Fervidicoccus fontis gen. nov., sp. nov., an anaerobic, thermophilic crenarchaeote from terrestrial hot springs, and proposal of *Fervidicoccaceae* fam. nov. and *Fervidicoccales* ord. nov.

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Two novel thermophilic and slightly acidophilic strains, Kam940^T and Kam1507b, which shared 99 % 16S rRNA gene sequence identity, were isolated from terrestrial hot springs of the Uzon caldera on the Kamchatka peninsula. Cells of both strains were non-motile, regular cocci. Growth was observed between 55 and 85 °C, with an optimum at 65–70 °C (doubling time, 6.1 h), and at pH 4.5–7.5, with optimum growth at pH 5.5–6.0. The isolates were strictly anaerobic organotrophs and grew on a narrow spectrum of energy-rich substrates, such as beef extract, gelatin, peptone, pyruvate, sucrose and yeast extract, with yields above 10⁷ cells ml⁻¹. Sulfate, sulfite, thiosulfate and nitrate added as potential electron acceptors did not stimulate growth when tested with peptone. H₂ at 100 % in the gas phase inhibited growth on peptone. Glycerol dibiphytanyl glycerol tetraethers (GDGTs) with zero to four cyclopentyl rings were present in the lipid fraction of isolate Kam940^T. The G + C content of the genomic DNA of strain Kam940^T was 37 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolates were archaea of the phylum *Crenarchaeota*, only distantly related to the cultured members of the class *Thermoprotei* (no more than 89 % identity), and formed an independent lineage adjacent to the orders *Desulfurococcales* and *Acidilobales* and clustering only with uncultured clones from hot springs of Yellowstone National Park and Iceland as the closest relatives. On the basis of their phylogenetic position and novel phenotypic features, isolates Kam940^T and Kam1507b are proposed to be assigned to a new genus and species, *Fervidicoccus fontis* gen. nov., sp. nov. The type strain of *Fervidicoccus fontis* is strain Kam940^T (=DSM 19380^T =VKM B-2539^T). The phylogenetic data as well as phenotypic properties suggest that the novel crenarchaeotes form the basis of a new family, *Fervidicoccaceae* fam. nov., and order, *Fervidicoccales* ord. nov., within the class *Thermoprotei*.

The *Crenarchaeota* are one of the two major phyla of the domain *Archaea*. Many crenarchaeotes have been isolated

Abbreviation: GDGT, glycerol dibiphytanyl glycerol tetraether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Kam940^T is EF552404.

Details of sequences from other orders used in the construction of the trees shown in Fig. 2 are available as supplementary material with the online version of this paper.

from hot marine and terrestrial environments (Huber & Stetter, 2006). The Kamchatka peninsula is an active volcanic area where many terrestrial hot springs occur. The Uzon caldera is located in the east of Kamchatka and contains numerous active hydrothermal fields with a wide variety of chemical compositions, temperature and pH (Perevalova *et al.*, 2008). Several organotrophic, anaerobic crenarchaeotes have been isolated from hot springs of Kamchatka, including *Desulfurococcus amylolyticus*

(Bonch-Osmolovskaya *et al.*, 1988), *Thermoproteus uzoniensis* (Bonch-Osmolovskaya *et al.*, 1990b), *Acidilobus aceticus* (Prokofeva *et al.*, 2000), *Desulfurococcus fermentans* (Perevalova *et al.*, 2005), *Vulcanisaeta* strains (Prokofeva *et al.*, 2005), *Desulfurococcus kamchatkensis* (Kublanov *et al.*, 2009b) and *Acidilobus saccharovorans* (Prokofeva *et al.*, 2009). All these organisms are hyperthermophiles, with optimum growth temperatures around 80–92 °C. However, archaea, primarily representatives of uncultured lineages of *Crenarchaeota*, have also been found in terrestrial hot springs with temperatures that are too low (55–75 °C) for hyperthermophiles (Pearson *et al.*, 2004; Huang *et al.*, 2007; Perevalova *et al.*, 2008). Furthermore, *in situ* enrichment cultures obtained in the presence of various polymeric substrates from hot springs of the Uzon caldera at temperatures of 68–77 °C demonstrated the presence of a novel group of *Crenarchaeota* (Kublanov *et al.*, 2009a). Here, we report the isolation and characterization of two representatives of this novel group of *Crenarchaeota* that inhabit terrestrial hot springs and grow at temperatures around 70 °C.

Samples of sediment mixed with water were collected from Treshchinnyi spring (54° 29' 56.3" N 160° 00' 55.9" E) and Sery spring (54° 29' 58" N 160° 00' 50" E) in the east thermal field of the Uzon caldera. The temperature and pH at the sampling sites were 80 °C, pH 6.3 and 75 °C, pH 6.5, respectively.

For enrichment cultures, anaerobically prepared basal medium (Perevalova *et al.*, 2005) was used supplemented with (l^{-1}) 1 mg resazurin (Sigma), 1 ml trace element solution (Pfennig & Lippert, 1966), 1 ml vitamin solution (Wolin *et al.*, 1963) and 0.2 g yeast extract. After boiling, the medium was cooled under an atmosphere of 80 % N_2 /20 % CO_2 and supplemented with 0.5 g $NaHCO_3$ and 0.5 g $Na_2S \cdot 9H_2O$ l^{-1} , pH 6.0–6.5 (adjusted with H_2SO_4), and dispensed into 15 ml Hungate tubes with butyl rubber stoppers, leaving 5 ml headspace.

An aliquot (10%) of the sediment/water mixture was incubated at 70 °C with chitin (crab chitin; Bioprogress) or β -keratin (ground feathers) at 2 g l^{-1} and pH 6.0–6.5. After 3–5 days of incubation, organisms with regular coccoid cells were enriched. The enrichment cultures were designated Kam940^T and Kam1507b. For the isolation of strain Kam940^T, the basal medium with peptone (2 g l^{-1}) was used, with the addition of 1.5 % agar. Colonies were obtained in shake-tubes incubated at 60 °C after about a week. Individual colonies were picked and transferred to the same liquid medium without agar. Strain Kam1507b was isolated by the serial dilution-to-extinction method, using the basal medium with peptone. The purity of the isolates was tested by phase-contrast microscopy of cultures grown under various conditions. In pure cultures, all cells were single cocci.

Cells of both isolates were regular cocci, 1–3 μm in diameter, without flagella (Fig. 1a). Thin sections (Bonch-Osmolovskaya *et al.*, 1990a) revealed a cell

Fig. 1. Electron micrographs of negatively stained whole cells (a) and thin cell sections (b) of strain Kam940^T. Bars, 1 μm .

envelope consisting of the cellular membrane covered with one layer of subunits (Fig. 1b).

Growth of the novel isolates was determined by direct cell counts using phase-contrast microscopy. All growth experiments were conducted in triplicate. Both isolates were obligate anaerobes, since no growth was observed under oxic conditions, as well as under anoxic conditions when the medium was not prereduced by the addition of sodium sulfide. The organisms were extreme thermophiles, growing in the temperature range of 55–85 °C, with optimum growth at 65–70 °C, and at pH 4.5–7.0, with optimum growth at pH 5.5–6.0. No growth was observed at 53 or 89 °C or at pH 4.0 or 8.0.

The nutritional spectrum of both strains was very narrow. Strains Kam940^T and Kam1507b grew by fermentation of beef extract, gelatin, peptone and yeast extract at 2 g l^{-1} , with final yields of 5.0×10^7 – 8.5×10^7 cells ml^{-1} . Weaker growth resulting in yields below 5.0×10^7 cells ml^{-1} was observed on albumin, amygdalin, arabinose, cellobiose, pyruvate, rhamnose, soy flour, sucrose and tryptone. No growth was detected on acetate, agarose, casein hydrolysate, chitin, cellulose (CM-cellulose and filter paper), dextran, glucose, fructose, fumarate, α -keratin, lactate, maltose, mannitol, methanol, pectin, xylan or H_2 . Both

strains required yeast extract (at a concentration of 20–50 mg l⁻¹). The final yield of strain Kam940^T on medium with yeast extract under optimal growth conditions was 8.4 × 10⁷ cells ml⁻¹, with a doubling time of 6.1 h. The growth products after growth on medium with sucrose, determined as described by Bonch-Osmolovskaya & Miroshnichenko (1994), were acetate, H₂ and CO₂. The ability of strain Kam940^T to use electron acceptors was tested for the culture grown on peptone. Sodium thiosulfate and sodium nitrate (at 5 mM) were not reduced, whereas sodium sulfate and sodium sulfite inhibited growth. H₂ (100% in the gas phase) inhibited growth of strain Kam940^T completely on peptone.

Biomass of a culture of strain Kam940^T was lyophilized and used for lipid analysis. Total lipid extraction was performed using a single-phase organic solvent procedure and transesterified according to Pearson *et al.* (2004) and Zhang *et al.* (2006). HPLC-MS analysis of archaeal core lipids was performed at the University of Bremen, Germany, according to a modified procedure of Hopmans *et al.* (2000) on a ThermoFinnigan LCQ Deca XP Plus ion-trap mass spectrometer coupled to an HPLC by an atmospheric pressure chemical ionization interface. The instrument conditions are described in detail by Lipp & Hinrichs (2009). The results showed that the majority (97%) of the archaeal lipids were glycerol dibiphytanyl glycerol tetraethers (GDGTs) that contained zero to four cyclopentyl rings; the remaining small proportion (3%) consisted of the diether archaeol. Of the five GDGT compounds, GDGT-0 was the most abundant (35.5%), followed by GDGT-4 (19.2%), GDGT-1 (15.8%), GDGT-3 (13.8%) and GDGT-2 (12.7%). No crenarchaeol was present in the lipid fraction.

The G+C content of the DNA of strain Kam940^T was determined by the thermal denaturation method (Marmur & Doty, 1962) to be 37 mol%. 16S rRNA genes were amplified by PCR using the archaea-specific primer A8F (5'-TCCGGTTGATCCTGCCGG-3') (Kolganova *et al.*, 2002) as the forward primer and the universal primer S-D-Bact-1492-a-A-21 (5'-TACGGYTACCTTGTTACGACTT-3') as the reverse primer (Alm *et al.*, 1996). PCR products were sequenced using a Big Dye Terminator version 3.1 kit on an automatic ABI 3730 sequencer according to the manufacturer's protocol (Applied Biosystems). Sequences of the 23S rRNA genes of strain Kam940^T and *Acidilobus saccharovorans* 345-15^T were obtained in the course of complete genome sequencing of these organisms in the Centre 'Bioengineering' RAS. New sequences were used as queries for BLAST searches at the NCBI site (<http://www.ncbi.nlm.nih.gov/blast>; Altschul *et al.*, 1997) for their preliminary identification and retrieval of similar sequences. The newly determined and retrieved sequences were aligned using the MULTALIN software (<http://bioinfo.genopole-toulouse.prd.fr/multalin/>) or MAFFT version 6 (<http://align.genome.jp/mafft/>). Pairwise evolutionary distances were calculated by using the correction of Jukes & Cantor (1969). Phylogenetic trees were reconstructed using three different algorithms implemented in the

TREECONW software package version 1.3b (Van de Peer & de Wachter, 1994). Since the tree topologies were very similar in all cases, the final results are presented as the neighbour-joining trees (Saitou & Nei, 1987). Bootstrap analysis (1000 replications) was used to validate the reproducibility of the branching patterns of the trees.

Phylogenetic analysis based on 16S rRNA and 23S rRNA gene sequences placed the novel crenarchaeotes in the phylum *Crenarchaeota* as a novel, deep lineage adjacent to the orders *Desulfurococcales* and *Acidilobales* (Prokofeva *et al.*, 2009) (Fig. 2). Strains Kam940^T and Kam1507b formed an independent cluster together with several uncultured clones from Yellowstone National Park (USA) and Iceland (Meyer-Dombard *et al.*, 2005; Kvist *et al.*, 2007; Perevalova *et al.*, 2008). Of taxa with validly published names or any other reportedly cultured organisms, none exhibited more than 89% 16S rRNA gene sequence identity to strain Kam940^T or Kam1507b. On the basis of its phylogenetic position and novel phenotypic features, isolate Kam940^T is proposed as the type strain of a new genus and species, *Fervidicoccus fontis* gen. nov., sp. nov., which forms the basis of a new family, *Fervidicoccaceae* fam. nov., and order, *Fervidicoccales* ord. nov., within the class *Thermoprotei* of the phylum *Crenarchaeota*.

Environmental clones of the *Fervidicoccus* group have been found in terrestrial hot springs in different geographical regions with a broad range of temperatures (Meyer-Dombard *et al.*, 2005; Kvist *et al.*, 2007; Perevalova *et al.*, 2008; Kublanov *et al.*, 2009a). The phylotypes YNP_ObP_A25 and YNP_SSp_A51, found in Obsidian Pool and Sylvan Spring (Yellowstone National Park) with temperatures around 80 °C, exhibit 94% 16S rRNA gene sequence identity to strain Kam940^T, and phylotypes from the Hveragerði region (Iceland), with temperatures of 55–81 °C, Hverd031N (Kvist *et al.*, 2007) and Is6 and V4 (Perevalova *et al.*, 2008), exhibit 92–94% 16S rRNA gene sequence identity to strain Kam940^T. Furthermore, it was found that representatives of *Fervidicoccus* are widespread in terrestrial hot springs of the Uzon caldera with temperatures of 68–77 °C (Kublanov *et al.*, 2009a). The environments where *Fervidicoccus* clones were found are characterized by low contents of sulfide (Spear *et al.*, 2005; Sun & Armannsson, 2000; our unpublished results).

The majority of cultured crenarchaeotes are hyperthermophiles and neutrophiles. The more unusual extreme thermophiles with lower growth temperature optima are acidophiles and are predominantly aerobes or facultative anaerobes belonging to the *Sulfolobales* (Huber & Prangishvili, 2006). Two novel aerobic, ammonia-oxidizing crenarchaeotes described by Hatzenpichler *et al.* (2008) and de la Torre *et al.* (2008) are also (extreme) thermophiles. *Thermocodium modestius* and the two species of *Caldisphaera* are the only known obligate anaerobes among the extremely thermophilic crenarchaeotes (Itoh *et al.*, 1998, 2003; Boyd *et al.*, 2007). Here, we report on a novel group of crenarchaeotes that grow optimally at tempera-

Fig. 2. Neighbour-joining phylogenetic trees based on 16S rRNA gene sequences (a) and 23S rRNA gene sequences (b) showing the positions of strains Kam940^T and Kam1507b (a) and strain Kam940^T (b) (*Fervidicoccus fontis* gen. nov., sp. nov.) within the phylum Crenarchaeota. Clusters of members of the orders *Thermoproteales*, *Sulfolobales*, *Desulfurococcales* and *Acidilobales* are indicated by triangles; sequences are detailed in Supplementary Table S1, available in IJSEM Online. Bootstrap values (from 1000 trials) of 90% and above are shown. GenBank accession numbers are given in parentheses. The G + C contents of 16S rRNA genes are shown in brackets in (a). Bars, 1 substitution per 10 sequence positions.

tures (65–70 °C) that are below the growth range of most cultured hyperthermophilic crenarchaeotes. The adaptation of the novel group of the Crenarchaeota to moderate-temperature habitats is evidenced by the G + C contents of their 16S rRNA genes, known to correlate with the temperature growth optima of the organisms (Galtier & Lobry 1997; Kimura *et al.*, 2006). For example, the G + C contents of the 16S rRNA genes of hyperthermophilic crenarchaeotes are higher than 66 mol% and, for comparison, that of marine non-thermophilic crenarchaeotes is around 53 mol%; extreme thermophiles have 16S rRNA gene G + C contents of 56–64 mol%. The G + C content of the 16S rRNA genes of isolates Kam940^T and Kam1507b

and their relatives is 62–64 mol% (Fig. 2a), which correlates with the temperature characteristics of their sources of isolation and with their temperature ranges for growth. The GDGT composition of strain Kam940^T also confirms the differences from other cultured hyperthermophilic crenarchaeotes. Cultured hyperthermophilic crenarchaeotes contain mostly GDGT-4 to -6 (Pearson *et al.*, 2008), whereas the most abundant GDGT in strain Kam940^T is GDGT-0.

Considering the outlying position of the *Fervidicoccus* group in the phylogenetic trees (Fig. 2), the presence in the 16S rRNA of specific signatures (Table 1) and phenotypic

Table 1. 16S rRNA sequence signatures that distinguish the *Fervidicoccales* ord. nov. from other orders of the phylum *Crenarchaeota*

Signatures were deduced from alignments retrieved from the GreenGenes database (updated 16 June 2008; 623 crenarchaeotal sequences). Sequence positions are given according to the *Escherichia coli* numbering. *Fervidicoccales* here refers to strain Kam940^T and environmental clones YNP_SSp_A51 and YNP_ObP_A25 (Hverd031N, Is6 and V4 sequences were too short for analysis). For all bases, the frequency was at least 97%.

Position(s)	<i>Fervidicoccales</i>	<i>Acidilobales</i>	<i>Desulfurococcales</i>	<i>Thermoproteales-Sulfolobales</i>
34	U	U	C	C
501:544	U-A	C-G	C-G	C-G
1244:1293	U-A	G-C	G-C	K-M, A-U

properties such as the temperature range and optimum, which distinguish representatives of this group from the phylogenetically adjoining members of the *Desulfurococcales*, we propose the taxonomic status of the *Fervidicoccus* group as being at the order level. The evolutionary divergence of this group may be related to adaptation to anaerobic environments in terrestrial hot springs with temperatures of 55–70 °C.

Description of *Fervidicoccus* gen. nov.

Fervidicoccus (Fer.vi'di.coc'cus. L. adj. *fervidus* hot; L. masc. n. *coccus* a berry and, in biology, a coccoid cell; N.L. masc. n. *Fervidicoccus coccus* which grows at high temperatures).

Cells are regular cocci. Cell envelope consists of an S-layer covering the cytoplasmic membrane. Non-motile. Temperatures of 65–70 °C and slightly acidic pH (5.5–6.0) are preferred for growth. Obligately anaerobic. Organotrophic. Additional electron acceptors are not necessary. The 16S rRNA gene sequence is typical of a crenarchaeote in sequence signature analysis. Phylogenetically, the genus represents an independent lineage. Known strains have been isolated from terrestrial hot springs. The type species is *Fervidicoccus fontis*.

Description of *Fervidicoccus fontis* sp. nov.

Fervidicoccus fontis (fon'tis. L. gen. masc. n. *fontis* of a spring or fountain, referring to the isolation of the type strain from a terrestrial hot spring).

Displays the following properties in addition to those in the genus description. Cells are 1–3 µm in diameter. Grows at 55–85 °C and pH 4.5–7.0. Growth occurs on beef extract, gelatin, peptone, pyruvate, rhamnose, sucrose and yeast extract with yields above 10⁷ cells ml⁻¹. Sulfate, sulfite, thiosulfate and nitrate do not stimulate growth. H₂ (100%) in the gas phase inhibits growth completely. The core lipid fraction consists of GDGTs with zero to four cyclopentyl rings. The DNA G+C content of the type strain is 37 mol%.

The type strain is Kam940^T (=DSM 19380^T =VKM B-2539^T), isolated from a hot spring of the east thermal field of the Uzon caldera, Kamchatka.

Description of *Fervidicoccaceae* fam. nov.

Fervidicoccaceae (Fer.vi'di.coc.ca'ce.ae. N.L. masc. n. *Fervidicoccus* the type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Fervidicoccaceae* the family of the genus *Fervidicoccus*).

Cocci. Anaerobic heterotrophs. Extreme thermophiles. Known habitats are terrestrial hot springs. The following pattern of 16S rRNA sequence signature nucleotides distinguishes the family *Fervidicoccaceae*: 34 (U), 501:544 (U-A), 1244:1293 (U-A). Members of the order *Fervidicoccales* in the class *Thermoprotei*. The type genus is *Fervidicoccus*.

Description of *Fervidicoccales* ord. nov.

Fervidicoccales (Fer.vi'di.coc.ca'les. N.L. masc. n. *Fervidicoccus* the type genus of the order; N.L. -ales ending to denote an order; N.L. fem. pl. n. *Fervidicoccales* the order of the genus *Fervidicoccus*).

The description is as for the family *Fervidicoccaceae*. The type genus is *Fervidicoccus*.

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