Thermogladius calderae gen. nov., sp. nov., an anaerobic, hyperthermophilic crenarchaeote from a Kamchatka hot spring

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An obligately anaerobic, hyperthermophilic, organoheterotrophic archaeon, strain 1633T, was isolated from a terrestrial hot spring of the Uzon Caldera (Kamchatka Peninsula, Russia). Cells were regular cocci, 0.5–0.9 μm in diameter, with one flagellum. The temperature range for growth was 80–95 ℃, with an optimum at 84 ℃. Strain 1633T grew on yeast extract, beef extract, peptone, cellulose and cellobiose. No growth was detected on other sugars or carbohydrates, organic acids, or under autotrophic conditions. The only detected growth products were CO2, acetate, and H2. The growth rate was stimulated by elemental sulfur, which was reduced to hydrogen sulfide. The in silico-calculated G+C content of the genomic DNA of strain 1633T was 55.64 mol%. 16S rRNA gene sequence analysis placed strain 1633T together with the non-validly published ‘Thermogladius shockii’ strain WB1 in a separate genus-level cluster within the family Desulfurococcaceae. Average nucleotide identity (ANI) results revealed 75.72 % identity between strain 1633T and ‘Thermogladius shockii’ WB1. Based on these results we propose a novel genus and species with the name Thermogladius calderae gen. nov., sp. nov. The type strain of the type species is 1633T (=DSM 22663T =VKM B-2946T).

The vast majority of cultured Crenarchaeota have been isolated from terrestrial geothermal environments (Huber & Stetter, 2006). Located in Far East of Russia, Kamchatka Peninsula is a volcanically active area with hundreds of associated volcanoes and areas with geothermal activity, among which Uzon Caldera is the largest. Molecular studies have documented the presence of a significant number of cultured and uncultured Crenarchaeota in Uzon Caldera thermal pools. It was reported that Crenarchaeota inhabited not only high-temperature springs (Wemheuer et al., 2013; Chernyh et al., 2015), but also springs with moderate temperatures (Perevalova et al., 2008; Burgess et al., 2012; Menzel et al., 2015). At the time of writing, many thermophilic and hyperthermophilic crenarchaeotes have been isolated from hot springs of Uzon Caldera, including members of genera Desulfurococcus (Bonch-Osmolovskaya et al., 1988; Perevalova et al., 2005; Kublanov et al., 2009), Thermoproteus (Bonch-Osmolovskaya et al., 1990), Acidilobus (Prokofeva et al., 2000; 2009), Vulcanisaeta (Prokofeva et al., 2005), Fervidicoccus (Perevalova et al., 2010) and Pyrobaculum (Slobodkina et al., 2015). Here we report the isolation of strain 1633T obtained from a hot spring of the Uzon Caldera, and found to be closely related to ‘Thermogladius shockii’, a species with a name that was effectively, but never validly, published (Osburn & Amend, 2011).

Strain 1633T was isolated from a sample of mixed water and black mud from a freshwater hot spring (54° 30.047’ N 159° 56.851’ E) located in the West Thermal Field of Uzon Caldera. The in situ water temperature and

Abbreviations: ANI, average nucleotide identity; GDGT, glycerol dibiphytanyl glycerol tetraether.
pH were 86 °C and 5.5, respectively. The following basal medium was used for enrichment and isolation of the novel strain (g l⁻¹): 0.66 NH₄Cl, 0.16 MgCl₂·6H₂O, 0.1 CaCl₂·6H₂O, 0.33 KCl and 0.5 KH₂PO₄. After boiling, the medium was flushed with N₂ and upon cooling the following compounds were added (l⁻¹): 1 ml trace element solution (Kevbrin & Zavarzin, 1992) and vitamin solution (Wolin et al., 1963); 0.5 g NaHCO₃, 0.5 g Na₂S·9H₂O, 0.2 g yeast extract and 0.2 g cysteine hydrochloride. Finally, the pH was adjusted to 6.5–7.0 with 6 M HCl. Ten-millilitre portions of the medium were dispensed into 18 ml Hungate tubes with butyl rubber stoppers under a N₂ atmosphere, and 10 % (v/v) inoculum (a mix of sediment and water) was added. The tubes were incubated at 82 °C with carboxymethyl-cellulose (Sigma, 2 g l⁻¹) as sole carbon substrate, and penicillin (100 μg ml⁻¹). After incubation for 5 days, single regular coccoid cells dominated the cultures. To isolate strain 1633ᵀ, flat 100 ml bottles (Bellco) containing 3 ml basal medium supplemented with peptone (1 g l⁻¹), glucose (1 g l⁻¹) and yeast extract (1 g l⁻¹) and solidified with 1.2 % (w/v) Gelrite (Gifco) and MgSO₄ (1 g l⁻¹) were used. Individual colonies were picked under a stream of 100 % N₂ and transferred to liquid medium. The purity of the isolate was tested by cultivation on basal medium with various complex carbon sources (e.g. yeast extract, beef extract, peptone) and electron acceptors and subsequent monitoring of the cultures by light and electron microscopy, as well as by 16S rRNA gene sequencing.

For phase-contrast light microscopy an Olympus CX41RF microscope was used. For electron microscopy (negative staining), cultures were fixed as described previously (Sokolova et al., 2002) and examined under a JEM-100B microscope (JEOL). Cells of strain 1633ᵀ were cocci, 0.5–0.9 μm in diameter, occasionally with straight thin protrusions and one long flagellum (Fig. 1a). Virus-like particles associated with strain 1633ᵀ were detected by electron microscopy before isolation of the strain on solid medium (Fig. 1b, c). Single- and occasionally two-tailed (data not shown) spindle-shaped particles were observed in cultures, maintained over 10 weeks with successive dilutions every 3–4 days. No evidence was found for virus-induced cell lysis. No virus-like particles were detected by electron microscopy in cultures after transferring from colonies.

Growth of the novel isolate was registered by direct cell counts using phase-contrast microscopy. All growth experiments were conducted in triplicates. The novel isolate is an obligate anaerobe, growing only under anoxic conditions with a reductant (Na₂S). The organism is a hyperthermophile and neutrophile growing in the temperature range from 80 to 95 °C (optimum 84 °C), and in the pH range from 6.5 to 8.2 (optimum pH 7.1). No growth was observed at 75 °C and 98 °C, or at pH 6.2 and pH 8.6. Strain 1633ᵀ grew only at low NaCl concentrations, up to 2.5 g l⁻¹, and the best growth was obtained in the absence of NaCl. The doubling time of growth on yeast extract (1 g l⁻¹) under optimal conditions was 7.7 h.

**Fig. 1.** Electron micrographs of negatively stained cells of a pure culture of strain 1633ᵀ (a) and infected by virus-like particles (b, c). Bars, 0.5 μm.

Strain 1633ᵀ grew well on yeast extract (YE), beef extract and peptone (all at 2.0 g l⁻¹) with the final cell yield ranging from 0.5–7.7 × 10⁸ cells ml⁻¹. Weaker growth was
observed on cellulose and various cellulosics [filter paper, microcrystalline cellulose (Chemapol, Czech Republic), carboxymethyl cellulose (Sigma)] (all at 2.0 g l⁻¹). For growth on cellulose, the addition of 1.0 g l⁻¹ YE was essential. No growth was detected on glucose, xylose, fructose, sucrose, maltose, lactose, agarose, starch, amorphous cellulose, keratin, xylan, chitin, formate, pyruvate, lactate, fumarate, glycerine, methanol, ethanol, under a H₂/CO₂ (4 : 1, v/v), N₂/CO (19 : 1, 5 : 1, 1 : 1, v/v) or 100 % CO atmosphere. Elemental sulfur (at final concentration 10 g l⁻¹) stimulated growth of strain 1633³ and was reduced to hydrogen sulfide. SO₄²⁻ and S₂O₃²⁻, added as sodium salts (20 mM) did not stimulate growth of the strain whereas sodium sulfate or nitrate (20 mM) inhibited growth. Under optimal growth conditions, the final cell yields of the strain were 2.6 ± 1.5 x 10⁷ cells ml⁻¹ on the medium supplemented with 1.0 g l⁻¹ YE; 7 ± 2 x 10⁷ cells ml⁻¹ on the medium with 1.0 g l⁻¹ YE and S²⁻; and 1.8 ± 1 x 10⁸ cells ml⁻¹ with 1.0 g l⁻¹ YE, S²⁻ and 2.0 g l⁻¹ cellulose. Strain 1633³ was not sensitive to kanamycin, chloramphenicol or penicillin (100 µg ml⁻¹). The main end-products of growth were determined by GLC using a Shaper chromatograph with UV/VIS detector at 220 nm and REZEX ROA 300 x 7.8 mm column (Phenomenex), maintained at 35 °C, and using 0.2 % H₃PO₄ solution as a solvent at a flow rate of 0.5 ml min⁻¹. The only growth products with YE as the carbon substrate were CO₂, acetate and H₂. During growth on cellulose, neither acetate nor ethanol was formed.

Cells of strain 1633³ were lyophilized for lipid analysis via hydrolysis in 5 % HCl/MeOH (Pitcher et al., 2011). Core lipid extracts were injected into an HPLC-APCI-MS system (HPLC Agilent 1290 series with Econosphere NH₂ column; Agilent 6530 Q-TOF detector). The results showed that the majority (89.9 %) of the archaeal lipids were glycerol dibiphytanyl glycerol tetraethers (GDGTs) that contained zero to four cyclopentyl rings; the remaining small proportion (11.1 %) consisted of the glycerol diether (m/z = 651.663), probably the macroyclic archaeol found in Methanosarcina barkeri (Sprott, 1992) and Methanothermbarcum igneus (Trincone et al., 1992). Of the key GDGT compounds, GDGT-4 was the most abundant (52.7 %), followed by GDGT-3 (16.9 %), GDGT-2 (7.4 %), GDGT-1 (4.1 %) and GDGT-0 (3.0 %). Small amounts of isomers GDGT-4 iso (3.9 %) and GDGT-3 iso (0.9 %) were detected, probably the antiparallel regioisomers based on their MS spectra being identical to those of GDGT-4 and GDGT-3 (Sinninghe Damsté et al., 2002). No crenarchaeol was detected in the lipids of strain 1633³.

The G+C content (55.64 mol%) of the genomic DNA of strain 1633³ was calculated in silico using the whole genome sequence (GenBank accession no. NC_017954, Mardanov et al., 2012). The genome has a single copy of the 16S rRNA gene, which was used for phylogenetic analysis. BLAST analysis against species with validly published names using the EzBioCloud/EzTaxon server (Kim et al., 2012) revealed the nearest sequences belonged to representatives of the crenarchaeal genus Staphylothermus. However, the most similar sequence to strain 1633³ belonged to another member of the family Desulfurococaceae, 'Thermogluadius shockii' WB1 (Osburn & Amend, 2011). In order to reconstruct the phylogenetic position of strain 1633³ and 'T. shockii' WB1, their 16S rRNA gene sequences as well as sequences of other members of the family Desulfurococaceae with validly published names were aligned (total 19 sequences) using MUSCLE (Edgar, 2004) and analysed. Phylogenetic analysis was performed in the MEGA 6 package (Tamura et al., 2013). Evolutionary history was inferred using the maximum-likelihood method with 1000 repetitions [bootstrap, Felsenstein (1985)]. Evolutionary distances were calculated using the General Time Reversible (G + I, 4 categories, Nei & Kumar (2000)) model. The phylogenetic tree with the highest log-likelihood (−4095.1183) placed the novel isolate into a cluster with 'T. shockii' WB1, Staphylothermus hellenicus DSM 12710T, Staphylothermus marinus F1T (Fig. 2). The distances calculated using the above model suggested that the closest relatives of strain 1633³ were 'T. shockii' (98.7 %), Staphylothermus hellenicus (96.1 %) and Staphylothermus marinus (95.9 %). The genome of 'T. shockii' WB1 was sequenced and assembled into a single chromosome in the course of this work (data not shown). Evaluation of the average nucleotide identity (ANI) between genomes of strain 1633³ and 'T. shockii' WB1 using ANI calculator (http://enve-omics.ce.gatech.edu/ani/) revealed the value to be 75.72 %, suggesting these strains represent two different species.

The closest cultivated relative of strain 1633³, ‘Thermogluadius shockii’ strain WB1 (Osburn & Amend, 2011), was isolated from ‘Boomerang Pool’ in Yellowstone National Park, USA. Two strains (2412Fs and 2412Pk) also very closely related to strain 1633³ with 99 % 16S rRNA gene similarity, were isolated from ‘Burlyasch’ hot spring of Uzon Caldera recently (Bidzhieva et al., 2014). All isolates are anaerobic, hyperthermophilic, organotrophic cocci, able to grow without sulfur, but stimulated by its presence. This is in contrast to related species of the genus Staphylothermus, which are obligately dependent on elemental sulfur (Table 1). Unlike other closely related isolates, strain 1633³ is able to grow not only on proteinaceous substrates, but also on cellulose (filter paper, microcrystalline and carboxymethyl-cellulose). However, the complete genome analysis did not reveal the presence of genes encoding glycolide hydrolyases or known exo/endobeta-1,4-glucanases (Mardanov et al., 2012), suggesting either the existence of novel mechanisms of cellulose hydrolysis in strain 1633³ or the utilization of unidentified thermal degradation products of cellulose by this organism. The ability to grow on biopolymers (proteins and carbohydrates) was previously reported for several representatives of the family Desulfurococaceae (Bonch-Osmolovskaya et al., 1988; Sako et al., 1996; Perevalova et al., 2005; Kublanov et al., 2009; Bidzhieva et al., 2014).
Strain 1633T is a novel member of the order Thermopro-teales that shares this capability.

The low level of genomic DNA identity between strains 1633T and ‘Thermogladius shockii’ WB1 (75.72 %), 16S rRNA gene-based phylogenetic analysis and phenotypic differences indicate that the strains represent two different species (Goris et al., 2007). Based on the previous characterization of ‘T. shockii’ (Osburn & Amend, 2011) and phylogenetic and physiological differences, we propose a novel genus and species represented by strain 1633T, with the name Thermogladius calderae gen. nov., sp. nov. This species will be the type species of the genus Thermogladius after valid publication of the genus name, instead of the earlier proposed ‘Thermogladius shockii’, a name that was never validated.

Description of Thermogladius gen. nov.

Thermogladius (Ther.mo.gla’di.us. Gr. adj. thermos hot; L. n. gladius sword; N.L. masc. n. Thermogladius the hot sword).

Cells are regular to slightly irregular cocci, with occasional straight, thin protrusions. The cell envelope consists of an S-layer covering the cytoplasmic membrane. Obligately anaerobic, showing organoheterotrophic metabolism with the ability to ferment a variety of complex organic substrates, including cellulose and keratin. Facultative $S^0$-reducer. Cells can thrive in high temperatures and in a broad pH range. Known strains have been isolated from terrestrial hot springs.

The type species is Thermogladius calderae.

Description of Thermogladius calderae sp. nov.

Thermogladius calderae (cal.de’rae. N.L. gen. n. calderae of a caldera, referring to the isolation of the type strain from the Uzon Caldera).

Displays the following properties in addition to those in the genus description. Cells are regular cocci, 0.5–0.9 μm in diameter, with one long flagellum. The temperature range for growth is 80–95 °C, with an optimum at 84 °C. The pH range for growth at 84 °C is pH 6.5–8.2 (optimum pH 7.1). Grows on yeast extract, beef extract, peptone and cellulose (filter paper, microcrystalline cellulose, carboxymethyl-cellulose) and cellobiose. No growth is
Table 1. Characteristics of strain 1633T in comparison with those of the closest phylogenetic relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape and size (μm)</td>
<td>Regular cocci, 0.5–0.9</td>
<td>Coci, 0.6–1.2</td>
<td>Coci, 0.5–1</td>
<td>Irregular cocci, 0.8–1.3</td>
</tr>
<tr>
<td>Flagellum</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth temperature (°C)</td>
<td>80/84/95</td>
<td>64/84/93</td>
<td>65/92/98</td>
<td>70/85/90</td>
</tr>
<tr>
<td>(min./opt./max.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimum doubling time (h)</td>
<td>7.7</td>
<td>4.9*</td>
<td>4.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Growth pH (min./opt./max.)</td>
<td>6.5/7.1/8.2</td>
<td>3.5/6–8.5</td>
<td>4.5/6.5/8.5</td>
<td>4.5/6.0/7.0</td>
</tr>
<tr>
<td>NaCl range (% w/v)</td>
<td>0/0.25</td>
<td>0/0.46</td>
<td>1/3.5</td>
<td>2/4/8</td>
</tr>
<tr>
<td>Growth substrates</td>
<td>Yeast extract, beef extract, peptone and cellulose (filter paper, microcrystalline cellulose, carboxymethyl-cellulose), cellobiose</td>
<td>Yeast extract, peptone, casein</td>
<td>Yeast extract, peptone</td>
<td>Yeast extract, peptone</td>
</tr>
<tr>
<td>Effect of sulfur</td>
<td>Stimulated</td>
<td>No effect</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>55.64</td>
<td>51.3</td>
<td>34.9–35.3</td>
<td>38</td>
</tr>
<tr>
<td>Resistance to antibiotics</td>
<td>Kanamycin, chloramphenicol, penicillin</td>
<td>Kanamycin, rifampicin, chloramphenicol, streptomycin</td>
<td>Kanamycin, chloramphenicol, streptomycin</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Doubling time was measured in the absence of elemental sulfur in medium.

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