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Alkalibacillus aidingensis sp. nov., an Bacterium Isolated from Aiding Lake in Xinjiang Province, North-West China

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Abstract

A bacterial strain, Gram staining negative, aerobic, long rod, motile bacterium with flagellum, designated strain YIM 98829^T, was isolated from the Aiding Lake in Xinjiang province, North-West China. The isolate produced oval subterminal endospores in swollen sporangia. The predominant menaquinone was MK-7. The cell wall peptidoglycan contained ornithine, serine, aspartic acid, glutamic acid, and alanine, while diaminopimelic acid could not be detected. The major whole-cell sugars contained xylose, glucose, galactose, and mannose. Diphosphatidylglycerol, phosphatidylglycerol, one unknown phospholipid, and two unidentified aminophospholipids were part of the polar lipid profile. Iso-$C_{15:0}$ and anteiso-$C_{15:0}$ were the major fatty acids. The DNA G + C content of the type strain was 38.0 mol%. Phylogenetic analysis indicated that the isolate belongs to the genus *Alkalibacillus*. However, it differed from its closest relatives, *A. haloalkaliphilus* DSM 5271^T (97.04%), *A. filiformis* 4AG^T (96.99%), and *A. silvisoli* BM2^T (96.95%) in some physiological characteristics. DNA–DNA hybridization result indicated low levels of relatedness between strain YIM 98829^T and *A. haloalkaliphilus* JCM 12303^T (16.9%). On the basis of physiological, phenotypic, and chemotaxonomic data, strain YIM 98829^T represents a novel species of genus *Alkalibacillus*, for which the name *Alkalibacillus aidingensis* sp. nov. is proposed. The type strain is YIM 98829^T (= NBRC 114103^T = CGMCC 1.17260^T = DSM 112470^T).

Introduction

Alkalibacillus as a novel genus was proposed by Jeon et al. [1], based on a reclassification of [*Bacillus*] [2]. Seven species have been described in this genus till now, which were isolated from salt lake, hypersaline soil, water of a mineral pool, non-saline forest soil, marine solar saltern, and inland solar saltern [1–7]. Most species of the genus *Alkalibacillus*

Rui Li and Li Yang have equally contributed to this work.

The NCBI GenBank accession number for the 16S rRNA gene sequence of strain YIM 98829^T is MN251019. The draft whole-genome sequence for YIM 98829^T has been deposited at DDBJ/ENA/GenBank under Accession Number GCA_014595945.1.

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are halophilic bacteria that grow optimally in media containing 6–20% (w/v) NaCl and show considerable industrial benefits, particularly for the production of enzymes (proteases, xylanases, glycosidases, etc.) [2, 8]. Strain YIM 98829^T was isolated from Aiding Lake in Xinjiang province, North-West China within the process of exploring microbial resources in extreme environments. The present paper reports a polyphasic characterization of the strain and its classification as a novel species of the genus *Alkalibacillus.*

Methods and Materials

Bacterial Isolation

Strain YIM 98829^T was isolated from hypersaline sediment of the Aiding Lake in Xinjiang province, North-West China (42 68′66″ N, 89 33′07″ E). The isolation medium contained the following components, 10% (*w/v*) NaCl, 7.5 g casein, 10 g yeast extract, 3 g sodium citrate, 10 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, 1 ml 4.98% $FeSO_4$,15 g agar, and 1000 ml H_2O, pH 7.4. Stock cultures of strain YIM 98829^T were prepared in Tryptone soya agar (TSA) medium (10%, w/v NaCl) with 20% glycerol(w/v) and stored at − 80 °C. Biomass for chemical analysis and molecular studies were obtained by cultivation in TSA without agar (10%, w/v NaCl; pH 7.4) at 37 °C and 150 rpm for 1 week.

Physiological, Morphological, and Biochemical Tests

Cell morphology, size, shape, and flagellation were examined by light microscopy (BX41,Olympus) and transmission electron microscope (Model JEM1010, JEOL), using cells from exponentially growing cultures after incubation of 3 days in TSA containing 10% (*w/v*) NaCl and stored in refrigerator for 1 week to stimulate endospore formation. The Gram stain reaction was carried out by the Burke method [9] and the result was confirmed by the KOH test [10]. Growth at various temperatures (4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 45, 50, and 55 °C) were evaluated in TSA (10%, w/v NaCl) by incubating the cultures for 7 days. A series of pH conditions (4.0–11.0, at 0.5 intervals) using the buffer system described by Xu et al. [11] and salt tolerance (0–30%, w/v, at 5% intervals) was examined at 37 °C for 14 days. Growth under anaerobic condition was determined using the GasPak anaerobic system (BBL) according to the manufacturer's instructions.

Analysis of catalase and urease activities, nitrate reduction, H_2S and melanin production, and production of indole followed procedures as described by Smibert and Krieg [12]. Hydrolysis of Tweens was examined as described by Harrigan and McCance [13]. Carbon source utilization and enzyme activities of strain YIM 98829T was analyzed using API 20E, API 20NE, and API ZYM kits according to the manufacturer (bioMérieux, France) instructions. The acid production from carbohydrates was determined using the API 50CH system (bioMérieux) according to the manufacturer's instructions. Antibiotic susceptibility tests were performed on TSA containing 10% (*w/v*) NaCl using disks impregnated with various antimicrobial compounds [14]. The data from strains of species *A. haloalkaliphilus* DSM 5271^T, *A. silvisoli* BM2^T, and *A. filiformis* 4AG^T were used as references.

16S rRNA Gene Sequence, Phylogenetic Analysis, DNA–DNA Hybridization, and G + C Content

The extraction of genomic DNA, PCR amplification, and sequencing of the 16S rRNA gene were carried out as described by Yang et al. [15] and Li et al. [16]. The values for sequence similarity among the most closely related strains were determined using the NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [17]. Multiple alignments with sequences of the most closely related bacteria were carried out using the CLUSTAL X 1.8 program [18]. Phylogenetic trees were reconstructed by the neighbor-joining [19], maximum-parsimony [20], and maximum-likelihood [21] tree-making algorithms using the software packages MEGA version 7.0 [22]. The significant level of branch stability was assessed by performing bootstrap analyses with 1000 resamplings [23]. The genome of YIM 98829^T was sequenced using an HiSeq X-ten at BGI.tec (Shenzhen, China). The sequenced reads were assembled using SOAPdenovo software version 1.05 (https://soap.genomics.org.cn/soapd enovo.html). The DNA–DNA hybridization (DDH) similarity between strains was calculated in silico with the Genome-to-Genome distance calculator server version 2.1 [24]. Average nucleotide identity (ANI) was calculated with OrthoANI [25]. Strain *A. haloalkaliphilus* DSM 5271^T (GCA_007991275.1) was used as the reference strain in the ANI value calculation and digital DDH [26]. Genome tree was constructed using RAxML [26] and fast bootstrapping [27] was used to generate the support values in the tree. The DNA G + C mol% value was obtained from the genomic sequence.

Biochemical Characteristics

Peptidoglycan was purified and hydrolyzed according to Schumann [28] and was analyzed by HPLC–MS as described previously [29]. Amino acid enantiomers present in the total hydrolysate were analyzed on an Infinity Poroshell Chiral T column and detected by mass spectrometry (Fig. S5). The sugars of whole-cell hydrolysates were detected by Hasegawa et al. [30] Polar lipids were extracted and then examined by two-dimensional TLC and identified

using previously described procedures [31, 32]. The isolate and separate menaquinones were isolated according to Collins et al. [33] and separated by HPLC [34]. For cellular fatty acid analysis, strain YIM 98829^T was grown on tryptic soya broth with 10% NaCl (TSB, Difco) at 37 °C and harvested after 7 days. Fatty acid methyl esters were extracted, methylated, and analyzed by using the Microbial Identification System (Sherlock version 6.1, MIDI database, TSBA6) according to the manufacturer's instructions.

Results and Discussion

Physiological, Morphological, and Biochemical Tests

Strain YIM 98829^T was motile, aerobic, produced terminal endospores in swollen sporangia. Cells were long rods with a width of 0.3–0.5 μm and length of 2–5 μm (Fig. S4). Strain YIM 98829^T grew at NaCl concentrations between 5.0 and 20.0% (*w/v*), with optimal growth occurring at 5.0–10.0% (*w/v*). The temperature range for growth extended from 10 to 50 °C; with an optimum at 37 °C, strain YIM 98829^T grew well in the slightly alkaline conditions of pH 7.5–8.0 in TSA containing 10% (w/v) NaCl, positive for production of catalase and nitrate reduction and negative for urease, indole, and hydrolysis of aesculin. Tweens 20, 40, and 80 were not hydrolyzed. All the above characteristics are consistent with those of genus *Alkalibacillus.* However, strain YIM 98829^T stained Gram negative, which was very different from other published species of the genus *Alkalibacillus.* Other physiological characteristics and biochemical characteristics are given in Table 1 and in the species description.

16S rRNA Gene Sequence and Phylogenetic Analysis

An almost complete 16S rRNA gene sequence (1553 bp) of strain YIM 98829^T was obtained. The closest relative of strain YIM 98829^T was *Alkalibacillus. haloalkaliphilus* DSM 5271^T with 97.0% 16S rRNA gene sequence similarity. The phylogenetic trees based on the 16S rRNA gene

Table 1 The biochemical and physiological characteristics of strain YIM 98829^T and the reference strains of genus *Alkalibacillus*

Characteristics	1	2	3	4
Gram stain reaction	−	+[a]	+	+
Morphology	Long rods	Rods[a]	Long rods	Long rods
Pigmentation	Brown	Cream white[a]	Cream	White, transparent
NaCl for growth (%)				
Range	5.0–20.0	0–25.0[a]	5.0–25.0	0–18.0
Optimum	5.0–10.0	5.0–10.0[a]	10.0–15.0	10.0
pH for growth				
Range	6.0–10.0	7.0–9.7[a]	7.0–10.0	7.0–10.0
Optimum	7.5–8.0	9.7[a]	9.0–9.5	9.0
Motility	+	+[a]	+	−
Catalase	+	+[a]	+	−
Nitrate reduction	+	+	−	−
Hydrolysis of				
Gelatin	−	−	+	+
Acid production from				
D-Fructose	−	W	−	−
D-galactose	+	−	+	−
D-Mannitol	+	−	+	−
Maltose	−	−	+	−
D-Trehalose	−	−	+	−
Meso-Diaminopimelic acid	−	+	+	+
Major fatty acids	iso-$C_{15:0}$, anteiso-$C_{15:0}$	iso-$C_{15:0}$	iso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{17:0}$
G + C content (mol%)	38.0	37.0–38.0[a]	37.0	39.5
Quinone composition	MK-7	MK-7[a]	MK-7	MK-7, DeMK-6 fully saturated

Strains: 1, YIM 98829^T; 2, *A. haloalkaliphilus* DSM 5271^T (All data were obtained from this study unless otherwise stated); 3, *A. silvisoli* BM2^T Usami et al. [4]; 4, *A. filiformis* 4AG^T; Romano et al. [3] and Pérez-Davó et al. [7]

+ positive, − negative, *w* weak

[a]Jeon et al. [1]

sequences showed that strain YIM 98829^T clustered with strain DSM 5271^T, *A. silvisoli* BM2^T (96.9% similarity), and *A. filiformis* 4AG^T (96.9% similarity) under the high bootstrap values (Fig. 1; Figs S1 and S2). Genome tree shows that YIM 98829^T is steadily clustered in a branch with the type strain of this genus under the 1000 bootstrap values (Fig. S3). However, the level of DNA–DNA relatedness between strain YIM 98829^T and *A. haloalkaliphilus* DSM 5271^T (GenBank accession no. GCA 007991275.1) was only 16.9%, which was well lower than the threshold value of 70.0% recommended for recognition of separate species [35]. The ANI value between these two strains was 73.2%. The low similarity and phylogenetic results clearly demonstrated that strain YIM 98829^T represents a different genomic species of genus *Alkalibacillus* and is distantly related to the type species of the genus as well as to the species *A. almallahensis* and *A. salilacus*. The latter two show a 16S rRNA gene sequence similarity value of 95.6% only as compared to the type species *A. haloalkaliphilus*. The DNA G + C content of strain YIM 98829^T was 38.0 mol%.

Biochemical Characteristics

The cellular fatty acid profile contained iso-$C_{15:0}$ (60.0%) and anteiso-$C_{15:0}$ (11.4%) as major fatty acids (> 10%), iso-$C_{17:0}$ (7.4%), iso-$C_{16:0}$ (8.0%), iso-$C_{14:0}$ (2.8%), $C_{16:0}$ (2.1%) and anteiso-$C_{17:0}$ (5.1%) as minor fatty acids (< 10%). *Meso*-, LL-, DL-diaminopimelic acid were absent in peptidoglycan purified from YIM 98829^T (Fig. S5) and L-ornithine was present as the diagnostic diamino acid. Besides D-glutamic acid and alanine, L-serine and D-aspartic acid could be detected. In a partial hydrolysate of the peptidoglycan, the dipeptides L-Ala—D-Glu, D-Glu—L-Orn, L-Orn—L-Ser and L-Orn—D-Ala and the longer peptides D-Glu—L-Orn—L-Ser, L-Orn—L-Ser—D-Asp, L-Orn—L-Ser—D-Asp—D-Ala, and L-Orn—L-Ser—D-Asp—D-Ala—L-Orn were detectable. Accordingly, the amino acid composition of the peptidoglycan of strain YIM 98829^T corresponds to the peptidoglycan type A4β L-Orn—L-Ser—D-Asp (A21.7, www.peptidoglycan-types.info). Xylose (34.8%), glucose (23.2%), galactose (15.6%), and mannose (13.2%) were detected as the major whole-cell sugars; minor amounts of ribose (7.0%) and fucose (6.1%) were also detected. The phospholipids were diphosphatidylglycerol, phosphatidylglycerol, one unknown phospholipid, and two unidentified aminophospholipid (Fig. S6). And the predominant isoprenoid quinone in strain YIM 98829^T was MK-7 (100%). The chemical characteristics are similar to those of other species of genus *Alkalibacillus* except the presence of L-ornithine as the diamino acid of the peptidoglycan.

Conclusion

Some obvious differences in physiological and biochemical characteristics, such as Gram stain reaction, absence of *meso*-diaminopimelic acid, and low level of DNA–DNA relatedness, distinguish strain YIM 98829^T from other species. Based on the polyphasic analysis it can be concluded that strain YIM 98829^T should belong to the genus *Alkalibacillus*, even though that there is evidence to reclassify the *A. almallahensis* cluster in the future. The data described above also indicated that strain YIM 98829 ^{T} represents a novel species of the genus *Alkalibacillus*, for which the name *Alkalibacillus aidingensis* sp. nov. is proposed.

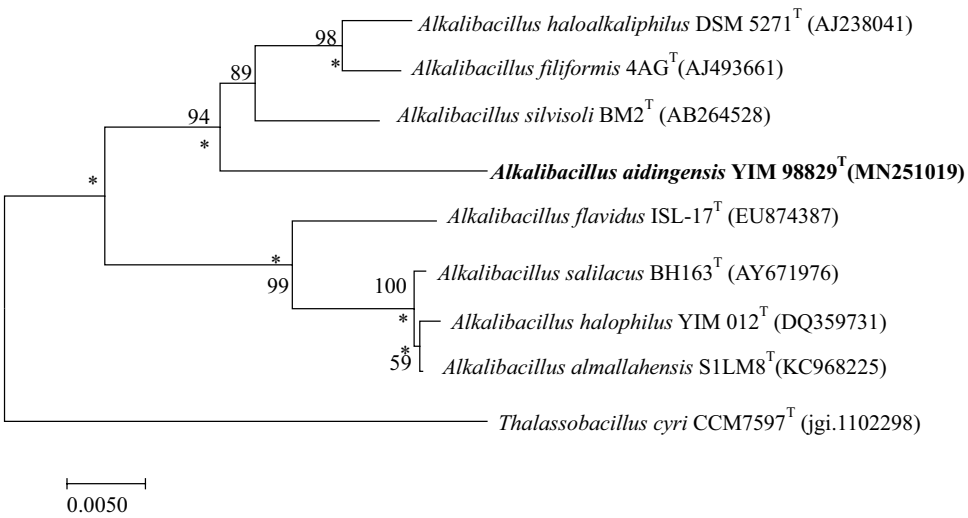

Fig. 1 The neighbor-joining (NJ) tree based on 16S rRNA gene sequence analysis showing phylogenetic relationships of strain YIM 98829^T and members of genus *Alkalibacillus*. Bootstrap values more than 50% based on 1000 replications are shown at branching points. Scale bar, 0.005 substitutions per nucleotide position

Description of *Alkalibacillus aidingensis* sp. nov.

Alkalibacillus aidingensis (ai.ding.en′sis; N.L. masc. adj. aidingensis of or belonging to Aiding lake, a salt lake in China, where the type strain was isolated).

Gram-stain-negative; results from API 50CH tests showed that acids was produced from D-galactose, D-mannitol, D-sorbitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, and potassium 5-ketogluconate. Sensitive to ampicillin (10 μg), bacitracin (10 μg), tetracycline (30 μg), novobiocin (5 μg), neomycin (30 μg), cefoxitin (30 μg), cefotaxime/clavulanic acid (30 μg), carbenicillin (100 μg), chloramphenicol (30 μg), gentamicin (10 μg), rifampicin (5 μg), streptomycin (10 μg), erythromycin (5 μg), and kanamycin (5 μg). Positive for alkaline phosphatase, esterase C4, esterase lipase C8, valine arylamidase, trypsin, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. The cell wall peptidoglycan contained ornithine, serine, aspartic acid, glutamic acid, and alanine. Mannose, galactose, glucose, and xylose are the major whole-cell sugars. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, unknown phospholipid and two unidentified aminophospholipids. The predominant menaquinone is MK-7. The major fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. The DNA G + C content of the type strain is 38.0 mol%. The type strain is YIM 98829^T (= NBRC 114103^T = CGMCC 1.17260^T = DSM 112470^T), isolated from the Aiding Lake in Xinjiang province, North-West China.

Emended Description of the Genus *Alkalibacillus* Jeon et al. 2005

Alkalibacillus (Al.ka.li.ba.cil′lus. N.L. n. alkali; L. n.bacillus rod; N.L. masc. n. *Alkalibacillus* bacillus living under alkaline conditions).

Cells are Gram positive or negative, spore-forming rods. Spherical endospores are produced terminally in swollen sporangia. Strictly aerobic and moderately halophilic. The whole-cell hydrolysates contain *meso*-diaminopimelic acid, or the cell wall peptidoglycan type is of the A4β type with L-Orn as the diagnostic diamino acid. Major isoprenoid quinone is MK-7. DNA G + C content is 38.0–41 mol% (HPLC). Predominant cellular fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$, and anteiso-$C_{17:0}$. Phylogenetically, the genus belongs to the family *Bacillaceae*. The type species of the genus is *Alkalibacillus haloalkaliphilus*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-021-02587-6.

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