Bacillus aurantiacus sp. nov., an alkaliphilic and moderately halophilic bacterium isolated from Hungarian soda lakes

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Three alkaliphilic and moderately halophilic strains designated K1-5^T, K1-10 and B1-1, characterized by optimal growth at pH 9.0–10.0 and at 3–7% (w/v) NaCl, were isolated from extremely shallow, alkaline soda lakes located in Hungary. Cells of the strains are Gram-positive, straight rods and form a central to subterminal, ellipsoidal endospore. The isolates are strictly aerobic, catalase-positive, oxidase-negative and contain a peptidoglycan of type A1 γ based on meso-diaminopimelic acid. In strain K1-5^T, menaquinone-7 (MK-7) is the predominant isoprenoid quinone and anteiso-C_{15:0} is the major cellular fatty acid. The DNA G+C content of strain K1-5^T is 42.9 mol%. 16S rRNA gene-based phylogenetic analysis revealed that the strains exhibit levels of sequence similarity of less than 95.8% to known *Bacillus* species. According to the polyphasic characterization, the strains represent a novel species, for which the name *Bacillus aurantiacus* sp. nov. is proposed. The type strain is K1-5^T (=DSM 18675^T =CCM 7447^T =NCAIM B002265^T).

Natural alkaline environments, especially the widely distributed soda lakes, contain dense populations of extremophile micro-organisms from phylogenetically diverse lineages (Jones *et al.*, 1998; Martins *et al.*, 2001; Rees *et al.*, 2004). Several alkaliphilic and/or halophilic *Bacillus* species originating from extreme habitats have been described (Agnew *et al.*, 1995; Nielsen *et al.*, 1995; Lim *et al.*, 2006a, b) and studied extensively not only because of their taxonomic and ecological significance but also for the applicability of the isolated strains for biotechnological and industrial purposes (Horikoshi, 1996; Nogi *et al.*, 2005). The shallow soda lakes (approx. 0.5–1.5 m deep) located in Hungary are the most western representatives of such lakes that lie across Eurasia and are structurally dissected by their extended reed coverage. In the last few years, the phylogenetic diversity of bacterial communities adapted to these special environments

has been studied thoroughly by cultivation-based methods. Different alkalitolerant and alkaliphilic *Bacillus* species were predominant within the cultivable bacteria not only from the water and sediment of the lakes but also from the periphyton communities of submerged stems of *Phragmites australis* (Borsodi *et al.*, 2005, 2007). The aim of the present work was a polyphasic characterization of strains of a novel alkaliphilic and moderately halophilic *Bacillus* species isolated from two extremely shallow alkaline soda lakes.

Samples for isolation of bacterial strains were collected from the upper 3–5 cm sediment layers of two extremely shallow soda lakes in May 2003. Strains K1-5^T and K1-10 were isolated from Kelemen-szék (46° 47' N 19° 11' E), while B1-1 was from Böddi-szék (46° 45' N 19° 09' E) situated in the Kiskunság National Park, Hungary. Serially diluted sediment samples were spread on modified seawater medium 246 (<http://www.dsmz.de>) consisting of 10.0 g beef extract, 10.0 g peptone, 20.0 g agar, 250.0 ml tap water and 750 ml seawater. Artificial seawater (ASW) contained 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (l⁻¹ distilled water). The pH of the medium was adjusted to 9.0. Single colonies that developed

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains K1-5^T, K1-10 and B1-1 are AJ605773, AJ605772 and AJ606036, respectively.

Detailed Biolog GP2 test results for the novel strains are available as supplementary material with the online version of this paper.

after 6 days of incubation at 28 °C were isolated. The same medium was used for maintenance of the strains.

Colony morphology was observed on modified seawater medium 246 by using a stereomicroscope. The shape, size and arrangement of the cells were studied in Gram-stained preparations (Claus, 1992). Endospore staining was carried out according to the method of Murray *et al.* (1994). Motility of 12 and 24 h old cells was observed by phase-contrast microscopy of hanging-drop preparations. The endospore morphology and the occurrence of flagella of the strains developed in modified seawater medium 246 (pH 9.0) were studied by transmission electron microscopy as described by Borsodi *et al.* (2003).

Physiological and biochemical tests such as catalase and Kovács' oxidase activity, Hugh and Leifson's oxidation-fermentation test of D-glucose, methyl-red and Barritt's Voges-Proskauer tests, nitrate reduction to nitrite or nitrogen (method 1), Baird-Parkers' phosphatase activity, production of H₂S from cysteine and indole from tryptophan, aesculin hydrolysis, tyrosine decomposition, phenylalanine deamination and Simmons' citrate utilization were performed as described by Barrow & Feltham (2003). Urease activity and hydrolysis of casein, gelatin, starch and Tween 80 were examined according to Smibert & Krieg (1994). Anaerobic growth was determined by incubation of strains on modified medium 246 for 7 days at 28 °C in a Forma Scientific anaerobic chamber. The pH range for growth was determined in nutrient broth (3.0 g beef extract and 5.0 g peptone l⁻¹, supplemented with 50 g NaCl) and pH was adjusted to 7.0–12.0 with KOH at intervals of 0.5 pH units. The NaCl requirement for growth was studied in nutrient broth adjusted to pH 9.0 and supplemented with 0–20 % (w/v) NaCl at intervals of 1 % NaCl. The influence of temperature on growth was investigated by incubation of strains cultivated on modified seawater medium 246 between 4 and 48 °C at intervals of 1 °C. All physiological tests were performed in duplicate at 28 °C except for temperature range determinations and were supplemented with ASW containing 5 % (w/v) NaCl.

For Biolog GP2 sole carbon source investigations, strains were cultivated on Biolog universal growth medium supplemented with 1 % glucose and 5 % NaCl (pH was adjusted to 9.0). After 16 h of incubation at 28 °C, the turbidity of the cell suspension (0.9 %, w/v, saline solution, pH 9.0) was set according to the instructions for Gram-positive spore-forming bacteria (GP-ROD SB) and the absence of endospores was checked microscopically. Development of the purple colour of the indicator due to substrate oxidation was measured as A₅₉₀ after 24 and 48 h of incubation at 28 °C. Carbon source utilization was regarded as positive when the given absorbance was higher than the mean absorbance on a microplate.

The cell-wall diamino acid of strain K1-5^T was determined from whole-cell hydrolysates as described by Hasegawa *et al.* (1983). Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977) and the profile was analysed

by HPLC (HP 9001) (Groth *et al.*, 1997) following the cultivation of cells in liquid rich medium (Yamada & Komagata, 1972) supplemented with 50 g NaCl and adjusted to pH 9.0. After 24 h of cell cultivation on tryptic soy agar medium (Difco) supplemented with 50 g NaCl and adjusted to pH 9.0, cellular fatty acids were extracted according to Stead *et al.* (1992) and were analysed by GC (Groth *et al.*, 1996). Polar lipids were determined according to the method described by Minnikin *et al.* (1979).

DNA for determination of the base composition of genomic DNA and for DNA–DNA hybridization was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). For determination of the base composition, the DNA was degraded to nucleosides by using P1 nuclease and bovine intestinal mucosa alkaline phosphatase as described by Mesbah *et al.* (1989). The nucleosides were separated by reversed-phase HPLC by using the HPLC system mentioned above according to the method described by Tamaoka & Komagata (1984). The G+C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine. DNA–DNA hybridization was carried out in 2× SSC buffer at 67 °C as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983), using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

DNA from the strains were extracted by using the Bacterial Genomic DNA Mini-prep kit (V-GENE) according to the manufacturer's instructions and 16S rRNA genes were amplified by PCR with the universal eubacterial primer pair of 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). PCR products were purified using the Viogene PCR-M Clean

Fig. 1. Longitudinal section showing a central to subterminal ellipsoidal endospore-forming cell of *Bacillus aurantiacus* sp. nov. K1-5^T. Bar, 1 µm.

Table 1. Differential characteristics between strain K1-5^T (*Bacillus aurantiacus* sp. nov.) and closely related species

Reference species: 1, *B. agaradhaerens* (data from Nielsen *et al.*, 1995); 2, *B. cellulosilyticus* (Nogi *et al.*, 2005); 3, *B. clarkii* (Nielsen *et al.*, 1995); 4, *B. salarius* (Lim *et al.*, 2006a); 5, *B. saliphilus* (Romano *et al.*, 2005); 6, *B. taeanensis* (Lim *et al.*, 2006b); 7, *B. vedderi* (Agnew *et al.*, 1995; Nogi *et al.*, 2005). +, Positive; –, negative; ND, no data available.

Characteristic	K1-5 ^T	1	2	3	4	5	6	7
Colony pigmentation	Orange	White	Cream	Yellow	Cream	Yellow	Cream	Yellow–white
Cell morphology	Rod	Rod	Rod	Rod	Rod	Cocci	Rod	Rod
Cell size (µm)	0.8–1.0 × 3.2–4.5	0.5–0.6 × 2.0–5.0	0.3–0.4 × 2.0–3.0	0.6–0.7 × 2.0–5.0	0.3–0.5 × 1.3–1.9	0.8–0.9	0.5–1.2 × 1.2–1.9	ND
Spore shape*	E	E	E	E	S	–	E	S
Spore location†	ST/C	ST	ST	T	T	–	T	T
Swollen sporangia	–	+	+	+	+	–	+	ND
Motility	–	+	+	ND	–	–	+	+
Flagellation	–	ND	Peritrichous	ND	–	–	Polar	Peritrichous
Oxidase	–	–	–	–	+	+	+	+
Anaerobic growth	–	–	ND	–	–	ND	ND	+
Nitrate reduction to nitrite	–	+	+	+	–	+	+	+
Hydrolysis of:								
Aesculin	–	+	ND	+	+	ND	+	+
Casein	–	+	–	+	–	–	–	–
Gelatin	–	+	–	+	ND	+	–	+
Starch	–	+	+	–	–	–	+	–
Tween 80	+	ND	ND	ND	–	ND	–	ND
Tyrosine decomposition	–	ND	ND	ND	–	+	+	ND
Phenylalanine deamination	–	–	ND	–	ND	–	ND	ND
Temperature optimum (°C)	28	30	37	30	30	37	35	ND
NaCl (% w/v) for growth								
Range	0–15	0–16	0–12	0–15	3–20	>0–25	0–12	0–8
Optimum	3–7	ND	ND	ND	10–12	16	2–5	ND
pH for growth								
Range	8.0–12.0	>7.0	8.0–10.0	>7.0	6.8–9.5	7.0–10.0	5.5–9.0	9.0–10.0
Optimum	9.5–10.0	10.0	9.0–10.0	10.0	8.0	9.0	7.5	10.0
DNA G + C content (mol%)	42.9	39.3–39.5	39.6	42.4–43.0	43.0	48.4	36.0	38.3

*E, Ellipsoidal; S, spherical.

†C, Central; ST, subterminal; T, terminal.

Up system following the manufacturer's instructions. Sequence analysis of the strains was accomplished by using the Big Dye Terminator cycle sequencing kit v. 3.1 according to the procedure provided by the manufacturer (Applied Biosystems). An ABI 310A Genetic Analyzer (Applied Biosystems) was used for automated sequence analysis. The sequences were aligned against the RDP database release 7.1 (Maidak *et al.*, 1996) using the ARB program package (Strunk & Ludwig, 1995). All sequences were also compared to 16S rRNA gene sequences available in GenBank by using the BLAST search program (<http://www.ncbi.nlm.nih.gov/blast/>). Evolutionary distances were calculated using Kimura's two-parameter and Jukes-Cantor methods (Kimura, 1980; Jukes & Cantor, 1969). Phylogenetic trees were constructed by three different treeing algorithms: neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and least-squares (De Soete, 1983). Tree topology was re-examined by the bootstrap method of resampling (Felsenstein, 1985) using 1000 bootstraps.

Strains K1-5^T, K1-10 and B1-1 formed circular, entire, smooth and convex colonies, goldish orange in colour on modified seawater medium 246. Straight, rod-shaped (0.8–1.0 × 3.2–4.5 µm) cells of the strains, occurring mainly singly or in pairs and occasionally in short chains, stained Gram-positive and were not motile in wet-mount preparations. Cells formed central to subterminal, ellipsoidal endospores in unswollen sporangia (Fig. 1). All three strains grew in nutrient broth medium as well as in this medium supplemented with up to 15 % NaCl (strain K1-5^T), 13 % NaCl (strain K1-10) or 12 % NaCl (strain B1-1). The differential characteristics of strain K1-5^T and type strains of related *Bacillus* species are presented in Table 1.

In Biolog GP2 microplates, all three strains oxidized Tween 40, α-D-glucose, 3-methylglucose, acetic acid, γ-hydroxybutyric acid, pyruvic acid and glycerol. Strain-dependent utilization of Tween 80, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, amygdalin, D-gluconic acid, myo-inositol,

Table 2. Fatty acid profiles of the novel strains and related species

Reference species: 1, *B. agaradhaerens* (data from Lim *et al.*, 2006b); 2, *B. cellulosilyticus* (Nogi *et al.*, 2005); 3, *B. clarkii* (Lim *et al.*, 2006a); 4, *B. salarius* (Lim *et al.*, 2006a); 5, *B. saliphilus* (Romano *et al.*, 2005); 6, *B. taeanensis* (Lim *et al.*, 2006b); 7, *B. vedderi* (Nogi *et al.*, 2005). Fatty acids representing less than 0.5 % are not shown. D, Detected; ND, not detected.

Fatty acid	K1-5 ^T	K1-10	B1-1	1	2	3	4	5	6	7
C _{14:0}	0.69	0.82	0.76	0.91	2	0.78				
C _{15:0}	ND	ND	ND			0.64			3.29	
C _{16:0}	2.43	2.77	3.22	5.28	42	2.25			5.41	3
C _{17:0}	ND	ND	ND		1			D		
C _{18:0}	ND	ND	ND	1.41	6					3
iso-C _{14:0}	1.53	1.71	1.61	0.91	2	9.67	8.95		2.46	3
iso-C _{15:0}	13.14	13.18	11.56	25.42	13	18.45	6.60	D	40.63	20
iso-C _{16:0}	7.40	7.00	8.26	1.66	4	11.35			0.96	9
iso-C _{17:0}	1.40	1.54	1.65	4.18	2	3.35	53.13		1.81	14
iso-C _{18:0}	ND	ND	ND							1
iso-C _{19:0}	ND	ND	ND							1
anteiso-C _{15:0}	55.49	53.41	52.63	40.18	23	35.64	18.67	89.0	25.89	15
anteiso-C _{17:0}	12.06	11.07	14.34	7.39	5	8.40				6
C _{16:1} ω7c	2.32	3.02	2.62							
C _{16:1} ω11c	ND	ND	ND	1.93					2.12	
C _{18:1} ω9c	ND	ND	ND	3.47						
iso-C _{16:1}	1.11	1.42	1.03							1
iso-C _{17:1}	ND	ND	ND							7
iso-C _{17:1} ω5c	0.70	0.83	0.59			1.09				
iso-C _{17:1} ω10c	ND	ND	ND	2.67						
anteiso-C _{17:1}	1.74	2.05	1.73							3
Summed features*										
2	ND	ND	ND			1.55				
3	ND	ND	ND			2.18			6.70	
4	ND	ND	ND	1.53					1.76	
5	ND	ND	ND	2.30						

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 comprises iso-C_{16:1} and/or C_{14:0} 3-OH; summed feature 3 comprises C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; summed feature 4 comprises iso-C_{17:1} and/or anteiso-C_{17:1}; and summed feature 5 comprises C_{18:2}ω6c and/or anteiso-C_{18:0}.

melibiose, stachyose, sucrose, trehalose, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, α -ketovaleric acid, propionic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, putrescine, 2,3-butanediol, 2'-deoxyadenosine, glucose 6-phosphate and DL- α -glycerol phosphate was detected (see Supplementary Table S1 in IJSEM Online). α -Cyclodextrin, β -cyclodextrin, dextrin, glycogen, inulin, mannan, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, α -D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, melezitose, methyl α -D-galactoside, methyl β -D-galactoside, methyl α -D-glucoside, methyl β -D-glucoside, methyl α -D-mannoside, palatinose, D-psicose, raffinose, L-rhamnose, D-ribose, salicin, sedoheptulosan, D-sorbitol, D-tagatose, turanose, xylitol, D-xylose, α -hydroxybutyric acid, β -hydroxybutyric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D- and L-malic acid, methyl pyruvate, monomethyl succinate, alaninamide, D- and L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyrogutamic acid, L-serine, adenosine, inosine, thymidine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, fructose 6-phosphate and glucose 1-phosphate were not utilized.

The cell wall of the type strain contained *meso*-diaminopimelic acid in the peptidoglycan, suggesting the occurrence of the peptidoglycan type A1 γ . The major isoprenoid quinone detected in strain K1-5^T was MK-7, and a minor amount of MK-6 was also detected. The cellular fatty acid compositions of strains K1-5^T, K1-10 and B1-1 were dominated by anteiso-C_{15:0}. Among the other major fatty acids, iso-C_{15:0} and anteiso-C_{17:0} were detected. The fatty acid profiles of the three strains were similar (Table 2). However, there are

characteristic differences between K1-5^T, K1-10 and B1-1 compared with their closest relatives (*Bacillus cellulosilyticus*, *Bacillus clarkii* and *Bacillus vedderi*). Strain K1-5^T contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unknown polar lipid.

The DNA G + C content of strain K1-5^T was 42.9 mol%. The three strains exhibited high levels of DNA–DNA reassociation with one another [K1-5^T with K1-10, 86.8 % (repetition 89.6 %); K1-5^T with B1-1, 88.0 % (repetition 91.8 %); K1-10 with B1-1, 95.3 % (repetition 97.9 %)], demonstrating that the strains belong to the same species. The phylogenetic tree constructed from evolutionary distances of Kimura's two-correction parameter by the neighbour-joining method showed that strains K1-5^T, K1-10 and B1-1 formed a cluster with *B. clarkii* DSM 8720^T, *B. cellulosilyticus* N-4^T, *B. vedderi* JaH^T, *Bacillus agaradhaerens* DSM 8721^T and *Bacillus saliphilus* 6AG^T, with sequence similarities of 94.7–95.8, 94.5–95.7, 94.3–95.4, 93.9–95.2 and 92.2–93.3 %, respectively (Fig. 2). The nearly complete 16S rRNA gene sequence of strain K1-5^T (1450 bp) had similarities of 98.7 and 97.6 % to strains K1-10 and B1-1 (1462 and 1459 bp), respectively. Sequence similarity between strains K1-10 and B1-1 was 98.9 %. Phylogenetic trees created by the maximum-likelihood and least-squares algorithms resulted in similar topologies of the strains (data not shown) to the tree obtained by neighbour-joining method.

The described results of phylogenetic distinctiveness and phenotypic disparities indicate that strains K1-5^T, K1-10 and B1-1 represent a novel species within the genus *Bacillus*, for which the name *Bacillus aurantiacus* sp. nov. is proposed.

Fig. 2. 16S rRNA gene sequence-based phylogenetic relationships of *Bacillus aurantiacus* sp. nov. strains K1-5^T, B1-1 and K1-10 and closely related members of the genus *Bacillus*. The tree was constructed using the neighbour-joining algorithm. GenBank accession numbers are given in parentheses. Only bootstrap values above 50 % are shown (1000 replications). Bar, 10 substitutions per 100 nucleotides.

Description of *Bacillus aurantiacus* sp. nov.

Bacillus aurantiacus (au.ran.ti.a'cus. N.L. masc. adj. *aurantiacus* goldish-orange coloured).

Cells are Gram-positive, straight rods. Central to subterminal, ellipsoidal endospores are formed in unswollen sporangia. Cells are non-motile. Colonies are goldish-orange, circular, entire, smooth and convex. Obligately aerobic. Catalase-positive, oxidase-negative. Acid is not produced from D-glucose and nitrate is not reduced to nitrite. Voges-Proskauer and methyl-red reactions are negative. Urea, aesculin, casein and starch are not hydrolysed. Tyrosine decomposition and phenylalanine deamination are negative. Simmons' citrate is not used. Production of H₂S and indole is negative. Phosphatase is not produced. Tween 80 is hydrolysed. No growth is detected at pH 7.0–7.5. Growth occurs between pH 8.0 and 12.0. Growth occurs optimally at pH 9.5–10.0 and with 3–7% (w/v) NaCl. Temperature range for growth is between 10 and 45 °C, with optimum growth at 28 °C. Major fatty acids are anteiso-C_{15:0}, iso-C_{15:0} and anteiso-C_{17:0}. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The DNA G + C content of the type strain is 42.9 mol%.

The type strain, K1-5^T (=DSM 18675^T =CCM 7447^T =NCAIM B002265^T), was isolated from sediment of Kelemen-szék, an extremely shallow soda lake situated in Hungary.

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