Desulfurococcus fermentans sp. nov., a novel hyperthermophilic archaeon from a Kamchatka hot spring, and emended description of the genus *Desulfurococcus*

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An obligately anaerobic, hyperthermophilic, organoheterotrophic archaeon, strain Z-1312^T, was isolated from a freshwater hot spring of the Uzon caldera (Kamchatka Peninsula, Russia). The cells were regular cocci, 1–4 µm in diameter, with one long flagellum. The cell envelope was composed of a globular layer attached to the cytoplasmic membrane. The temperature range for growth was 63–89 °C, with an optimum between 80 and 82 °C. The pH range for growth at 80 °C was 4.8–6.8, with an optimum at pH 6.0. Strain Z-1312^T grew by hydrolysis and/or fermentation of a wide range of polymeric and monomeric substrates, including agarose, amygdalin, arabinose, arbutin, casein hydrolysate, cellulose (filter paper, microcrystalline cellulose, carboxymethyl cellulose), dextran, dulcitol, fructose, lactose, laminarin, lichenan, maltose, pectin, peptone, ribose, starch and sucrose. No growth was detected on glucose, xylose, mannitol or sorbitol. Growth products when sucrose or starch were used as the substrate were acetate, H₂ and CO₂. Elemental sulfur, thiosulfate and nitrate added as potential electron acceptors for anaerobic respiration did not stimulate growth when tested with starch as the substrate. H₂ at 100% in the gas phase did not inhibit growth on starch or peptone. The G+C content of the DNA was 42.5 mol%. 16S rRNA gene sequence analysis placed the isolated strain Z-1312^T as a member of the genus *Desulfurococcus*, where it represented a novel species, for which the name *Desulfurococcus fermentans* sp. nov. (type strain Z-1312^T=DSM 16532^T=VKM V-2316^T) is proposed.

Anaerobic, hyperthermophilic prokaryotes with fermentative metabolism have been identified in both archaeal kingdoms, *Euryarchaeota* and *Crenarchaeota* (Blöchl *et al.*, 1995; Stetter, 1996; Huber *et al.*, 2000). Usually, these organisms grow on complex media with proteins or peptides as the energy substrates, producing volatile fatty acids, CO₂ and H₂ as fermentation products. Molecular hydrogen often inhibits growth of fermentative archaea; elimination of H₂ by reduction of elemental sulfur to H₂S, by flushing the cultures with an inert gas (Fiala & Stetter, 1986) or by cultivation in co-cultures with H₂-consuming

methanogens (Bonch-Osmolovskaya & Stetter, 1991) supports the growth of fermentative archaea. Thus, growth of most fermentative archaea in closed vessels is either obligately dependent on elemental sulfur or stimulated by its presence. An exception is the fermentation observed with two representatives of the *Crenarchaeota*, *Sulfophobococcus zilligii* (Hensel *et al.*, 1997) and *Thermosphaera aggregans* (Huber *et al.*, 1998), which do not require elemental sulfur and are inhibited by its presence. All members of the genus *Desulfurococcus* reported to date (Zillig *et al.*, 1982; Bonch-Osmolovskaya *et al.*, 1988) grow exclusively or preferably on peptides, concomitantly reducing elemental sulfur to H₂S. Their growth on peptides is stimulated significantly by the presence of elemental sulfur and inhibited by H₂ (Slobodkin & Bonch-Osmolovskaya, 1994). *Desulfurococcus amylolyticus* (Bonch-Osmolovskaya *et al.*, 1988; Tourova

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et al., 2000) can grow on starch, glycogen or pectin, but its growth on these substrates is much weaker in comparison with that on peptides and also depends on the presence of S^0 . Here, we describe a strain representing a novel hyperthermophilic species of the genus *Desulfurococcus*, *Desulfurococcus fermentans*, isolated from a Kamchatka hot spring. The organism grows by fermentation of diverse carbohydrates, including cellulose, does not require the presence of elemental sulfur and is not inhibited by H_2 . To our knowledge, this is the first report on an archaeon that is able to grow on cellulosic substrates.

Samples of water and mud from a freshwater hot spring of the Uzon caldera (Kamchatka Peninsula, Russia) were used for inoculation of anaerobically prepared basal medium of the following composition (mg l^{-1} unless indicated): KCl, 330; NH_4Cl , 330; KH_2PO_4 , 330; $MgCl_2 \cdot 6H_2O$, 330; $CaCl_2 \cdot 2H_2O$, 330; $Na_2S \cdot 9H_2O$, 500; starch, 5000; yeast extract (Difco), 200; resazurin, 1; trace element solution (Pfennig & Lippert, 1965), 1 ml l^{-1} ; vitamin solution (Wolin *et al.*, 1963), 1 ml l^{-1} ; pH 6.5 (adjusted with H_2SO_4). The medium was prepared anaerobically under an atmosphere of 80% N_2 + 20% CO_2 and dispensed into 15 ml Hungate tubes with butyl rubber stoppers, leaving 5 ml as the headspace.

After 3–5 days incubation at 85 °C, an organism with regular coccoid cells, often forming sarcina-like aggregates, was enriched from the Uzon caldera sample. In pure culture obtained by serial dilutions of the initial enrichment, the cells were present only as single coccoid cells. The new isolate was designated strain Z-1312^T.

Cells of strain Z-1312^T were regular cocci, 1–4 µm in diameter (Fig. 1a). On electron micrographs of whole cells, a long single flagellum is present. Thin sections (Bonch-Osmolovskaya *et al.*, 1990) revealed a cell-wall structure consisting of a cellular membrane covered by one layer of subunits (Fig. 1b). Strain Z-1312^T was an obligate anaerobe, since no growth was observed under oxic conditions as well as under anoxic conditions when the medium was not pre-reduced by the addition of sodium sulfide. The organism was a hyperthermophile, growing in the temperature range 63–89 °C with an optimum at 80–82 °C. Strain Z-1312^T grew over a pH range of 4.8–6.8, with an optimum at pH 6.0.

Strain Z-1312^T grew by fermentation of starch (5 g l^{-1}) or cellulose (2 g l^{-1}) (filter paper, microcrystalline cellulose, carboxymethyl cellulose) as well as of a wide range of monomeric and polymeric substrates (2 g l^{-1}), including agarose, arabinose, arbutin, casein hydrolysate, dulcitol, pectin, peptone, sucrose and yeast extract, with a final cell yield of $2.5\text{--}5.0 \times 10^7$ cells ml^{-1} . Weaker growth resulting in a cell yield around or below 2.0×10^7 cells ml^{-1} was observed on amygdalin, dextran, fructose, lactose, laminarin, lichenan, maltose and ribose. No growth was detected on glucose, xylose, mannitol or sorbitol. Growth curves of strain Z-1312^T on media with starch and microcrystalline cellulose are shown in Fig. 2. The final cell yield on starch

Fig. 1. Electron micrographs of cells of strain Z-1312^T. (a) Negatively stained whole cell showing a single flagellum. (b) Thin section. Bars, 0.5 µm.

Fig. 2. Growth of strain Z-1312^T on media containing 0.2 g yeast extract l^{-1} alone (Δ) or additionally supplemented with 5 g starch l^{-1} ($\diamond$) or 5 g microcrystalline cellulose l^{-1} ($\circ$). The incubation temperature was 82 °C and the pH of the media was 6.0.

was 5×10^7 cells ml^{-1} , while the doubling time was 6.3 h. On microcrystalline cellulose, the final cell yield was around 2.5×10^7 cells ml^{-1} and the doubling time was 10.7 h. The only growth products (Bonch-Osmolovskaya & Miroshnichenko, 1994) detected during growth on media with starch or sucrose were acetate, H_2 and CO_2 . Growth on cellulosic substrates, peptone, starch and sucrose was found to be stimulated by yeast extract (optimum concentration 200 mg l^{-1}). The ability of strain Z-1312^T to use elemental sulfur as an electron acceptor was tested in cultures growing on starch and peptone. Although sulfur was reduced to hydrogen sulfide, no effect of the presence of sulfur on the cell yield was observed. Thiosulfate, sulfate and nitrate were not reduced and did not influence growth on starch or peptone. H_2 (100 % in the gas phase) did not inhibit growth of strain Z-1312^T on starch or peptone.

The G+C content of the DNA of strain Z-1312^T was determined by the denaturation method (Owen & Lapage, 1976) as 42.5 mol%. Analysis of partial 16S rRNA gene sequences of strain Z-1312^T (1378 nucleotides) was done as described previously (Sanger *et al.*, 1977) using 5'-AG-AGTTTGATCCTGGCTCAG-3' as the forward primer and 5'-TACGGTTACCTTGTACGACTT-3' as the reverse primer (Lane, 1991). This sequence has a high G+C content (65.0 %), as observed previously for the 16S rRNA genes of other thermophilic prokaryotic organisms. Primary analysis of 16S rRNA gene nucleotide sequence similarity of the new isolate was carried out using the BLASTA server (<http://www.ncbi.nlm.nih.gov/blast>). The sequences were aligned against the corresponding 16S rRNA gene sequences of related organisms using the CLUSTAL program (Thompson *et al.*, 1994). Positions that had not been sequenced in one or more reference organisms were omitted and a total of 1193 nucleotides were used in the analysis. The phylogenetic tree rooted by the outgroup *Methanococcus vannielii* was constructed by the neighbour-joining method with bootstrap analysis of 100 trees using the programs of the TREECON package (Van de Peer & De Wachter, 1994). This analysis revealed that strain Z-1312^T was a member of the family *Desulfurococcaceae* (Burggraf *et al.*, 1997) (kingdom *Crenarchaeota*, domain *Archaea*). Additional

sequence alignments and phylogenetic analysis performed with members of this family revealed that strain Z-1312^T was closely related to the species of the genus *Desulfurococcus* (95.5–96.4 % sequence similarity). In the phylogenetic tree (Fig. 3), strain Z-1312^T formed a single cluster with *Desulfurococcus* species with a high level of bootstrap probability of this branching point (96 %).

The genus *Desulfurococcus* (Zillig *et al.*, 1982) at present consists of three species with validly published names: *Desulfurococcus mucosus*, the type species of the genus, *Desulfurococcus mobilis* (Zillig *et al.*, 1982) and *D. amylolyticus* (Bonch-Osmolovskaya *et al.*, 1988; Tourova *et al.*, 2000). Nucleotide sequences of the 16S rRNA gene have been obtained only for the last two species. Representatives of these species could be detected by specific oligonucleotide probes (Perevalova *et al.*, 2003). The affiliation of isolate Z-1312^T to the genus *Desulfurococcus* is in good agreement with common phenotypic features that exist between strain Z-1312^T and other representatives of the genus *Desulfurococcus* (Table 1). However, although it shows the same cell morphology and growth characteristics, strain Z-1312^T differs significantly in growth substrates and its relation to elemental sulfur and H_2 . In contrast to other *Desulfurococcus* species, which prefer proteinaceous substrates, it grows well on different monomeric and polymeric carbohydrates. Elemental sulfur is not required for growth, which is in agreement with the lack of an inhibitory effect of H_2 on growth.

Peptides are the common substrates of anaerobic, organotrophic, hyperthermophilic archaea, while carbohydrates are metabolized by a limited number of hyperthermophilic archaeal species (Schönheit & Schäfer, 1995). *Pyrococcus furiosus* can ferment maltose and cellobiose (Fiala & Stetter, 1986; Schäfer & Schönheit, 1992), *Pyrococcus woesei*, *D. amylolyticus*, *Thermococcus stetteri* and *Acidilobus aceticus* were able to grow fermentatively on starch (Zillig *et al.*, 1987; Bonch-Osmolovskaya *et al.*, 1988; Miroshnichenko *et al.*, 1989; Prokofeva *et al.*, 2000) and *Pyrococcus glycovorans* was able to grow fermentatively on glucose, cellobiose and starch (Barbier *et al.*, 1999). The range of growth

Fig. 3. Phylogenetic tree generated by the neighbour-joining method on the basis of 16S rRNA gene sequences showing the position of *Desulfurococcus fermentans* sp. nov. Z-1312^T among members of the family *Desulfurococcaceae*. Bootstrap values (of 100 replications) are shown at branch points; values greater than 95 were considered significant. Bar, 10 nucleotide substitutions per 100 nucleotides.

Table 1. Characteristics of strain Z-1312^T and species of the genus *Desulfurococcus*

Characteristic	<i>D. mucosus</i>	<i>D. mobilis</i>	<i>D. amylolyticus</i>	Z-1312 ^T
Shape and size of cells	Cocci	Cocci	Irregular cocci	Regular cocci, 1–4 µm
Flagellation	No flagella	One flagellum	No flagella	One flagellum
Growth temperature (°C) (min./opt./max.)	ND/85/ND	ND/85/ND	68/90–92/97	63/80–82/89
Growth pH (min./opt./max.)	4·5/6·0/7·0	4·5/6·0/7·0	5·7/6·4/7·5	4·8/6·0/6·8
Growth substrates	Peptides	Peptides	Peptides, starch	Peptides, monosaccharides, polysaccharides including cellulose
Influence of sulfur on growth	Stimulating	Stimulating	Stimulating	No effect
Influence of hydrogen on growth	Inhibiting*	ND	Inhibiting*	No effect
G + C content of DNA (mol%)	51·3	50·8	41·2	42·5

ND, No data.

*Data from Slobodkin & Bonch-Osmolovskaya (1994).

substrates of strain Z-1312^T is much wider than that of other hyperthermophilic archaea, including cellulose and other mono-, di- and polysaccharides. In this capacity it resembles '*Desulfurococcus saccharovorans*' (Stetter, 1986); however, the name of this species was never validly published.

A unique feature of the new isolate is its ability to grow on cellulosic substrates (microcrystalline cellulose, carboxymethyl cellulose, filter paper). To our knowledge, strain Z-1312^T is the first hyperthermophilic archaeon able to grow on such substrates. So far, genes for endoglucanase that could be involved in cellulose degradation have been found in several hyperthermophilic archaea. Endoglucanase genes detected in the genomes of *P. furiosus*, *Pyrococcus horikoshii* and *Sulfolobus solfataricus* have been cloned and expressed in *Escherichia coli* (Bauer *et al.*, 1999; Limauro *et al.*, 2001; Ando *et al.*, 2002). However, growth of these microorganisms on cellulosic substrates has never been reported. Taking into consideration both phenotypic and genosystematic differentiating features between strain Z-1312^T and other *Desulfurococcus* species, we propose that it represents a novel species, *Desulfurococcus fermentans* sp. nov. The description of this novel species necessitates the emendation of the description of the genus.

Description of *Desulfurococcus fermentans* sp. nov.

Desulfurococcus fermentans (fer.men'tans. L. part. adj. *fermentans* fermenting).

Cells are cocci, 1–4 µm in diameter, with one polar flagellum. Obligate anaerobe. Temperature growth range from 63 to 89 °C, with optimum at 82 °C. pH growth range from 4·8 to 6·8, with optimum at pH 6·0. Obligate chemoorganoheterotroph; obtains energy by fermentation of arabinose, agarose, amygdalin, arbutin, casein hydrolysate, cellulose, dextran, dulcitol, fructose, lactose, laminarin, lichenan, maltose, pectin, peptone, ribose, starch and sucrose. No growth is observed on glucose, mannitol,

sorbitol or xylose. Fermentation products detected are acetate, H₂ and CO₂. Growth is not inhibited by 100 % H₂. Elemental sulfur, sulfate, thiosulfate and nitrate do not stimulate growth and are not used as electron acceptors. The G + C content of DNA of the type strain is 42·5 mol%.

The type strain, strain Z-1312^T (=DSM 16532^T=VKM V-2316^T), was isolated from a hot spring of Uzon caldera, Kamchatka peninsula, Russia. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of the type strain is AY264344.

Emended description of genus *Desulfurococcus* Zillig and Stetter 1983

Desulfurococcus (De.sul.fu.ro.coc'cus. N.L. pref. *de* from; L. n. *sulfur* sulfur; Gr. n. *coccus* berry; N.L. masc. n. *Desulfurococcus* the sulfur-reducing coccus).

Archaea of the kingdom *Crenarchaeota*. Cells are regular or irregular cocci, with or without flagella, with a cell envelope of globular structure. Hyperthermophiles with optimum growth temperature of 80–90 °C. Neutrophiles or moderate acidophiles with optimum pH for growth of 6·0–6·5. Obligate anaerobes. Organotrophs utilizing a wide range of organic substrates: peptides and monomeric and polymeric carbohydrates. Fermentative type of metabolism. Growth of representatives of some species is inhibited by molecular hydrogen and stimulated by the presence of elemental sulfur. The G + C content of the DNA is 41–51 mol%. Reported species inhabit terrestrial hot springs. The type species is *Desulfurococcus mucosus* Zillig and Stetter 1983 (type strain ATCC 35584^T=DSM 2162^T=JCM 9187^T).

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