Caldimicrobium rimae gen. nov., sp. nov., an extremely thermophilic, facultatively lithoautotrophic, anaerobic bacterium from the Uzon Caldera, Kamchatka

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An extremely thermophilic, strictly anaerobic, facultatively chemolithoautotrophic bacterium designated strain DS^T was isolated from Treshchinnyi Spring, one of the hottest springs of the Uzon Caldera (Kamchatka, Russia). Cells of the novel organism were Gram-negative rods, about 1.0–1.2 µm long and 0.5 µm wide. The temperature range for growth was 52–82 °C, with an optimum at 75 °C. Growth was observed at pH 6.8–7.4, and the optimum pH was 7.0–7.2. Strain DS^T was able to grow lithoautotrophically with hydrogen in the presence of CO₂ as a carbon source and thiosulfate or elemental sulfur as an electron acceptor. It also grew well with ethanol, fumarate, succinate or malate in the presence of thiosulfate. Yeast extract was not required for growth and did not stimulate growth. The genomic DNA G+C content was 35.2 mol%. Phylogenetic analysis of the 16S rRNA gene sequence indicated that the novel organism was a member of the family *Thermodesulfobacteriaceae*. On the basis of phylogenetic and physiological considerations, it is proposed that strain DS^T represents a new genus and species, *Caldimicrobium rimae* gen. nov., sp. nov. The type strain of *Caldimicrobium rimae* is DS^T (=DSM 19393^T =VKM B-2460^T).

The phylum ‘*Thermodesulfobacteria*’ (Garrity & Holt, 2001) represents one of the deeply branching lineages within the domain *Bacteria* and comprises Gram-negative, rod-shaped, anaerobic, thermophilic, sulfate-reducing microorganisms. At present, the phylum includes a single class *Thermodesulfobacteria*, a single order *Thermodesulfobacteriales* and a single family *Thermodesulfobacteriaceae*, consisting of the genera *Thermodesulfobacterium* (Zeikus *et al.*, 1983) and *Thermodesulfatator* (Moussard *et al.*, 2004) with validly published names and the genus ‘*Geothermobacterium*’ (Kashefi *et al.*, 2002), which as yet has no standing in nomenclature. The group of microorganisms belonging to the family *Thermodesulfobacteriaceae* is metabolically diverse and includes both

obligate chemolithotrophs (Jeanthon *et al.*, 2002; Kashefi *et al.*, 2002; Moussard *et al.*, 2004) and chemo-organo-trophs (Zeikus *et al.*, 1983; Rosanova & Pivovarov, 1988; Sonne-Hansen & Ahring, 1999). All of them, except the obligate iron-reducer ‘*Geothermobacterium ferrireducens*’, are able to reduce sulfate. In this paper, we report the isolation and characterization of a novel extremely thermophilic, anaerobic, facultatively lithoautotrophic bacterium that belongs to the family *Thermodesulfobacteriaceae* and, similarly to ‘*G. ferrireducens*’, is not capable of dissimilatory sulfate reduction.

A sample of sediment mixed with water was collected from Treshchinnyi (‘Crack’) Spring in the eastern solfataric field of the Uzon Caldera. The temperature and pH of the water at the sampling site were 80 °C and pH 6.3. For the initial enrichment, the following basal medium was used (l⁻¹): NH₄Cl, 0.33 g; MgCl₂·6H₂O, 0.33 g; CaCl₂·6H₂O, 0.1 g; KCl, 0.33 g; KH₂PO₄, 0.33 g; resazurin, 0.001 g; trace-

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DS^T is EF554596.

A maximum-parsimony tree based on 16S rRNA gene sequences is available as supplementary material with the online version of this paper.

element solution (Pfennig & Lippert, 1966), 1 ml; and vitamin solution (Wolin *et al.*, 1963), 1 ml. After being boiled, the medium was cooled under CO₂ and supplemented with 2.0 g Na₂S₂O₃, 1.5 g NaHCO₃ and 0.5 g Na₂S · 9H₂O l⁻¹. NaOH (6 ml of a 10 % solution l⁻¹) was added to the medium before autoclaving to adjust the pH to 7.2. The medium was dispensed in 5 ml portions into 20 ml Balch tubes and the headspace was filled with 100 % H₂ at atmospheric pressure. After autoclaving, the tubes were inoculated with a mixture of water and sediment and incubated at 82 °C. After 5 days of incubation, the growth of small, oval rods was observed. Growth was accompanied by formation of sulfide, determined as described by Trüper & Schlegel (1964). The culture was purified using the dilution-to-extinction technique and designated strain DS^T. Electron microscopy (Bonch-Osmolovskaya *et al.*, 1990) indicated that cells of isolate DS^T were small rods, about 1–1.2 µm long and 0.5 µm wide, with two polar flagella (Fig. 1a). They occurred singly or formed chains of varying length (up to eight cells). Electron micrographs of thin sections of cells revealed that the cell-wall structure was typical of Gram-negative bacteria (Fig. 1b).

After demonstrating that strain DS^T grew well with fumarate (10 mM) and thiosulfate (10 mM), we chose this donor–acceptor pair for growth experiments. In this case, the headspace of the tubes was filled with 100 % CO₂. The cell density was determined by direct cell counting. The pH range for growth was determined using various buffers (MES for pH 6.0–6.5, PIPES for pH 6.5–7.0, HEPES for pH 7.0–7.5 and Tris for pH 7.5–8.0) at a concentration of 10 mM. NaHCO₃ was omitted from the medium. The pH was determined at room temperature. Strain DS^T was a neutrophilic bacterium, growing in a pH range of 6.8–7.4 with an optimum at pH 7.0–7.2; no growth was detected at pH 7.5 or 6.5. The novel isolate grew at 52–82 °C with an optimum at 75 °C; no growth was detected at 85 or 50 °C. The doubling time under optimum growth conditions was about 4 h. The final cell concentration under optimal growth conditions was 3.2 × 10⁷ cells ml⁻¹.

Strain DS^T was a strictly anaerobic organism. It was able to grow lithoautotrophically with hydrogen in the presence of CO₂ as a carbon source, ammonium as the source of nitrogen and thiosulfate or elemental sulfur as the electron acceptor. It grew well with ethanol, fumarate, succinate or malate in the presence of thiosulfate. Compounds tested but not utilized as electron donors by strain DS^T were acetate, propionate, glucose, sucrose, galactose, lactate, pyruvate (all at 20 mM), methanol (10 mM), starch, yeast extract and peptone (all at 10 g l⁻¹). In the presence of hydrogen, fumarate or ethanol, strain DS^T could use elemental sulfur (10 g l⁻¹) as an alternative electron acceptor; however, the cell yield was lower than that with thiosulfate. Nitrate (10 mM), sulfate (10 mM), sulfite (2 mM), nitrite (2 mM), amorphous Fe(III) oxide (90 mM) (Slobodkin *et al.*, 1999) and Fe(III) citrate (20 mM) were not used as electron acceptors. Pyruvate, lactate and fumarate were not fermented. The end product of anaerobic respiration with thiosulfate or sulfur was H₂S. Yeast extract (100 mg l⁻¹) was not required for growth of strain DS^T and, when added, did not stimulate growth.

DNA was isolated as described by Marmur (1961). The DNA G+C content was 35.2 mol% as determined by melting point analysis (Marmur & Doty, 1962), using *Escherichia coli* K-12 DNA as a reference. For amplification and sequencing of the 16S rRNA gene, DNA was obtained by standard phenol/chloroform extraction. The 16S rRNA gene was selectively amplified using 5'-AGAGTTGA-TCCTGGCTCAG-3' and 5'-TACGGTACCTTGTTACG-ACTT-3' as the forward and reverse primers, respectively. PCR products were purified using the Wizard PCR-Prep kit (Promega) according to the manufacturer's instructions. Sequencing was performed using a Big Dye Terminator v.3.1 sequencing reaction kit and an ABI 3730 DNA automatic sequencer (Applied Biosystems). In various phylogenetic trees that were reconstructed by using either maximum-likelihood, neighbour-joining or parsimony methods, the novel isolate DS^T formed a distinct branch within the family *Thermodesulfobacteriaceae*, thereby confirming its separate taxonomic status. A

Fig. 1. Electron micrographs of cells of strain DS^T showing a negatively stained whole cell (a) and a cell thin section (b). Bars, 0.5 µm.

representative maximum-likelihood tree is shown in Fig. 2 and a parsimony tree containing bootstrap values (1000 resamplings) is shown in Supplementary Fig. S1 (available in IJSEM Online).

The phylogenetic affiliation of strain DS^T was examined according to the method described by Vatsurina *et al.* (2008). '*G. ferrireducens*' FW-1a was the most closely related strain to strain DS^T, with 97.0 % 16S rRNA gene sequence similarity. '*G. ferrireducens*' is the only species of this genus and the name has not yet been validly published. Strain DS^T differs significantly from '*G. ferrireducens*' in the temperature characteristics of growth and the electron acceptors and donors used (Table 1). '*G. ferrireducens*' is an obligate Fe(III)-reducer, gaining energy exclusively by hydrogen oxidation and formation of magnetite as the end product. Strain DS^T is nutritionally more versatile: it is able to use ethanol and some non-fermentable electron donors in the presence of thiosulfate. In addition, the novel isolate is unable to utilize crystalline Fe(III) oxides and, hence, it is clearly distinguishable from '*G. ferrireducens*' (Table 1). Based on phylogenetic considerations, strain DS^T is also related to the species of the genus *Thermodesulfobacterium*: *Thermodesulfobacterium hydrogeniphilum* (95.8 % 16S rRNA gene sequence similarity to the type strain), *Thermodesulfobacterium commune* (95.0 %), *Thermodesulfobacterium hveragerdense* (93.9 %) and *Thermodesulfobacterium thermophilum* (93.7 %). Of these species, *Thermodesulfobacterium hydrogeniphilum* is the only one able to grow autotrophically with hydrogen in the presence of sulfate. However, strain DS^T does not exhibit the ability to reduce sulfate, which is the most important common characteristic of all members of the genus *Thermodesulfobacterium*. Other distinctions between strain DS^T and related species are presented in Table 1.

A characteristic feature of isolate DS^T is its ability to use elemental sulfur as an electron acceptor in the course of growth. This capacity is an intrinsic metabolic feature of micro-organisms of the family *Desulfurobacteriaceae* and the genus *Thermosulfidibacter* (L'Haridon *et al.*, 2006; Nunoura *et al.*, 2008). These organisms are anaerobic, thermophilic, highly specialized hydrogen-oxidizers that readily reduce S⁰; for members of *Thermosulfidibacter*,

sulfur is the sole electron acceptor. In contrast to strain DS^T, they are unable to oxidize organic acids or alcohols and cannot use thiosulfate as an electron acceptor. Phylogenetically, isolate DS^T is remote from the family *Desulfurobacteriaceae* and the genus *Thermosulfidibacter* (the 16S rRNA gene sequence similarity is about 85 %).

In various phylogenetic trees that were reconstructed by using either maximum-likelihood, neighbour-joining or parsimony methods, the novel isolate DS^T formed a distinct branch within the family *Thermodesulfobacteriaceae*, thereby confirming its separate taxonomic status (Fig. 2; Supplementary Fig. S1).

On the basis of physiological traits in combination with the results of the 16S rRNA gene sequence comparison, we propose that strain DS^T should be assigned to a new genus and species within the family *Thermodesulfobacteriaceae*, for which we propose the name *Caldimicrobium rimae* gen. nov., sp. nov.

Description of *Caldimicrobium* gen. nov.

Caldimicrobium (Cal.di.mi.cro'bi.um. L. adj. *caldus* hot; N.L. neut. n. *microbium* microbe; N.L. neut. n. *Caldimicrobium* microbe living in hot places).

Extremely thermophilic. Strictly anaerobic. Cells are Gram-negative, oval rods, about 1.0–1.2 µm long and 0.5 µm wide, with two polar flagella. Neutrophilic. Able to grow chemolithoautotrophically with molecular hydrogen in the presence of thiosulfate or elemental sulfur with formation of H₂S. Able to utilize organic acids and alcohols in the presence of the same electron acceptors. The DNA G + C content of the type strain of the type species is 35.2 mol%. 16S rRNA gene sequence analysis places the genus in the family *Thermodesulfobacteriaceae*. The type species is *Caldimicrobium rimae*.

Description of *Caldimicrobium rimae* sp. nov.

Caldimicrobium rimae (ri'mae. L. gen. n. *rimae* of a crack or fissure, referring to the English translation of Treshchinnyi, the hot spring from which the type strain was isolated).

Fig. 2. Phylogenetic tree based on an alignment of almost-complete 16S rRNA gene sequences, showing the affiliation of strain DS^T to the family *Thermodesulfobacteriaceae*. GenBank/EMBL/DBJ accession numbers are shown in parentheses. The tree was reconstructed using the fastDNAmI algorithm included in the ARB program package version 03_08_22 (<http://www.arb-home.de>). The sequence of *Desulfovibrio desulfuricans* ATCC 27774 (GenBank accession no. M34113; not shown) was used to define the root. Bar, 10 % estimated sequence divergence.

Table 1. Characteristics that differentiate strain DS^T from members of the family *Thermodesulfobacteriaceae*

Reference species: 1, '*Geothermobacterium ferrireducens*' (data from Kashefi *et al.*, 2002); 2, *Thermodesulfobacterium hydrogeniphilum* (Jeamthong *et al.*, 2002); 3, *Thermodesulfobacterium hveragerdense* (Sonne-Hansen & Ahring, 1999); 4, *Thermodesulfobacterium commune* (Zeikus *et al.*, 1983); 5, *Thermodesulfobacterium thermophilum* (Rosanova & Pivovarova, 1988); 6, *Thermodesulfatator indicus* (Moussard *et al.*, 2004). NR, Not reported.

Characteristic	DS ^T	1	2	3	4	5	6
Temperature range (°C)	52–82	65–100	50–80	55–74	45–85	45–85	55–80
Optimum temperature (°C)	75	85–90	75	70–74	70	65	70
pH range	6.8–7.4	NR	6.3–6.8	NR	6.0–8.0	NR	6.0–6.7
pH optimum	7.0–7.2	NR	6.5	NR	7.0	7.0	6.25
DNA G + C content (mol%)*	35.2 ^a	NR	28 ^b	40 ^b	34.4 ^a	38 ^a	46 ^b
Electron acceptors							
Sulfate	–	–	+	+	+	+	+
Thiosulfate	+	–	–	+	+	+	–
Sulfur	+	–	–	–	–	–	–
Sulfite	–	–	–	+	–	+	–
Fe(III) oxide	–	+	–	–	–	–	NR
Capacity for lithoautotrophic growth with H ₂ †	+	+	+	–	–	–	+
Electron donors							
Formate	–	–	–	–	–	+	–
Fumarate	+	–	–	–	–	–	–
Malate	+	–	–	–	–	–	–
Ethanol	+	–	–	–	–	–	–
Fermentation of:							
Pyruvate	–	–	–	+	+	+	–
Lactate	–	–	–	–	–	+	–

*Determined by: *a*, melting-point analysis; *b*, HPLC.

†Growth with hydrogen in the presence of an organic carbon source.

Displays the following characteristics in addition to those given for the genus. Growth occurs at 52–82 °C, with an optimum at 75 °C; the pH range for growth is pH 6.8–7.4, with an optimum at pH 7.0–7.2. Grows chemo-organotrophically with ethanol, fumarate, malate or succinate.

The type strain, DS^T (=DSM 19393^T =VKM B-2460^T), was isolated from the terrestrial neutral hot spring Treshchinnyi, located in the Uzon Caldera, Kamchatka, Russia.

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