRNA gene sequences of strain MC28^T and related taxa were performed as described by Zhang *et al.* [20] using MEGA version 5 [21]. Phylogenetic trees were reconstructed from 1000 replicates each for bootstrap analysis with the neighbour-joining (Fig. 1), maximum-likelihood (Fig. S1, available in the online version of this article) and maximum-parsimony (Fig. S2) methods, which all illustrated that strain MC28^T clustered into the clade of genus *Halomonas* by forming a distinct lineage among the most closely related species: *H. desiderata* DSM 9502^T (96.4 %), *Halomonas heilongjiangensis* DSM 26881^T (96.2 %) and *Halomonas urumqiensis* JCM 30202^T (95.2 %). For this reason, these three type strains were selected as reference strains in this study and obtained from the DSMZ and the JCM, unless otherwise stated, all strains were incubated in MA or MB at 28 °C.

The whole genomes of strain MC28^T, *H. heilongjiangensis* DSM 26881^T, and *H. urumqiensis* JCM 30202^T were sequenced by Solexa PE150 sequencing technology with the HiSeq platform (Beijing Genomics Institute). The sequencing generated 1.175, 1.134 and 1.171 GB of clean data, respectively,(approximate 250-fold genome coverage). The *de novo* assembly of the reads was performed using ABySS 1.5.2 [22]. The assembly k-value was tested from 32 to 64 to find the optimal k-value by using the abyss-pe script. The quality of microbial genomes was assessed using the bioinformatic tool CheckM [23]. The genome sequences of *H. desiderata* DSM 9502^T (MUMZ00000000) were retrieved from the GenBank database. The average nucleotide identity (ANI) was calculated using the OrthoANIu algorithm of Chun laboratory's online average nucleotide identity calculator [24]. *In silico* DNA–DNA hybridization (DDH) values were calculated by using the Genome-to-Genome Distance Calculator (GGDC) [25]. The average amino acid identity (AAI) was calculated using Kostas laboratory's online AAI calculator [26].

Growth was tested at total Nacl concentrations of 0–15.0 % (w/v) at intervals of 0.5 % in MB (pH 7.0). Temperature range for growth was tested at 4, 10, 15, 20, 25, 28, 30, 35, 40, 45, 50 °C in MB (pH 7.0). The pH range for growth was determined at 0.5 pH intervals by supplementing 30 mM buffering agents in MB at 28 °C, including sodium 4-morpholin-1-ylethylsulphonate (MES, pH 5.5–6.4), 3-(4-Morpholino) propanesulfonic acid sodium salt (MOPS, pH 6.5–7.9), Tricine (pH 8.0–8.9) and Bis-Tris propane (pH 9.0–9.5) [27]. Optimal growth was measured after 5 days of incubation and the growth limits were tested after 14 days of incubation. [20]. Growth was monitored by measuring OD_{590} in a UV/visible spectrophotometer (Ultrospec 6300 pro; Amersham Biosciences). Strain MC28^T was incubated at 28 °C for 3 days, cell morphology was examined and observed by transmission electron microscopy (JEM-1230; Jeol) after uranyl acetate (0.5 %, w/v) staining under 80KV and by optical microscopy (BX40; Olympus) after Gram staining.

Degradation of starch, oxidase and catalase activities, hydrolysis of Tween 20, 40, 60, 80, hypoxanthine and xanthine, nitrate reduction and urease activity, the ability to hydrolyse casein, chitin, carboxymethyl cellulose, filter paper and gelatin, H_2S production, methyl red and Voges–Proskauer reactions were tested as described previously [17]. Degradation of tyrosine was measured on MA with 5 g l^{-1} tyrosine. The presence of flexirubin-type pigments was investigated using 20 % (w/v) KOH solution. Anaerobic growth was determined with an anaerobic system (AnaeroPack-MicroAero, 2.5 l, MGC) using MA, to which 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite and 20 mM sodium nitrate were respectively added as electron acceptors [28]. Utilization of carbon substrates was tested at concentration of 0.5 % (w/v) according to the protocol of Dong and Cai [29] using whole components of soluble material of MB, yeast extract (0.01 %, w/v) was added as growth factors. Acid production was tested using marine oxidation–fermentation medium supplemented with 1 % sugars [30]. Enzyme activities and other physiological and biochemical traits were tested using API ZYM and API 20NE strips (bioMérieux) according to the manufacturer's instructions. The three reference strains, *H. desiderata* DSM 9502^T, *H. heilongjiangensis* DSM 26881^T and *H. urumqiensis* JCM 30202^T, were used as controls in the above tests.

Cells of strain MC28^T and reference strains were harvested during the third quadrants on MA (28 °C, 3 days) for cellular fatty acid analysis. Whole-cell fatty acids were analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID) with the standard MIS Library Generation Software version 4.5. Isoprenoid quinones were extracted from freeze-dried cells (500 mg) with chloroform/

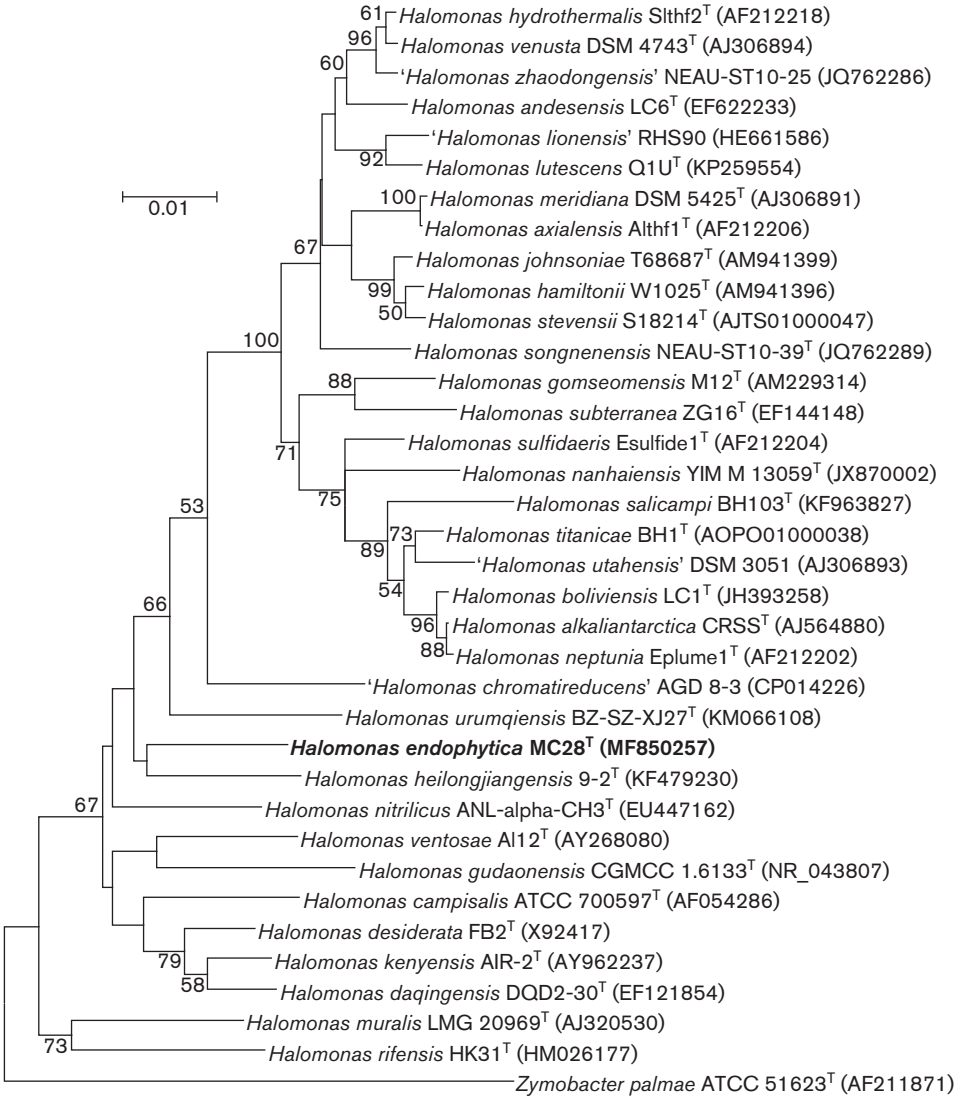

Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain MC28^T and representatives of related taxa. Bootstrap values were expressed as a percentage of 1000 replicates and only those higher than 50 % were given at the branch points. Bar, 0.01 substitutions per nucleotide position.

methanol (2 : 1, v/v) and analysed by liquid chromatography–mass spectrometry (Agilent) [31]. Polar lipids were extracted with chloroform/methanol (1 : 2, v/v) and identified by two-dimensional thin-layer chromatography on silica gel 60 F_{254} plates (Merck). Spraying molybdophosphoric acid, ninhydrin reagent, molybdenum blue and α-naphthol/H_2SO_4 reagent, used for the detection of all lipids, lipids containing free aminolipids, phosphorus containing lipids and glycolipids, respectively [32].

Strain MC28^T was Gram-stain-negative and motile. Colonies were yellow-coloured, circular, smooth and 1–2 mm in diameter after 3 days of incubation at 28 °C on MA. As shown in Fig. S3, cells of strain MC28^T were rod-shaped, about 0.5 µm wide and 1.0–1.5 µm long. Growth of strain MC28^T was observed at 10–45 °C (optimum 40 °C), pH 6.0–9.0 (optimum pH 8.5) and 0.5–6.0 % (w/v) NaCl (optimum 3.0 %). The DNA G+C content of strain MC28^T was 64.1 mol%, which is similar to those of *H. desiderata* DSM 9502^T (64.1 mol%), *H. heilongjiangensis* DSM 26881^T (69.7 mol%) and *H. urumqiensis* JCM 30202^T (61.7 mol%).

The genome completeness of strain MC28^T, *H. heilongjiangensis* DSM 26881^T, and *H. urumqiensis* JCM 30202^T were 99.6, 99.2, and 100 %, with 1.66, 1.28 and 1.35 % contamination, respectively. The genome sequences considered as good reference genomes for deeper analyses ($\geq$95 % completeness, $\leq$5 % contamination) [23]. The DNA G+C content of strain MC28^T was 62.1 %, which was similar to the genus *Halomonas* and distinct from *H.*

heilongjiangensis DSM 26881^T (66.7 %), *H. urumqiensis* JCM 30202^T (62.6 %) and *H. desiderata* DSM 9502^T (64.7 %). The *in silico* DDH values, ANI values and AAI values between strain MC28^T and *H. heilongjiangensis* DSM 26881^T, *H. urumqiensis* JCM 30202^T and *H. desiderata* DSM 9502^T were 23.0, 21.6, 25.6; 79.5, 77.2, 80.3 %; and 76.8, 74.6, 65.8 %, respectively, which were below the 70 % threshold value for GGDC, 95 % for ANI and 95 % for AAI proposed for the delineation of bacterial species, indicating that strain MC28^T belongs to a novel species of the genus *Halomonas* [33–35].

Results of chemotaxonomic analyses revealed that Q-9 (89 %) and Q-8 (10 %) were the predominant respiratory quinones in strain MC28^T. The detailed fatty acid profiles of strain MC28^T and the reference strains are shown in Table S1. The major fatty acids (>10 %) detected in strain MC28^T included summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$, 22.7 %), summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, 18.3 %), $C_{16:1}\omega9c$ (16.1 %), $C_{16:0}$ (11.2 %) and $C_{17:1}\omega9c$ (10.6 %). The fatty acid profiles obtained in this study for the reference strains were similar to those given in the original descriptions in terms of the major fatty acids [3, 4, 36], despite some differences in their proportions. As showed in Fig. S4, the polar lipid profile of strain MC28^T was composed of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, phosphatidylinositol mannoside, an aminophospholipid, an unidentified phospholipid, an unidentified aminolipid and two unidentified lipids.

The chemotaxonomic data obtained in this study support the results of the phylogenetic analysis and exhibits common characteristic of the genus *Halomonas*. The predominant respiratory quinones found in strain MC28^T were Q-9 (89 %) and Q-8 (10 %), which similar to *H. desiderata* DSM 9502^T (Q9) [36], *H. heilongjiangensis* DSM 26881^T (Q9, 75 %; Q8,25%) [3] and *H. urumqiensis* JCM 30202^T (Q9, 89 %; Q8,10%) [4], and in line with all other members of the genus *Halomonas*. Summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$, 22.7 %), summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, 18.3 %), $C_{16:1}\omega9c$ (16.1 %) and $C_{16:0}$ (11.2 %) were the major fatty acids in strain MC28^T, which was similar to the reference strains (18.5–31.3, 3.7–15.2, 5.9–11.2 %, respectively). Although the overall profiles of acids were similar, the component ratio of strain MC28^T and references strains were significantly different, such as $C_{17:1}\omega9c$ (10.6 %) also being the major fatty acid found in strain MC28^T which was a trace amount (<1 %) in *H. urumqiensis* JCM 30202^T, *H. desiderata* DSM 9502^T and *H. heilongjiangensis* DSM 26881^T (Table S1).

The phenotypic data (Table 1) showed many common grounds between strain MC28^T and the references strains, such as all strains being positive for oxidase, catalase, esterase (C4), esterase lipase (C8), H_2S production, aerobic and motile and require Na+ or sea water for growth; all strains are negative for α-mannosidase, α-fucosidase, arginine dihydrolase, indole production, methyl-red test, degradation of starch, casein and gelatin. Concurrently, a number of phenotypic differences could also be found between strain MC28^T and the reference strains. For example, strain MC28^T was positive for degradation of Tween 20, 40, 60 and 80, but none of the references strains were able to degrade all of those Tweens. In the API ZYM tests, lipase and trypisn were positive in strain MC28^T, but were negative in all references strains. Strain MC28^T was isolated from storage liquid in the stems of *Populus euphratica*, a plant with saline–alkali tolerance and drought resistance. Reference strains were isolated from municipal sewage, lake water and soil of saline and alkaline, respectively. In addition, strain MC28^T was the first plant endophytic bacteria identified as a new species belonging to genus *Halomonas*. There are also other physiological and biochemical characteristics shown in Table 1 which are distinctive in strain MC28^T.

In conclusion, 16S rRNA gene sequence analyses and chemotaxonomic characterizations supported the placement of strain MC28^T within the genus *Halomonas*. However, strain MC28^T could be distinguished from the type strains of the most closely related species based on phenotypic, chemotaxonomic and genotypic characterizations. On the basis of the results presented here, strain MC28^T is considered to represent a novel species of the genus *Halomonas*, for which the name *Halomonas endophytica* sp. nov. is proposed.

DESCRIPTION OF *HALOMONAS ENDOPHYTICA* SP. NOV.

Halomonas endophytica (en.do.phy′ti.ca. Gr. pref. *endo* within; Gr. n. *phyton* plant; L. fem. suff. *-ica* adjectival suffix used with the sense of belonging to; N.L. fem. adj. *endophytica* within plant, pertaining to the endophytic nature of the strain and its isolation from internal plant tissues).

Cells are Gram-stain-negative, aerobic, motile, short rod-shaped, 0.5–0.8 µm wide and 1.0–1.5 µm long. Colonies are yellow-coloured, circular, smooth and 1–2 mm in diameter after 3 days of incubation at 28 °C on MA. Growth occurs at 10–45 °C (optimum 40 °C), pH 6.0–9.0 (optimum pH 8.5) and 0.5–6.0 % (w/v) NaCl (optimum 3.0 %). Na^+ ion is required for growth. Positive for catalase, oxidase, production of H_2S, glucose fermentation, hydrolysis of nitrate, Tween 20, 40, 60 and 80. Negative for methyl-red test and Voges–Proskauer test, flexirubin-type pigments, hydrolysis of casein, L-tyrosine, starch, gelatin, CM-cellulose and crystalline cellulose (filter paper). According to the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase activities are present. The following compounds are utilized as sole carbon and energy source: citric acid, D-fructose, D-ribose, L-arabinose, succinic acid, maltose, D-mannitol, trehalose, D-galactose, D-mannose, sucrose, D-tagatose, L-sorbose, D-sorbitol, D-glucose, D-xylose and sodium gluconate. The DNA G+C content of the type strain is 62.1 mol%. The principal fatty acids are summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$), summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:1}\omega9c$, $C_{16:0}$ and $C_{17:1}\omega9c$. The respiratory quinone is Q-9

Table 1. Differential phenotypic characteristics of strain MC28^T and its most closely related species

Taxa: 1, strain MC28 ^{T}; 2, *Halomonas urumqiensis* JCM 30202^T [4]; 3, *Halomonas heilongjiangensis* DSM 26881^T [3]; 4, *Halomonas desiderata* DSM 9502^T [36]. All data were obtained from this study. All strains are positive for oxidase, catalase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase; production of acid from glycerol, D-ribose, D-glucose, D-fructose, D-lyxose, H2S production, aerobic and motile, require Na^+ or sea water for growth. All strains are negative for α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase; indole production, methyl-red test, degradation of starch, casein and gelatin. +, Positive; −, negative.

Characteristic	1	2	3	4
Voges–Proskauer reaction	−	+	+	−
Flexirubin-type pigment	−	−	+	−
Degradation of:				
Tween 20	+	−	−	−
Tween 40	+	+	+	−
Tween 60	+	−	−	+
Tween 80	+	−	+	−
Tyrosine	−	−	+	+
Tests of API ZYM:				
Alkaline phosphatase	+	−	+	+
Lipase	+	−	−	−
Trypisn	+	−	−	−
Acid phosphatase	+	+	+	−
Tests of API 20NE:				
Reduction of nitrates to nitrites	+	−	+	+
Reduction of nitrates to nitrogen	+	−	+	+
Glucose fermentation	+	−	+	+
UREase	−	−	+	−
Acid production from (API 50CH):				
D-Arabinose	+	−	+	−
L-Arabinose	−	−	−	+
D-Xylose	+	+	−	+
D-Mannose	+	−	+	−
L-Sorbose	+	−	−	+
Maltose	−	+	−	+
Sucrose	−	+	−	+
Trehalose	−	+	−	+
Turanose	−	+	−	+
D-Tagatose	+	−	−	−
D-Fucose	−	−	+	+
D-Arabitol	+	+	−	+
Major fatty acids (%):				
Summed feature 8*	22.7	31.3	18.5	20.4
Summed feature 3†	18.3	15.2	11.6	3.7
$C_{16:1}\omega 9c$	16.1	11.2	8.6	5.9
$C_{16:0}$	11.2	25.9	21.1	18.8
$C_{17:1}\omega 9c$	10.6	<1.0	<1.0	<1.0
G+C (mol%)	64.1	61.7	69.7	66.0
Habitat	Storage liquid of plant	Saline and alkaline lake	Saline and alkaline soil	Municipal sewage

*Summed features are groups of two or three fatty acids that could not be separated by the MIDI system. Summed feature 8 is composed of $C_{18:1}\omega 6c$ and/or $C_{18:1}\omega 7c$.

†Summed feature 3 is composed of $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$.

and Q-8. The polar lipid profile is composed of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylinositol, phosphatidylinositol mannoside, an aminophospholipid, an unidentified phospholipids, an unidentified aminolipids and two unidentified lipids.

The type strain is MC28^{T} (=KCTC 52999^{T}=MCCC 1K03343^{T}), which was isolated from stems of *Populus euphratica*, a plant with saline–alkali tolerance and drought resistance in Ebinur Lake Wetland Nature Reserve (44° 25′ N, 83° 41′ E), Xinjiang Province, China.

Funding information
This work was supported by National Natural Science Foundation of China (Nos 41406174 and 41506183), the National Key Basic Research Program of China (2014CB441503), the Natural Science Foundation of Zhejiang Province (LR17D060001), the Science Foundation of Zhejiang Sci-Tech University (16042186-Y), the Scientific Research Fund of Zhejiang Provincial Education Department (Y201636535).

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

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