 DOI 10.1099/ijs.0.018259-0

Amphibacillus jilinensis sp. nov., a facultatively anaerobic, alkaliphilic bacillus from a soda lake

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A facultatively anaerobic, alkaliphilic, spore-forming, Gram-positive-staining rod, designated $Y1^T$, was isolated under strictly anaerobic conditions from sediment of a soda lake in Jilin province, China. The strain was not dependent on Na^+ but was highly halotolerant and grew optimally in medium JY with 0.5 M Na^+ (0.06 M $NaHCO_3$ and 0.44 M NaCl). The optimum pH for growth was 9.0, with a range of pH 7.5–10.5. No growth occurred at pH 7.0 or 11.0. The strain was mesophilic, with a temperature range of 15–45 °C and optimum growth at 32 °C. Strain $Y1^T$ was able to use certain mono- and oligosaccharides. Soluble starch and casein were hydrolysed. The methyl red test, Voges–Proskauer test and tests for catalase and oxidase activities were negative. The predominant fatty acids were anteiso-$C_{15:0}$ and iso-$C_{15:0}$. Comparative 16S rRNA gene sequence analysis revealed 93.4–96.8 % sequence similarity to members of the genus *Amphibacillus*. The DNA G+C content was 37.7 mol% (T_m method). The DNA–DNA relatedness of strain $Y1^T$ with respect to *Amphibacillus tropicus* DSM 13870^T and *Amphibacillus sediminis* DSM 21624^T was 48 and 37 %, respectively. On the basis of its phylogenetic position and the DNA–DNA relatedness data as well as its physiological and biochemical properties, strain $Y1^T$ represents a novel species of the genus *Amphibacillus*, for which the name *Amphibacillus jilinensis* sp. nov. is proposed. The type strain is $Y1^T$ (=CGMCC 1.5123^T =JCM 16149^T).

The genus *Amphibacillus* currently comprises four recognized species, *Amphibacillus xylanus* (Niimura *et al.*, 1990), *Amphibacillus fermentum*, *Amphibacillus tropicus* (Zhilina *et al.*, 2001) and *Amphibacillus sediminis* (An *et al.*, 2007). Members of the genus *Amphibacillus* are Gram-positive, moderately alkaliphilic, facultatively anaerobic rods (An *et al.*, 2007).

Strain $Y1^T$ was isolated from sediment of a soda lake (44° 45′ N 123° 34′ E) in Jilin province, China, in November 2007. There is no river to supply the lake with water, and atmospheric water and groundwater are the only sources of water. The lake was rich in (mg l^{-1}) Na^+ (257.2), CO_3^{2-} (50.7), Cl^- (10.1), HCO_3^- (6.5) and SO_4^{2-} (4.4), with the pH of the water sample being 10.0. About 3 g mud sample was incubated anaerobically in 40 ml enrichment culture (see below) at 32 °C for 3 days and pure cultures were obtained by the Hungate roll-tube technique (Hungate, 1969) under a gas phase of O_2-free N_2. The enrichment culture contained (l^{-1} distilled water) 10.0 g NaCl, 10.0 g Na_2CO_3, 5.0 g $NaHCO_3$, 0.2 g KH_2PO_4, 0.33 g $MgCl_2 . 6H_2O$, 0.5 g NH_4Cl, 0.2 g KCl, 2.0 g yeast extract (Difco), 5.0 g glucose, 0.5 g $Na_2S . 9H_2O$, 0.4 g L-cysteine, 10 ml trace element solution 141 (see DSMZ medium 141) and 0.001 g resazurin. After sterilization at 121 °C for 20 min, the pH of the medium was 9.5–10.0. Experiments then showed that $Y1^T$ also grew aerobically in the same medium without $Na_2S . 9H_2O$ and L-cysteine. The strain was routinely cultivated aerobically in optimized medium JY [l^{-1} distilled water: 2.0 g yeast extract (Difco), 5.0 g sucrose, 0.2 g KCl, 0.2 g KH_2PO_4, 0.1 g $MgCl_2 . 6H_2O$, 0.5 g NH_4Cl, 0.1 g $CaCl_2$, 0.06 M $NaHCO_3$ and 0.44 M NaCl; final pH 9.0].

Cell morphology and motility were examined by using optical (BX 40; Olympus) and transmission electron (H-600; Hitachi) microscopy. Cells of strain $Y1^T$ were straight to slightly curved rods (0.4–0.6 × 2–3.2 μm) with peritrichous flagellation (Supplementary Fig. S1, available in IJSEM Online) and stained Gram-positive. In the late-exponential and stationary phases of growth, the rods formed terminal endospores (Supplementary Fig. S1).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain $Y1^T$ is FJ169626.

A transmission electron micrograph of cells of strain $Y1^T$ and a 16S rRNA gene sequence-based maximum-parsimony tree are available as supplementary material with the online version of this paper.

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In the study of growth dependence on the medium pH, the total concentration of $NaHCO_3$ in the medium was 0.06 M and the optimal sodium concentration was maintained with NaCl. Sterile solutions (10 %) of HCl or NaOH were used to adjust the pH of the medium with the buffers MES (pH 5.5–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), CAPSO (pH 9.0–9.5) and CAPS (pH 10.0–11.5) at a concentration of 25 mM. No growth of strain $Y1^T$ occurred at pH 7.0, as is also the case for the type species of the genus *Amphibacillus*, and the optimum pH for growth was 9.0.

In order to establish NaCl dependence, different amounts of NaCl (0–0.9 M at 0.1 M intervals and 1.0–3.7 M at 0.3 M intervals) were added separately to the medium, with 0.06 M $NaHCO_3$ at every concentration except 0 M Na^+. The need for Cl^- was tested in a medium where NaCl was replaced with an equimolar amount of $NaHCO_3$ and all other chlorides were replaced with sulfates. To determine whether HCO_3^- was required for growth, it was replaced with an equimolar amount of NaCl, while the pH was maintained at 9.0 with 20 mM Tris (BBI). The temperature for growth was assessed at 4, 10, 15, 20, 25, 30, 32, 37, 40, 45, 50, 55 and 60 °C. Cl^- was not required. Strain $Y1^T$ was not dependent on Na^+ or HCO_3^- ions, but was highly halotolerant and could tolerate Na^+ to 2.8 M.

Single carbon source utilization tests were performed by using a minimal medium based on medium JY with 0.2 g yeast extract l^{-1} and without glucose. UV-sterilized sugars (25 mM), alcohols and glycerol (0.1 %), organic acids and amino acids (20 mM) and Casamino acids (BBI), yeast extract (Difco), tryptone (Difco) and starch (each at 10 g l^{-1}) were added separately to the minimal medium and under static culture.

Catalase activity was tested by adding a drop of 3 % H_2O_2 to a single colony and was recorded as positive when the development of bubbles was observed. Oxidase activity was determined by transferred strains to filter paper with freshly prepared 1 % *N*,*N*-dimethyl-*p*-phenylenediamine hydrochloride. H_2S and indole production were tested by using API 20E strips (bioMérieux). The methyl red and Voges–Proskauer reactions were examined as described by Lányí (1987). The results of these physiological tests are given in Table 1 and the species description.

For determination of the G+C content of the genomic DNA and for 16S rRNA gene sequence determination and phylogenetic analysis, DNA was prepared and purified as described by Marmur (1961). The DNA G+C content, determined by thermal denaturation (T_m) (Marmur & Doty, 1962) using *Escherichia coli* K-12 DNA as the calibration standard, was found to be 37.7 mol%. DNA–DNA hybridizations were performed by using the thermal denaturation and renaturation method of De Ley *et al.* (1970), with a Beckman DU 800 spectrophotometer. DNA–DNA relatedness of strain $Y1^T$ with respect to *A. tropicus* DSM 13870^T and *A. sediminis* DSM 21624^T was 48 and 37 %, respectively.

The 16S rRNA gene was amplified by PCR using the universal primers Bac 27F and 1492R, complementary to positions 8–27 and 1492–1513, respectively, of the 16S rRNA gene of *E. coli* (Winker & Woese, 1991). An almost-complete 16S rRNA gene sequence (1532 nt) of strain $Y1^T$ was aligned with sequences deposited in GenBank using CLUSTAL_X software (version 1.8). Preliminary comparisons with 16S rRNA gene sequences revealed that the isolate showed relatively high sequence similarity to the type strains of *Amphibacillus* species: *A. sediminis* Shu-P-Ggiii25-2^T (96.8 % similarity), *A. tropicus* Z-7792^T (95.7 %), *A. fermentum* Z-7984^T (94.4 %) and *A. xylanus* JCM 7361^T (93.4 %). Similarity values were calculated and converted to a distance matrix by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with the MEGA 4 program package (Tamura *et al.*, 2007). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method. Both the neighbour-joining tree (Fig. 1) and the maximum-parsimony tree (Supplementary Fig. S2) indicated that the isolate clustered most closely with members of the genus *Amphibacillus*.

Fatty acid methyl esters were prepared from lipids extracted from cells grown in DSMZ medium 529 under optimum conditions until OD_{600} 0.6 and analysed by using GC/MS (Kuykendall *et al.*, 1988). Data are given in Table 2. Quinones were extracted and analysed by HPLC according to Tindall (1990). No quinone was detected from strain $Y1^T$ or the type strains of the four recognized species of the genus *Amphibacillus*. The major distinguishing chemotaxonomic characteristic was the relatively large amount of anteiso-$C_{15:0}$ and iso-$C_{15:0}$ in strain $Y1^T$. In addition, comparisons of phenotypic properties (Table 1) also indicated differences between strain $Y1^T$ and recognized *Amphibacillus* species in motility, Na^+ and HCO_3^- dependence and optimum temperature for growth, hydrolysis of starch and casein, utilization of carbohydrates and antibiotic susceptibility. On the basis of phenotypic and genotypic characteristics and phylogenetic analysis, it is suggested that strain $Y1^T$ represents a novel species of the genus *Amphibacillus*, for which the name *Amphibacillus jilinensis* sp. nov. is proposed.

Description of *Amphibacillus jilinensis* sp. nov.

Amphibacillus jilinensis (ji.lin.en′sis. N.L. masc. adj. *jilinensis* pertaining to Jilin, a province of north-east China, from which the sample that yielded the type strain was collected).

Cells are straight to slightly curved, peritrichously flagellated rods (0.4–0.6 × 2.0–3.2 μm) that stain Gram-positive. In the late-exponential and stationary phases of growth, the rods form terminal endospores. The optimal temperature for growth is 32 °C; growth occurs at 15–45 °C, but not at 10 or 50 °C. The pH range for growth is 7.5–10.5 (optimum pH 9.0). Cl^- and HCO_3^- are not required.

Table 1. Phenotypic and genotypic characteristics useful for distinguishing strain Y1^T from type strains of *Amphibacillus* species

Strains: 1, Y1^T; 2, *A. tropicus* DSM 13870^T; 3, *A. sediminis* DSM 21624^T; 4, *A. fermentum* DSM 13869^T; 5, *A. xylanus* DSM 6626^T. Data are from this study. Tests for Na^+ dependence used 0.06 M $NaHCO_3$ with different concentrations of NaCl. The methyl red and Voges–Proskauer tests and tests for oxidase and catalase activity were negative for all strains. Cellobiose, D-fructose, D-glucose, maltose, sucrose, trehalose, D-xylose and yeast extract were utilized by all strains. None of the strains utilized Casamino acids, sorbose, organic acids (acetate, butyrate, formate, lactate, malate, propionate, pyruvate, succinate, tartrate and oxalate), several amino acids (L-alanine, L- arginine, L-aspartate, L-cysteine, L-glycine, L-histidine, L-methionine and L-serine) or glycerol. All five strains were sensitive to (μg per disc unless indicated) ampicillin (10), amoxicillin (10), carbenicillin (100), cefotaxime (30), cefoxitin (300), chloromycetin (30), nitrofurantoin (300), oxacillin (1), penicillin (10), phosphonomycin (200), rifampicin (5), tetracycline (30) and vancomycin (30) and not sensitive to bacitracin (0.04 U) or polymyxin B (300 U).

Characteristic	1	2	3	4	5
Motility	+	+	−	+	−
Bicarbonate dependence	−	+	−	+	−
Optimum temperature (°C)	32	37	30	37	40
Optimum pH	9.0	9.0	8.5	9.0	9.5
Na^+ tolerance (M)	0–2.8	0.06–2.5	0–1.6	0.06–3.6	0–0.9
Optimum Na^+ concentration (M)	0.5	0.5	0.1	0.7–0.9	0.1
Utilization of:					
Tryptone	−	−	+	−	−
L-Arabinose	+	−	+	+	+
D-Galactose	+	+	−	+	−
Lactose	+	+	+	+	−
Mannose	+	−	+	+	+
Mannitol	+	−	+	−	−
Melibiose	+	+	−	−	−
D-Raffinose	+	+	−	−	−
Rhamnose	+	+	+	−	+
Sorbitol	+	−	−	+	−
Hydrolysis of:					
Starch	+	+	−	+	+
Casein	+	−	+	−	−
Sensitivity to (per disc):					
Amikacin (30 μg)	−	+	+	−	+
Azithromycin (15 μg)	+	+	+	+	−
Erythromycin (15 μg)	+	+	+	+	−
Gentamicin (10 μg)	−	+	+	−	−
Kanamycin (30 μg)	+	+	+	−	+
Polymyxin B (300 U)	−	−	+	−	−
Streptomycin (10 μg)	+	+	+	+	−
Tenebrimycin (10 μg)	+	+	+	−	−
Nalidixic acid (30 μg)	−	−	+	−	−
Neomycin (30 μg)	+	+	+	−	−
Novobiocin (30 μg)	+	−	+	−	+
Nystatin (100 μg)	−	−	−	+	−

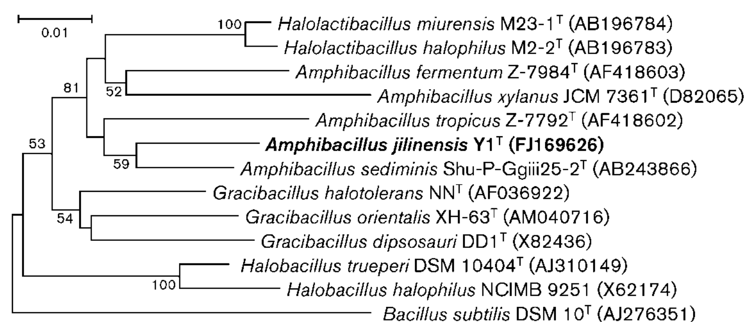

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain Y1^T and related taxa. Bootstrap values based on 1000 replications are listed as percentages at branching points. Only values >50 % are shown. Bar, 0.01 substitutions per nucleotide position. A maximum-parsimony tree is available as Supplementary Fig. S2.

Table 2. Fatty acid contents of strain Y1^T and type strains of *Amphibacillus* species

Strains: 1, Y1^T; 2, *A. tropicus* DSM 13870^T; 3, *A. sediminis* DSM 21624^T; 4, *A. fermentum* DSM 13869^T; 5, *A. xylanus* DSM 6626^T. Data are from this study. Values are percentages of total fatty acids; major components (>10 %) are in bold. −, Not detected; tr, trace amount (<0.5 %). Fatty acids present at less than 0.5 % in all strains are not shown.

Fatty acid methyl ester	1	2	3	4	5
Unbranched					
$C_{14:0}$	1.8	5.8	11.0	3.9	2.0
$C_{15:0}$	tr	2.0	2.3	tr	tr
$C_{16:0}$	7.3	**38.2**	**36.3**	**31.3**	7.6
$C_{18:0}$	tr	2.9	1.9	1.1	1.4
Branched					
iso-$C_{13:0}$	0.5	3.7	7.4	0.8	0.9
anteiso-$C_{13:0}$	tr	2.3	**19.7**	1.2	1.1
iso-$C_{14:0}$	2.5	3.3	2.3	2.4	5.4
iso-$C_{15:0}$	**22.1**	**10.3**	3.5	9.0	**14.1**
anteiso-$C_{15:0}$	**51.4**	**22.3**	**14.7**	**36.1**	**38.6**
iso-$C_{16:0}$	4.9	3.5	−	3.5	**13.2**
iso-$C_{17:0}$	1.0	1.5	−	1.1	1.8
anteiso-$C_{17:0}$	7.5	2.0	−	8.3	**11.1**
iso-$C_{13:0}$ 3-OH	−	0.7	−	−	−
$C_{16:0}$ N alcohol	tr	1.6	0.9	tr	1.4
Unsaturated					
$C_{18:1}\omega 9c$	−	−	−	tr	0.6
Summed feature 1*	−	−	−	tr	1.1

*Summed features are groups of two or three fatty acids that cannot be separated by using the MIDI system. Summed feature 1 contains $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH.

Na^+ is not required for growth but can be tolerated at concentrations of up to 2.8 M but not at 3.1 M, with optimum growth at 0.5 M (0.06 M $NaHCO_3$ and 0.44 M NaCl). Facultatively anaerobic. The methyl red and Voges–Proskauer tests and tests for catalase and oxidase activities are negative. Isoprenoid quinones are not detected. H_2S and indole are not produced. The following substrates are utilized for growth as sole carbon sources: L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, mannose, D-mannitol, melibiose, D-raffinose, rhamnose, D-sorbitol, sucrose, trehalose and D-xylose. Growth occurs with yeast extract, but not Casamino acids or tryptone. Soluble starch and casein can be hydrolysed, but not tyrosine, xanthine or hypoxanthine. The following compounds are not utilized: glycerol, L-alanine, L-arginine, L-cysteine, L-glycine, L-histidine, L-methionine, organic acids (including acetate, butyrate, citrate, fumarate, formate, lactate, malate, pyruvate, succinate, tartrate and oxalate), methanol and ethanol. Sensitive to amoxicillin, ampicillin, azithromycin, carbenicillin, cefotaxime, cefoxitin, chloromycetin, erythromycin, kanamycin, neomycin, nitrofurantoin, novobiocin, oxacillin, penicillin, phosphonomycin, rifampicin, streptomycin, tenebrimycin, tetracycline and vancomycin, but not amikacin, bacitracin, gentamicin, nalidixic acid, polymyxin B or nystatin. The major cellular fatty acids are anteiso-$C_{15:0}$ and iso-$C_{15:0}$. The G+C content of the type strain is 37.7 mol% (T_m).

The type strain, Y1^T (=CGMCC 1.5123^T =JCM 16149^T), was isolated from sediment from a soda lake in Jilin province, China.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (grant no. 30970002).

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