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Alkaliphilus namsaraevii sp. nov., an alkaliphilic iron- and sulfur-reducing bacterium isolated from a steppe soda lake

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Abstract

A novel alkaliphilic spore-forming bacterium was isolated from the benthic sediments of the highly mineralized steppe Lake Khilganta (Transbaikal Region, Russia). Cells of the strain, designated X-07-2^T, were straight to slightly curved rods, Gram-stain-positive and motile. Strain X-07-2^T grew in the pH range from 7.0 to 10.7 (optimum pH 9.6–10.3). Growth was observed at 25–47 °C (optimum 30 °C) and at an NaCl concentration from 5 to 150 g l^{-1} with an optimum at 40 g l^{-1}. Strain X-07-2^T was a chemo-organoheterotroph able to reduce amorphous ferric hydroxide, Fe(III) citrate and elemental sulfur in the presence of yeast extract as the electron donor. It used tryptone, peptone and trypticase with Fe(III) citrate as the electron acceptor. The predominant fatty acids in cell walls were $C_{16:1}\omega8$, iso-$C_{15:0}$, $C_{14:0}$ 3-OH and $C_{16:0}$. The DNA G+C content was 32.6 mol%. 16S rRNA gene sequence analysis revealed that strain X-07-2^T was related most closely to members of the genus *Alkaliphilus* within the family *Clostridiaceae*. The closest relative was *Alkaliphilus peptidifermentans* Z-7036^T (96.4 % similarity). On the basis of the genotypic, chemotaxonomic and phenotypic data, strain X-07-2^T represents a novel species in the genus *Alkaliphilus*, for which the name *Alkaliphilus namsaraevii* sp. nov. is proposed. The type strain is X-07-2^T (=VKM B-2746^T=DSM 26418^T).

Steppe lakes are typically shallow and their physicochemical parameters such as temperature, pH and mineral composition are subjected to large changes due to extreme climatic conditions. High levels of water evaporation from the surface of these lakes lead to large fluctuations in salinity. It can range from 25 to 270 g l^{-1} and in some rare cases the water can evaporate completely. pH in these steppe lakes can vary between 9.9 and 7.1 [1, 2]. Despite the constant fluctuations in salinity, alkalinity and water level, a number of anaerobic halophilic and alkaliphilic bacteria including representatives of the genus *Alkaliphilus* of the family *Clostridiaceae* have been isolated from these lakes. At the time of writing, the genus *Alkaliphilus* consists of five validly published species names: *A. transvaalensis* [3], *A. crotonatoxidans* [4], *A. oremlandii* [5], *A. peptidifermentans* [6] and *A. halophilus* [7]. Another species isolated from a hydrothermal chimney in Prony Bay, New Caledonia, and described as '*A. hydrothermalis*' [8] and the well-studied '*A. metalliredigens*' [9, 10] from alkaline borax leachate ponds have not yet been validly published. Among all characterized representatives of *Alkaliphilus* only '*A. metalliredigens*' is a chemo-organotroph incapable of fermentation. It obtains energy during anaerobic respiration with Co(III)-EDTA, Cr(VI), selenate and ferric iron as the electron acceptors [11].

In this paper, we describe a novel representative of the genus *Alkaliphilus* capable of reducing iron and sulfur up to pH 10.7, isolated from the sediments of alkaline Lake Khilganta located in the South Siberia steppe (50° 42′ 535″ N 115° 06′ 086″ E).

The benthic sediment samples were collected at approximately 0.4 m depth (salinity 41.0 g l^{-1}, pH 9.1). The enrichment and isolation of the Fe(III)-reducing bacterium, designated as strain X-07-2^T, were performed using anaerobic techniques [12]. The alkaliphilic growth medium (AGM) contained (per litre): 1.2 g Na_2CO_3, 1.85 g $NaHCO_3$, 15 g NaCl, 0.5 g K_2HPO_4, 0.1 g $MgCl_2.6H_2O$, 0.5 g NH_4Cl, 0.025 g yeast extract (Difco) and 10 ml trace element solution SL-10 (medium 320; DSMZ). Sodium acetate (20 mM) served as the carbon source and as the electron donor while amorphous ferric hydroxide (AFH) served as the electron acceptor [added at about 90 mM Fe (III)]. The

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Keywords: soda lake; anaerobes; iron reduction; *Alkaliphilus* sp.
Abbreviations: AFH, amorphous ferric hydroxide; AGM, alkaliphilic growth medium; AQDS, anthraquinone-2,6-disulfonate; DMA, dimethylacetal.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain X-07-2^T is KJ196388.
One supplementary table is available with the online Supplementary Material.

final pH was adjusted to 9.6. High-purity nitrogen was used as the gas phase. The enrichments were incubated at 30 °C for 14 days. Strain X-07-2^T was isolated by a serial tenfold dilution and by applying the Hungate roll tube technique to separate colonies in AGM solidified with 2 % (w/v) Bacto agar. After 4 weeks of incubation colonies of strain X-07-2^T were round, small, dark brown, smooth and convex, with a homogeneous structure. The pure culture was maintained in AGM with acetate (20 mM) and yeast extract (0.05 g l^{-1}) or yeast extract (2.5 g l^{-1}) and Fe(III) citrate (30 mM) at pH 9.6.

A. peptidifermentans Z-7036^T (=VKM B-2502^T), used as a reference strain, was grown in AGM1 containing (per litre): 3.0 g Na_2CO_3, 10.0 g $NaHCO_3$, 1.0 g NaCl, 0.2 g K_2HPO_4, 0.1 g $MgCl_2.6H_2O$, 0.5 g NH_4Cl, 0.2 g KCl, 0.2 g yeast extract (Difco), 2.0 g peptone (Difco) and 10 ml trace element solution SL-10 (medium 320; DSMZ). The pH was adjusted to 9.5.

Morphology and ultrathin structure were examined using phase-contrast microscopy (Olympus BX41) at ×1300 magnification and a JEOL JEM-100C electron microscope. For ultrathin sectioning, cells were harvested by centrifugation and then fixed in a solution of 1.5 % (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4 °C for 1 h. The cells were washed three times in the same buffer and post-fixed in 1 % OsO_4 in 0.05 M cacodylate buffer (pH 7.2) for 3 h at 20 °C. The preparation was dehydrated using a series of different ethanol concentrations and embedded in Epon 812 epoxy resin. The ultrathin sections were mounted on grids and post-stained in 3 % (w/v) uranyl acetate in 70 % (v/v) ethanol for 30 min and subsequently they were additionally stained in lead citrate following the methodology of Reynolds [13]. Cells of strain X-07-2^T were straight to slightly curved rods (0.3×5.6–8.9 µm), motile by a single polar flagellum (Fig. 1a). They occurred singly. In the late-exponential and stationary phases of growth, the rods formed terminal endospores (Fig. 1b). Gram staining was performed following standard protocols [14] and cells of strain X-07-2^T stained Gram-positive.

The optimal growth conditions for strain X-07-2^T were tested in AGM with acetate (20 mM), yeast extract (0.05 g l^{-1}) and Fe(III) citrate (30 mM). Bacterial biomass (measured as OD_{600} on a spectrophotometer) and Fe(II) concentrations were determined at the beginning and end of growth. All tests were performed in triplicate and confirmed by two additional transfers.

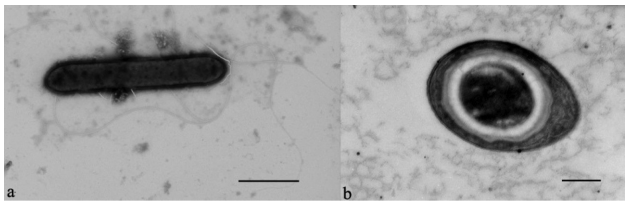

Fig. 1. Electron micrographs of negatively stained cells of strain X-07-2^T in the exponential phase of growth (a; bar, 2 µm), and a longitudinal ultrathin section of a spore (b; bar, 0.2 µm).

The temperature range for growth was determined by incubating at 4, 10, 20, 25, 30, 36, 40, 47, 50 and 55 °C. The effect of pH was examined at pH 6.0, 6.5, 7.0, 8.0, 8.5, 9.0, 9.3, 9.6, 10.0, 10.3, 10.7 and 11.0. To study the pH dependence, the following buffer solutions were used: for pH 6.0–6.5 imidazole/HCl; for pH 7.0–8.0 HEPES/NaOH; for pH 8.5–10.0 a mixture of sodium bicarbonate/sodium carbonate; for pH 10.0–11.0 carbonate/NaOH. To confirm the need for Na^+ for growth, sodium salts in AMG were replaced with potassium salts in the presence of NaCl at 0, 5, 10, 20, 30, 40, 60, 100, 150 and 200 g l^{-1}. pH was adjusted to 9.6 using 10 % (w/v) KOH. Dependence on Cl^- was investigated in AMG, where chlorides were substituted by sulfates. The effect of carbonates was determined by replacing them with equimolar amounts of NaCl and maintaining the pH with 50 mM CAPS.

Strain X-07-2^T grew optimally at 30 °C. Growth and iron reduction were not observed below 25 °C or above 47 °C. The isolate was an alkaliphile growing in a pH range between 7.0 and 10.7 (optimum pH 9.6–10.3). The isolate grew at NaCl concentrations from 5 to 150 g l^{-1} with an optimum at 40 g l^{-1}. Strain X-07-2^T required Na^+ and Cl^-. Carbonates were also needed for growth and could not be substituted for by organic buffer.

The capacity for microaerophilic growth was assessed by the addition of O_2 (0.1, 0.6, 1.5 and 20 %) to the AGM prepared under anaerobic conditions in a nitrogen atmosphere. Strain X-07-2^T did not grow under aerobic conditions but it was an aerotolerant anaerobe with the upper limit for growth of 1.5 % O_2 in the gas phase.

The effect of antibiotics on strain X-07-2^T and *A. peptidifermentans* Z-7036^T was determined by transferring the culture into fresh medium containing (per litre) bacitracin (250 mg), vancomycin (250 mg), kanamycin (250 mg), penicillin G (1000 mg), ampicillin (1000 mg), streptomycin (1000 mg), cefotaxime (100 mg) and rifampicin (25 mg). Tests were performed in duplicate with a non-antibiotic control for 1 week at the optimum temperature and pH. Both strains were sensitive to penicillin, ampicillin, streptomycin and bacitracin but *A. peptidifermentans* Z-7036^T was resistant to rifampicin and kanamycin while cefotaxime and vancomycin did not affect growth of strain X-07-2^T.

The utilization of proteinaceous compounds, carbohydrates, alcohols or organic acids as sole carbon sources by strain X-07-2^T was tested in AGM in which acetate was omitted. The organic substrates were added to a final concentration of 0.25 % (w/v) of the respective carbohydrates, sugars and proteinaceous compounds, 0.25 % (v/v) alcohols, or 0.1 % (w/v) of the respective organic acids in the presence of Fe (III) citrate (30 mM) as the terminal electron acceptor. All tests were performed in duplicate and confirmed by two transfers.

The strain was isolated on medium with acetate and AFH in the presence of yeast extract (0.025 g l^{-1}), but subsequent study showed that better growth was observed in the medium with yeast extract (2.5 g l^{-1}) and Fe(III) citrate as the electron acceptor. The isolate used peptone, tryptone and trypticase in the presence of Fe(III) citrate or AFH. It did not oxidize casein, albumin, Casamino acids, arabinose, galactose, glucose, xylose, lactose, maltose, mannose, sucrose, sorbose, rhamnose, ribose, trehalose, fructose, fucose, raffinose, cellobiose, melibiose, inositol, sorbitol, lactate, citrate, ethanol, methanol, molecular hydrogen, lysine, glutamine, arginine, histidine, valine, ornithine, uracil, alanine, choline, proline, citrulline or serine. The strain did not use the following pairs of amino acids for growth (glycine +alanine, proline+leucine, valine+ornithine, histidine+arginine, alanine+tryptophan, alanine+asparagine, proline+isooleucine, valine+glycine). Therefore, it was not able to perform the Stickland reaction.

To test for the use of electron acceptors (mM) Na_2SO_3 (2 and 20), $Na_2S_2O_3.5H_2O$ (10), Na_2SO_4 (20), $NaNO_2$ (2), $NaNO_3$ (10), anthraquinone-2,6-disulfonate (AQDS; 20), Mn(IV) (in the form of artificially synthesized MnO_2) (25), S^o (10), crotonate (10) or fumarate (5) were added to the sterile AGM with yeast extract (2.5 g l^{-1}) used as the electron donor. Growth was assessed by measuring the changes in OD_{600} and by hydrogen sulfide production [15]. The reduction of nitrogen compounds was determined using Nessler reagent. AFH was prepared by titration of acidic $FeCl_3$ solution with 10 % (w/v) NaOH to a pH of 7.0 and was added at about 90 mM Fe(III). Reduction of Fe^{3+} and AQDS was studied as described by Lovley and Phillips [16] and Lovley *et al.* [17].

Strain X-07-2^T and the reference strain *A. peptidifermentans* Z-7036^T were able to reduce AFH in the presence of yeast extract as the electron donor with formation of 11.2 and 13.0 mM Fe(II), respectively, by day 14 of incubation. Unlike *A. peptidifermentans* Z-7036^T, strain X-07-2^T demonstrated good growth with yeast extract and Fe(III) citrate and Fe(III) citrate + cysteine (0.25 g l^{-1}) as the electron acceptor. After 2 weeks of incubation it formed 4.0 and 6.2 mM Fe(II), respectively, while there was 0.3 and 0.9 mM Fe(II), respectively, in abiotic controls. Besides AFH and Fe(III) citrate the isolate reduced S^o. In the third transfer after 14 days of incubation the number of cells increased from 5.0×10^6 to 3.0×10^7 and it was accompanied by the formation of 6.3 mM hydrogen sulfide. We did not observe growth of strain X−07-2^T with thiosulfate, sulfite, sulfate, nitrate, nitrite, fumarate, crotonate, MnO_2 or AQDS in the presence of yeast extract nor reduction of the test compounds.

Metabolic end products were determined using HPLC. Organic acids were separated on a Knauer HPLC system equipped with Inertsil ODS-3, 5 µm column (4.6×250 mm; GL Sciences) at 35 °C and speed of 1 ml min^{-1}, with 20 mM H_3PO_4 used as eluent. Fractions were detected by absorbance at 210 nm, and identified using analytical standards (Sigma-Aldrich). The main product of strain X-07-2^T grown with yeast extract and Fe(III) citrate or AFH was acetate (8.2 and 10.5 mM, respectively) with a trace amount of propionate (<1 mM). No organic acids were observed in the medium during incubation of the strain without electron acceptors. In contrast, *A. peptidifermentans* Z-7036^T produced acetate and formate (3.8 and 4.4 mM, respectively) from yeast extract, which is consistent with previous data [6].

For analysis of the cellular fatty acids, cells of strain X-07-2^T and *A. peptidifermentans* Z-7036^T were grown in AGM1 medium with AFH at 30 and 35 °C, respectively, and harvested in the late exponential growth phase. Cellular fatty acid profiles were determined by GC-MS as described by Slobodkina [18] and fatty acid content was determined as the percentage of the total ion current peak area. The cellular fatty acid composition of strain X-07-2^T and *A. peptidifermentans* Z-7036^T (Table S1, available in the online Supplementary Material) showed the presence of iso-$C_{15:0}$ (36.2 and 27.1 %, respectively), which is characteristic of the genus *Alkaliphilus*. The dominant fatty acids of strain X-07-2^T were $C_{16:1}\omega8$, iso-$C_{15:0}$, $C_{14:0}$ 3-OH and $C_{16:0}$.

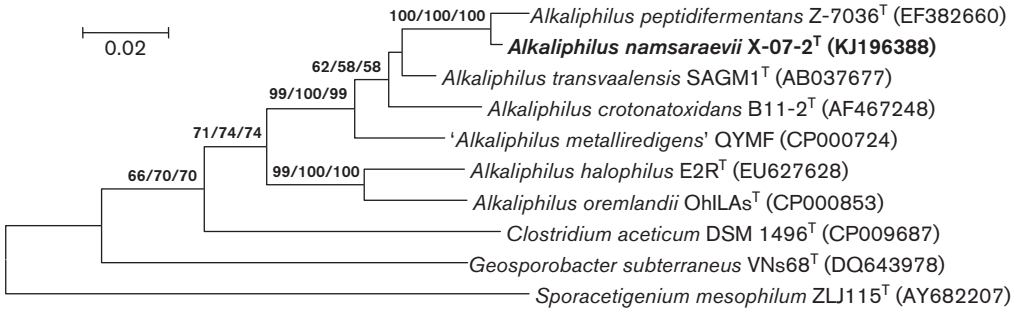

Fig. 2. Phylogenetic tree based on a maximum-likelihood analysis of nearly complete 16S rRNA gene sequences showing the position of strain X-07-2^T and the type strains of related species of the *Clostridiaceae*. Bootstrap values (only values ≥50 % are shown) were obtained with the maximum-likelihood/minimum-evolution/neighbour-joining methods based on 1000 replicates. Bar, 0.02 substitutions per nucleotide sequence position.

For 16S rRNA gene sequencing, DNA G+C content determination and DNA–DNA hybridization, DNA was prepared and purified as described by Marmur [19]. The 16S rRNA gene was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG) and 1492R (5′-TACGG YTACCTTGTTACGATT). The PCR product was purified using a Wizard PCR Preps DNA Purification System. The sequencing reactions were performed using a CEQ Dye Terminator Cycle Sequencing kit according to the protocols provided by the manufacturer and analysed in a Beckman Coulter CEQ 2000 XL automatic DNA sequencer. The NCBI GenBank BLAST utility [20, 21] was used to reveal the closest relatives of strain X-07-2^T. Phylogenetic trees were reconstructed by using three different methods: the neighbour-joining, maximum-likelihood and minimum-evolution methods implemented in MEGA6 [22]. The phylogenetic trees were evaluated by bootstrap analysis based on 1000 replications. The analysis involved 10 nucleotide sequences. There were a total of 1332 positions in the final dataset. The consensus tree inferred by using the maximum-likelihood method based on the general time reversible model is presented in Fig. 2. Bootstrap values obtained for trees using the maximum-likelihood/minimum-evolution/neighbour-joining methods are shown at branch points. Phylogenetic analysis demonstrated that strain X-07-2^T was a member of the *Alkaliphilus–Clostridiaceae* cluster with the closest relative *A. peptidifermentans* Z-7036^T (96.4 % 16S rRNA gene sequence similarity).

The DNA G+C content of the novel isolate was determined by thermal denaturation (T_m) as described by Marmur and Doty [23] with *A. peptidifermentans* Z-7036^T DNA as the calibration standard; the DNA G+C content of strain X-07-2^T was 32.6 mol%. DNA–DNA hybridization (four replications) was performed as described by De Ley *et al.* [24] and modified by Huß *et al.* [25] using a PyeUnicam SP 1800 spectrophotometer equipped with a thermoprogrammer and hermetically sealed thermocuvettes. The standard deviations of the hybridization experiments were between 5.5 and 9.0 %. The level of DNA–DNA relatedness brtween the new isolate and *A. peptidifermentans* Z-7036^T was 52.8 %.

Table 1. Differential characteristics between strain X-07-2^T and strains of *Alkaliphilus* species

Strains: 1, X-07-2^T; 2, *A. peptidifermentans* Z-7036^T [6]; 3, *A. transvaalensis* SAGM1^T [3]; 4, *A. crotonatoxidans* B11-2^T [4]; 5, '*A. metalliredigens*' QYMF [9]; 6, *A. halophilus* E2R^T [7]; 7, *A. oremlandii* OhILAs^T [5]. ND, No data; DMA, dimethylacetal.

Characteristic	1	2	3	4	5	6	7
Cell size (μm)	0.3×5.6–8.9	0.4×1.2–3.0	0.4–0.7×3.0–6.0	0.4–0.6×2.0–3.0	0.5×3.0–6.0	0.5–0.9×1.6–6.0	0.5×2.0
Temperature (°C) (optimum)	25–47 (30)	6–40 (35)	20–50 (40)	15–45 (37)	4–45 (35)	15–42 (32)	32–44 (37)
NaCl (g l^{-1}) (optimum)	5–150 (40)	0–50 (20)	0–33 (5)	0–70 (10)	0–80 (20)	5–150 (75)	0.1–2.5 (1.0)
pH (optimum)	7.0–10.7 (10.3)	7.5–9.7 (9.1)	8.5–12.5 (10)	5.5–9.0 (7.5)	7.0–11.0 (9.5)	5.5–9.0 (8.0)	8.0–8.8 (8.4)
Electron acceptors with yeast extract	Fe(III)- citrate, AFH, S^o	Fumarate, crotonate, $S_2O_3^{2-}$, AQDS, AFH, Fe(III)-EDTA	Fumarate, crotonate, S^o, $S_2O_3^{2-}$	Crotonate	Fe(III) citrate, Fe(III)-EDTA, Co(III)-EDTA, Cr(VI)	$S_2O_3^{2-}$	Arsenate, $S_2O_3^{2-}$
Substrates:							
Tryptone or peptone	+	+*	+	+	+	+	−
Casamino acids	−	+*	−	ND	−	+	ND
Trypticase	+	+*	−	ND	ND	ND	ND
Cellobiose	−	−*	−	+	ND	−	−
Fructose	−	−*	−	+	ND	+	+
Maltose	−	−*	−	+	ND	−	−
Ribose	−	−*	−	+	ND	+	−
Sucrose	−	−*	−	−	ND	+	−
Trehalose	−	−*	−	+	ND	−	−
Xylose	−	−*	−	+	ND	+	−
Lactate	−	−*	−	−	+	+	+
Major fatty acids	$C_{16:1}\omega8$, iso-$C_{15:0}$, $C_{14:0}$ 3-OH, $C_{16:0}$	iso-$C_{15:0}$, iso-$C_{17:1}$, iso-$C_{17:1}$ DMA*	iso-$C_{15:0}$, $C_{14:0}$, $C_{16:0}$	$C_{14:0}$, $C_{16:0}$	$C_{14:1}$, $C_{16:0}$, $C_{16:1}\omega7c$	iso-$C_{15:0}$, iso-$C_{15:1}$, iso-$C_{13:0}$	ND
DNA G+C content (mol %)	32.6	33.8	36.4	30.6	36.8	28.5	36.1

*Obtained in the present study.

The studied strain is an anaerobic, alkaliphilic, halophilic peptide-utilizing bacterium involved in the proteolytic pathway within the microbial community of the soda lake. It is not capable of growth on amino acids and it does not drive the Stickland reaction. Peptides of dead cells are possible substrates of its trophic specialization in the microbial community, perhaps because oligopeptides are more available to bacteria than free amino acids due to transport limitations [26]. According to the phylogenetic position of the isolated bacterium, it belongs to the genus *Alkaliphilus*, which currently includes five validly described species. Only for *A. peptidifermentans* Z-7036^T [6] and '*A. metalliredigens*' [9] has the capacity for metal reduction been demonstrated. Like '*A. metalliredigens*' QYMF, strain X-07-2^T is a chemo-organoheterotroph that utilizes ferric iron as an electron acceptor and yeast extract or peptones as electron donors and is incapable of fermentation. Similar to *A. peptidifermentans* Z-7036^T the novel bacterium is able to reduce AFH. Furthermore it is able to grow on yeast extract by reducing elemental sulfur to hydrogen sulfide. In contrast to the closely related halotolerant species *A. peptidifermentans* Z-7036^T and *A. transvaalensis* SAGM1^T, strain X-07-2^T requires NaCl for growth and tolerates NaCl up to 150 g l^{-1} (Table 1).

On the basis of the genotypic, phenotypic and phylogenetic characteristics presented, strain X-07-2^T should be classified as a member of a novel species of the genus *Alkaliphilus*, for which the name *Alkaliphilus namsaraevii* sp. nov. is proposed.

DESCRIPTION OF *ALKALIPHILUS NAMSARAEVII* SP. NOV.

Alkaliphilus namsaraevii (nam.sa.ra.e'vi.i. N.L. gen. n. *namsaraevii* named in honour of the late Bair Namsaraev, a great microbiologist and researcher of microbial communities of Transbaikalia soda lakes).

Cells are Gram-stain-positive, straight to slightly curved rods (0.3×5.6–8.9 µm). Cells are motile by means of a polar flagellum and form endospores. Aerotolerant anaerobe. The temperature range for growth is 25–47 °C (optimum 30 °C). The pH range for growth is 7.0–10.7 (optimum 9.6–10.3). Obligately dependent on NaCl: grows at NaCl concentrations of 5–150 g l^{-1} (optimum 40 g l^{-1}). Chemo-organoheterotroph. Able to reduce AFH Fe(III) citrate and elemental sulfur in the presence of yeast extract as the electron donor, but does not use thiosulfate, sulfite, sulfate, nitrate, nitrite, fumarate, crotonate, MnO_2 or AQDS as electron acceptors. Oxidizes peptone, tryptone, trypticase and yeast extract, but not casein, albumin, Casamino acids, arabinose, galactose, glucose, xylose, lactose, maltose, mannose, sucrose, sorbose, rhamnose, ribose, trehalose, fructose, fucose, raffinose, cellobiose, melibiose, inositol, sorbitol, lactate, citrate, ethanol, methanol, molecular hydrogen, lysine, glutamine, arginine, histidine, valine, ornithine, uracil, alanine, choline, proline, citrulline or serine; does not carry out the Stickland reaction. The main product from yeast extract and Fe(III) is acetate with trace amounts of propionate. Sensitive to penicillin, ampicillin, streptomycin, bacitracin, rifampicin and kanamycin. Resistant to cefotaxime and vancomycin. The predominant cellular fatty acids are $C_{16:1}\omega8$, iso-$C_{15:0}$, $C_{14:0}$ 3-OH and $C_{16:0}$.

The type strain, X-07-2^T (=VKM B-2746^T=DSM 26418^T), was isolated from the benthic sediments of the highly mineralized steppe Lake Khilganta, Transbaikal Region, Russia. The DNA G+C content of the type strain is 32.6 mol% (T_m).

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Conflicts of interest
There are no conflicts of interest related to this article.

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