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Bacillus tamaricis sp. nov., an alkaliphilic bacterium isolated from a *Tamarix* cone soil

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Abstract

A Gram-stain-positive, alkaliphilic bacterium, designated EGI 80668^T, was isolated from a *Tamarix* cone soil in Xinjiang, north-west China. Cells were facultatively anaerobic, terminal endospore-forming and motile by means of peritrichous flagella. Colonies were yellowish and the cells showed oxidase-negative and catalase-positive reactions. Strain EGI 80668^T grew at pH 8.0–10.0 and with 0–10 % (w/v) NaCl (optimally at pH 9.0 and with 1–2 % NaCl) on marine agar 2216. The predominant menaquinone was MK-7. The major fatty acids were anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$. The cellular polar lipids contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, four unknown phospholipids and one unknown aminophospholipid. The G+C content of the genomic DNA was 38.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain EGI 80668^T was affiliated to the genus *Bacillus*. The highest 16S rRNA gene sequence similarity between strain EGI 80668^T and a member of the genus *Bacillus* was 96.83 % with *Bacillus cellulosilyticus* JCM 9156^T. A polyphasic taxonomic study based on morphological, physiological, biochemical and phylogenetic data indicated that strain EGI 80668^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus tamaricis* sp. nov. (type strain EGI 80668^T=KCTC 33703^T=CGMCC 1.15917^T) is proposed.

The genus *Bacillus* was first described by Cohn in 1872 and is the largest genus in the family *Bacillaceae* [1]. At the time of writing, the genus contains 227 recognized species (http://www.bacterio.net/bacillus.html). The core characteristics of the genus are Gram-stain-positive, endospore-forming and rod-shaped cells. *Bacillus* species are ubiquitously distributed in diverse environments, such as neutral, acidic, saline and alkaline habitats [1, 2]. Alkaliphilic *Bacillus* strains have attracted great interest because of their industrial potential for production of alkaline-active enzymes [3]. The first reported alkaliphilic *Bacillus* species was *Bacillus alcalophilus* [4], and many novel alkaliphilic *Bacillus* species have since been isolated from various environments, such as *Bacillus alkalitelluris* and *Bacillus bogoriensis* [5]. During a study on the diversity of alkaliphilic bacteria, a novel alkaliphilic bacterium, designated EGI 80668^T, was isolated from a *Tamarix* cone soil collected from Xinjiang, north-west China. In this study, strain EGI 80668^T was subjected to a polyphasic taxonomic investigation, and the resulting data indicated that it represents a novel member of the genus *Bacillus*.

Strain EGI 80668^T was isolated by the dilution plating method on marine agar 2216 adjusted to pH 9.0 with autoclaved 10 M NaOH and then incubated at 30 °C for 4 weeks. The strain was maintained on the same medium as described above, and preserved as a 20 % glycerol (w/v) suspension at −80 °C. Biomass for chemical and molecular studies was obtained by cultivation on modified marine agar 2216 for 7 days.

To characterize strain EGI 80668^T, standard phenotypic tests were performed according to the recommended minimal standards for describing new taxa of aerobic, endospore-forming bacteria [6]. The reference strains *Bacillus vedderi* CGMCC 1.3496^T and *Bacillus clarkii* CGMCC 1.3674^T were obtained from China General Microbiological Culture Collection (CGMCC), and *Bacillus cellulosilyticus*

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Keywords: *Bacillus tamaricis* sp. nov.; alkaliphilic; polyphasic taxonomy; *Tamarix* cone soil.
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One supplementary table and three supplementary figures are available with the online version of this article.

JCM 9156^T and *Bacillus polygoni* JCM 14604^T were obtained from Japan Collection of Microorganisms (JCM).

Colony morphology was observed by incubating strain EGI 80668^T on marine agar 2216 plates adjusted to pH 9.0. Cell morphology and flagella type were observed by transmission electron microscopy (JEM-2100; JEOL) of 5-day-old cells. Endospores were observed by phase-contrast light microscopy (DM 2000; Leica). Gram-staining was performed by using a bioMérieux Gram stain kit according to the manufacturer's instructions and the result was confirmed with the KOH lysis test [7]. To determine the oxygen requirements of strain EGI 80668^T, the isolate was inoculated on modified marine agar 2216 adjusted to pH 9.0 with autoclaved NaOH and cultured at 30 °C for 7 days in a Whitley A45 Workstation (DWS) charged with mixed gases (10 % CO_2, 10 % H_2, 80 % N_2). The pH range for growth was determined between pH 4.0 and 12.0 (at intervals of 1.0 pH unit) in marine broth 2216 by using the buffer system described by Xu *et al.* [8]. The NaCl requirement for growth was tested on marine agar 2216 adjusted to pH 9.0 with autoclaved 10 M NaOH and supplemented with 0–15 % (w/v) NaCl at intervals of 1 % NaCl. The effect of temperature on growth was investigated by incubation on marine agar 2216 (pH 9.0) at 5–60 °C (at intervals of 5 °C). Catalase activity was tested by determination of bubble production in a 3 % (v/v) aqueous hydrogen peroxide solution. Oxidase activity was detected by using the bioMérieux oxidase reagent according to the manufacturer's instructions. General biochemical tests including H_2S production, nitrate reduction, and hydrolysis of Tweens 20, 40, 60 and 80, cellulose, starch and casein were carried out according to previously described methods [9, 10]. Carbon source utilization tests were performed as described by Shirling and Gottlieb [11]. Sole nitrogen source utilization tests were performed as described by Williams *et al.* [9]. All media were adjusted to pH 9.0 with autoclaved 10 M NaOH.

Strain EGI 80668^T formed circular, smooth, convex, yellowish colonies with diameters of 1–3 mm after inoculation for 3 days on marine agar 2216 at 30 °C. Cells were Gram-stain-positive, motile by peritrichous flagella and rod-shaped (0.4–0.5 µm in width and 7.0–11.0 µm in length) (Fig. 1). Ellipsoidal endospores were located terminally in swollen sporangia. Strain EGI 80668^T was able to grow under anaerobic conditions, but only very sparsely under aerobic conditions, which indicated that strain EGI 80668^T was facultatively anerobic. Growth occurred at pH 8.0–10.0 and 25–40 °C, optimally at pH 9.0 and 30 °C. The isolate was able to tolerate up to 10 % (w/v) NaCl on marine agar 2216 (optimum concentration 2 % NaCl). The oxidase reaction was negative and catalase reaction was positive. Nitrate could be reduced to nitrite. Hydrolysis of cellulose, Tweens 40 and 60 was positive, hydrolysis of casein was weakly positive, while reactions for H_2S production, and hydrolysis of starch and Tweens 20 and 80 were negative. Other physiological and biochemical characteristics of the isolate are given in Table 1 and the species description.

Respiratory quinones were extracted with chloroform/methanol (2 : 1, v/v) from dried cells and purified by TLC [12]. The purified contents were analysed by reversed-phase HPLC [13]. Polar lipids were extracted and separated by two-dimensional silica gel TLC with chloroform/methanol/water (65 : 25 : 4, by vol.) in the first direction followed by chloroform/methanol/acetic acid/water (80 : 12 : 18 : 5, by vol.) in the second direction. The spots were identified by spraying with appropriate detection reagents as previously described [14]. For fatty acid analysis, strain EGI 80668^T was cultured on tripticase soy agar (TSA) medium adjusted to pH 9.0 with NaOH at 30 °C for 4 days. Cellular fatty acid analysis was performed as described by Sasser [15] according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System. The prepared fatty acid methyl esters were separated using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID) and analysed with the Microbial Identification software package (Sherlock Version 6.1). During the procedure, an Agilent Technologies 7890A GC system (gas chromatographic column, 0.2 mm × 25 m, ultra 25 % Phenyl Methyl Silox; Agilent) and MIDI database (TSBA6) were used. To determine the G+C content, the genomic DNA was extracted with an E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek) and sequenced using a HiSeq 2500 sequencer (Illumina) at Beijing Biomarker Technologies. The paired-end reads were assembled using SOAPdenovo [16]. The G+C content (mol%) was calculated from the genome sequence.

The predominant menaquinone detected was MK-7. Cellular polar lipids extracted contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, four unidentified phospholipids and one unidentified aminophospholipid (Fig. S1, available in the online version of this article). The major fatty acids (>10 %) were anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$. The fatty acid profiles of strain EGI

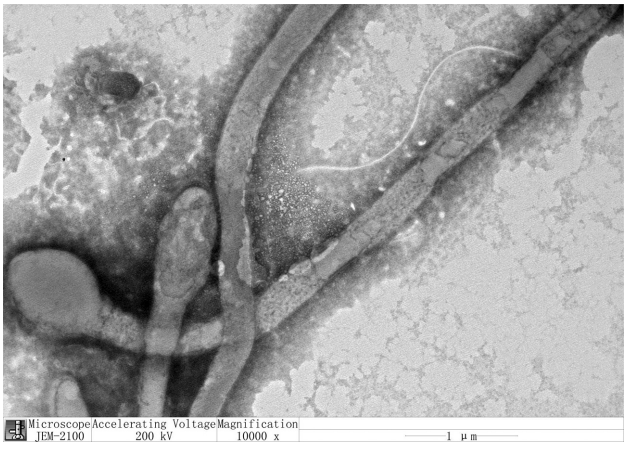

Fig. 1. Transmission electron micrograph showing the cell morphology and flagella type of strain EGI 80668^T grown on modified marine agar 2216 with pH adjusted to 9.0 with autoclaved 10 M NaOH at 30 °C for 5 days. Bar, 1 µm.

Table 1. Differential characteristics between strain EGI 80668^T and closely related members of the genus *Bacillus*

Strains: 1, EGI 80668^T; 2, *B. cellulosilyticus* JCM 9156^T; 3, *B. vedderi* CGMCC 1.3496^T; 4, *B. clarkii* CGMCC 1.3674^T; 5, *B. polygoni* JCM 14604^T; 6, *B. subtilis* subsp. *subtilis* DSM 10^T. +, Positive, utilized; −, negative, not utilized; w, weakly positive. All are positive for catalase, nitrate reduction and hydrolysis of Tween 40. They can utilize cellobiose, dulcitol, D-mannose and D-sorbitol as sole carbon source, and utilize L-isoleucine, L-threonine, L-proline and L-valine as sole nitrogen source.

Characteristic	1	2	3	4	5	6
Cell size (μm)	0.4–0.5×7.0–11.0	0.3–0.4×2.0–3.0*	0.4–0.5×1.0–1.2*	0.6–0.7×2.0–5.0*	0.4–0.5×1.0–3.5*	0.7–0.8×2.0–3.0*
NaCl tolerance range (w/v, %)	0–10	0–15	0–10	0–20	3–15	0–15
pH range	8–10	8–11	6–11	7–10	8–11	6–9
Optimum pH	9	9	9–10	8	9–10	7–8
Temperature range (°C)	25–40	15–35	20–45	15–40	15–45	10–55
Oxidase	−	−	+	−	−	+
H_2S production	−	−	−	+	+	−
Hydrolysis of:						
Casein	w	−	−	+	−	−
Cellulose	+	+	−	−	−	+
Starch	−	−	−	−	−	+
Tween 20	−	−	−	+	+	+
Tween 60	+	+	−	−	+	+
Tween 80	−	+	+	+	+	+
Carbon source utilization:						
D-Arabinose	−	−	+	−	+	+
L-Arabinose	−	+	−	−	+	+
Citric acid	+	+	−	−	−	+
D-Fructose	+	+	+	−	+	+
D-Galactose	−	+	+	+	−	+
D-Glucose	+	+	−	−	+	+
Glycerol	+	+	+	−	+	+
Lactose	−	+	−	+	+	+
Maltose	+	+	−	+	+	+
D-Mannitol	+	−	−	−	+	+
Melibiose	+	+	−	+	−	+
Raffinose	−	+	+	−	+	+
L-Rhamnose	+	+	−	+	−	+
Sodium acetate	+	−	+	+	−	+
Sucrose	+	+	−	−	+	+
Trehalose	+	+	+	−	+	+
D-Xylitol	−	−	−	+	−	+
D-Xylose	−	+	−	−	+	+
Nitrogen source utilization:						
L-Alanine	−	−	−	+	+	+
L-Arginine	−	−	−	−	−	+
L-Aspartic acid	−	−	−	−	−	+
L-Cysteine	−	−	−	−	+	+
L-Glutamic acid	−	−	+	+	−	+
L-Glycine	−	−	−	−	−	+
L-Histidine	−	−	−	+	−	−
L-Leucine	+	+	+	+	+	−
L-Methionine	−	−	+	+	+	+
L-Lysine	−	+	+	+	+	+
L-Phenylalanine	+	+	+	+	+	−
L-Serine	−	+	−	+	−	−
L-Tyrosine	+	+	+	−	+	+
L-Tryptophan	−	−	−	+	−	+
Hypoxanthine	−	−	−	−	−	+

Table 1. cont.

Characteristic	1	2	3	4	5	6
DNA G+C content (mol %)	38.3	39.6*	38.3*	38.6*	42.9*	42.9*

*DNA G+C contents and cell size data for the reference strains are from previously published articles [26, 4, 28, 27, 1].

80668^T and related reference strains are given in Table S1. The draft genome size of strain EGI 80668^T was 5 152 141 bp, and its G+C content was 38.3 mol% according to the genome.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out using procedures described by Li *et al.* [17]. The 16S rRNA gene was amplified by using the universal eubacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′). The PCR product was purified with a PCR purification kit in accordance with the manufacturer's protocol. The purified product was linked into the pEASY-T1 vector and transformed into *Escherichia coli* DH 5α by using the pEASY-T1 cloning kit (Transgen Biotechnology). The 16S rRNA gene sequence was determined by Sangon Biotech. Multiple alignments with 16S rRNA gene sequences of members of the genus *Bacillus* and calculations of levels of sequence similarity were carried out using the EzTaxon-e server (https://www.ezbiocloud.net/) [18]. Phylogenetic analysis was performed using three tree-making algorithms, namely the neighbour-joining [19], maximum-likelihood [20] and maximum-parsimony [21] methods, by using the software MEGA 6 [22] to determine the taxonomic position of strain EGI 80668^T. Evolutionary distances were calculated using Kimura's two-parameter method [23]. The topologies of the resultant trees were evaluated by using the bootstrap resampling method of Felsenstein [24] with 1000 replicates.

The almost-complete 16S rRNA gene sequence (1549 bp) was obtained from strain EGI 80668^T. 16S rRNA gene sequence analysis indicated that strain EGI 80668^T shared highest similarity values of 96.83, 96.61, 96.51 and 96.29 % with *Bacillus cellulosilyticus* JCM 9156^T, *Bacillus vedderi* DSM 9768^T, *Bacillus clarkii* DSM 8720^T and *Bacillus polygoni* JCM 14604^T, respectively. Gene sequence similarities to other species of the genus *Bacillus* were lower than 96.25 %, which has been proposed as a cut-off for determination of separate genomic species of *Bacillus*. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain EGI 80668^T fell within the clade of the genus *Bacillus*. In the neighbour-joining phylogenetic tree, strain EGI 80668^T clustered with *B. vedderi* DSM 9768^T and formed a single clade (Fig. 2). A similar tree topology appeared using the other two tree-making algorithms in this study (Figs S2 and S3).

According to the morphological, physiological, biochemical and phylogenetic data, strain EGI 80668^T shows typical characteristics of the genus *Bacillus*: Gram-stain-positive, endospore-forming, rod-shaped, MK-7 as the predominant quinone, and anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$ as major fatty acids. Furthermore, phylogenetic analysis based on 16S rRNA gene sequences also indicates that strain EGI 80668^T has the closest phylogenetic relationship with members of the genus *Bacillus*. Therefore, the new isolate belongs to the genus *Bacillus*. However, strain EGI 80668^T has distinct features from other species of the genus *Bacillus*: cell length is 7.0–11.0 µm, which is much higher than those of its closest phylogenetically related neighbours; the isolate is slightly halophilic and alkaliphilic, while its closest phylogenetic neighbours are alkaliphilic; and anteiso-$C_{17:0}$ is the predominant fatty acid for strain EGI 80668^T but not for its closest phylogenetic neighbours (Table S1). Phenotypically, strain EGI 80668^T also clearly differs from its phylogenetic neighbours based on physiological characteristics and the carbon and nitrogen sources utilized (Table 1). Therefore, based on the phenotypic, chemotaxonomic and phylogenetic characteristics described above, it is concluded that strain EGI 80668^{T}represents of a novel species of the genus *Bacillus*, for which the name *Bacillus tamaricis* sp. nov. is proposed.

DESCRIPTION OF *BACILLUS TAMARICIS* SP. NOV.

Bacillus tamaricis (ta.ma′ri.cis. L. gen. fem. n. *tamaricis* of *Tamarix*, referring to the isolation of the type strain from a *Tamarix* cone soil).

Cells are facultatively anaerobic, Gram-stain-positive, motile by peritrichous flagella and rod-shaped (0.4–0.5×7.0–11.0 µm). Terminal, ellipsoidal endospores are formed in swollen sporangia. Colonies are circular, smooth, convex and yellowish. Growth occurs at pH 8.0–10.0 (optimum, pH 9.0), at 25–40 °C (optimum, 30 °C) and in the presence of 10 % (w/v) NaCl (optimum, 2 %) on marine agar 2216. Catalase reaction is positive but oxidase reaction is negative. Positive for nitrate reduction and hydrolysis of cellulose and Tweens 40 and 60, weakly positive for hydrolysis of casein, but negative for H_2S production, and hydrolysis of starch, Tweens 20 and 80. Cellobiose, citric acid, dulcitol, D-fructose, D-glucose, D-glycerol, maltose, D-mannitol, D-mannose, melibiose, L-rhamnose, sodium acetate, D-sorbitol, sucrose and trehalose are utilized as sole carbon source for growth, but not D-arabinose, L-arabinose, D-galactose, lactose, raffinose, D-xylitol or D-xylose. L-Leucine, L-isoleucine, L-phenylalanine, L-threonine, L-tyrosine, L-proline and L-valine are utilized as sole nitrogen source, but not L-alanine, L-arginine, L-aspartic acid, L-cysteine,

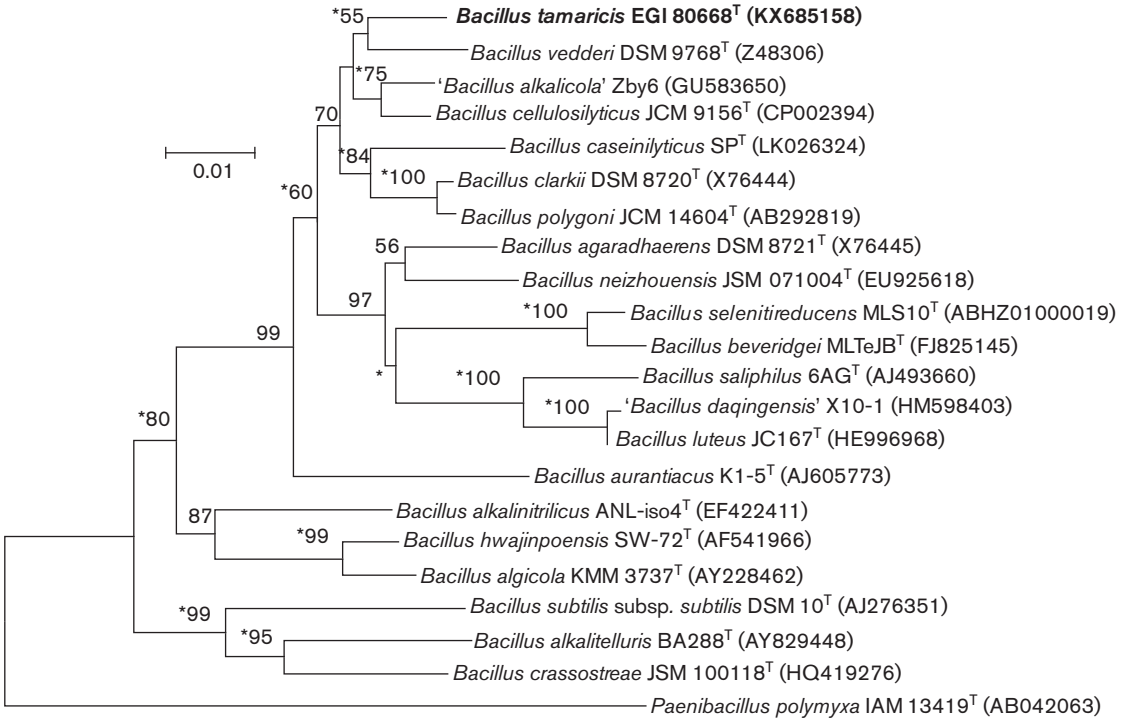

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain EGI 80668^T. Bootstrap values (expressed as percentages of 1000 replications) of above 50 % are shown at branch nodes. Asterisks indicate that the clades were conserved when the maximum-parsimony and maximum-likelihood methods were used to reconstruct the phylogenetic trees. Bar, 0.01 sequence divergence.

L-glutamic acid, L-glycine, L-histidine, L-methionine, L-lysine, L-tryptophan, L-serine or hypoxanthine. The predominant menaquinone is MK-7. The cellular polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, four unknown phospholipids and one unknown aminophospholipid. The major fatty acids are anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$.

The type strain is EGI 80668^T (=KCTC 33703^T=CGMCC 1.15917^T), isolated from a *Tamarix* cone sample in Xinjiang, north-west China. The genomic DNA G+C content of the type strain is 38.3 mol%.

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

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