Caloribacterium cisternae gen. nov., sp. nov., an anaerobic thermophilic bacterium from an underground gas storage reservoir

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A novel anaerobic, moderately thermophilic bacterium (strain SGL43\textsuperscript{T}) was isolated from Severo-Stavropolskoye underground gas storage reservoir (Russia). Cells of strain SGL43\textsuperscript{T} were motile straight rods, 0.4 μm in diameter and 2.0–3.0 μm in length. The temperature range for growth was 28–65 °C, with optimum growth at 50 °C. The pH range for growth was 5.5–8.0, with optimum growth at pH 7.0–7.5. Growth of strain SGL43\textsuperscript{T} was observed at NaCl concentrations of 0–4.0 % (w/v) with optimum growth at 1.0 % (w/v) NaCl. Substrates utilized by strain SGL43\textsuperscript{T} included peptone, yeast extract, glucose, fructose, maltose, galactose, pyruvate and citrate. Products of glucose or citrate fermentation were acetate, hydrogen and CO\textsubscript{2}. Thiosulfate was reduced to sulfide. The DNA G+C content of strain SGL43\textsuperscript{T} was 43.1 mol%. 16S rRNA gene sequence analysis revealed that strain SGL43\textsuperscript{T} belongs to the order Thermoanaerobacterales (phylum 'Firmicutes'). The closest relative of strain SGL43\textsuperscript{T} was Thermoanaerobacterium saccharolyticum (86.2 % 16S rRNA gene sequence similarity with the type strain). Based on the data presented here, strain SGL43\textsuperscript{T} is considered to represent a novel species of a new genus, for which the name Caloribacterium cisternae gen. nov., sp. nov. is proposed. The type strain of Caloribacterium cisternae, the type species of the genus, is SGL43\textsuperscript{T} (\textsuperscript{5}DSM 23830\textsuperscript{T} = VKM B-2670\textsuperscript{T}).

Underground reservoirs for the storage of natural gas represent anthropogenic, carbon-rich environments that are often characterized by elevated temperatures. The microbial communities of these habitats have been poorly studied. The presence of viable methanogenic, acetogenic, sulfate-, nitrate- and iron-reducing, fermentative mesophilic prokaryotes has been demonstrated for deep subsurface gas storage reservoirs in Russia and France. Both culturing and molecular techniques have shown that members of the 'Firmicutes' and 'Proteobacteria' dominate the bacterial portions of the community (Ivanova et al., 2007; Basso et al., 2009). To our knowledge, only one thermophilic anaerobic micro-organism, 'Moorella perchloratireducens' (strain An10\textsuperscript{\textsuperscript{ATCC BAA-1531 = JCM 14829}}, has been isolated from an underground gas storage reservoir (Balk et al. 2008). Here, we report the isolation and characterization of a novel anaerobic, moderately thermophilic, organotrophic bacterium (strain SGL43\textsuperscript{T}) from production water of Severo-Stavropolskoye (North Caucasus Region, Russia), the world’s largest such reservoir.

Details of the sampling location and procedures are described by Ivanova et al. (2007). An enrichment culture was initiated by inoculation of 10 % (w/v) of the water sample into anaerobically prepared, bicarbonate-buffered, sterile (135 °C, 1 h) liquid medium of the following composition (per litre distilled water): 0.33 g NH\textsubscript{4}Cl, 0.33 g KCl, 0.33 g MgCl\textsubscript{2} . 6 H\textsubscript{2}O, 0.33 g CaCl\textsubscript{2}, 0.33 g KH\textsubscript{2}PO\textsubscript{4}, 2.0 g NaHCO\textsubscript{3}, 1 ml trace element solution (Slobodkin et al., 1997), 1 ml vitamin solution (Wolin et al., 1963) and 0.2 g yeast extract (Sigma). The medium did not contain any reducing agents; the pH of the autoclaved medium was 6.7–6.9 at 20 °C. Glucose (15 mM) was added as substrate from anaerobically prepared sterile stock solution before inoculation. Incubation temperature was 50 °C. After three subsequent transfers, the enrichment was purified by serial 10-fold dilutions in the same medium followed by the selection of well-separated colonies that had developed in anaerobic agar blocks (1.5 % agar in

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SGL43\textsuperscript{T} is JF262044.

A supplementary table is available with the online version of this paper.
Cells of strain SGL43 T were straight rods, 0.4 μm in diameter. Cells occurred singly or in pairs and were motile by means of several non-polar flagella (Fig. 1a, b). Spores were not observed for cultures that had been grown under optimal or suboptimal conditions (different pH, temperature and salinity). In addition, cultures incubated at 100 °C for 20 min or at 121 °C for 60 min could not be further subcultured, suggesting the absence of heat-resistant bodies such as spores. Ultrathin sectioning of cells of strain SGL43 T revealed the presence of a distinct electron-dense material characteristic of a peptidoglycan layer of Gram-positive type cell walls (Fig. 1c). The temperature range for growth of strain SGL43 T was 28–65 °C, with an optimum at 50 °C. No growth was detected at pH values <5.0 or >8.5. Growth of strain SGL43 T was observed at NaCl concentrations of 0–4.0 % (w/v) with an optimum at 1.0 % (w/v), but no growth was evident at 5.0 % (w/v) NaCl or above. Yeast extract (0.2 g l−1) was essential for growth. The doubling time under optimal conditions was 2.1 h. Potential electron donors and acceptors were tested on the same medium as used for isolation but supplemented with 1.0 % (w/v) NaCl. Soluble substrates and electron acceptors were added from sterile anaerobic stock solutions before incubation. Insoluble substrates and electron acceptors were added directly into each test tube with liquid medium prior to sterilization. Medium with poorly crystalline iron (III) oxide (ferrihydrite) was prepared as described previously (Slobodkin et al., 1999). Substrates utilized by strain SGL43 T included glucose, fructose, galactose, maltose, pyruvate (15 mM each), peptone, yeast extract (2.0 g l−1 each) and citrate (10 mM). Products of glucose or citrate fermentation were acetate, hydrogen and CO2. From 1 mM of glucose consumed, approximately 2 mM acetate and 1 mM H2 were produced; CO2 was not quantified. No growth was observed on tryptone, beef extract (1.0 g l−1 each), glycerol (20 mM), D-ribulose, cellobiose, lactose, sucrose, xylose (15 mM each), cellulose, carboxymethyl-cellulose, filter paper, dextran, starch (2.5 g l−1 each), lactate, fumarate, malate, maleate, succinate (10 mM each), olive oil (5 g l−1) or H2/CO2 (80 : 20, v/v) without an electron acceptor. Potential
electron acceptors were tested with glycerol (20 mM) or peptone (2.0 g l\(^{-1}\)) as the energy sources in the presence of yeast extract (0.2 g l\(^{-1}\)). Thiocyanate enhanced growth slightly and was reduced to sulfide. Ferrihydrite [90 mmol Fe(III) l\(^{-1}\)], nitrate (10 mM), nitrite (2.5 mM), sulfate (14 mM), sulfite (5 mM), elemental sulfur (10 g l\(^{-1}\)), 9,10-anthraquinone-2,6-disulfonate, fumarate (20 mM each) and oxygen [3 or 20 % (v/v) in the gas phase] were not reduced and did not support growth (on glycerol) or stimulate growth (on peptone). Penicillin, ampicillin, novobiocin, kanamycin and neomycin (each at 100 \(\mu\)g ml\(^{-1}\)) inhibited growth of strain SGL43\(^{T}\). The cellular fatty acids comprised a mixture of monounsaturated and saturated straight-chain and branched components (see Table S1 in IJSEM Online). The major fatty acids were iso-C\(_{15}:0\), C\(_{16}:0\), C\(_{18}:0\) and iso-C\(_{17}:1\) \(\alpha\) \(\omega\) (19.8, 16.6, 15.9 and 15.3 %, respectively).

The G + C content of the genomic DNA of strain SGL43\(^{T}\) was 43.1 mol% (\(T_{m}\)). A comparison of 1437 nt of the 16S rRNA gene sequence of strain SGL43\(^{T}\) with those available in GenBank showed that strain SGL43\(^{T}\) belonged to the order Thermoanaerobacterales, class Clostridia, and shared highest pairwise similarity with the type strains of the type strains of Thermoanaerobacterium saccharolyticum and Thermoanaerobacterium aotearoense (86.2 and 86.0 %, respectively) (Fig. 2). The novel isolate was almost equidistantly placed between the genera Thermoanaerobacterium (84.6–86.2 % 16S rRNA gene sequence similarity) and Caldanaerobius (84.1–85.1 %). At the time of writing, the numbers and genera making up the families within the order Thermoanaerobacterales are not firmly defined (Euzéby, 2011; Wang et al., 2007; Wiegel, 2009). According to RDP Release 10 (http://rdp.cme.msu.edu/), the order Thermoanaerobacterales consists of two families, Thermoanaerobacteraceae and Thermodesulfobiaceae. Strain SGL43\(^{T}\) shares morphological and physiological traits with members of the family Thermoanaerobacteraceae. As with the majority of representatives of Thermoanaerobacteraceae, strain SGL43\(^{T}\) is an anaerobic, thermophilic, chemo-organotrophic micro-organism with a fermentative type of metabolism. It grows on carbohydrates as energy sources, although utilizing a more narrow range of sugars (Table 1). Similar to many species of different genera of the order Thermoanaerobacterales, strain SGL43\(^{T}\) reduces thiosulfate to sulfide (Wiegel, 2009). In contrast to representatives of the closest related genera, strain SGL43\(^{T}\) produces acetate and hydrogen but not ethanol, lactate or formate as products of glucose fermentation. It also differs from its closest relatives based on temperature and salinity ranges for growth. The optimal growth temperature is 10–15 °C lower than that for its closest relatives. The optimal NaCl concentration is 1 % and strain SGL43\(^{T}\) can grow in up to 4 % NaCl whereas the optimal concentration of NaCl for its closest relatives is about 0.1 %. In addition to carbohydrates, strain SGL43\(^{T}\) is able to ferment citrate; this feature has been not reported for representatives of the order Thermoanaerobacterales. Remarkable differences exist in cellular fatty acid profiles for the novel isolate and representatives of closely related genera. iso-C\(_{15}:0\), iso-C\(_{17}:0\) and, to a lesser extent, C\(_{16}:0\) have been reported to be the major fatty acids for some strains of the genera Thermoanaerobacterium, Caldanaerobius, Thermoanaerobacter and Moorella (Cann et al., 2001; Yamamoto et al., 1998). For strain SGL43\(^{T}\), iso-C\(_{15}:0\) and C\(_{16}:0\) are also the predominant components but iso-C\(_{17}:0\) is present at a low level (4.3 %). Unsaturated fatty acids and straight-chain fatty acids longer than C\(_{17}:0\) were not detected or were present at low or trace amounts for strains of the genera Thermoanaerobacterium, Caldanaerobius, Thermoanaerobacter and Moorella. In contrast, C\(_{18}:0\) and iso-C\(_{17}:1\) \(\alpha\) \(\omega\) are among the major fatty acids for strain SGL43\(^{T}\). In addition to these data, the significant phylogenetic distance (80–86 % 16S rRNA gene sequence similarity) precludes assignment of strain SGL43\(^{T}\) to any described genera of the order Thermoanaerobacterales. Therefore, on the basis of its phylogenetic position, and phenotypic and physiological properties we propose that strain SGL43\(^{T}\) represents a novel species of a new genus in the family Thermoanaerobacteraeae, for which the name Caloribacterium cisternae gen. nov., sp. nov. is proposed.

**Description of Caloribacterium gen. nov.**

*Caloribacterium* (Ca.lo.ri.bac.tel.ri.um. L. n. *calor* heat; L. neut. n. *bacterium* a small rod; L. neut. n. *Caloribacterium* a thermophilic rod).
Cells are rod-shaped. Anaerobic and moderately thermophilic chemo-organotroph. Member of the family *Thermoanaerobacteraceae*, order *Thermoanaerobacterales*, class *Clostridia*. Ferments monosaccharides and disaccharides. Reduces thiosulfate. The type species is *Caloribacterium cisternae*.

### Description of *Caloribacterium cisternae* sp. nov.

*Caloribacterium cisternae* (cis.ter'nae. L. fem. n. cisterna reservoir; L. gen. n. cisternae of/from a reservoir, referring to the source of the type strain).

Has the following characteristics in addition to those given for the genus. Cells are motile, straight rods, 0.4 µm in diameter and 2.0–3.0 µm in length. Spores are not produced. Gram-positive cell wall. Grows at 28–65 °C (optimum, 50 °C), at pH 5.5–8.0 (optimum, pH 7.0–7.5) and in the presence of 0–4.0 % (w/v) NaCl (optimum, 1.0 %). Yeast extract is essential for growth. Utilizes glucose, fructose, galactose, maltose, peptone, yeast extract, pyruvate and citrate. Products of glucose or citrate fermentation are acetate, hydrogen, and CO₂. No growth is observed on beef extract, tryptone, glycerol, L-arabinose, cellobiose, lactose, sucrose, xylose, cellulose, carboxymethyl-cellulose, filter paper, dextran, starch, lactate, fumarate, maleate, succinate, olive oil or H₂/CO₂ (80:20, v/v) without an electron acceptor. Thiosulfate is reduced to sulfide. Poorly crystalline iron (III) oxide, nitrate, nitrite, sulfate, sulfite, elemental sulfur, 9,10-anthraquinone-2,6-disulfonate, fumarate and oxygen are not reduced and do not support growth. The major fatty acids are iso-C₁₅ : 0, C₁₆ : 0, C₁₈ : 0, and iso-C₁₇ : 1Vo₈.

The type strain, SGL43T (=DSM 23830T=VKM B-2670T), was isolated from production water of an underground gas storage reservoir. The DNA G+C content of the type strain is 43.1 mol% (Tm).

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References


