**Natran aerobaculum magadiense** gen. nov., sp. nov., an anaerobic, alkalithermophilic bacterium from soda lake sediment

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An obligately alkaliphilic, anaerobic, thermo- and halotolerant, spore-forming bacterium was isolated from sediments of soda lake Magadi (Kenya) and designated strain Z-1001T. Cells of strain Z-1001T were straight, Gram-positive rods, slowly motile. Strain Z-1001T was found to be an obligate anaerobe. It grew within a pH range from 7.5 to 10.7 with an optimum at 9.25–9.5 (at 40 °C), a temperature range from 20 to 57 °C with an optimum at 45–50 °C, and a NaCl concentration range from 0 to 1.55 M with an optimum at 1.2–1.4 M. Peptides, such as meat and yeast extracts, peptone and tryptone, were fermented by Z-1001T. Carbohydrates did not support growth. With yeast extract as an electron donor, strain Z-1001T reduced S2O3$^2$-, NO3–, AsO4$^3$–, Fe(III) citrate and anthraquinone-2,6-disulfonate (AQDS) as electron acceptors. The isolate was able to grow oligotrophically with a very small amount of yeast extract: 0.03 g l$^{-1}$. The main fatty acids were C$_{16:0}$, C$_{16:1\omega7c}$, C$_{18:0}$ and C$_{18:1\omega9c}$. The DNA G+C content of the isolate was 35.6 mol%. 16S rRNA gene sequence analysis showed that strain Z-1001T is a member of family Natran aerobiaceae, clustering with the type strain of **Natran aerobius thermophilus** (95.8–96.0% sequence similarity). On the basis of physiological and phylogenetic data it is proposed that strain Z-1001T (=DSM 24923$^T$=VKM B-2666$^T$) represents a novel genus and species, *Natran aerobaculum magadiense* gen. nov., sp. nov.

The hypothesis that soda lakes harbouring prokaryotic communities can be considered as analogues of the terrestrial communities of the ancient continents of the early Proterozoic was advanced by Zavarzin (1993). Modern soda lakes may represent a refuge for the relict microbial communities; because of their high salt concentration and alkalinity, they are practically unoccupied by eukaryotes. The microbial community of soda lake Magadi (East-African Rift Valley, Kenya) has been extensively investigated over more than twenty years by many microbiologists (Tindall, 1988; Grant et al., 1990; Zhilina & Zavarzin, 1994; Duckworth et al., 1996; Jones et al., 1998; Rees et al., 2004). Such a keen interest can be explained by the great taxonomic and phenotypic diversity of the alkaliphilic microbial community of this lake. The unique geochemical conditions combined in lake Magadi, such as volcanic activity in the rift zone and the geothermal gradient, leaching of fresh volcanic products, precipitation of trona (NaHCO$_3$*Na$_2$CO$_3$*2H$_2$O) and an extremely high content of phosphate (about 0.04 g l$^{-1}$), make lake Magadi an ideal habitat for the evolution of extremophiles and their isolation. Usually, extremophiles are well adapted to one or two extreme environmental factors. A combination of three extreme factors: high salt concentration, high pH and elevated temperature developing due to tropical heliothermic heating or geothermal activity should be...
considered extreme even for micro-organisms. It is no surprise that the physiological group of anaerobic halophilic alkalithermophiles was described only recently (Mesbah et al., 2007). Described were three members of this group, namely *Natrananaerobius thermophilus*, *Natrananaerobius trueperi* and *Natronovirga wadinatrunensis* (Mesbah et al., 2007; Mesbah & Wiegel, 2009). All of them belong to the order *Natrananaerobiales* and family *Natrananaerobiaceae*.

In this work we present the taxonomic description of a novel halotolerant, alkalithermophilic bacterium isolated from sediments of soda lake Magadi. It represents a novel genus and species within the family *Natrananaerobiaceae*.

Samples of sediments and water from soda lake Magadi (Kenya, East-African Rift) were collected by G. A. Zavarzin in 1992. The pH of water at the sampling site was 10.0 and the temperature was 40 °C. Samples were put into glass vials with screw caps, without free volume, and transported to the laboratory at ambient temperature, where they were stored at 4 °C.

The initial enrichment was obtained by inoculating anaerobically prepared medium with the slurry obtained by mixing sediments and water from soda lake Magadi. The goal of our work was to isolate alkaliphilic, dissimilatory iron-reducing bacteria; therefore, the composition of the basal medium used for the enrichment cultures was as follows (g l⁻¹): NH₄Cl, 0.5; KH₂PO₄, 0.2; MgCl₂·6H₂O, 0.1; KCl, 0.2; yeast extract (Difco), 0.05; Na₂CO₃ 68; NaHCO₃, 38.0; NaCl, 60.0; 1 ml trace element solution (Kevbrin & Zavarzin, 1992); cysteine hydrochloride, 0.3; sodium acetate, 2.0; ferrihydrite, synthesized as described previously (Zavarzina et al., 2006), 90 mmol Fe(III) l⁻¹. The pH was 9.5 owing to the presence of sodium carbonate–sodium bicarbonate buffer. The medium was dispensed in 10 ml portions into 15 ml Hungate tubes with screw caps; the head space (5 ml) was filled with N₂ at atmospheric pressure. Inoculated tubes were incubated at 35 °C. Pure culture was obtained by serial dilutions in optimized medium, where sodium acetate and ferrihydrite were omitted and yeast extract and anthraquinone-2,6-disulfonate (AQDS) were added to concentrations of 2 g l⁻¹ and 2 mM, respectively. The last positive dilution was used to obtain colonies in roll-tubes with the same medium solidified with Bacto-agar (2.0 g l⁻¹; Difco) and prepared as described previously (Garnova et al., 2003). Single colonies were transferred to the liquid medium and an isolate was obtained designated strain Z-1001T.

The colonies of strain Z-1001T that appeared in agar roll-tubes after 10 to 14 days of incubation were round, white–cream and 0.2–0.6 mm in diameter. A Zetopan light microscope (Reichert) was used to examine cell morphology, make cell size measurements and take phase-contrast photomicrographs. Ultrathin sections were obtained and electron microscopy was conducted as previously (Zavarzina et al., 2002). Cells of strain Z-1001T were straight to slightly curved rods, 0.2–0.5 μm in diameter and 3.0–7.0 μm in length (Fig. 1a). The diameter and length of the cells were dependent on the amount of substrate. When the concentration of yeast extract was lower than 0.3 g l⁻¹, cells of strain Z-1001T were thin (0.2–0.3 μm in diameter). Cells exhibited slight tumbling motility, but no flagella were observed under the electron microscope. Strain Z-1001T formed spores of clostridial type (Fig. 1b). Ultrathin sections revealed cell walls with the Gram-positive cell envelope profile (Fig. 1c).

Strain Z-1001T was an obligate anaerobe and grew only in anaerobically prepared medium where O₂ was removed by boiling. In aerobically prepared medium or under a gas phase containing 1.5 or 2% O₂ no growth occurred. Catalase activity was judged from the formation of O₂ bubbles on the cell pellet treated with 3% (v/v) H₂O₂ solution. The strain was catalase-positive. Bacterial growth was monitored by direct cell counting under a phase-contrast microscope and by measuring OD₆₀₀ (Spectrophotometer-2100, UNICO).

The optimal conditions for growth of strain Z-1001T were tested in optimized carbonate-buffered medium (see
above) with 0.2 % yeast extract and 10 mM Na$_2$S$_2$O$_3$ added as an electron acceptor. Growth of strain Z-1001$^T$ occurred at temperatures ranging from 20 to 57 °C, with an optimum between 45 and 50 °C (Fig. S1, available in IJSEM Online).

The pH range for growth was determined at 40 °C as described previously (Garnova et al., 2003). Strain Z-1001$^T$ was an obligate alkaliphile. The pH range for growth was 7.5–10.7 with an optimum at pH 9.25–9.5. No growth occurred at pH lower than 7.5 or higher than 10.7 (Fig. S2).

The Cl$^-$ concentration range suitable for growth was determined in carbonate-buffered, optimized medium. In cases where the concentration of NaCl was below 60 g l$^{-1}$, the salinity shortage was compensated for by adding an equivalent amount of Na$_2$SO$_4$ (e.g. in the variant of medium with 1 g NaCl l$^{-1}$, 59 g Na$_2$SO$_4$ l$^{-1}$ was added). Strain Z-1001$^T$ was halotolerant: it did not require Cl$^-$ for growth. The Cl$^-$ ion concentration range suitable for growth was from 0 to 1.55 M with an optimum at 1.2–1.4 M Cl$^-$. The dependence of the growth rate upon the total salts content and composition was determined as described previously (Zhilyina et al., 2012). Strain Z-1001$^T$ could grow within the ranges of 0.46–2.73 M for Na$^+$, 0.22–1.35 M for CO$_3^{2-} +$ HCO$_3^-$ and 1.2–1.4 M for Cl$^-$. Optimum growth was observed in the medium containing 0.9 M Na$^+$, 0.45 M CO$_3^{2-} +$ HCO$_3^-$ and 0.1 M Cl$^-$. In order to determine the dependence of growth upon sodium carbonates, the isolate was inoculated into media with TRIS or CAPS buffers as described previously (Zavarzina et al., 2006). Strain Z-1001$^T$ was an obligate natronophile and did not grow on these media.

For substrate utilization tests, the culture was inoculated into mineral medium with 0.05 g yeast extract l$^{-1}$. All organic substrates (peptides, carbohydrates, alcohols and organic acids) were added to a final concentration of 0.2 % (w/v) or 0.2 % (v/v) for alcohols, introduced as filter-sterilized (0.2 μm; Millipore) concentrated solutions. Medium without substrates but with yeast extract (0.05 g l$^{-1}$) was used as a control. In growth-positive variants, three successive transfers to the same medium were performed. Bacterial growth was monitored by direct cell counting under a phase-contrast microscope and by estimating an increase in OD$_{600}$. Strain Z-1001$^T$ fermented peptides such as meat extract, tryptone and peptone. Growth on yeast extract was most probably also due to peptide fermentation. Strain Z-1001$^T$ did not utilize carbohydrates: fructose, glucose, galactose, lactose, maltose, mannose, ribose, sucrose, trehalose or xylose. It did not utilize butanol, ethanol, glycerol, methanol, 2-propanol, acetate, betaine, formate, lactate, mannitol, succinate, propionate, pyruvate, oxalate, Casamino acids or trimethylamine.

The main fermentation products formed from 0.2 % yeast extract were (mM): acetate, 4.1; succinate, 1.3; formate, 0.7; lactate, 0.5; propionate, 0.4; isovalerate, 0.5; isobutyrate, 0.4; 2-methylbutyrate, 0.3; NH$_4^+$, 4.2. Acetate and formate were assayed with a GC Crystal-5000.2 (Chromatec) equipped with a 1 m glass column filled with the Carbopack C with 0.3 % Carbowax 20 M and 0.1 % H$_3$PO$_4$ (Supelco) stationary phase. Other products were detected by HPLC on a Stayer chromatograph (Aquilon) equipped with an Aminex HPX 87H column (Bio-Rad) and a Smartline 2300 refractometric detector (Knauer); the elution was with 5 mM H$_2$SO$_4$. Samples for chromatography were obtained by culture centrifugation at centrifuge CM-50 (ELMI) at 15000 r.p.m. for 3 min followed by acidification of the clear supernatant with 5 M H$_2$SO$_4$ to pH 2.0. Ammonium was detected by the microdiffusion method with Nessler reagent.

The capacity for anaerobic respiration with various electron acceptors was studied in media containing 0.2 % yeast extract and judged from an increase in OD$_{600}$ and in the levels of reduction products determined as follows: by measuring sulfide (Trüper & Schlegel, 1964) in the case of sulfur compounds as acceptors; ammonium in the case of nitrate by the microdiffusion method with Nessler reagent; Fe(II)-ferrozine complex at A$_{562}$ (Stokey, 1970) in case of Fe(III) citrate and Fe(III) hydroxide and by colour changes of AQDS. Arsenate was detected as described previously (Zavarzina et al., 2009). Strain Z-1001$^T$ reduced S$_2$O$_3^{2-}$ (10 mM), NO$_3^-$ (20 mM), fumarate (5 mM; Fluka), AQDS (2 mM), Fe(III) citrate (20 mM), slightly amorphous Fe(III)-hydroxide [90 mM Fe(III)] and AsO$_4^{3-}$ (5 mM). Addition of S$_2$O$_3^{2-}$ (10 mM) or NO$_3^-$ (20 mM) stimulated growth of strain Z-1001$^T$. SeO$_4^{2-}$ (5 mM), SeO$_3^{2-}$ (2.5 mM), SO$_3^{2-}$ (20 mM), Fe(III)-EDTA, S$^0$ (1%, w/v) and crotonate (10 mM; Sigma) were not reduced by strain Z-1001$^T$. The addition of S$_2$O$_3^{2-}$ (20 mM) or NO$_3^-$ (2 mM) completely inhibited growth of strain Z-1001$^T$.

Strain Z-1001$^T$ could grow oligotrophically with a very low concentration of yeast extract. When the concentration of yeast extract was less than 0.3 g l$^{-1}$, no cell density could be observed; however, growth could be assessed by direct counting of cells. Strain Z-1001$^T$ grew within a yeast extract concentration range of 3.0–0.03 g l$^{-1}$ with an optimum at 1.0–3.0 g l$^{-1}$. The growth yield versus yeast extract curve reached saturation plateau when the concentration of yeast extract reached 1 g l$^{-1}$ (see Fig. S4).

Streptomycin, vancomycin and rifampicin completely inhibited growth of strain Z-1001$^T$ at concentrations of 100 μg l$^{-1}$. Kanamycin, novobiocin and benzylpenicillin at the same concentration did not inhibit growth.

For fatty acid analysis, cells of strain Z-1001$^T$ were grown on optimized mineral medium with 0.2 % yeast extract as the carbon source and electron donor and (10 mM) as an electron acceptor at pH 9.5 and temperature of 40 °C. Cells were harvested in the late exponential growth phase. Fatty acid extraction was carried out by dry biomass (5 mg) acid methanolysis in 0.4 ml 1.2 M HCl in methanol at 80 °C for 1 h. The resulting fatty acid methyl esters were extracted twice with 0.2 ml hexane and processed on an Agilent Technologies AT-5850/5973 GC-MS system. The dominant fatty acids of strain
Z-1001T were C_{16:0} (21.5 %), C_{16:1ω7c} (17.4 %), C_{18:0} (15.0 %) and C_{18:1ω9} (12.6 %).

For polar lipids analysis, approximately 50 mg cells were freeze-dried, extracted according to Tindall (1990), and the extract was analysed by two-dimensional TLC on high-efficient 100 × 100 mm plates (Sorbfil, Krasnodar) using the eluent systems recommended by Christie (2011). Specific spray reagents for lipid phosphate (Vaskovsky & Kostetsky, 1968), free amino groups (ninhydrin in acetic acid/butanol), sugars (z-naphthol in sulfuric acid with subsequent charring) and for quaternary nitrogen substances (Dragendorff’s reagent) were used. TLC standards were phosphatidylinositol, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine, all from Sigma-Aldrich. A photograph of polar lipids TLC is shown in Fig. S5. Polar lipids of strain Z-1001T included two unknown aminophospholipids (APL1–2), four unknown phospholipids (PL1–4) and three unknown polar lipids (UL1–3). The reaction of the unknown polar lipids with all specific spray reagents tested was negative.

The G+C content of the DNA was determined by the thermal denaturation/reassociation technique (Marmur, 1961) using a Cary 100 Bio (Varian) spectrophotometer at a rate of 0.5 °C min$^{-1}$ and calculated according to Owen et al. (1969). The genomic DNA G+C content of strain Z-1001T was 35.6 ± 0.1 mol%.

Genomic DNA was extracted according to a previously described procedure (Boulygina et al., 2002). PCR of the 16S rRNA gene and sequencing of PCR products of the 16S rRNA genes was performed using universal 16S rRNA gene primers (Lane, 1991). PCR fragments were prepared for sequencing and cloning using standard Wizard PCR Preps protocols (Promega).

Direct sequencing of the nearly complete 16S rRNA gene revealed an ambiguous region at position 1137 (Escherichia coli numbering). Therefore, we performed cloning of the 16S rRNA gene PCR products in the pGEM-T vector followed by ABI3730 sequencing of 50 independent clones. The resulting sequences were aligned using the CLUSTAL W (Thompson et al., 1994) algorithm implemented in the BioEdit v.7.0.9.0 software package (Hall, 1999). Detailed analysis revealed the presence of three different copies among the sequenced clones, with approximately equal representation. The obtained sequences were compared with all GenBank entries by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

The obtained 16S rRNA gene sequences of strain Z-1001T were aligned with those of representatives of the family Natranaerobiaceae. A phylogenetic tree was reconstructed by using the MEGA4 software package (Tamura et al., 2007). Evolutionary distances were calculated by using the Jukes–Cantor algorithm and branching order was determined via the neighbour-joining algorithm. The tree is a consensus of 1000 replicate trees (Fig. 2). The three 16S rRNA gene sequences of strain Z-1001T formed a separate cluster with similarity between sequences from 99.6 to 99.9 %. Among species with validly published names, the highest 16S rRNA gene sequence similarity levels were with the members of the genus Natranaerobius, particularly with the type strain Natranaerobius thermophilus JW/NM-WN-LF of this genus (95.8–96.0 %). Thus, it may be concluded that strain Z-1001T is a representative of a novel genus belonging to the family Natranaerobiaceae.

According to phylogenetic analysis, strain Z-1001T belongs to the class Clostridia of the phylum Firmicutes. Its closest relative with a validly published name is Natranaerobius thermophilus, a member of the family Natranaerobiaceae (Mesbah et al., 2007). Strain Z-1001T and Natranaerobius thermophilus are close to each other by their phylogenetic position. Similarly to Natranaerobius thermophilus, whose genome contains two different copies of the 16S rRNA gene (Mesbah et al., 2007), three different gene copies encoding 16S rRNA were found among the cloned PCR fragments of the genomic 16S rRNA gene of strain Z-1001T. However, in both cases, the presence of multiple 16S rRNA-encoding genes does not affect the results of phylogenetic analysis because these genes form compact separate clusters on the dendrogram. Both micro-organisms are anaerobic alkali-thermophiles. Both of them can reduce SO$_4^{2-}$, NO$_3^-$ and Fe(III) citrate. However, the 16S rRNA gene similarity between strain Z-1001T and Natranaerobius thermophilus was 95.8–96.0 %, suggesting the status of strain Z-1001T as a representative of a separate genus.

This conclusion is supported by significant phenotypic differences between the two organisms (Table 1): strain Z-1001T is unable to utilize carbohydrates. The results of tests for carbohydrate utilization performed for the description of Natranaerobius thermophilus, N. trueperi and Natronovirga wadinatrunensis (Mesbah et al., 2007; Mesbah &Wiegel, 2009) are difficult to interpret, because carbohydrates were added against the background of high concentrations of yeast extract and tryptone, which themselves supported growth of all of these organisms. In our opinion, carbohydrate utilization should be tested with the lowest possible concentration of yeast extract. However, we tested the ability of strain Z-1001T to utilize carbohydrates under the same conditions used for testing the related organisms (in the presence of a high concentration of yeast extract, 1 g L$^{-1}$), and the results were negative. Strain Z-1001T is not so extremely halophilic as Natranaerobius thermophilus and may be classified as halotolerant. On the basis of physiological properties and phylogenetic analysis data, we propose strain Z-1001T as the type strain of a novel species of a new genus, Natranaerobaculum magadiense gen. nov., sp. nov.

**Description of Natranaerobaculum gen. nov.**

*Natranaerobaculum* [Natr.an.a.e.r.o.ba’cu.lum. N.L. n. *natron* derived from the Arabic n. *natrun* or *natron*) soda; Gr. pref. an not; Gr. n. aer air; L. neut. n. baculum small stick; N.L. neut. n. *Natranaerobaculum* a soda-requiring anaerobic rod].
Cells are spore-forming rods, Gram-type positive. Obligately alkaliphilic. Halotolerant. Thermophilic. Obligately anaerobic. Chemo-organotrophic. The DNA G+C content is approximately 34 mol%. The type species is \textit{Natranaerobaculum magadiense}.

\textbf{Description of \textit{Natranaerobaculum magadiense}}

\textit{Natranaerobaculum magadiense} (ma.ga.di.en\textsuperscript{9} N.L. neut. adj. magadiense named after its habitat, soda Lake Magadi, Kenya).

Cells are straight to slightly curved rods, 0.2–0.5 \(\mu\)m in diameter and 3.0–7.0 \(\mu\)m in length. Diameter and length of the cells are dependent on the amount of substrate. Cells form endospores of clostridial type. The cell wall has Gram-positive structure. Obligately anaerobic. Obligately alkaliphilic: pH range for growth is from 7.5 to 10.7 with an optimum at 9.25–9.5 (at 40°C). Thermophile: growth temperature range is from 20 to 57°C with an optimum at 45–50°C. Halotolerant: Cl\textsuperscript{-} concentration range for growth is 0–1.55 M with an optimum at 1.2–1.4 M. Obligately natronophilic: Na\textsuperscript{+} concentration range for growth is 0.5–2.7 M with an optimum at 0.9 M. Cannot grow without carbonates. Chemo-organotrophic. Utilizes peptides such as meat and yeast extracts, peptone and tryptone. The main fermentation products formed from yeast extract are acetate, succinate, formate, lactate, propionate, isovalerate, isobutyrate, 2-methylbutyrate and NH\textsubscript{4}\textsuperscript{+}. Carbohydrates do not support growth. S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-}, NO\textsubscript{3}–, AsO\textsubscript{4}\textsuperscript{3-}, Fe(III) citrate and AQDS are reduced as electron acceptors with yeast extract as an electron donor. Able to grow oligotrophically with a small amount of yeast extract – 0.03 g l\textsuperscript{-1}. The main fatty acids are C\textsubscript{16}:0, C\textsubscript{16}:1\(\omega7\)c, C\textsubscript{18}:0 and C\textsubscript{18}:1\(\omega9\).

The type strain is Z-1001\textsuperscript{T} (\textit{DSM 24923}\textsuperscript{T} VKM B-2666\textsuperscript{T}), isolated from bottom sediments of soda lake Magadi, Kenya. The G+C content of DNA of the type strain is 35.6 ± 0.1 mol%.

\begin{table}[h]
\caption{Characteristics that differentiate strain Z-1001\textsuperscript{T} from other members of the family \textit{Natranaerobiaceae}}
\label{tab:characteristics}
\begin{tabular}{|l|c|c|c|c|}
\hline
Characteristic & 1 & 2 & 3 & 4 \\
\hline
Cell size (\(\mu\)m) & 0.2–0.5 \(\times\) 3–7 & 0.2–0.4 \(\times\) 3–5 & 0.6 \(\times\) 2–3 & 0.3–0.4 \(\times\) 4–5 \\
Spore formation & + & – & – & – \\
Temperature range (optimum) (°C) & 20–57 (40–50) & 35–56 (53) & 26–56 (51) & 26–55 (52) \\
\textit{pH} range (optimum) & 7.5–10.7 (9.25–9.5) & 8.3–10.6 (9.5) & 8.5–11.5 (9.9) & 8.0–10.8 (9.5) \\
NaCl range (optimum) (M) & 0–1.55 (1.2–1.4) & 1.5–3.2 (1.6–2.3) & 1.7–3.7 (2.3) & 1.5–3.8 (2.2) \\
Utilization of carbohydrates & – & + & + & + \\
Reduction of & + & – & – & – \\
G + C content of DNA (mol%) & 35.6 & 41.0 & 41.7 & 40.4 \\
Major fatty acids & \(\text{C}_{16}:0\), \(\text{C}_{16}:1\text{\(\omega7\)c}\), \(\text{C}_{18}:0\), \(\text{C}_{18}:1\text{\(\omega9\)}\), \(\text{i-C}_{15}:0\), \(\text{i-C}_{17}:0\), \(\text{i-C}_{16}:0\), \(\text{i-C}_{15}:\text{ai-C}_{15}:0\), \(\text{i-C}_{15}:\text{ai-C}_{15}:0\) & & & \\
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