Ammonifex thiophilus sp. nov., a hyperthermophilic anaerobic bacterium from a Kamchatka hot spring

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A novel anaerobic, extremely thermophilic, facultatively chemolithoautotrophic bacterium designated strain SR^T was isolated from a terrestrial hot spring in Kamchatka (Russia). The cells of the novel strain were spore-forming rods with a Gram-positive type of cell wall. The novel isolate grew at 60–82 °C (optimum 75 °C) and pH 6.0–7.5 (optimum 6.8). Molecular hydrogen and formate were used as electron donors. Thiosulfate, sulfate or elemental sulfur served as electron acceptors yielding hydrogen sulfide. No growth was observed on either substrate in the presence of nitrate as the electron acceptor. The G+C content of the DNA was 56.2 mol%. Phylogenetic analyses of the 16S rRNA gene showed that strain SR^T was most closely related to *Ammonifex degensii* (96.4% gene sequence similarity). However, the novel isolate possessed phenotypic characteristics that differed significantly from those of *A. degensii*, the only other recognized species of the genus *Ammonifex*. It is concluded that strain SR^T (=DSM 19636^T=VKM B-2461^T) represents the type strain of a novel species of the genus *Ammonifex*, for which the name *Ammonifex thiophilus* sp. nov. is proposed. An emendation of the genus *Ammonifex* is proposed based on the phenotypic characteristics of the novel species.

Geothermal areas in the Uzon Caldera, Kamchatka, have created a diverse array of extreme microbial habitats. Over the years, many novel anaerobic thermophilic or hyperthermophilic archaea and bacteria have been isolated from this unique ecosystem. The majority of these isolates are chemoorganoheterotrophs utilizing complex proteinaceous substrates (Bonch-Osmolovskaya *et al.*, 1988, 1990, 1991; Zavarzina *et al.*, 2000, 2002; Perevalova *et al.*, 2005; Kublanov *et al.*, 2007). However, hot springs in the Uzon Caldera are rich in CO₂ with an admixture of CH₄, S, CO and H₂, which may strongly support chemolithotrophic metabolism. Radioisotopic experiments aimed at determining the rates of the main microbial processes (lithotrophic and acetoclastic methanogenesis, acetate oxidation and CO₂ assimilation and sulfate reduction) have also indicated the presence of lithotrophic prokaryotes in the Uzon Caldera (Bonch-Osmolovskaya *et al.*, 1999). However, to date only few thermophilic anaerobes capable of chemolithoautotrophic growth have been isolated from the Uzon Caldera hot springs. These are moderately thermophilic, sulfur-reducing bacteria of the genus *Desulfurella* (Bonch-Osmolovskaya

et al., 1990; Miroshnichenko *et al.*, 1998). In this paper, we describe novel extremely thermophilic, anaerobic, facultatively lithoautotrophic bacteria inhabiting one of the hottest springs of the Uzon Caldera.

A sample of sediments mixed with water was collected from Treshchinnyi Spring, East Thermal Field. The temperature and pH at the sampling site were 80 °C and 6.3, respectively. The evolving gases consisted of CO₂, N₂, H₂S, CH₄ and H₂ (N. V. Pimenov, personal communication).

For the enrichment culture, anaerobically prepared modified Pfennig medium (Miroshnichenko *et al.*, 1998) was used supplemented with 1 mg l⁻¹ resazurin (Sigma), 1 ml l⁻¹ trace element solution (Pfennig & Lippert, 1966) and 1 ml l⁻¹ vitamin solution (Wolin *et al.*, 1963). After boiling, the medium was cooled under CO₂ and supplemented with 2 g l⁻¹ Na₂S₂O₃, 1.5 g l⁻¹ NaHCO₃ and 0.5 g l⁻¹ Na₂S·9H₂O. To adjust the pH of the medium to 6.8, 10% NaOH was added at a concentration of 5 ml l⁻¹. The medium was dispensed as 5 ml samples into 20 ml Balch tubes; the headspace was filled with 100% H₂.

After 5 days incubation at 82 °C, the growth of rod-shaped cells was observed. The culture was purified using the dilution-to-extinction technique and the resulting isolate

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SR^T is EF554597.

was designated as strain SR^T. Electron microscopy (Bonch-Osmolovskaya *et al.*, 1990) indicated that cells of the novel isolate had peritrichous flagellation and a cell wall typical of Gram-positive bacteria (Fig. 1). The cells were 0.6–0.7 µm wide, 3–6 µm long and appeared singly or in pairs. Strain SR^T formed round terminal endospores after long (5–7 days) incubation at 75 °C. The percentage of sporulated cells, though, was less than 1–2%.

After demonstrating that strain SR^T grew well with formate (10 mM) and thiosulfate (10 mM), this donor–acceptor pair was chosen for growth experiments carried out with CO₂ (100%) filling the head space of the tubes (see results in Table 1).

Isolate SR^T grew only on the prereduced medium over a temperature range from 60 to 82 °C (optimally at 75 °C) and over a pH range from 6.0 to 7.5 (optimally at pH 6.8–7.0). In the presence of thiosulfate under optimized conditions, the following compounds did not support the growth of strain SR^T: acetate, ethanol, propionate, methanol, glucose, fructose, pyruvate, fumarate, malate, succinate or lactate (all at 10 mM) and yeast extract, starch or peptone (at 10 g l⁻¹). These data suggested that molecular hydrogen and formate were the only energy substrates used by strain SR^T. The electron acceptors tested for their ability to support the growth of strain SR^T were selected from the following: S⁰ (10 g l⁻¹), NO₃⁻ (10 mM), SO₄²⁻ (10 mM), SO₃²⁻ (2 mM) and NO₂⁻ (10 mM). At the optimum temperature and pH values, the best growth for strain SR^T (8.3 × 10⁷ cells ml⁻¹) was observed with formate

and thiosulfate. The final cell density on medium with formate and S⁰ or SO₄²⁻ as electron acceptors was 7.5 × 10⁶ cells ml⁻¹. The final end product of anaerobic respiration (Trüper & Schlegel, 1964) was H₂S.

DNA was prepared as described by Marmur (1961). The G + C content of the DNA was determined by melting point analysis (Marmur & Doty, 1962) using *Escherichia coli* K-12 DNA as a reference. The DNA G + C content for strain SR^T was 56.2 mol%. For amplification and sequencing of the 16S rRNA genes, DNA was obtained by standard phenol/chloroform extraction. The 16S rRNA genes were selectively amplified using 5'-AGAGTTGATCCTGGCTCAG-3' and 5'-TACGGTTACCTTGTTACGACTT-3' as the forward and reverse primers, respectively. The PCR products were purified using the Wizard PCR Prep kit (Promega) according to the manufacturer's instructions. Sequencing was performed using Big Dye Terminator v.3.1 sequencing reaction kit and an ABI 3730 DNA automatic sequencer (Applied Biosystems). Preliminary phylogenetic analysis of the new sequences was conducted using the NCBI BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>). The nucleotide sequences were aligned with sequences from the GenBank using CLUSTAL W. The phylogenetic tree was reconstructed using the neighbour-joining algorithm as implemented in the TREECONW software package (Van de Peer & De Wachter, 1994). A comparison of the almost complete 16S rRNA gene sequence (1536 nucleotides) of isolate SR^T with the database suggested that the novel strain belonged to the Gram-positive bacteria of the *Moorella* group and was most related to the genus *Ammonifex*, with similarity as high as 96.4% (Fig. 2).

The new isolate was compared with *Ammonifex degensii* DSM 10501^T, the only recognized member of this genus and isolated from an Indonesian volcanic hot spring (Huber *et al.*, 1996). Both organisms had rod-shaped cells with a Gram-positive type cell wall. Although *A. degensii* has been characterized as a Gram-negative bacterium (Huber *et al.*, 1996), no outer membrane characteristic for this type of cells has been documented. Both strains were thermophilic obligate anaerobes that were capable of growing autotrophically by the oxidation of H₂ or formate, using CO₂ as the carbon source. Both strains were able to grow weakly by the reduction of sulfate or sulfur. The DNA G + C contents of *A. degensii* and strain SR^T were very similar at 54 and 56.2 mol%, respectively. However, comparisons of metabolic and physiological characteristics of the two strains revealed a number of significant differences (Table 1).

It was found that *A. degensii* DSM 10501^T and strain SR^T used different sets of electron acceptors. Strain SR^T reduced thiosulfate as the preferential electron acceptor, but did not use nitrate. *A. degensii* reduced nitrate to ammonium (this trait is reflected in the name of the genus), but was not able to grow with thiosulfate as the electron acceptor. Strain SR^T was not capable of fermentative growth, whereas *A. degensii* grew by pyruvate fermentation. In addition, the growth rate of strain SR^T was higher than that of *A. degensii*

Fig. 1. Electron micrographs of cells of strain SR^T. Negative staining of whole cell (a) and thin section (b). Bars, 0.5 µm.

Table 1. Comparative characteristics of strain SR^T and *Ammonifex degensii* DSM 10501^T

Data for *A. degensii* are from Huber *et al.* (1996). +, Positive; –, negative.

Characteristic	Strain SR ^T	<i>A. degensii</i>
Cell shape	Rods (0.6–0.7 × 3–6 µm)	Rods (0.6 × 2–4.5 µm)
Cell wall	Gram-positive	Gram-negative*
Flagellation	Peritrichous	Terminal and lateral
Ability to form spores	+	–
Electron acceptors used:		
Thiosulfate	+	–
Nitrate	–	+
Fermentative growth with pyruvate	–	+
Temperature for growth (°C)		
Range	60–82	57–77
Optimum	75	70
DNA G + C content (mol%)	56.2	54

*However, no outer membrane has been documented.

and its temperature range and optima were shifted to higher values when compared with those of *A. degensii*.

While both organisms were isolated from terrestrial hot springs, recent environmental 16S rRNA gene sequence analyses have shown that the deep-subsurface environment is dominated by representatives of the so-called *Ammonifex* group. An abundant group of bacterial clones (83 % clonal frequency) related to *A. degensii* as the closest relative (91 % gene sequence similarity) was found in ridge flank oceanic crusts (Nakagawa *et al.*, 2006). Based on the

metabolic capacities of *A. degensii*, speculations have been made on the ecological function of these organisms as regards the production of ammonium from nitrate (Cowen *et al.*, 2003). Although strain SR^T belongs to the same group of organisms, it was not able to reduce nitrate indicating that this group of organisms could accomplish other functions in microbial communities.

Isolate SR^T differed from *A. degensii* in 16S rRNA gene sequence (96.4 % similarity) and in some important phenotypic features. On the basis of these differences, it

Fig. 2. The position of strain SR^T of the order *Thermoanaerobacterales* in a phylogenetic tree constructed by a comparison of the nucleotide sequence of the 16S rRNA genes. *Escherichia coli* was used as outgroup. Tree topology and evolutionary distances are given by the neighbour-joining method with Jukes & Cantor distances. Bootstrap values (expressed as percentage of 100 replications) are shown as branch points; values >70 were considered significant. Bar, 5 nucleotide substitutions per 100 nucleotides.

is suggested that the new strain is assigned as the type strain of a novel species, for which the name *Ammonifex thiophilus* sp. nov. is proposed.

Emended description of the genus *Ammonifex*

Ammonifex [Am.mo.ni.fex. L. neut. n. *sal ammoniacum* salt of Ammon (NH₄Cl); L. v. *facere* to make; N.L. masc. n. *Ammonifex* the ammonium-maker].

The description is based on that given by Huber *et al.* (1996). Cells are Gram-positive rods. Some species form spores. Facultatively chemolithoautotrophic. Able to oxidize hydrogen and formate, reducing nitrate to ammonium, or, alternatively, reducing thiosulfate, sulfate or elemental sulfur to hydrogen sulfide. The DNA G+C content is in the range 54–56 mol%.

Description of *Ammonifex thiophilus* sp. nov.

Ammonifex thiophilus (thi.o'phi.lus. Gr. n. *thion* sulfur; Gr. adj. *phylos* loving; N.L. neut. adj. *thiophilus* sulfur-loving).

Cells are peritrichously flagellated, non-motile rods of 0.6–0.7 µm in diameter and 3–6 µm in length. Cell wall is of the Gram-positive type. Spore-forming. Obligately anaerobic. Growth occurs between 60 and 82 °C, with an optimum at 75 °C. The pH range for growth is 6.0–7.5, with an optimum at 6.8–7.0. Facultatively chemolithoautotrophic. Hydrogen- and formate-oxidizing. Reduces thiosulfate, sulfate or elemental sulfur to H₂S.

The type strain, SR^T (=DSM 19636^T=VKM B-2461^T), was isolated from Treshchinnyi hot spring, Uzon Caldera, Kamchatka, Russia. The DNA G+C content of the type strain is 56.2 mol%.

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