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TAXONOMIC DESCRIPTION

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Bacillus kiskunsagensis sp. nov., a novel alkaliphilic and moderately halophilic bacterium isolated from soda soil

Andrea K. Borsodi,[1,*] Erika Tóth,[1] Júlia M. Aszalós,[1] Ágnes Bárány,[1] Peter Schumann,[2] Cathrin Spröer,[2] Attila L. Kovács,[3] Károly Márialigeti[1] and Tibor Szili-Kovács[4]

Abstract

An alkaliphilic and moderately halophilic strain characterized by optimal growth at pH 9.0–10.0 and 7 % (w/v) NaCl, and designated B16-24^T, was isolated from the rhizosphere soil of the bayonet grass *Bolboschoenus maritimus* at a soda pond in the Kiskunság National Park, Hungary. Cells of the strain were Gram-staining-positive, non-motile, straight rods, and formed central, ellipsoidal endospores with slightly swollen sporangia. The isolate was facultative anaerobic, catalase positive, oxidase negative, and contained a peptidoglycan of type A1γ based on *meso*-diaminopimelic acid. Menaquinone-7 (MK-7) was the predominant isoprenoid quinone, and anteiso-$C_{15:0}$ the major cellular fatty acid. The DNA G+C content of strain B16-24^T was 36.6 mol%. The 16S rRNA gene-based phylogenetic analysis revealed that the novel isolate had the greatest similarities to the type strains of *Bacillus okhensis* Kh10-101^T (97.8 %), *B. akibai* 1139^T (97.4 %), *B. alkalisediminis* K1-25^T (97.3 %) and *B. wakoensis* N-1^T (97.1 %). The DNA–DNA relatedness of strain B16-24^T and the closely related *Bacillus* species ranged between 24±6 % and 35±3 %. The distinctive phenotypic and genetic results of this study confirmed that strain B16-24^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus kiskunsagensis* sp. nov. is proposed. The type strain is B16-24^T (=DSM 29791^T=NCAIM B.02610^T).

The first aerobic alkaliphilic Gram-staining-positive and endospore-forming bacterium, denominated *Bacillus alcalophilus*, was isolated by Vedder [1]. Since then, several novel facultative and obligate alkaliphilic *Bacillus* species with different salt tolerance have been isolated and characterized by polyphasic taxonomic methods [2–6]. Alkaliphilic and/or halophilic *Bacillus* strains have been of great importance not only for the development of special adaptation mechanisms but to produce extracellular enzymes stable and active in alkaline and/or saline environments [7–10]. Athalassohaline soda lakes and soda soils of the Earth's warm semi-arid areas serve as typical habitats for these extremophiles. A phenotypically and phylogenetically non-coherent group of alkaliphilic *Bacillus* species were described from soda environments in the years of the new millennium. It includes the species of, for example, *B. alkalicola* [11], *B. alkalinitrilicus* [12], *B. alkalisediminis* [13], *B. aurantiacus* [14], *B. bogoriensis* [15], *B. caseinilyticus* [16], *B. chagannorensis* [17], *B. daliensis* [18], *B. lindianensis* [19] and *B. lonarensis* [20].

In the present study, a Gram-staining-positive, endospore-forming strain, designated B16-24^{T}and isolated from soda soil, is examined by polyphasic taxonomic methods and described as a novel species of the genus *Bacillus*.

The sample was collected from the rhizosphere soil of *Bolboschoenus maritimus* on the shores of the Böddi-szék soda pond (N46° 46′, E019° 08′) in the Kiskunság National Park, Hungary. The measured physical and chemical parameters of the soda soil at the time of sampling are presented by Bárány *et al.* [21]. The serially diluted sample was spread on Horikoshi alkaline medium 940 (www.dsmz.de) consisting of 10.0 g D-glucose, 5.0 g peptone, 5.0 g yeast extract, 1.0 g KH_2PO_4, 0.2 g $MgSO_4 \times 7H_2O$, 5.0 g Na_2CO_3, 15.0 g agar and 1000.0 ml distilled water. The pH of the medium was adjusted to 9.0. Single colonies developed after 6 days of incubation at 28 °C and were isolated in September 2013. DSM medium no. 940 was used to maintain the isolate.

DNA from strain B16-24^T was extracted using the Bacterial Genomic DNA Mini-prep Kit (V-GENE) according to the

Author affiliations: [1]Department of Microbiology, ELTE Eötvös Loránd University, Pázmány P. sétány 1/C, H-1117 Budapest, Hungary; [2]Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Inhoffenstrasse 7 B, 38124 Braunschweig, Germany; [3]Department of Anatomy-, Cell- and Developmental Biology, ELTE Eötvös Loránd University, Pázmány P. sétány 1/C, 1117 Budapest, Hungary; [4]Institute for Soil Sciences and Agricultural Chemistry, Agricultural Research Center, Hungarian Academy of Sciences, Herman Ottó út 15, H-1022 Budapest, Hungary.
***Correspondence:** Andrea K. Borsodi, borsodi.andrea@ttk.elte.hu
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Abbreviation: NCAIM, National Collection of Agricultural and Industrial Microorganisms.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B16-24^T is LN610501.
Four supplementary figures and one supplementary table are available with the online Supplementary Material.

manufacturer's instructions and 16S rRNA genes were amplified by PCR with the primers 27F (5′−AGA GTT TGA TCM TGG CTC AG−3′), 519F (5′−CAG CAG CCG CGG TAA TAC−3′) and 1492R (5′−TAC GGY TAC CTT GTT ACG ACT T−3′). Purification and sequencing of the PCR product were carried out by LGC Genomics GmbH (Berlin, Germany). The sequence was compared to 16S rRNA gene sequences available in the EzBioCloud database [22]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6.0 [23]. Following multiple alignments of sequences by ClustalW, phylogenetic trees were constructed by neighbour-joining (NJ) [24], maximum-likelihood (ML) [25] and maximum-parsimony (MP) [26] treeing algorithms. Kimura's two-parameter and general time-reversible models were selected for NJ and ML analyses, respectively. Tree topology was re-examined by the bootstrap method of resampling using 500 bootstraps. The DNA–DNA hybridization and the base composition of genomic DNA were performed following the methods given in Borsodi *et al.* [13].

The nearly complete 16S rRNA gene sequence of strain B16-24^T (1444 bp) had the closest sequence similarities with *Bacillus okhensis* Kh10-101^T (97.8 %), *B. akibai* 1139^T (97.4 %), *B. alkalisediminis* K1-25^T (97.3 %), *B. wakoensis* N-1^T (97.1 %), *B. alcalophilus* DSM 485^T (97.1 %), *B. nanhaiisediminis* NH3^T (97.1 %) and *B. haemicellulosilyticus* C-11^T (97.0 %). On the phylogenetic tree constructed by the ML method (Fig. 1), strain B16-24^T formed a distinct phyletic lineage within the genus *Bacillus* only with four of the seven closely related type strains showing higher than 97.0 % sequence similarity to strain B16-24^T. However, the type strain of *B. krulwichiae* was positioned in the same phyletic lineage despite its less than 97.0 % pairwise sequence similarity to strain B16-24^T. The phylogenetic trees created by NJ and MP algorithms resulted in similar topologies of the strains as shown in Figs S1 and S2 (available in the online Supplementary Material). The mean±SD values for DNA–DNA reassociation between strain B16-24^T and the closely related type strains of *B. okhensis*, *B. akibai*, *B. alkalisediminis* and *B. wakoensis* were 24±6, 35±3, 34 ±9 and 31±9 %, respectively. Considering the 70 % DNA–DNA relatedness threshold accepted for species delineation [27], strain B16-24^T occupied a distinct position within the genus *Bacillus*. The DNA G+C content of strain B16-24^T was 36.6 mol%.

The phenotypic characterization of strain B16-24^T was achieved by following the minimal standards for describing new taxa of aerobic, endospore-forming bacteria [28]. The type strains of *Bacillus okhensis* DSM 23308^T, *B. akibai* DSM 21942^T, *B. alkalisediminis* K1-25^T and *B. wakoensis* DSM 2521^T were used as reference strains for comparison of phenotypic properties of strain B16-24^T under the same laboratory conditions. The growth of strain B16-24^T was tested on modified nutrient (DSM medium no. 1) and R2A (DSM medium no. 830) media supplemented with 5 % (w/v) NaCl (the pH was adjusted to 9.0). Colony morphology of a 24–48 h culture was observed on nutrient medium (pH 9.0) supplemented with 5 % (w/v) NaCl using a stereomicroscope. The shape, size and arrangement of the cells were studied in native and Gram-stained preparations [29]. Endospore staining was carried out according to the method of Murray *et al.* [30]. Motility of 24-hour-old cells

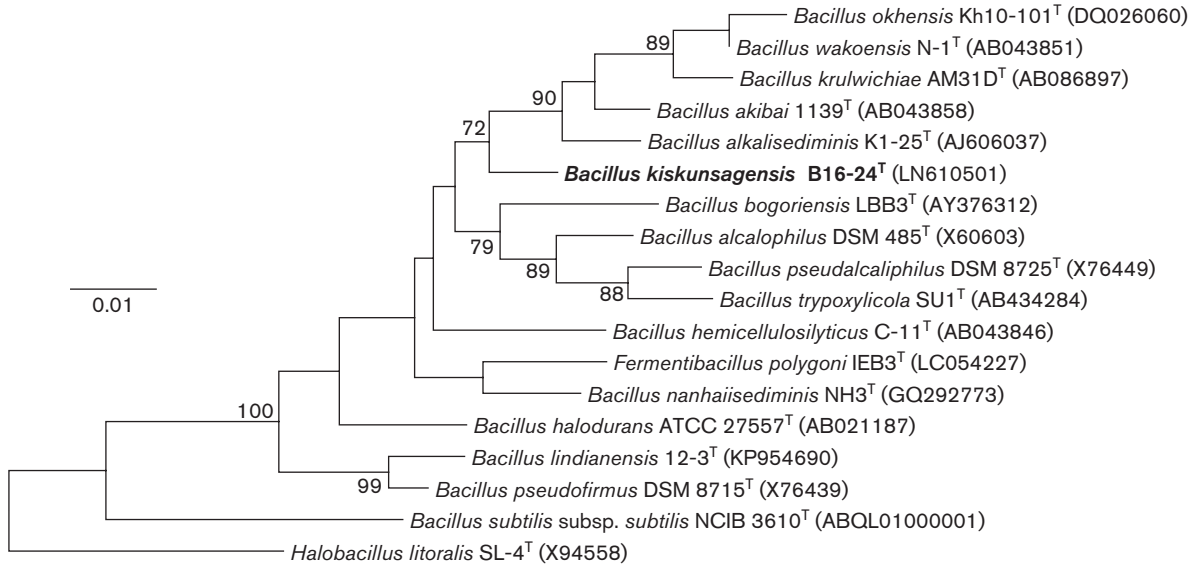

Fig. 1. 16S rRNA gene sequence based phylogenetic relations of strain B16-24^T and closely related *Bacillus* species. The tree was constructed using the maximum-likelihood algorithm. GenBank accession numbers are given in parentheses. Only bootstrap values above 70 % are shown (500 replications). Bar, 1 nucleotide substitution per 100 nucleotides.

was observed by phase-contrast microscopy of hanging-drop preparations. The morphology of sporulated cells developed on nutrient medium (pH 9.0) supplemented with 5 % (w/v) NaCl following 72 h of incubation at 28 °C was also studied using electron microscopy as described by Borsodi *et al.* [31]. Phenotypic tests such as catalase and Kovács' oxidase activity, Hugh-Leifson's oxidation–fermentation test of D-glucose as well as methyl red and Barritt's Voges-Proskauer tests, nitrate reduction to nitrite or nitrogen, Baird-Parkers' phosphatase activity, production of H_2S from cysteine and indole from tryptophan, aesculin hydrolysis and Simmons' citrate utilization were performed as described by Barrow and Feltham [32]. Urease activity, hydrolysis of casein, gelatin, starch and Tween 80 were examined according to Smibert and Krieg [33]. Physiological and biochemical tests were performed in media supplemented with 5 % (w/v) NaCl and pH was adjusted to 9.0 where possible. Growth was determined by incubation of strains on Horikoshi alkaline, furthermore on modified nutrient and R2A media both supplemented with 5 % (w/v) NaCl, and when required an anaerobic atmosphere was created by an Anaerocult A Mini (Merck) gas generator system. Utilization of various carbon compounds (1 % w/v) as sole carbon and energy sources was determined in a basal medium containing $(NH_4)_2SO_4$ 2.64 g, KH_2PO_4 2.38 g, $K_2HPO_4\times3H_2O$ 5.65 g, $MgSO_4\times7H_2O$ 1.00 g, $CuSO_4\times5H_2O$ 0.064 g, $FeSO_4\times7H_2O$ 0.011 g, $MnCl_2\times4H_2O$ 0.079 g, $ZnSO_4\times7H_2O$ 0.015 g, agar 20 g in 1000 ml deionized water as described by Makk *et al.* [34] supplemented with 5 % (w/v) NaCl, and adjusted to pH 9.0. Carbon compounds tested involved sodium formate, sodium acetate, sodium pyruvate, D-ribose, D-xylose, L-arabinose, D-fructose, D-glucose, D-galactose, D-mannose, D-rhamnose, lactose, maltose, D-sucrose, trehalose, glycerol, inositol, dulcitol, DL-alanine, asparagine and L-serine. The influence of temperature on growth was investigated by incubation of strains cultivated on nutrient medium (pH 9.0) supplemented with 5 % (w/v) NaCl at 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 28 °C, 37 °C, 40 °C and 45 °C. The NaCl requirement for growth was studied in nutrient broth (3.0 g beef extract

Table 1. Differential characteristics of strain B16-24^T and closely related *Bacillus* species

Strains: 1, B16-24^T; 2, *B. okhensis* DSM 23308^T; 3, *B. akibai* DSM 21942^T; 4, *B. alkalisediminis* K1-25^T; 5, *B. wakoensis* DSM 2521^T. Characters are scored as: +, positive; –, negative; w, weak positive reaction; CW, creamish white; CR, cream; Y, yellow; CRB, creamish brown; C, central; ST, subterminal; T, terminal; E, ellipsoidal; S, slightly swollen; NA, not applicable. All data were obtained from this study unless otherwise indicated.

Characteristic	1	2	3	4	5
Colony pigmentation	CW	CR	Y	CRB	Y
Spore formation	+	–	+	+	+
Spore location	C	NA	ST	C	T
Spore shape	E	NA	E	E	E
Swollen sporangia	S	NA	S	+	+
Motility	–	+	+	–	+
Anaerobic growth	+	–	–	–	–
Nitrate reduction to nitrite	+	–	+	+	+
Phosphatase activity	–	–	+	–	–
Hydrolysis of:					
Casein	–	+	–	–	–
Gelatin	–	+	–	+	–
Starch	–	+	+	–	+
Tween 80	–	–	+	+	–
Temperature (°C):					
Range	10–40	25–40	20–45	15–37	10–40
Optimum	25–28	37	37	25–28	37
NaCl (% w/v) for growth:					
Range	5–10	0–10	0–7	2–12	0–10
Optimum	5–7	5	7	5	10
pH for growth:					
Range	7–12	7–10	8–10	7–12	8–10
Optimum	9–10	9	9–10	9–11	9–10
DNA G+C content (mol%)	36.6	41.6*	34.4†	39‡	38.1§

*Data from Nowlan *et al.* [6].

†Data from Nogi *et al.* [9].

‡Data from Borsodi *et al.* [13].

§Data from Nogi *et al.* [9].

and 5.0 g peptone l^{-1}) adjusted to pH 9.0 and supplemented with 0–15 % (w/v) NaCl at intervals of 1 % NaCl. The pH range for growth was determined in nutrient broth supplemented with 50 g l^{-1} NaCl. The pH was adjusted to 4.0–13.0 at intervals of 1.0 pH unit using the following buffer systems: Na_2HPO_4/citric acid (at pH $\leq$7.0), Tris/HCl (at pH 8.0–9.0) and Na_2CO_3/$NaHCO_3$ (at pH $\geq$10.0). All physiological tests were performed in duplicate at 28 °C.

Strain B16-24^T formed circular, entire, smooth and convex colonies of creamish white on the Horikoshi alkaline medium within 24–48 h at 25–28 °C. Straight rod-shaped cells of the strain (Fig. S3a), occurring singly or in pairs and occasionally in short chains, stained Gram-positive. No motility was observed by phase-contrast microscopy. Cells formed central ellipsoidal endospores in slightly swollen sporangia (Fig. S3b). The taxonomically relevant dissimilar morphological and physiological characteristics of strain B16-24^T and the type strains of *Bacillus okhensis*, *B. akibai*, *B. alkalisediminis* and *B. wakoensis* are shown in Table 1. Most sole carbon source utilization tests of strain B16-24^T were negative except for D-fructose, D-glucose, trehalose, DL-alanine and L-serine as presented in Table S1.

The cell-wall diamino acid was determined from whole-cell hydrolysates as described by Hasegawa *et al.* [35]. Isoprenoid quinones were extracted according to the method of Collins *et al.* [36] and the profile was analysed by HPLC (HP 9001) [37]. After 48 h of cultivation on Horikoshi alkaline medium (DSM medium no. 940) at 30 °C, cellular fatty acids were extracted according to Stead *et al.* [38] and analysed by GC [39]. For polar lipid analysis strain B16-24^T was cultivated in modified nutrient broth (supplemented with 5.0 g l^{-1} Na_2CO_3 and the pH was adjusted to 9.0) for 24 h at 28 °C. Polar lipids were identified according to the method described by Minnikin *et al.* [40].

The cell wall of strain B16-24^T contained *meso*-DAP in the peptidoglycan, indicating the existence of the peptidoglycan type A1γ. The major isoprenoid quinone of strain B16-24^T was MK-7 (90 %) as it is typical of the species *Bacillus* [41] but in strain B16-24^T a relatively high amount of MK-6 (9 %) was also detected. The fatty acid profile of strain B16-24^T was dominated by anteiso-$C_{15:0}$ as in the case of most members of the genus *Bacillus*. On the other hand, characteristic differences could be observed in the amounts of the other detected fatty acids compared to its closest relatives (Table 2). Summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{15:0}$ iso 2-OH) proved to be the third most abundant fatty acids, and anteiso-$C_{17:1}$ was present only in strain B16-24^T. In contrast, $C_{14:0}$ and $C_{16:1}\omega11c$ fatty acids were detected only in its closest relatives. Strain B16-24^T contained phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylserine and eight unknown phospholipids (Fig. S4). The composition of major polar lipids of strain B16-24^T (PG, DPG and PE) was identical to those found in alkaliphilic *Bacillus* species described from soda environments [11, 14, 16–20] but differences were detected in the number of other (e.g. unknown) polar lipids.

Table 2. Fatty acid profiles of strain B16-24^T and the closely related *Bacillus* species

Strains: 1, B16-24^T; 2, *B. okhensis* DSM 23308^T; 3, *B. akibai* DSM 21942^T; 4, *B. alkalisediminis* K1-25^T; 5, *B. wakoensis* DSM 2521^T. All data were obtained from this study. Fatty acids comprising less than 1 % are not shown. ND, not detected.

Fatty acid	1	2	3	4	5
$C_{14:0}$	ND	1.7	4.8	1.6	2.5
$C_{15:0}$	ND	1.7	1.7	ND	ND
$C_{16:0}$	6.6	10.5	14.0	4.8	9.8
iso-$C_{14:0}$	2.6	5.0	9.3	14.6	3.4
iso-$C_{15:0}$	5.5	5.3	12.1	19.5	8.2
iso-$C_{16:0}$	4.2	6.6	3.5	4.7	3.4
iso-$C_{17:0}$	2.4	1.5	2.5	2.3	1.8
anteiso-$C_{15:0}$	50.0	52.0	26.3	36.5	50.8
anteiso-$C_{17:0}$	15.8	11.4	3.5	1.1	6.6
anteiso-$C_{17:1}$	1.4	ND	ND	ND	ND
$C_{16:1}\omega7c$ alcohol	ND	ND	2.6	3.6	ND
$C_{16:1}\omega11c$	ND	1.1	18.2	6.8	3.8
Summed feature 3*	9.0	2.3	ND	4.2	6.6

*Summed feature represents groups of two or three fatty acids that could not be separated by GLC with the MIDI system: summed feature 3 comprises $C_{16:1}\omega7c$ and/or C_{15} iso 2-OH.

Based on the presented genetic and phenotypic distinctiveness, strain B16-24^T represents a novel species within the genus *Bacillus* for which the name *Bacillus kiskunsagensis* sp. nov. is proposed.

DESCRIPTION OF *BACILLUS KISKUNSAGENSIS* SP. NOV.

Bacillus kiskunsagensis (kis.kun.sag.en′sis N. L. masc. adj. *kiskunsagensis* referring to the name of Kiskunság National Park in Hungary, the location of the sampling site).

Cells of a 24–48 h culture are Gram-staining-positive straight rods (0.5–0.7×1.5–2.5 µm in size). Central, ellipsoidal endospores are formed in slightly swollen sporangia. Cells are non-motile. Colonies are creamish white, circular, entire, smooth and convex. Temperature range for growth is between 10 °C and 40 °C, with optimum at 25–28 °C. The range of salt concentration for growth is 5–10 % (w/v), optimally with 5–7 % (w/v) NaCl. Growth occurs between pH 7.0 and 12.0, optimally at pH 9.0–10.0. Facultative anaerobic. Catalase positive, oxidase negative. No acid and gas are produced from D-glucose. Voges-Proskauer and methyl red reactions are negative. Nitrate is reduced to nitrite but not to nitrogen. Hydrolysis of aesculin is positive. Casein, gelatin, starch, Tween 80 and urea are not hydrolysed. Citrate is not used. Production of H_2S, indole and phosphatase is negative. The following compounds are utilized as sole carbon

sources: D-fructose, D-glucose, trehalose, DL-alanine and L-serine. The following compounds are not utilized as sole carbon sources: sodium formate, sodium acetate, sodium pyruvate, D-ribose, D-xylose, L-arabinose, D-galactose, D-mannose, D-rhamnose, lactose, maltose, D-sucrose, glycerol, inositol, dulcitol and asparagine. The major fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The predominant quinone is menaquinone with seven isoprene units (MK-7). Phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine are the major polar lipids. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The DNA G+C content is 36.6 mol%.

The type strain, B16-24^T (=DSM 29791^T=NCAIM B.02610^T), was isolated from the rhizosphere soil of *Bolboschoenus maritimus* on the shores of the Böddi-szék soda pond in the Kiskunság National Park, Hungary.

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

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