Anoxynatronum buryatiense sp. nov., an anaerobic alkaliphilic bacterium from a low mineralization soda lake in Buryatia, Russia

Yana Ryzhmanova,* Victoria Oshurkova, Olga Troshina, Tatyana Abashina, Elena Ariskina, Alexander Avtukh and Viktoriya Shcherbakova

Abstract

An anaerobic alkaliphilic, proteolytic bacterium, strain Su22Ti, was isolated from the bottom sediment of the alkaline low mineralization lake Sulphatnoe (Selenginsky district, Buryatia, Russia). A comparative analysis of the 16S rRNA gene sequence revealed that this bacterium was closely related to Anoxynatronum sibiricum Z-7981T with a similarity of 98.1%. Strain Su22T differed from A. sibiricum Z-7981T in its inability to use carbohydrates, peptone and amino acids as carbon sources. Strain Su22T grew over a temperature range of 20–40 ºC with an optimum at 30 ºC and within the pH range 7.4–11.0 with an optimum at pH 9.6. Sodium cations stimulated the growth of the strain considerably with an optimal concentration at 0.76–1.09 M. The whole-cell fatty acid profile included C16:1ω7c, C16:0 and C16:1ω9c ALDE. The G+C content was 46.1 mol%. Based on the DNA–DNA hybridization level (53.2 %) and phenotypical differences between strains Su22T and Z-7981T, the new isolate is thus considered to represent a novel species, for which the name Anoxynatronum buryatiense sp. nov. is proposed. The type strain is Su22T (=VKM B-2510T=CECT 8731T).

Proteolytic bacteria decompose biomass of primary producers and supply volatile fatty acids and ammonia to microbial community trophic chains. They are ubiquitous in harsh environments such as soda lakes. A number of different bacterial representatives, such as Alkaliphilus [1, 2], Anaerovirgula [3], Anoxynatronum [4], Natronaerobius [5], Natronincola [6, 7], Spirochaeta [8], Anaerobranca [9], Tindallia [10–12] and Proteinivorax [13], that are able to decompose proteins have been isolated from alkaline lakes of different locality. The majorities of these bacteria are alkaliphiles and belong to the order Clostridiales. Some of them, namely Alkaliphilus peptidofermentans and Anoxynatronum sibiricum, can grow on cyanobacterial biomass [2, 4]. Recently it has been conclusively demonstrated that the haloalkaliphilic bacterium Proteinivorax tanatarense in addition to cyanobacterial dead cells is able to use archaeal biomass as a carbon and energy source [14]. Photosynthesis and decomposition of the accumulated biomass of primary producers by alkaliphilic bacteria lead to the appearance of autochthonous organic matter and allow microbial communities of soda lakes to exist autonomously, carrying out the basic cycles of biogenic nutrients.

Strain Su22T was a stable member of the sulfate-reducing enrichments from the bottom sediments of the alkaline low mineralization lake from which Desulfitonatronum lacustris strain Su2 (=VKM B-2475T) was isolated earlier [15]. Pure culture of Su22T was obtained by serial dilutions using Hungate tubes with anaerobic medium AM1 containing (g l−1): Na2CO3, 1.6; NaHCO3, 0.6; KCl, 0.2; MgCl2×6H2O, 0.1; NH4Cl, 0.5; K2HPO4, 0.2; yeast extract, 2.0; Na2S×9H2O, 0.25; trace element solution SL-10 (320 medium, DSMZ), 1.0 ml; vitamin solution (141 medium, DSMZ), 10.0 ml. Sterile sodium sulfide, carbonate and bicarbonate solutions were added to the medium before inoculations and pH was adjusted to 9.6. The culture purity was assessed by observing the uniform cell types under a phase-contrast microscope. The material for the examination were single colonies developing on Difco agar (15 g l−1) under 100 % nitrogen atmosphere at 28–30 ºC. Colonies appearing 2 weeks after plating were small, smooth, convex, glistening, non-pigmented and circular in shape with entire edges. Anoxynatronum sibiricum Z-7981T=VKM B-2327T [4] was used for comparative studies and grown in the AM1 medium amended with 5.0 g l−1 glucose at pH 9.0.

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Keywords: proteolytic bacteria; soda lake; alkaliphiles; Anoxynatronum buryatiense.

Abbreviations: AHF, amorphous ferric hydroxide; ALDE, aldehyde; 10-OH, 10-hydroxy; OMe, methyl ester.

The GenBank accession number for the 16S rRNA gene sequence of strain Su22T is EU315116.

Two supplementary tables and one supplementary figure are available with the online Supplementary Material.

DOI 10.1099/ijsem.0.002365

002365 © 2017 IUMS
For determining the 16S rRNA gene sequence, genomic DNA was isolated by the standard method [16]. The 16S rRNA gene was amplified using universal bacterial primers 27f and 1492r [17]. The PCR product was purified by DNA isolation using agarose gels on a SiOx2-coated Magnetic Particles reagent kit (Silex) according to the manufacturer’s instructions. DNA was sequenced by using an ABI PRISM BigDye Terminator version 3.1 reagent kit followed by analysis of reaction products in an ABI PRISM 3730 Applied Biosystems automated DNA sequencing machine.

The NCBI GenBank BLAST utility [18, 19] was used to reveal the closest relatives of strain Su22T. The sequences were selected and manually aligned with CLUSTAL_W [20]. Phylogenetic trees were reconstructed based on nucleotide sequences of the 16S rRNA gene fragments using MEGA 6 software packages [21] by the neighbour-joining [22], minimum evolution [23] and maximum likelihood methods [24] and had similar topology. The 16S rRNA gene sequence analyses of strain Su22T (1507 bp) and relevant organisms showed that it was affiliated with the Cluster XI in the family Clostridiaceae with a high bootstrap support and had the highest sequence similarity (98.1 %) to A. sibiricum Z-7981T. The phylogenetic tree indicated that these strains represented a separate lineage within the group mainly related to alkaliphilic proteolytic and saccharolytic bacteria isolated from different soda lakes (Fig. 1).

Gram-staining was performed by following the standard protocol [25]. Cells of strain Su22T stained Gram-positive. Cell morphology was examined using phase-contrast microscopy (1-2; Lumam) at ×1350 magnification and using electron microscopy (JEM-100; JEOL, Japan) with ultrathin sections as described earlier [26]. Cells of strain Su22T were non-motile, slightly curved rods (Fig. 2a) with slightly pointed ends, and 0.4–0.7 µm wide and 2.0–6.5 µm long. Cells divided by the formation of septa. The microscopic study showed that Su22T formed nubs in the middle of the cell in the stationary phase. Although the ultrathin sections did not show the presence of classic spores, we observed a microscopic structure resembling a ‘lysing prosopore’ in the cell (Fig. 2b). Pasteurization of strain Su22T at 80 °C for 10 and 20 min with the following inoculation of pasteurized culture and incubation under optimal conditions showed that the cells were resistant to temperature. Thus, the results did not give a clear answer concerning the spore formation by the new isolate. Subsequent studies on the selection of sporulation conditions and/or the genome study of Su22T are needed. The micrographs of negatively stained preparations confirmed the absence of flagella and the presence of a well-expressed S-layer with a thickness of 0.15–0.2 µm (Fig. 2c).

To determine the optimal parameters for growth the liquid medium, AM1 was used. The effects of temperature, salinity and pH on growth were tested in triplicate and confirmed by two additional transfers. Growth was assayed by measuring an increase in optical density at 600 nm. Kinetic parameters of growth were determined at different pH values (7.0, 7.4, 7.8, 8.4, 8.8, 9.2, 9.6, 10, 10.5, 11.0 and 12.0), temperatures (10, 15, 20, 25, 30, 35, 40, 45 and 50 °C) and NaCl concentrations (0.001, 0.06, 0.21, 0.42, 0.54, 0.65, 0.76, 0.87, 1.09, 1.3, 2.6 and 3.4 M). The pH dependence was examined using the following filter-sterilized buffer systems: for pH 7.0–8.0, HEPES/NaOH; for pH 8.5–10.5, a mixture of sodium bicarbonate/sodium carbonate; for pH 11.0–12.0, carbonate/NaOH buffer systems. To measure NaCl effect on growth, all sodium salts were replaced by potassium ones in AM1 medium. NaCl was added to the medium before inoculations in the required concentration. The dependence on chloride ions was investigated in medium AM1, where NaCl was replaced with an equimolar amount of sodium carbonate and sodium bicarbonate, but all other 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 2-(Cyclohexamino)ethanesulfonic acid (CHES).

The new bacterium grew in the temperature range of 20–40 °C, optimally at 30 °C. Strain Su22T was an alkaliphile and grew in the range of pH from 7.4 to 11.0 with optimum at pH 9.6 (Fig. S1a, available in the online Supplementary Material). It required NaCl for growth, with the optimal concentration being 0.76–1.09 M (Fig. S1b). The weak growth observed without the addition of NaCl could be due to the presence of sodium ions in the yeast extract. Strain Su22T did not need chloride ions, but had an obligatory requirement for sodium carbonates. Their replacement with CHES buffer (pH 9.6) inhibited the growth of strain Su22T in the second transfer.

Oxidase and catalase tests were carried out according to standard methods [25]. Strain Su22T was catalase-negative and oxidase-positive. The growth of Su22T was not inhibited in the presence of 0.9–4.5 % (v/v) gaseous O2 but the isolate was not able to grow aerobically.

The ability to use different substrates as a source of carbon and energy was checked in AM1 medium with or without 0.1 g l−1 yeast extract at optimal temperature and pH. The substrate was added at a concentration of 1.0 g l−1 of amino acids or 2.0 g l−1 of the other tested compounds.

The growth of the strain was observed with yeast extract as a sole carbon and energy source. The biomass yield was proportional to the concentrations of yeast extract in the range of 0.1–10 g l−1. The strain grew with tryptone, sodium caseinate and casamino acids in the presence of yeast extract (0.1 g l−1). Strain Su22T did not use peptone, trypticase, fructose, glucose, mannose, xylose, formate, acetate, propionate, butyrate, caproate, heptanoate, fumarate, malate, succinate, citrate, sorbitol, methanol or butanol as sources of carbon and energy.

Amino acids, ammonium and methylamines were determined using Biotronics LC-6000E in hydrolysed culture supernatants before and after cultivation in AM1 medium with yeast extract (2.0 g l−1). The analysis showed that asparagine, glutathione, glycine, histidine, lysine, phenylalanine, serine, threonine and tyrosine were fully utilized from
the yeast extract during growth (Table S1). At the same time, alanine, isoleucine and valine accumulated in the supernatant. Nevertheless, strain Su22\textsuperscript{T} did not grow on individual amino acids (alanine, arginine, asparagine, citrulline, glutamine, glycine, histidine, lysine, ornithine, phenylalanine, proline, serine, threonine, tyrosine and valine) or pairs of amino acids (alanine+asparagine, alanine+glycine, alanine+tryptophan, arginine+histidine, proline+leucine, proline+isoleucine, valine+glycine, valine+ornithine). Also strain Su22\textsuperscript{T} did not use choline, glutathione or uracil for growth.

Organic acid metabolites were determined in broth culture supernatants (72 h) using high-performance liquid chromatography (HPLC). Metabolites were separated on a Knauer HPLC system equipped with an Inertsil ODS-3, 5 µm column (4.6 x 250 mm, GL Sciences) at 35 °C and 1 ml min\textsuperscript{-1} speed, with 20 mM H\textsubscript{3}PO\textsubscript{4} used as eluent. Fractions were detected by absorbance at 210 nm, and identified using analytical standards (Sigma-Aldrich). Alcohols were analysed using a Pye-Unicam 304 gas chromatograph equipped with a glass column (1 m x 2 mm ID) packed with Porapak QS, 80–100 mesh (Fluka). The temperatures of the column, injector and flame-ionization detector were 90, 150 and 180 °C, respectively. The carrier gas was nitrogen at a flow rate of 20 ml min\textsuperscript{-1}. Hydrogen was measured with a JIXM80 gas chromatograph (MPO Manometr) using a katharometer and a glass pillar (1m x 3 mm) filled with...
molecular sieves (30–40 mesh). The temperature of the pillar, injector and detector was 40 °C. Argon was used as a gas carrier; the flow rate was 20 ml min⁻¹. Su22°T fermented yeast extract (2.0 g l⁻¹) to ammonium (9.5 mM), acetate (6.7 mM) and propionate (6.5 mM). Trace amounts of succinic acid, iso-valerate and hydrogen were also detected. Strain Su22°T did not produce methylamines, lactate, ethanol, formate, n-butyrate, n-valerate or iso-butyrate.

Some proteolytic bacteria isolated from soda lakes have been shown to grow on biomass of algae, cyanobacteria and archaea [4, 13, 14]. The ability of strain Su22°T to grow on the autoclaved biomass of its companion sulfate-reducing bacterium, D. lacustris strain Su2, was checked. Two grams of wet biomass of D. lacustris Su2 was diluted in 10 ml of 9.0 g l⁻¹ NaCl solution and autoclaved twice. A 0.1 ml sample of the above solution was then added to 10 ml of the AM1 growth medium without yeast extract. Good growth of Su22°T (3.5×10⁸ cells per 1 ml) was observed after 48 h of incubation in the second transfer.

To test the strain’s ability to use electron acceptors, the following compounds were added to the medium: Na₂SO₄ (30.0 mM); Na₂S₀₃ (5.0 mM); Na₂S₀₃ (10.0 mM); DMSO (2.0 g l⁻¹); elemental sulfur (1.0 g l⁻¹); fumarate (10.0 mM); amorphous ferric hydroxide (AHF) converting for Fe(III) (–60.0 mM); and NaNO₃ (10.0 mM). The AHF was prepared as described earlier [27]. All tests were performed in duplicate and confirmed by growth with two following inoculations. The growth was determined by either the increase of optical density or by hydrogen sulfide and Fe(II) production as described earlier [27, 28]. Sulfur-containing electron acceptors, particularly thiosulfate and sulfur, stimulated the growth of strain Su22°T. Elemental sulfur inhibited the growth of A. sibiricum Z-7981°T while thiosulfate stimulated the growth of the reference strain with 2.9 mM sulfide formation. The growth of Su22°T using thiosulfate and sulfur was accompanied by sulfide production (2.0 and 3.2 mM, respectively). Electron acceptors such as DMSO, fumarate, Fe(III) and NaNO₃ did not stimulate growth of either of the strains.

To determine the cellular fatty acids (CFA), strains Su22°T and Z-7981°T were grown in AM1 medium with the addition of glucose (5.0 g l⁻¹) at the optimal temperature and pH 9.6 for Su22°T, pH 9.0 for Z-7981°T. Biomass was harvested during the late exponential growth phase. CFA were extracted from the cell biomass (3–5 mg of dry cells) by acidic methanolysis. The sample of lyophilized biomass (5 mg) was processed by 0.4 ml 1 M hydrogen chloride in a methanol solution at 80 °C for 3 h. Reaction mixture sample (2 µl) was analysed by using a Sherlock system (MIDI) as described previously [15]. The comparison of CFAs showed a predominance of C₁₆:₁ω7c, C₁₆:₀ and C₁₆:₀ ALDE for both strains, which in total were 42.5 and 54.3% of the total CFA for Z-7981°T and Su22°T, respectively. The CFA profiles of the investigated strains differed by the composition of minor components. Thus, cells of Su22°T contained unsaturated acids C₁₅:₁ω6, C₁₇:₁ω8 and C₁₇:₁ω9, while the cells of Z-7981°T contained C₁₄:₁ω5, C₁₆:₁ OMe, C₁₆:₁ ALDE, C₁₈:₀ ALDE and C₁₈ 10-OH (Table S2).

For determination of the DNA base composition and DNA–DNA hybridization, DNA was isolated by using Mar- mur’s method [16]. The nucleotide content was assessed by thermal denaturation of the DNA using a spectrophotometer PyeUnicam SP1800 spectrophotometer with a heating rate of 0.5 °C min⁻¹ [29]. The DNA–DNA hybridization was performed by the De Ley method [30] using a Beckman Coulter DU800 spectrophotometer equipped with a thermo programmer and hermetically sealed thermostuvettes. The G + C contents of the total DNA of strain Su22°T was 46.1 ±0.3 mol% (mean ±SD of four determinations). The DNA–DNA hybridization value between strains Su22°T and Z-7981°T was 53.2±1.3 % (mean ±SD of six determinations).

Taken together, all the above data indicate that strain Su22°T represents a novel species of the genus Anoxynatronum that, at the time of writing, includes a single described species, A. sibiricum Z-7981°T. The name proposed for this organism is Anoxynatronum buryatiense sp. nov. This conclusion is based on the phylogeny and the moderate 16S rRNA gene sequence similarity (98.1%) between strains Su22°T and A. sibiricum Z-7981°T, as well as on distinct differences between the strains at the phenotypic and chemotaxonomic levels. A key feature of the novel species is its inability to ferment sugars. Both strains are strict alkaliphiles and grew optimally at pH 9.6 (Su22°T) and 9.1 (Z-7981°T). Strain Su22°T, in contrast to A. sibiricum Z-7981°T, did not utilize sugars and peptone; nor did it grow using amino acids. Furthermore, strain Su22°T reduced elemental sulfur. It also differed from A. sibiricum Z-7981°T in its growth temperature optimum and NaCl tolerance as well as in its cell motility (Table 1).

Proteolytic bacteria play an important role in functioning alkaliphilic microbial communities, providing substrates for the micro-organisms involved in the carbon and nitrogen cycles in these ecosystems. The dissimilatory reduction of oxidized sulfur compounds resulting in sulfide production (sulfidogenesis) is a major biogeochemical process in soda lakes [31]. Elemental sulfur reduction seems to be very active in soda lakes [32, 33], probably due to a unique feature of the sulfur chemistry at high pH. Here we have described a new alkaliphilic proteolytic bacterium, which is able to reduce sulfur compounds and is a closely associated with the sulfate reducing bacterium D. lacustris Su2. This finding indicates that the novel bacterium can participate in the sulfur cycle in low mineralization soda lakes.

**DESCRIPTION OF ANOXYNATRONUM BURYATIENSE SP. NOV.**

Anoxynatronum buryatiense (bu.ri.a.ti.en′se. N.L. neut. adj. buryatiense, of or belonging to the Buryatia region, referring to the region of isolation).

Cells are non-motile rods with sharpened ends (0.4–0.7×2.0–6.5 µm). Multiplication is performed by binary
fission. Gram-positive with an S-layer. Moderately aerotolerant, catalase-negative and oxidase-positive anaerobe. Obligate alkaliphilic, grows within the range of pH 7.4–11.0 with an optimum at 9.6. Mesophilic. Grows at a temperature range between 20 and 40 °C with an optimum at 30 °C. Halotolerant. Able to grow at Na⁺ concentrations of up to 2.4 M, optimally at 0.76–1.09 M. Fermenter. Utilizes yeast extract and casein, tryptophane, casamino acids in the presence of 0.1 g l⁻¹ yeast extract. Does not utilize single amino acids, peptone and sugars. Does not carry out the Stickland reaction. Does not utilize pyruvate, fumarate, lactate, propionate, citrate, methanol, butanol, chitin, uracil, choline or glutathione. The main products of yeast extract fermentation are acetate, propionate and ammonium. Reduces elemental sulfur and thiosulfate with yeast extract as an electron donor. The predominant fatty acids are C₁₆:₀, C₁₆:₁ω7c, C₁₆:₀ and C₁₆:₀ ALDE. The DNA G+C content of the type strain is 46.1 mol% (Tₘ).

The type strain, Su22T (=VKM B-2510=CECT 8731T), was isolated from the bottom sediments of the alkaline low mineralization lake Sulphatnoe (Selenginsky district, Buryatia, Russia).

Funding information
This work was supported by the Russian Federation Federal Agency of Scientific Organizations for fundamental studies under topic no. 01201350918.

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

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Table 1. Comparative characteristics of strains Su22T and Anoxyonatronum sibiricum Z-7981T.

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